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(71) Applicant: **MEMORIAL SLOAN KETTERING CANCER CENTER** [US/US]; 1275 York Avenue, New York, New York 10065 (US).

(72) Inventors: **SCHEINBERG, David A.**; c/o Memorial Sloan Kettering Cancer Center, 1275 York Avenue, New York, New York 10065 (US). **GARDNER, Thomas J.**; c/o Memorial Sloan Kettering Cancer Center, 1275 York Avenue, New York, New York 10065 (US). **TAN, Derek S.**; c/o Memorial Sloan Kettering Cancer Center, 1275 York Avenue, New York, New York 10065 (US). **LEE, Jonghan**; c/o Memorial Sloan Kettering Cancer Center, 1275 York Avenue, New York, New York 10065 (US). **LORENZ, Ivo**; c/o Memorial Sloan Kettering Cancer Center, 1275 York Avenue, New York, New York 10065 (US).

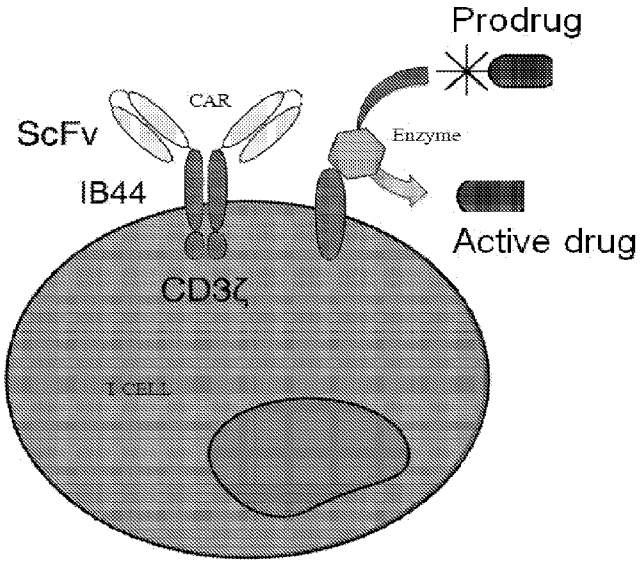
(74) Agent: **EWING, James F.** et al.; Foley & Lardner LLP, Washington, District of Columbia 20007 (US).

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(54) Title: COMPOSITIONS AND METHODS FOR ADOPTIVE CELL THERAPY FOR CANCER

FIG. 1



(57) Abstract: Provided herein are compositions and methods for adoptive cell therapy comprising engineered immune cells that express a tumor antigen-targeted chimeric antigen receptor and a prodrug converting enzyme.

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[Continued on next page]

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COMPOSITIONS AND METHODS FOR ADOPTIVE CELL THERAPY FOR CANCER

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the benefit of and priority to U.S. Provisional Patent Application No. 62/527,932, filed June 30, 2017, the entire contents of which are incorporated herein by reference.

SEQUENCE LISTING

[0002] The instant application contains a Sequence Listing which has been submitted electronically in ASCII format and is hereby incorporated by reference in its entirety. Said ASCII copy, created on July 1, 2018, is named 115872-0384_SL.txt and is 119,572 bytes in size.

STATEMENT OF GOVERNMENT SUPPORT

[0003] This invention was made with government support under AI073736, AI095692, AR068118, R01 CA55349, P01 CA23766, GM100477, and AI118224 awarded by the National Institutes of Health. The government has certain rights in the invention.

BACKGROUND OF THE INVENTION

[0004] Adoptive and engineered T cell therapies, including chimeric antigen receptor (CAR) T cells, T cell receptor (TCR) engineered T cells, and antigen adopted T cells, have emerged recently as important therapies for cancer. First generation CARs were designed by fusing the scFv to the intracellular signaling domain of the CD3-ζ chain, whereas second generation CARs added CD28-CD80 costimulation in which the CD28 signaling domain was incorporated into the CAR construct (*i.e.*: "28ζ CAR") for improved T cell activation and efficacy. However, greater potency and mechanisms to defeat the immunosuppressive tumor microenvironment are still needed for many cancer types. Resistance to the activity of these cells, relapse, and toxicity are still important hurdles to their success. Recent reports of significant toxicities and even deaths after CAR T cell therapy, and TCR T cell therapy, and the continuing incidence of GVHD with its associated short- and long-term morbidity and

mortality, call for new methods to potentiate efficacy, and at the same time, to better control the various adoptive cell therapies.

SUMMARY OF THE INVENTION

[0005] Provided herein are compositions and methods for adoptive cell therapy comprising engineered immune cells that express a receptor that binds to a target antigen and a first prodrug converting enzyme, wherein the first prodrug converting enzyme is expressed in the cytoplasm (*e.g.*, intracellularly) of the engineered immune cells to allow selective destruction of the cells. In some embodiments, the engineered immune cells further comprise a second prodrug converting enzyme expressed on the surface of the engineered immune cell or secreted by the engineered immune cell. In some embodiments, the receptor is a T cell receptor. In some embodiments, the receptor is a native T cell receptor. In some embodiments, the receptor is a non-native T cell receptor, for example, an engineered receptor, such as a chimeric antigen receptor (CAR). In some embodiments, the engineered immune cells comprise a chimeric antigen receptor and/or nucleic acid encoding the chimeric antigen receptor.

[0006] In some embodiments, the engineered immune cells comprise a first prodrug converting enzyme and/or a nucleic acid encoding the first prodrug converting enzyme, wherein the first prodrug converting enzyme is expressed in the cytoplasm of the immune cell. In some embodiments, the engineered immune cells further comprise a second prodrug converting enzyme and/or a nucleic acid encoding the second prodrug converting enzyme, wherein the second prodrug converting enzyme is expressed on the surface of the immune cell or is secreted.

[0007] In some embodiments, the engineered immune cells comprise: (a) first prodrug converting enzyme and/or a nucleic acid encoding the first prodrug converting enzyme, wherein the first prodrug converting enzyme is expressed in the cytoplasm of the immune cell and is selected from among a carboxypeptidase, a β-lactamase, a glucosidase, a nitroreductase, and carboxypeptidase A; and (b) a receptor that binds to a target antigen and/or nucleic acid encoding the receptor, such as a native receptor or a chimeric antigen receptor and/or nucleic acid encoding the receptor. In some embodiments, the engineered immune cells further comprise a second prodrug converting enzyme and/or a nucleic acid

encoding the second prodrug converting enzyme, wherein the second prodrug converting enzyme is expressed on the surface of the immune cell or is secreted.

[0008] In some embodiments, the engineered immune cells comprise: (a) a first prodrug converting enzyme and/or a nucleic acid encoding the first prodrug converting enzyme, wherein the first prodrug converting enzyme is expressed in the cytoplasm of the immune cell; (b) a second prodrug converting enzyme and/or a nucleic acid encoding the second prodrug converting enzyme, wherein the second prodrug converting enzyme is expressed on the surface of the immune cell or is secreted; and (c) a receptor that binds to a target antigen and/or nucleic acid encoding the receptor. In some embodiments, the first prodrug converting enzyme and/or second prodrug converting enzyme is selected from among a carboxypeptidase, a β-lactamase, a glucosidase, a nitroreductase, and carboxypeptidase A.

[0009] In some embodiments, the second prodrug converting enzyme is fused to a transmembrane domain. In some embodiments, the transmembrane domain of the prodrug converting enzyme comprises a CD8 transmembrane domain. In some embodiments, the second prodrug converting enzyme is attached to the surface of the cell by a GPI anchor. In some embodiments, the nucleic acid encoding the second prodrug converting enzyme comprises a leader sequence for secretion of the prodrug converting enzyme.

[0010] In some embodiments, the first prodrug converting enzyme and the second prodrug converting enzyme have the same enzymatic activity and/or are the same enzyme. In some embodiments, the first prodrug converting enzyme and the second prodrug converting enzyme have different enzymatic activities and/or are different enzymes. In some embodiments, the first prodrug converting enzyme is a carboxypeptidase (*e.g.*, a *Pseudomonas sp.* Carboxypeptidase G2 (CPG2)). In some embodiments, the first prodrug converting enzyme is a β-lactamase (*e.g.*, an *Enterobacter cloacae* β-lactamase). In some embodiments, the second prodrug converting enzyme is a carboxypeptidase (*e.g.*, a *Pseudomonas sp.* Carboxypeptidase G2 (CPG2)). In some embodiments, the second prodrug converting enzyme is a β-lactamase (*e.g.*, an *Enterobacter cloacae* β-lactamase). In some embodiments, the nucleic acid encoding the first prodrug converting enzyme and/or the second prodrug converting enzyme is operably linked to a promoter. In some embodiments, the promoter is a constitutive promoter. In some embodiments, the promoter is a conditional

promoter. In some embodiments, the conditional promoter is inducible by binding of the receptor (*e.g.*, a CAR) to an antigen, such as a tumor antigen.

[0011] In some embodiments, the chimeric antigen receptor comprises (i) an extracellular antigen binding domain; (ii) a transmembrane domain; and (iii) an intracellular domain. In some embodiments, the extracellular antigen binding domain binds to a tumor antigen. In some embodiments, the tumor antigen is selected from among CD19, WT1, and PRAME. In some embodiments, the extracellular antigen binding domain comprises a single chain variable fragment (scFv). In some embodiments, the extracellular antigen binding domain comprises a human scFv. In some embodiments, the extracellular antigen binding domain comprises a CD19 scFv of SEQ ID NO: 19. In some embodiments, the extracellular antigen binding domain comprises a CD19 scFv having at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% sequence identity to SEQ ID NO: 19. In some embodiments, the extracellular antigen binding domain comprises a signal peptide that is covalently joined to the N-terminus of the extracellular antigen-binding domain. In some embodiments, the transmembrane domain comprises a CD8 transmembrane domain. In some embodiments, the intracellular domain comprises a costimulatory domain. In some embodiments, the one or more costimulatory domains are selected from a CD28 costimulatory domain, a CD3ζ-chain, a 4-1BBL costimulatory domain, or any combination thereof. In some embodiments, the immune cell is a lymphocyte. In some embodiments, the lymphocyte is a T-cell, a B cell or a natural killer (NK) cell. In some embodiments, the T cell is a CD4+ T cell or a CD8+ T cell. In some embodiments, the immune cell is a tumor infiltrating lymphocyte. In some embodiments, the immune cell is derived from an autologous donor or an allogenic donor.

[0012] Also provided are nucleic acids encoding any of polypeptides disclosed herein. In some embodiments, the nucleic acid encoding the polypeptide is operable linked to a promoter. In some embodiments, the promoter is a constitutive promoter. In some embodiments, the promoter is a conditional promoter. In some embodiments, the conditional promoter is inducible by the CAR binding to an antigen.

[0013] Also provided are vectors comprising any of nucleic acids disclosed herein. In some embodiments, the vector is a viral vector or a plasmid. In some embodiments, the vector is a retroviral vector.

[0014] Also provided are host cells comprising a polypeptide, a nucleic acid or a vector disclosed herein.

[0015] Also provided are methods for treating cancer in a subject in need thereof comprising administering an effective amount of any of the engineered immune cells provided herein. Also provided herein are methods for treating of inhibiting tumor growth or metastasis in a subject comprising contacting a tumor cell with an effective amount of any of the engineered immune cells provided herein. In some embodiments, the prodrug is administered subsequent to administration of the engineered immune cells. In some embodiments, the methods further comprise administering to the subject a prodrug that is converted to an active drug by the prodrug converting enzyme. In some embodiments, the methods further comprise administering to the subject a first prodrug that is converted to an active drug by the first prodrug converting enzyme. In some embodiments, the first prodrug is administered subsequent to administration of the engineered immune cells. In some embodiments, the methods further comprise administering to the subject a second prodrug that is converted to an active drug by the second prodrug converting enzyme. In some embodiments, the second prodrug is administered prior to the administering the first prodrug. In some embodiments, the engineered immune cells are administered are administered intravenously, intraperitoneally, subcutaneously, intramuscularly, or intratumorally. In some embodiments, the cancer or tumor is a carcinoma, sarcoma, a melanoma, or a hematopoietic cancer. In some embodiments, the cancer or tumor is selected from among adrenal cancers, bladder cancers, blood cancers, bone cancers, brain cancers, breast cancers, carcinoma, cervical cancers, colon cancers, colorectal cancers, corpus uterine cancers, ear, nose and throat (ENT) cancers, endometrial cancers, esophageal cancers, gastrointestinal cancers, head and neck cancers, Hodgkin's disease, intestinal cancers, kidney cancers, larynx cancers, leukemias, liver cancers, lymph node cancers, lymphomas, lung cancers, melanomas, mesothelioma, myelomas, nasopharynx cancers, neuroblastomas, non-Hodgkin's lymphoma, oral cancers, ovarian cancers, pancreatic cancers, penile cancers, pharynx cancers, prostate cancers, rectal cancers, sarcoma, seminomas, skin cancers, stomach cancers, teratomas, testicular cancers, thyroid cancers, uterine cancers, vaginal cancers, vascular tumors, and metastases thereof. In some embodiments, the methods further comprise administering an additional cancer therapy. In some embodiments, the additional cancer therapy is selected

from among chemotherapy, radiation therapy, immunotherapy, monoclonal antibodies, anti-cancer nucleic acids or proteins, anti-cancer viruses or microorganisms, and any combinations thereof. In some embodiments, the methods further comprise administering a cytokine to the subject. In some embodiments, the cytokine is administered prior to, during, or subsequent to administration of the one or more engineered immune cells. In some embodiments, the cytokine is selected from a group consisting of interferon α, interferon β, interferon γ, complement C5a, IL-2, TNFalpha, CD40L, IL12, IL-23, IL15, IL17, CCL1, CCL11, CCL12, CCL13, CCL14-1, CCL14-2, CCL14-3, CCL15-1, CCL15-2, CCL16, CCL17, CCL18, CCL19, CCL19, CCL2, CCL20, CCL21, CCL22, CCL23-1, CCL23-2, CCL24, CCL25-1, CCL25-2, CCL26, CCL27, CCL28, CCL3, CCL3L1, CCL4, CCL4L1, CCL5, CCL6, CCL7, CCL8, CCL9, CCR10, CCR2, CCR5, CCR6, CCR7, CCR8, CCRL1, CCRL2, CX3CL1, CX3CR, CXCL1, CXCL10, CXCL11, CXCL12, CXCL13, CXCL14, CXCL15, CXCL16, CXCL2, CXCL3, CXCL4, CXCL5, CXCL6, CXCL7, CXCL8, CXCL9, CXCL9, CXCR1, CXCR2, CXCR4, CXCR5, CXCR6, CXCR7 and XCL2.

[0016] Also provided are methods for preparing immune cells for cancer therapy, comprising isolating immune cells from a donor subject, transducing the immune cells (*e.g.*, T cells) with a nucleic acid that encodes a first prodrug converting enzyme, wherein the first prodrug converting enzyme is expressed in the cytoplasm of the immune cell; and optionally, nucleic acid that encodes (i) a second prodrug converting enzyme, wherein the second prodrug converting enzyme is expressed on the surface of the immune cell or is secreted and/or (ii) nucleic acid that encodes a receptor that binds to a target antigen and/or nucleic acid encoding the receptor. Also provided are methods for treating a cancer comprising administering the transduced engineered immune cells to a recipient subject. In some embodiments, the donor subject and the recipient subject are the same (i.e., autologous). In some embodiments, the donor subject and the recipient subject are different (i.e., allogenic). In some embodiments, the lymphocytes comprise a T-cell, a B cell, and/or a natural killer (NK) cell. In some embodiments, the T cell is a CD4+ T cell or a CD8+ T cell. In some embodiments, the immune cells isolated from the donor subject comprise tumor infiltrating lymphocytes (TILs).

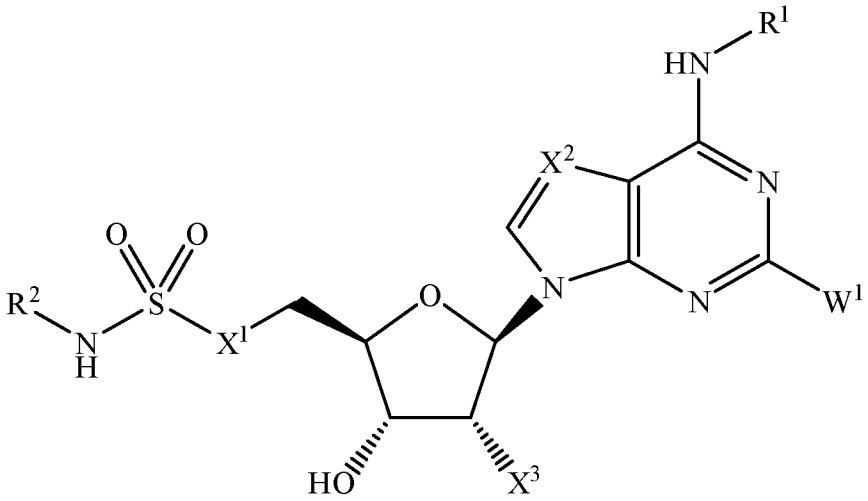
[0017] Also provided are methods for treatment comprising isolating T-cells from a donor subject, transducing the immune cells (*e.g.*, T cells) with a nucleic acid of provided

herein or the vector provided herein, and administering the transduce immune cells (*e.g.*, T cells) to a recipient subject. In some embodiments, the donor subject and the recipient subject are the same (i.e., autologous). In some embodiments, the donor subject and the recipient subject are different (i.e., allogenic). In some embodiments, the immune cells isolated from the donor subject comprise one or more lymphocytes. In some embodiments, the lymphocytes comprise a T-cell, a B cell, and/or a natural killer (NK) cell. In some embodiments, the T cell is a CD4+ T cell or a CD8+ T cell. In some embodiments, the immune cells isolated from the donor subject comprise tumor infiltrating lymphocytes (TILs).

[0018] Also provided are uses of any of the engineered immune cells provided herein for treating a cancer.

[0019] Also provided are uses of any of the engineered immune cells provided herein the preparation of a medicament for the treatment of a cancer.

[0020] Also provided are compounds of Formula I:



(I) or a zwitterion, pharmaceutically acceptable salt, and/or solvate thereof, wherein

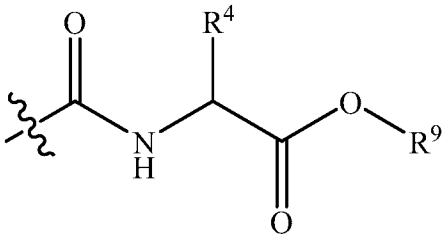
X^1 is O or NH;

X^2 is N or CH;

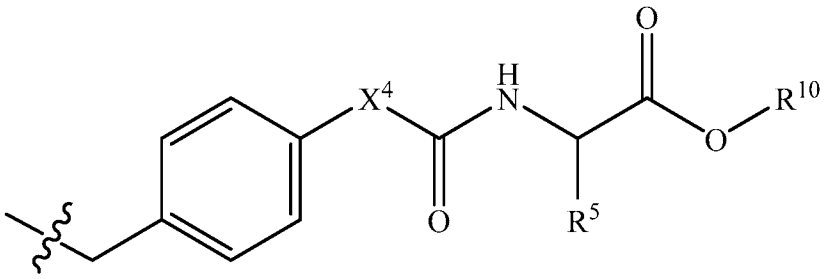
X^3 is OH or H;

W^1 is H or NH-R^3;

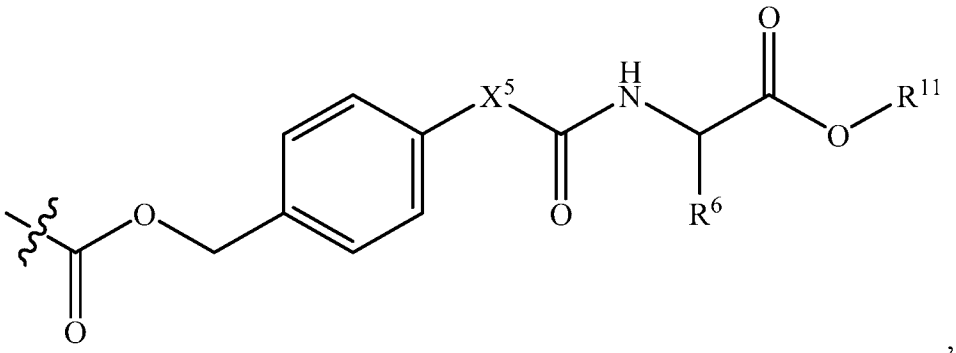
two of R^1 ,R^2, and R^3 are H and the remaining R^1 ,R^2, and R^3 is



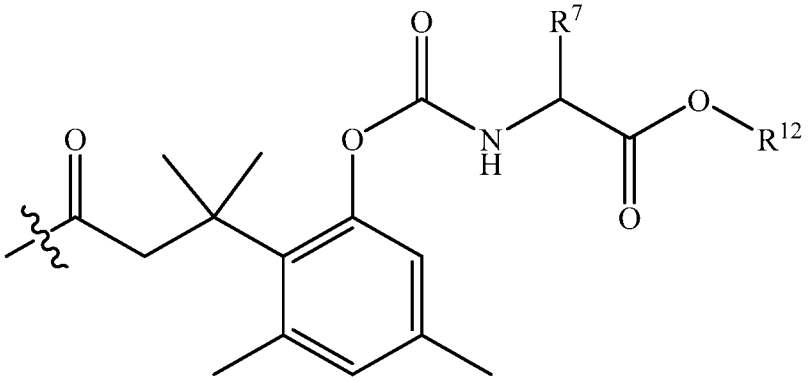
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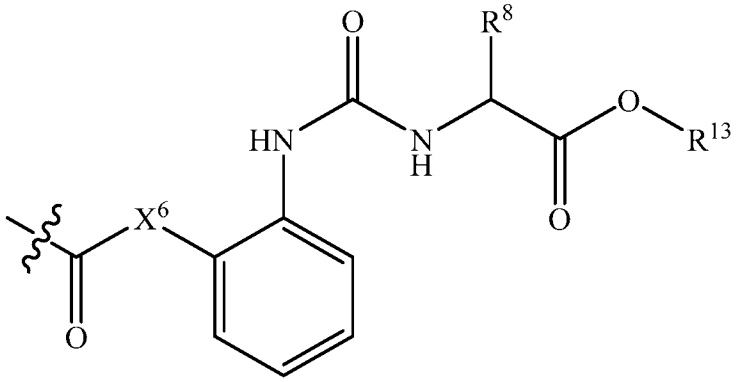
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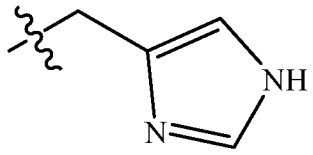
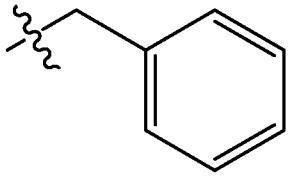


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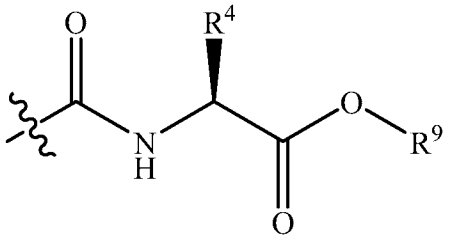


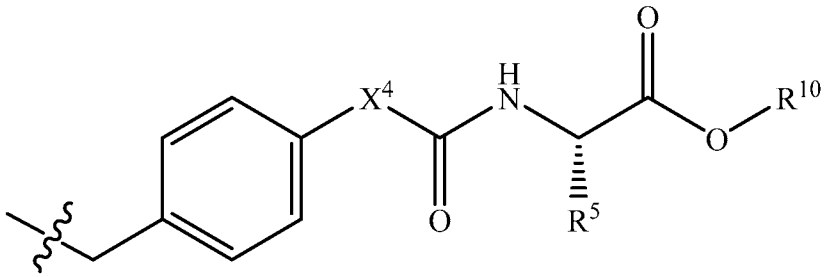
; X^4 and X^5 are each independently O or NH;

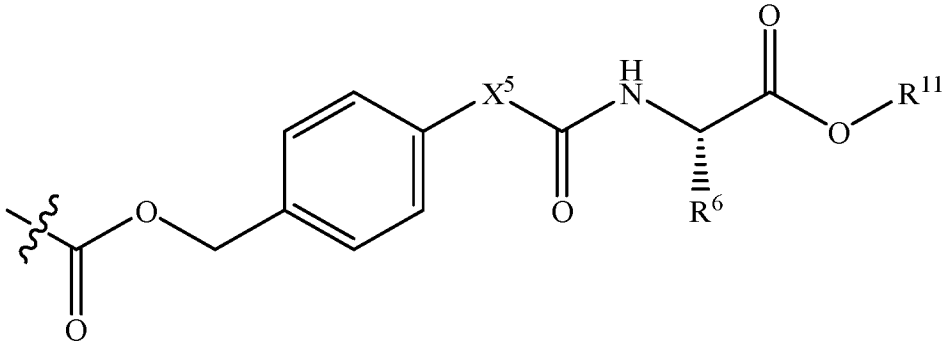
X^6 is O, NH, CH_2, or $C(Me)_2$;

R^4, R^5, R^6, R^7, and R^8 are each independently H, $-(CH_2)_3-NH(NH)-NH_2$, $-(CH_2)_3-NH_2$, $-(CH_2)_4-NH_2$, $-CH_2-C(O)OR^{13a}$, $-(CH_2)_2-C(O)OR^{13b}$, , or ; and

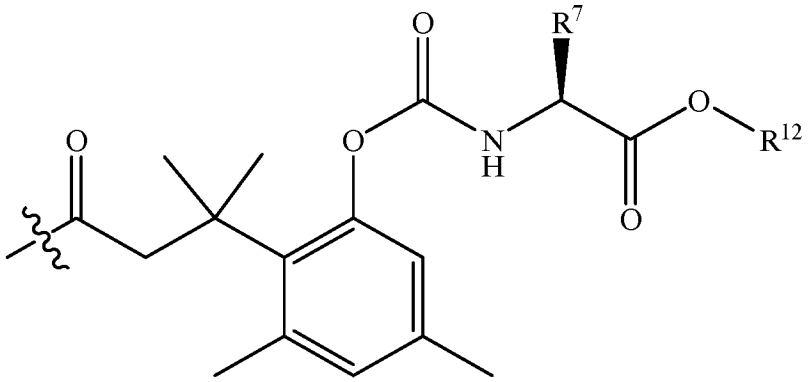
R^9, R^{10}, R^{11}, R^{12}, R^{13}, R^{13a}, and R^{13b} are each independently alkyl, aryl, heteroaryl, aralkyl, or heteroaralkyl. In some embodiments, two of R^1 ,R^2, and R^3 are H and the remaining R^1 ,R^2, and R^3 is

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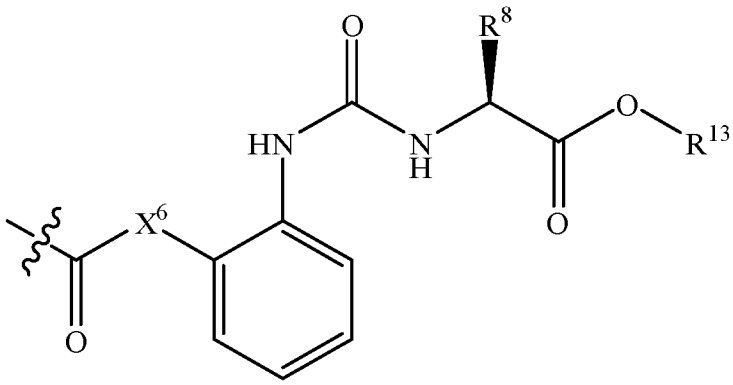
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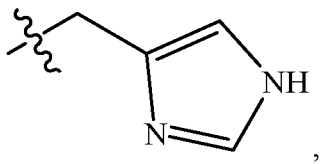


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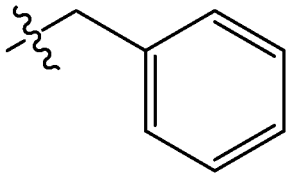


; and

R^4, R^5, R^6, R^7, and R^8 are each independently $-(CH_2)_3-NH(NH)-NH_2$, $-(CH_2)_3-NH_2$, $-(CH_2)_4-NH_2$, $-CH_2-C(O)OR^{13a}$, $-(CH_2)_2-C(O)OR^{13b}$,

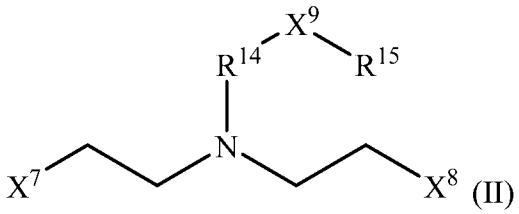


, or



. In some embodiments, R^9, R^{10}, R^{11}, R^{12}, R^{13}, R^{13a}, and R^{13b} are each independently neopentyl.

[0021] Also provided are compounds of Formula II:



(II)

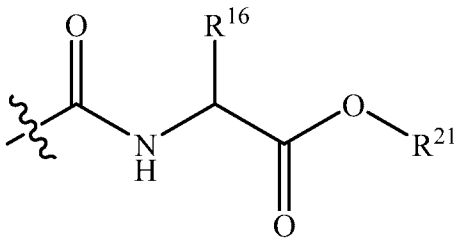
or a zwitterion, pharmaceutically acceptable salt, and/or solvate thereof, wherein

X^7 and X^8 are each independently Cl, Br, I, O-S(O)$_2$CH$_3$, or O-S(O)$_2$-tolyl;

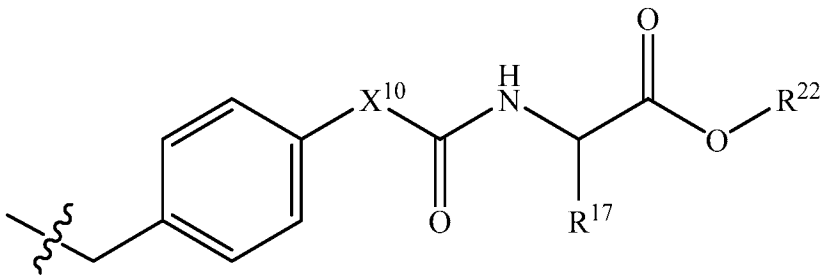
X^9 is –C(O)-O, -C(O)-NH, O, or NH;

R^{14} is a bond, alkylene, arylene, aralkylene, heteroarylene, or heteroaralkylene;

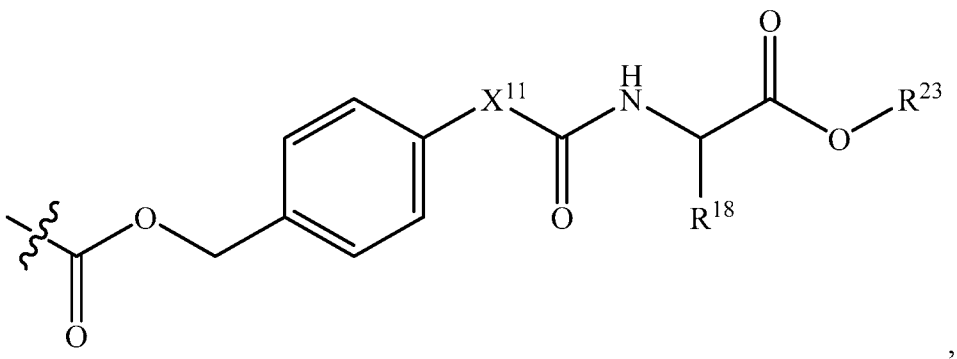
R^{15} is



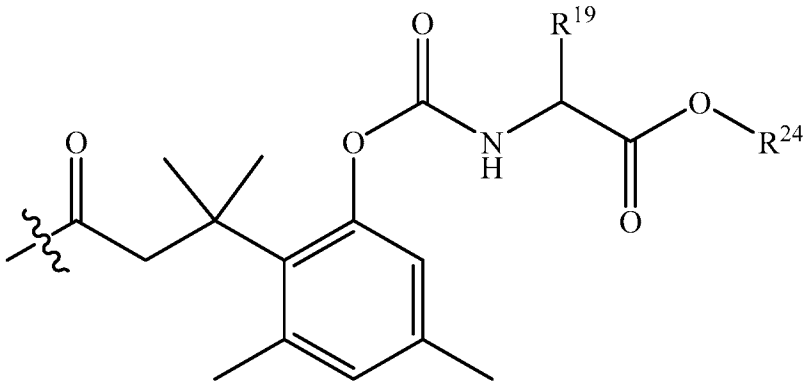
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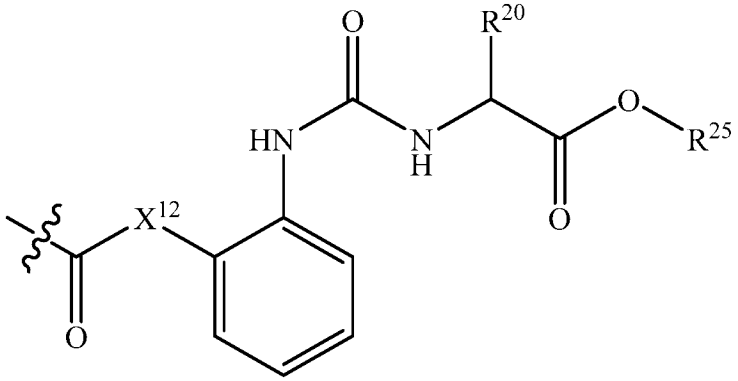
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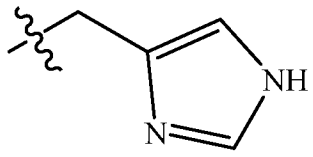


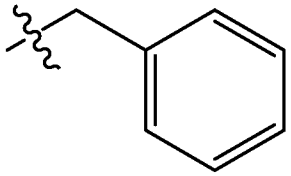
;

X^{10} and X^{11} are each independently O or NH;

X^{6} is O, NH, CH_2, or $C(Me)_2$;

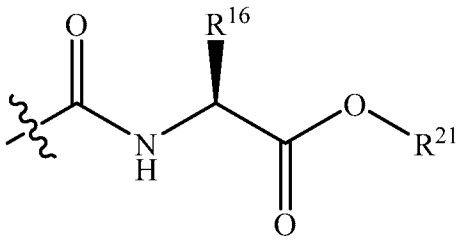
X^{12} is O, NH, CH_2, or $C(Me)_2$;

R^{16}, R^{17}, R^{18}, R^{19}, and R^{20} are each independently H, $-(CH_2)_3-NH(NH)-NH_2$, $-(CH_2)_3-NH_2$, $-(CH_2)_4-NH_2$, $-CH_2-C(O)OR^{25a}$, $-(CH_2)_2-C(O)OR^{25b}$, , or

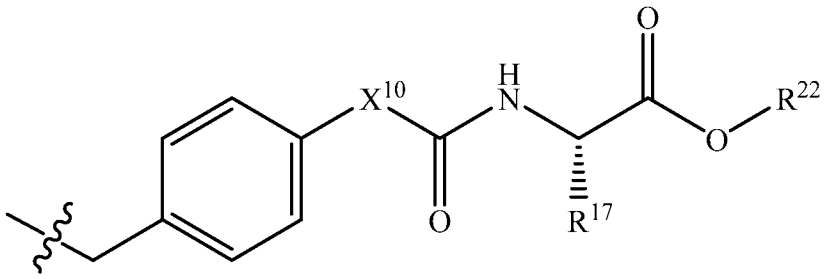


; and

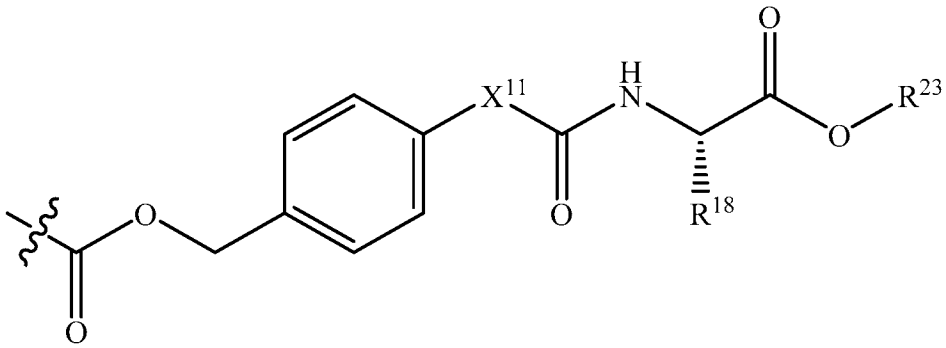
R^{21}, R^{22}, R^{23}, R^{24}, R^{25}, R^{25a}, and R^{25b} are each independently alkyl, aryl, heteroaryl, aralkyl, or heteroaralkyl. In some embodiments, R^{15} is



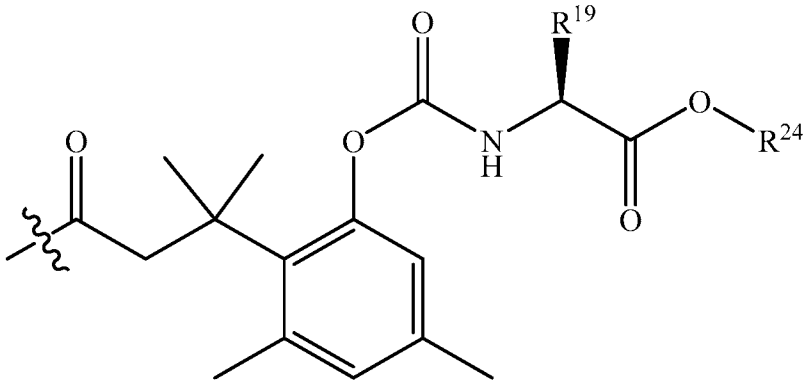
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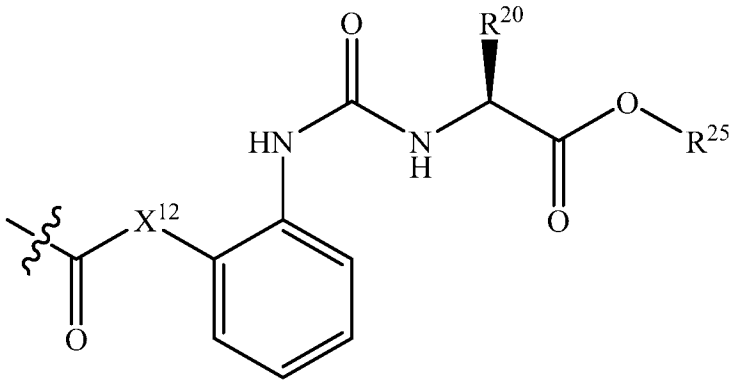
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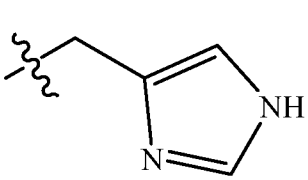


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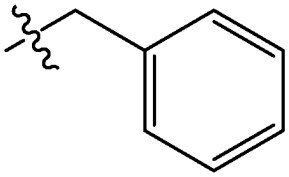


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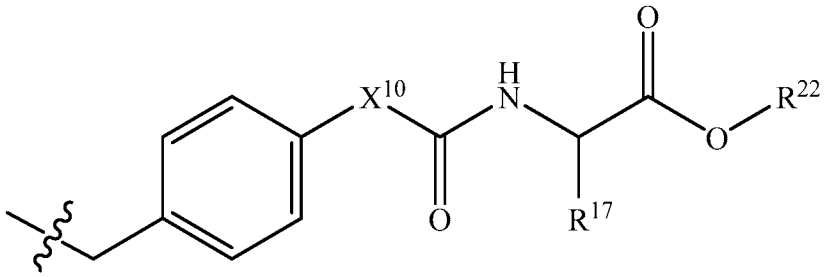
R^{16}, R^{17}, R^{18}, R^{19}, and R^{20} are each independently $-(CH_2)_3-NH(NH)-NH_2$, $-(CH_2)_3-NH_2$, $-(CH_2)_4-NH_2$, $-CH_2-C(O)OR^{25a}$, $-(CH_2)_2-C(O)OR^{25b}$,



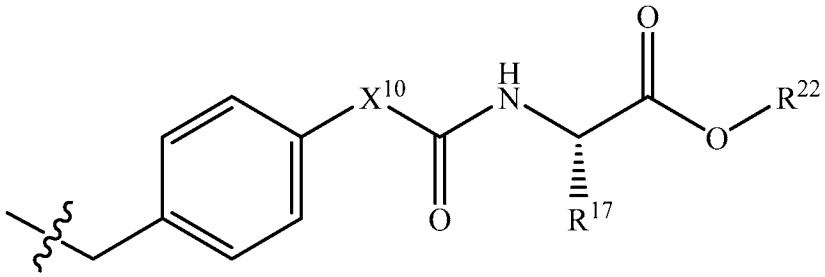
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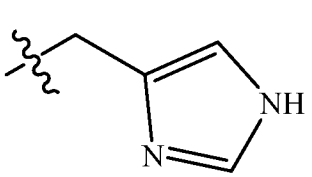
. In some embodiments, R^{21}, R^{22}, R^{23}, R^{24}, R^{25}, R^{25a}, and R^{25b} are each independently neopentyl. In some embodiments, X^9 is –C(O)-O, R^{15} is

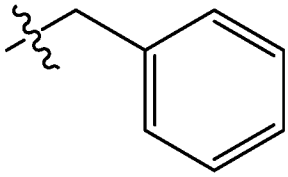


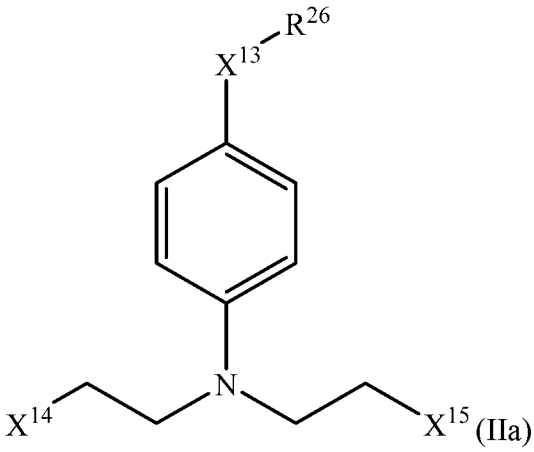
. In some embodiments, X^9 is –C(O)-O, R^{15} is



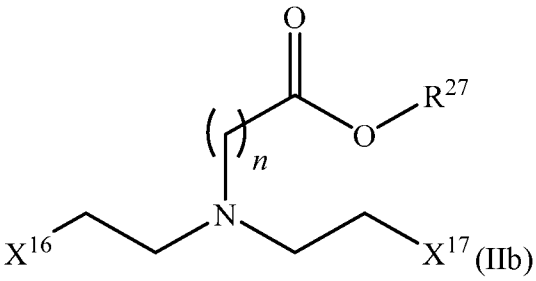
, and R^{17} is $-(CH_2)_3-NH(NH)-NH_2$, $-(CH_2)_3-NH_2$, $-(CH_2)_4-NH_2$, $-CH_2-C(O)OR^{25a}$, $-(CH_2)_2-C(O)O\ R^{25b}$,



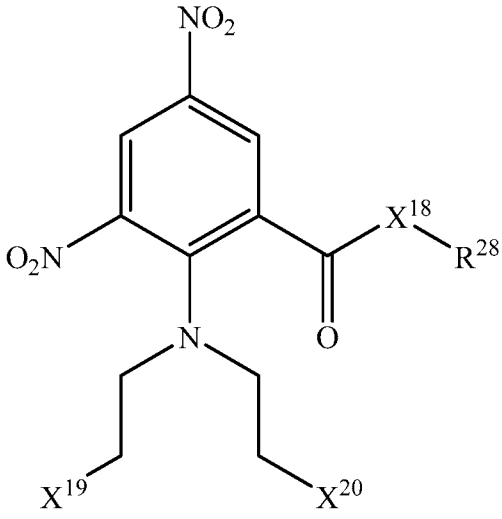
, or . In some embodiments, the compound of Formula II is a compound of any one of Formulas IIa-IIe



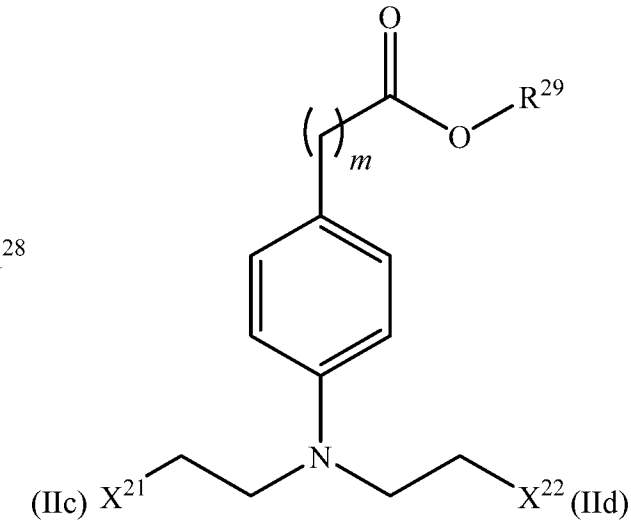
(IIa)



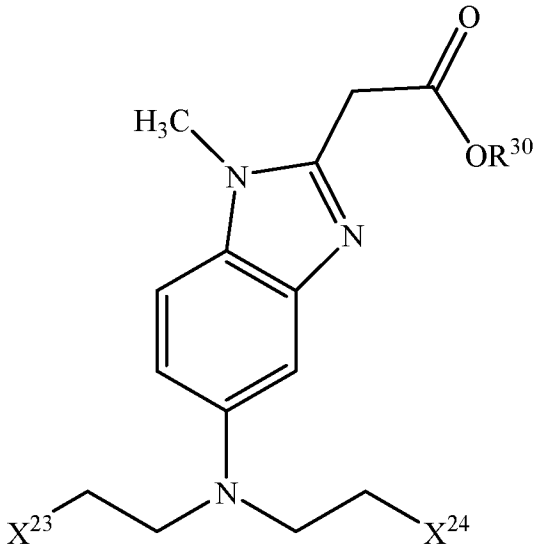
(IIb)



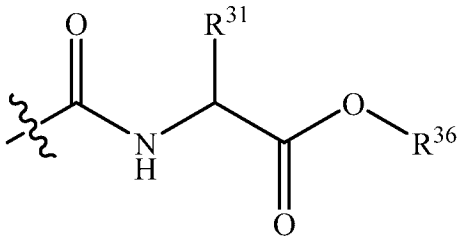
(IIc)



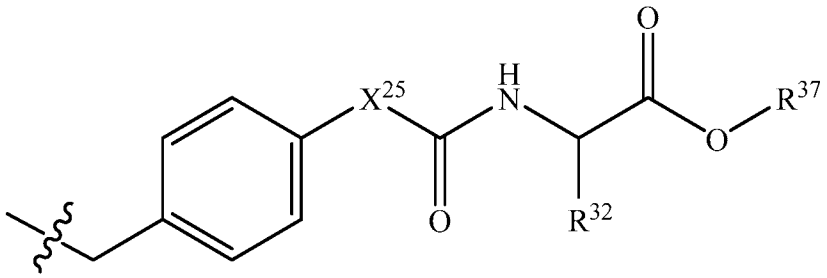
(IId)



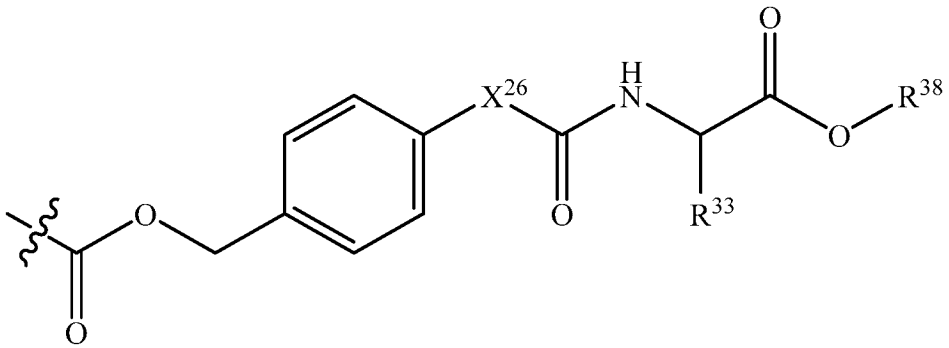
(IIe) or a zwitterion, pharmaceutically acceptable salt, and/or solvate thereof, wherein X^{13} and X^{18} are each independently O or NH; X^{14}, X^{15}, X^{16}, X^{17}, X^{19}, X^{20}, X^{21}, X^{22}, X^{23} and X^{24} are each independently Cl, Br, I, O-$S(O)_2CH_3$, or O-$S(O)_2$-tolyl; *n* and *m* are each independently 0, 1, 2, 3, 4, or 5; R^{26}, R^{27}, R^{28}, R^{29}, and R^{30} are each independently



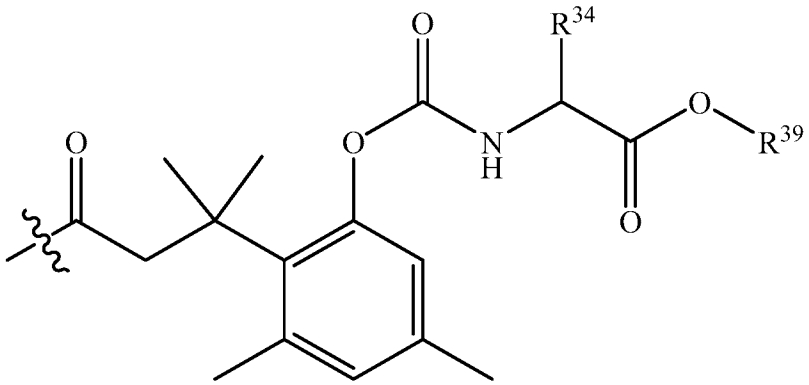
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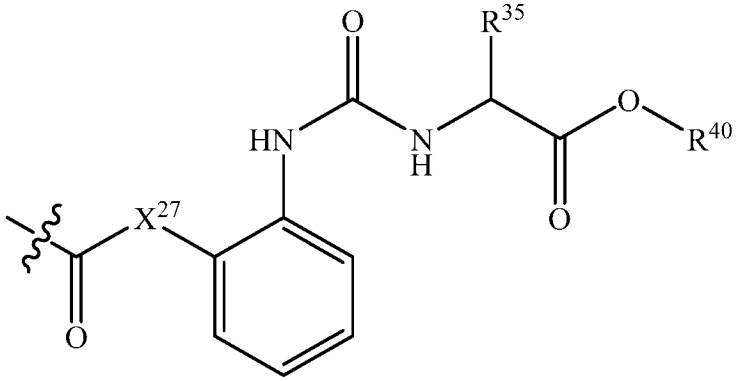
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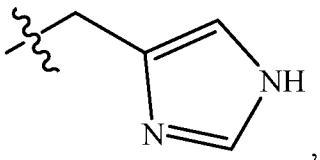
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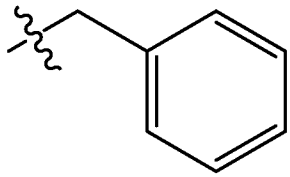
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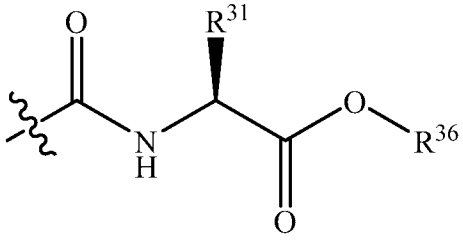
; X^{25} and X^{26} are each independently O or NH; X^{27} is O, NH, CH_2, or $C(Me)_2$; R^{31}, R^{32}, R^{33}, R^{34}, and R^{35} are each independently H, $-(CH_2)_3-NH(NH)-NH_2$, $-(CH_2)_3-NH_2$, $-(CH_2)_4-NH_2$, $-CH_2-C(O)OR^{40a}$, $-(CH_2)_2-C(O)OR^{40b}$,



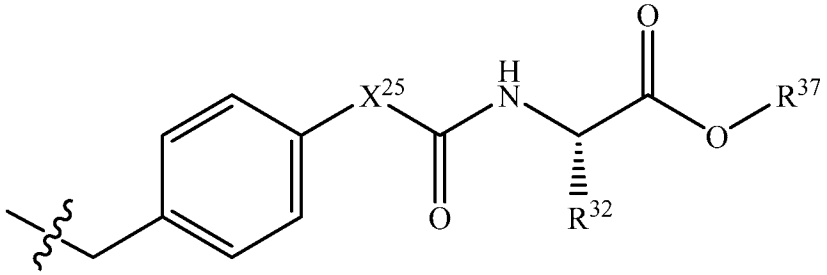
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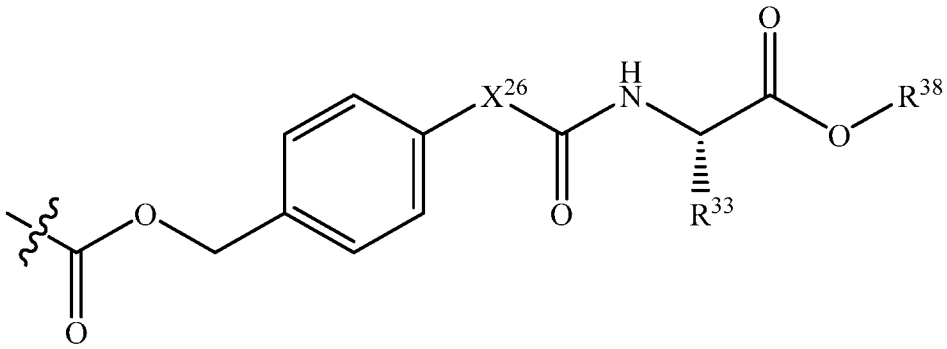
; and R^{36}, R^{37}, R^{38}, R^{39}, R^{40}, R^{40a}, and R^{40b} are each independently alkyl, aryl, heteroaryl, aralkyl, or heteroaralkyl. In some embodiments, R^{26}, R^{27}, R^{28}, R^{29}, and R^{30} are each independently



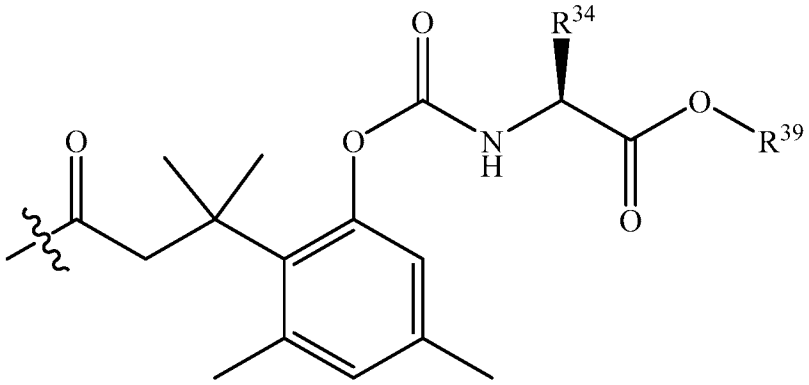
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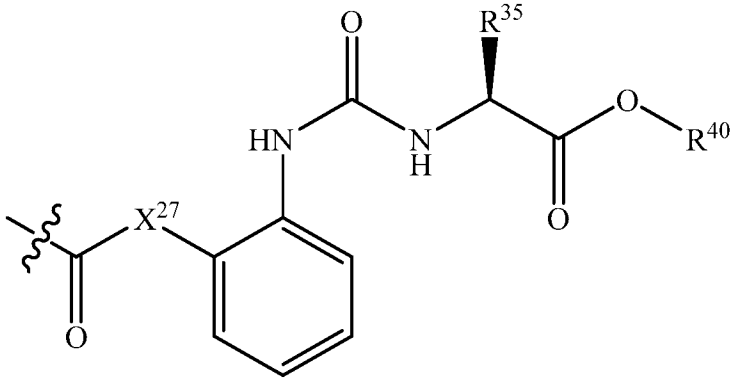
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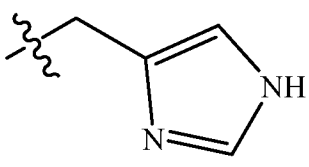


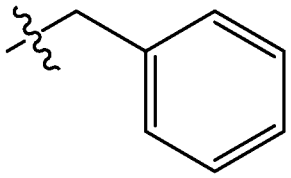
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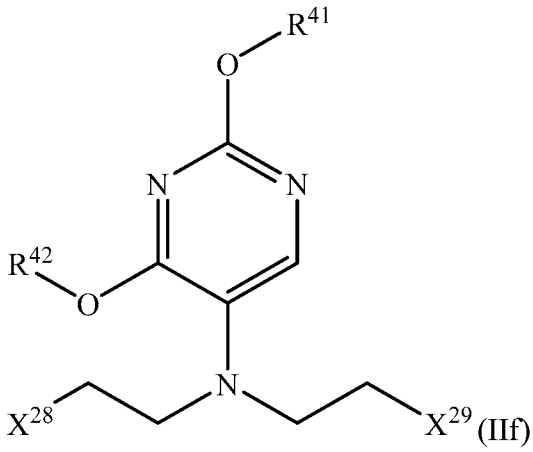
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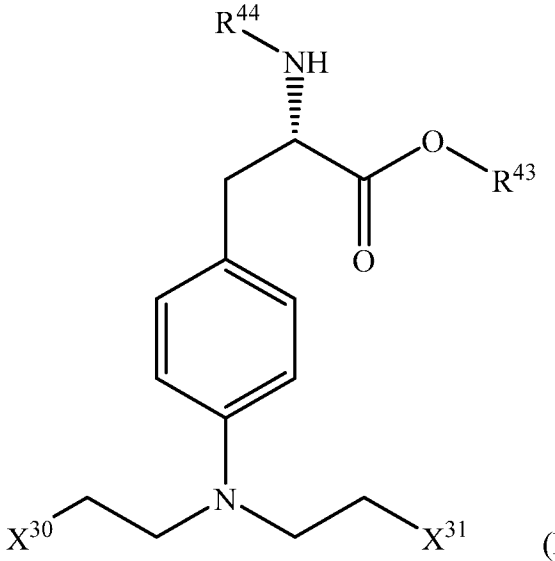
R^{31}, R^{32}, R^{33}, R^{34}, and R^{35} are each independently $-(CH_2)_3-NH(NH)-NH_2$, $-(CH_2)_3-NH_2$, $-(CH_2)_4-NH_2$, $-CH_2-C(O)OR^{40a}$, $-(CH_2)_2-C(O)O\,R^{40b}$, , or



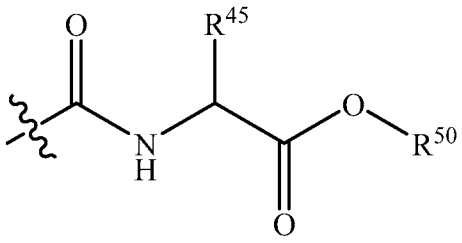
. In some embodiments, R^{36}, R^{37}, R^{38}, R^{39}, R^{40}, R^{40a}, and R^{40b} are each independently neopentyl. In some embodiments, the compound of Formula II is a compound of Formula IIf or Formula IIg



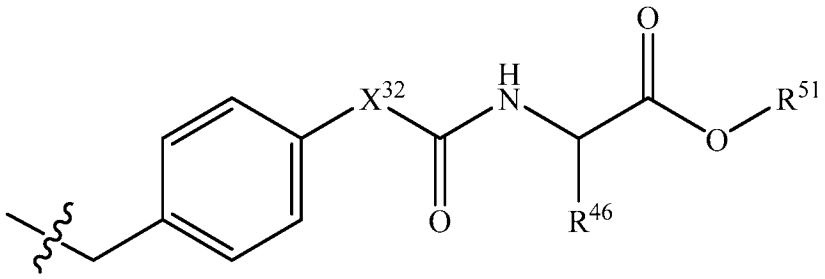
(IIf)



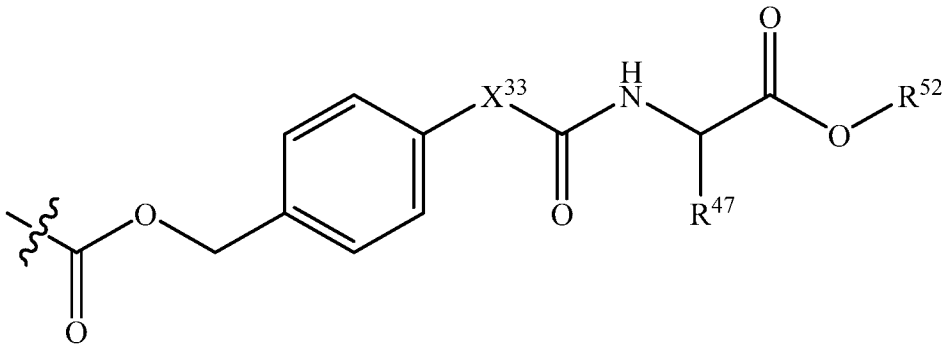
(IIg) or a zwitterion, pharmaceutically acceptable salt, and/or solvate thereof, wherein X^{28}, X^{29}, X^{30}, and X^{31} are each independently Cl, Br, I, O-S(O)$_2$CH$_3$, or O-S(O)$_2$-tolyl; one of R^{41} and R^{42} is H and one of R^{43} and R^{44} is H and the remaining R^{41} and R^{42} (for Formula IIf) and R^{43} and R^{44} (for Formula IIg) are each independently



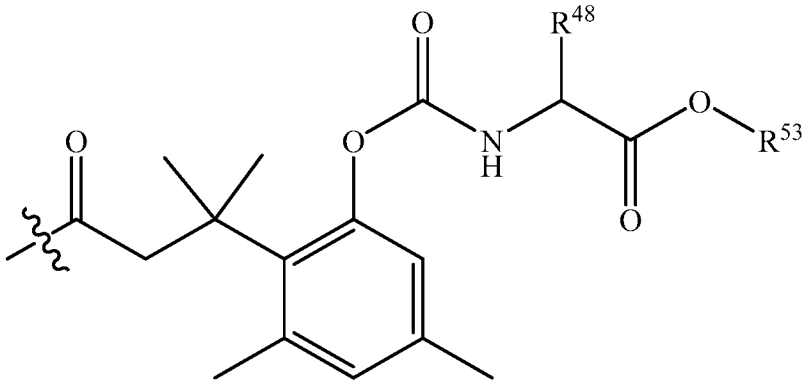
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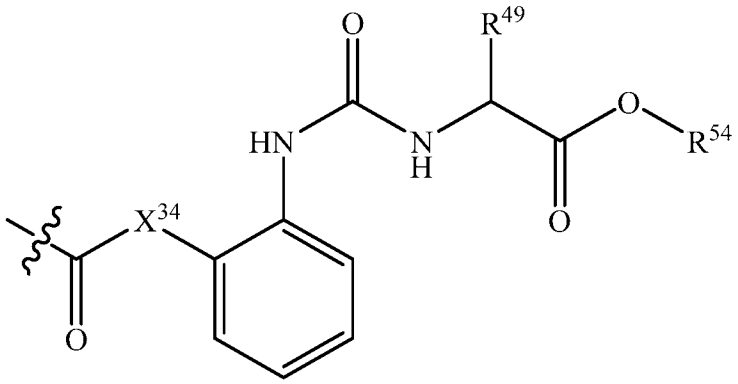
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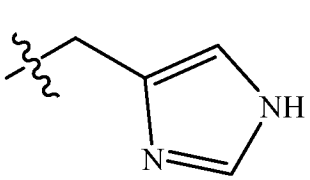
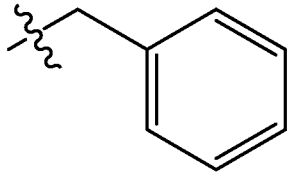


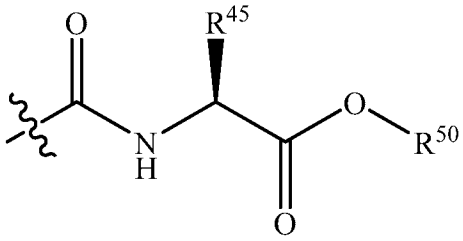
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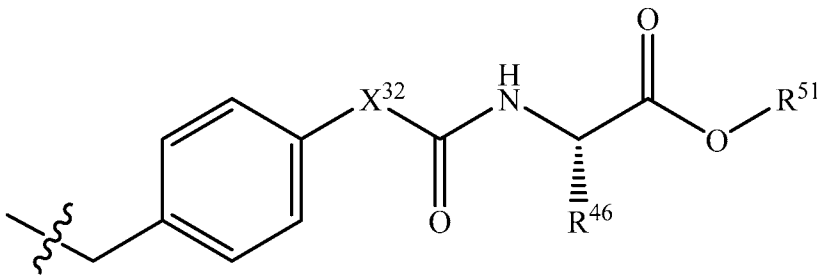


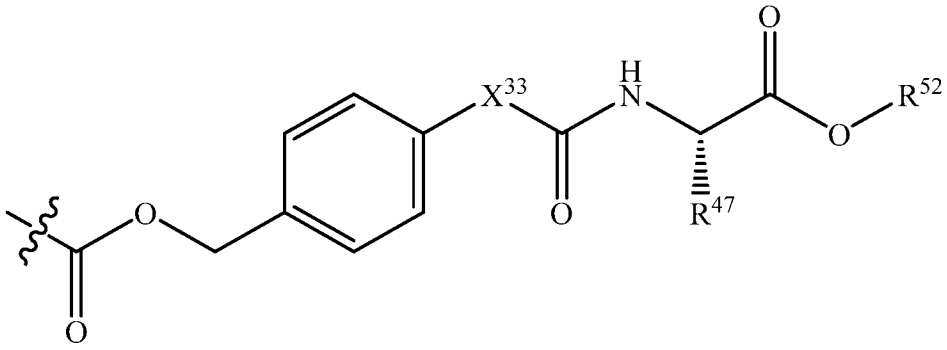
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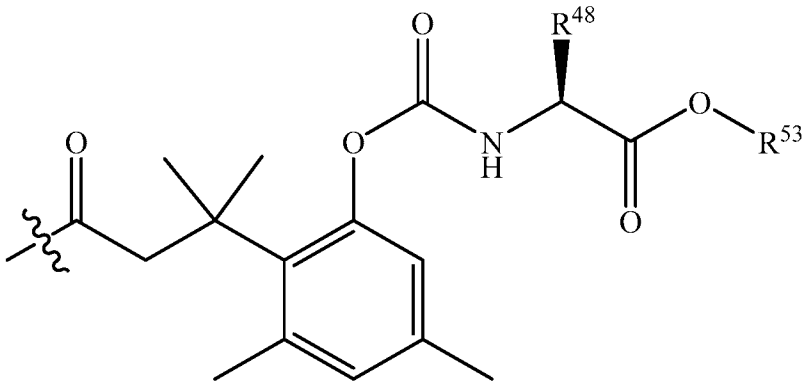
; X^{32} and X^{33} are each independently O or NH; X^{34} is O, NH, CH_2, or $C(Me)_2$; R^{45}, R^{46}, R^{47}, R^{48}, and R^{49} are each independently H, $-(CH_2)_3-NH(NH)-NH_2$, $-(CH_2)_3-NH_2$, $-(CH_2)_4-NH_2$, $-CH_2-C(O)OR^{54a}$, $-(CH_2)_2-C(O)OR^{54b}$, , or ; and R^{50}, R^{51}, R^{52}, R^{53}, R^{54}, R^{54a}, and R^{54b} are each independently alkyl, aryl, heteroaryl, aralkyl, or heteroaralkyl. In some embodiments, one of R^{41} and R^{42} is H and one of R^{43} and R^{44} is H and the remaining R^{41} and R^{42} (for Formula IIf) and R^{43} and R^{44} (for Formula IIg) are each independently

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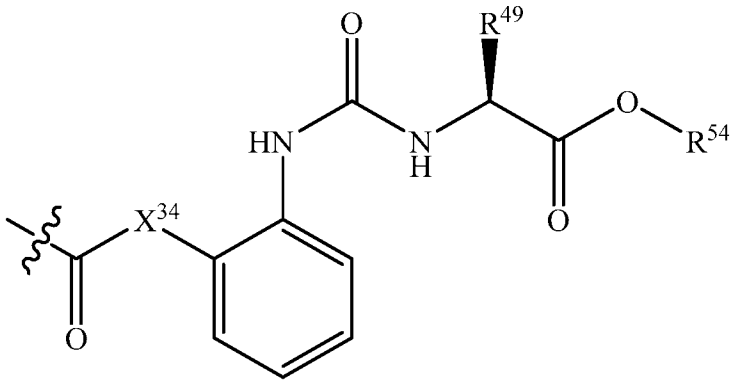
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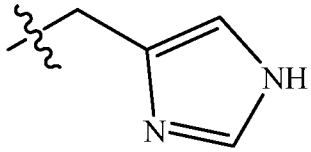
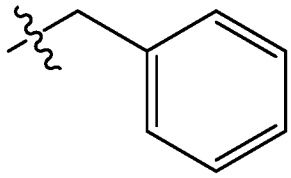


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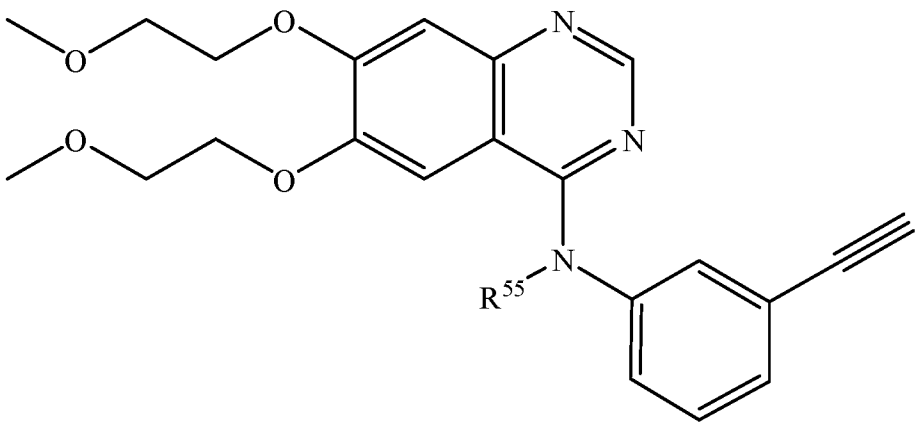


, or



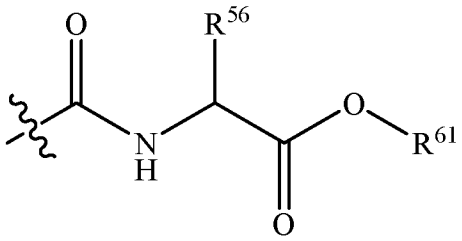
; and R^{45}, R^{46}, R^{47}, R^{48}, and R^{49} are each independently $-(CH_2)_3-NH(NH)-NH_2$, $-(CH_2)_3-NH_2$, $-(CH_2)_4-NH_2$, $-CH_2-C(O)OR^{54a}$, $-(CH_2)_2-C(O)O\,R^{54b}$, , or . In some embodiments, R^{50}, R^{51}, R^{52}, R^{53}, R^{54}, R^{54a}, and R^{54b} are each independently neopentyl.

[0022] Also provided are compounds of Formula III:

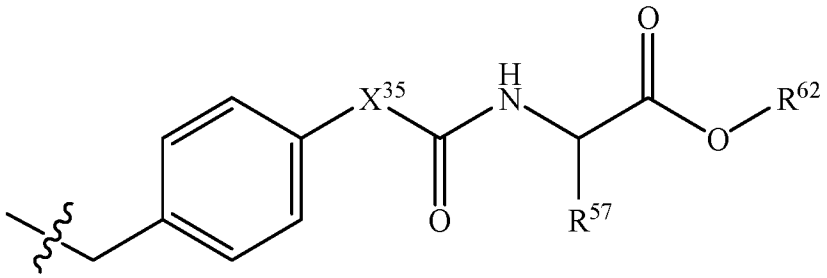


(III)

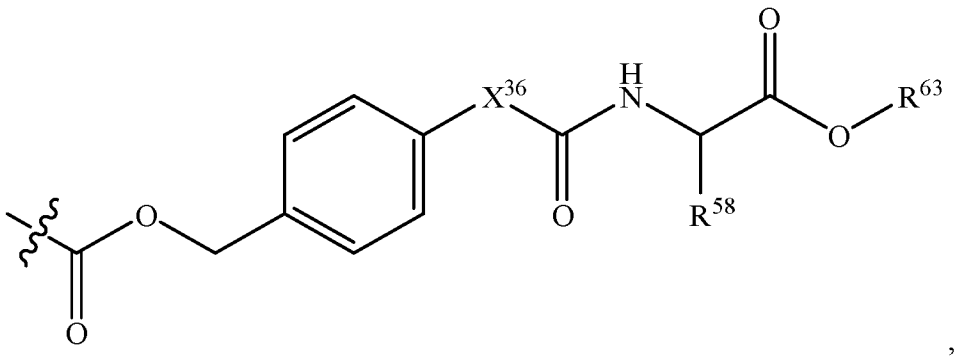
or a zwitterion, pharmaceutically acceptable salt, and/or solvate thereof, wherein R^{55} is



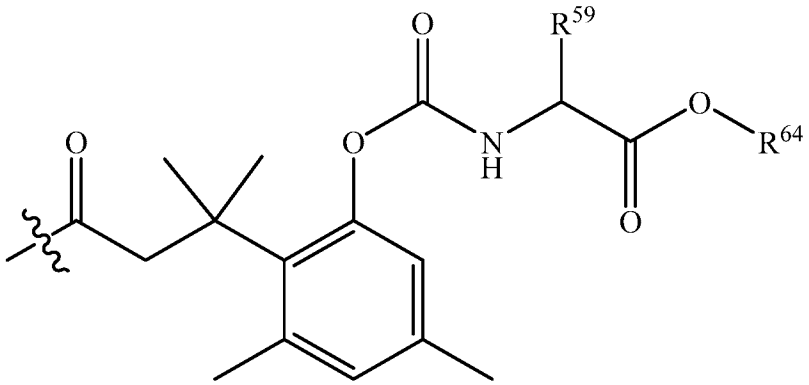
,



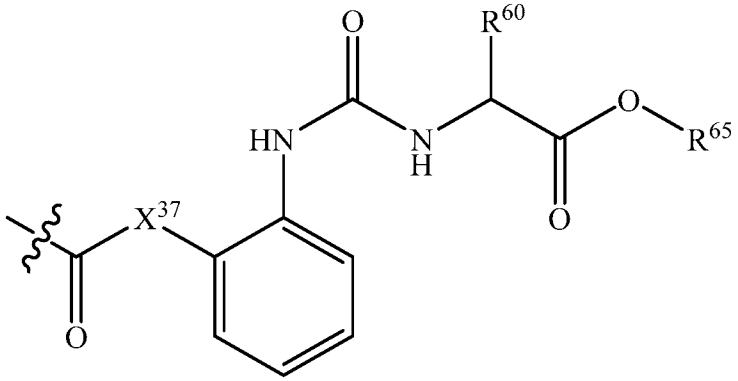
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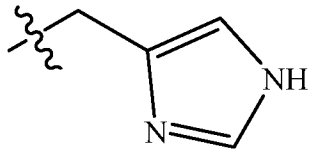


;

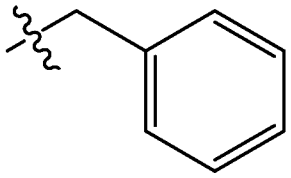
X^{35} and X^{36} are each independently O or NH;

X^{37} is O, NH, CH_2, or $C(Me)_2$;

R^{56}, R^{57}, R^{58}, R^{59}, and R^{60} are each independently H, $-(CH_2)_3-NH(NH)-NH_2$, $-(CH_2)_3-NH_2$, $-(CH_2)_4-NH_2$, $-CH_2-C(O)OR^{65a}$, $-(CH_2)_2-C(O)OR^{65b}$,

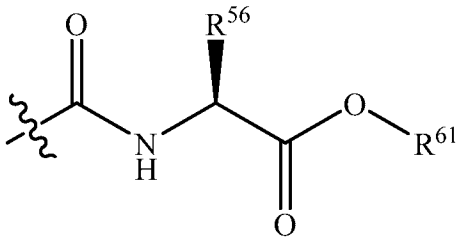


, or

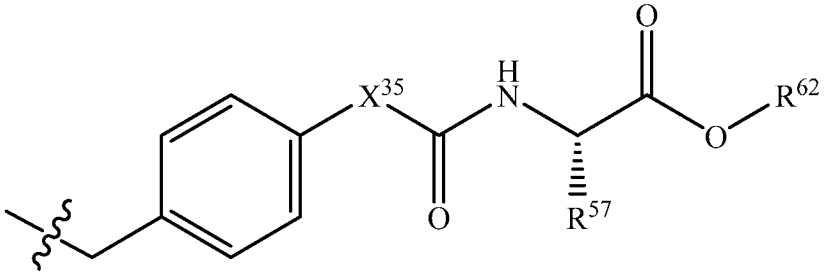


; and

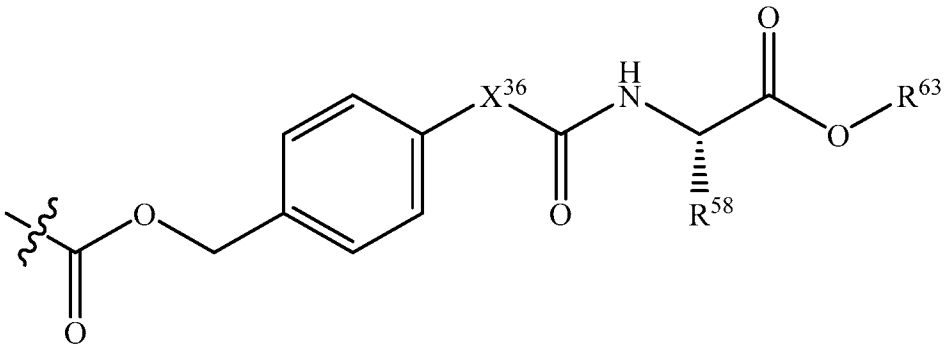
R^{61}, R^{62}, R^{63}, R^{64}, R^{65}, R^{65a}, and R^{65b} are each independently alkyl, aryl, heteroaryl, aralkyl, or heteroaralkyl. In some embodiments, R^{55} is



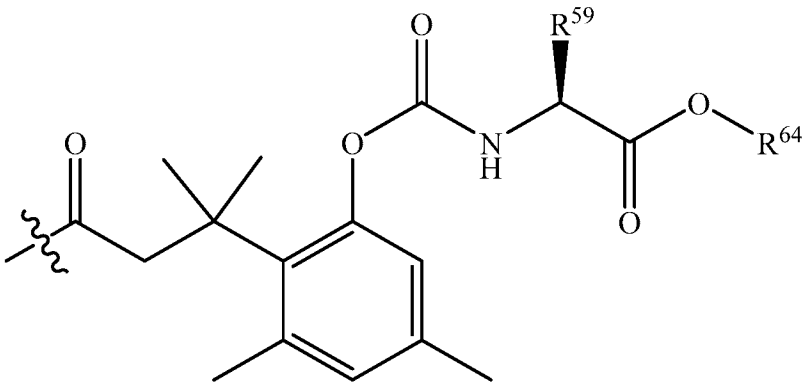
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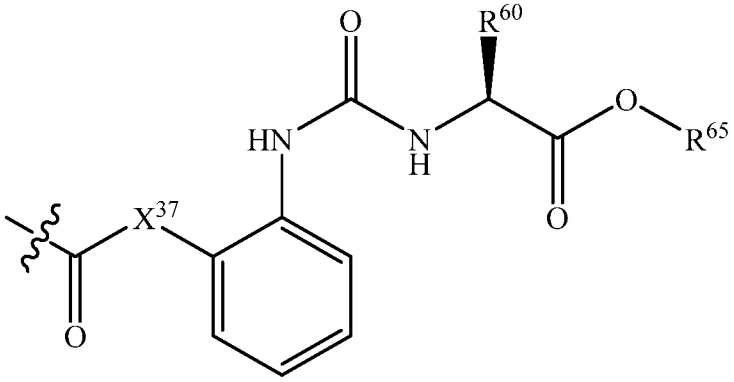
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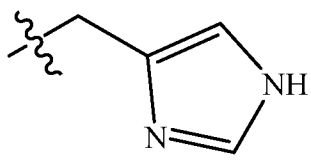
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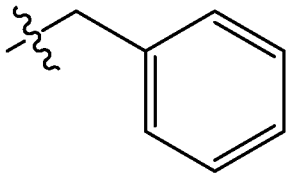


, or



; and

R^{56}, R^{57}, R^{58}, R^{59}, and R^{60} are each independently $-(CH_2)_3-NH(NH)-NH_2$, $-(CH_2)_3-NH_2$, $-(CH_2)_4-NH_2$, $-CH_2-C(O)OR^{65a}$, $-(CH_2)_2-C(O)OR^{65b}$, , or


. In some embodiments, R^{61}, R^{62}, R^{63}, R^{64}, R^{65}, R^{65a}, and R^{65b} are each independently neopentyl.

BRIEF DESCRIPTION OF THE DRAWINGS

[0023] **FIG. 1** provides an exemplary overview of the Synthetic Enzyme Activated Killer (SEAKER) Cell technology.

[0024] **FIG. 2** illustrates an exemplary scheme for the production of CPG2-labile glutamate prodrugs. (a) Inactive prodrugs undergo cleavage of glutamate (blue) by CPG2 then spontaneous decomposition of linker (gray) to unmask active drug (red). (b) Various self-immolative linkers that can be used to tune prodrug stability and cleavage rates based on electronic and steric considerations. (c) Prodrug variants 1a ("P-AMS") and 2a of the cytotoxic sulfamoyladenosine ("AMS"; 1c) and nitrogen mustard ZD2767 (2c), respectively, and a prodrug variant of AMS (1d) designed for cell permeability. (d) Synthetic precursors. (e) A prodrug variant of erlotinib (6a) with an alkyl PAB linker to avoid spontaneous hydrolysis of conventional amide-based linkers (*cf.* N,N-diarylamide). (CO = carbonyl linker; PAB = p-aminobenzyl linker; TML = trimethyl lock linker; IND = indanone-forming linker).

[0025] **FIG. 3** provides a schematic depiction of exemplary SEAKER constructs. In exemplary embodiments of the SEAKER cell technology, a glutamated prodrug is administered systemically but activated to its cytotoxic form only at sites of CAR-T cell accumulation. (A) $CPG2_{transmembrane}$ expresses a CPG2 enzyme tethered to the surface of the CAR-T cell through the CD8 transmembrane region. (B) $CPG2_{secreted}$ expresses a CPG2 enzyme that is secreted directly into the surrounding environment. These exemplary CAR-T cells express an antigen-specific scFv coupled to the 4-1BB costimulatory molecule and CD3ζ chain. A self-cleaving P2A peptide sequence permits co-translational cleavage of the polyprotein. (C) An exemplary $CPG2_{inducible}$ CAR-T cell utilizes a syn-notch system to activate expression of a secreted form of CPG2 only upon engagement with a tumor antigen.

[0026] **FIG. 4** provides exemplary data showing activation of P-AMS (*see* FIG. 2) by recombinant CPG2 expressed and purified from *E. coli*. (A) Human Jurkat T cells were incubated with or without P-AMS in the presence of increasing concentration of purified

CPG2. Cell viability was measured at 48 hours by CellTitre-Glo luminescence. (B) Recombinant CPG2 was exposed to various concentrations of glutamated AMS prodrug. Following incubation at 37°C (0.5, 1, 2, and 4 hr.), the reaction was combined with Amplex® Red (converts free glutamate to a fluorescent species) and fluorescent emission was analyzed using a fluorescence multiwell plate scanner. The data indicate that at high concentrations (~10μM), the majority of the prodrug is converted within 1 hour.

[0027] **FIG. 5** provides exemplary data showing surface expression of a membrane-anchored CPG2. HEK293T cells transfected with a membrane-tethered CPG2 (CPG2TM) expressing a hemagglutinin tag were exposed to surface staining with an anti-HA antibody and compared to cells stained with isotype control antibody.

[0028] **FIG. 6** provides exemplary data showing expression of CPG2 in mammalian cells. Human embryonic kidney (293t) cells transfected with constructs encoding a secreted form of CPG2 (CPG2) and a membrane bound CPG2 (CPG2-TM) with or without hemagglutinin (HA) tags were assayed for CPG2 expression by immunoblot of total cell lysates (Lanes 1-5, 11-15), or cell supernatant (Lanes 1-10, 16-20) for CPG2 (top row) or HA (bottom row).

[0029] **FIG. 7** provides exemplary data showing that cell-secreted CPG2 activates an AMS prodrug. Jurkat T cells were exposed to increasing concentrations of AMS (red circle) or P-AMS with media alone (blue triangle) or supernatant from CPG2-secreting 293t cells (black square). Cell viability was measured at 48 hours by CellTitre-Glo luminescence.

[0030] **FIG. 8** provides exemplary data showing that cell-secreted CPG2 activates a Mustard prodrug. Jurkat T cells were exposed to increasing concentrations of ZD2767P with media (blue triangle) or supernatant from CPG2-secreting 293t cells (black square). Cell viability was measured at 48 hours by CellTitre-Glo luminescence.

[0031] **FIG. 9** provides exemplary data showing that that cell-secreted and transmembrane CPG2 expressed in stably transfected cells activates an AMS prodrug. Expression of CPG2 without a signal peptide (CPG2nosigp) was employed as a control. 293t cells stably transfected with CPG2 variants were exposed to P-AMS (1μM) for 48 hours and analyzed by bright field microscopy (FIG. 9A). Cells were exposed to varying concentrations of P-AMS and cell viability was analyzed at 48hr post treatment (FIG. 9B).

[0032] **FIG. 10** provides exemplary data showing expression of the anti-CD19 CAR in a human T cell line, Jurkat, and primary human T cells following retroviral transduction of constructs encoding the anti-CD19 CAR in combination with the transmembrane or secreted forms of CPGS. A human T cell line, Jurkat (top panel) and primary human T cells (bottom panel) were transduced with retrovirus encoding an anti-CD19 CAR with 4-1BB costimulatory element (BBz) and either a CPG gene with no signal peptide (CPGnosigp) or a CPG transmembrane gene as described above. Transduction levels were assessed by staining for anti-CD19 ScFv expression on the cell surface demonstrating that the CAR protein is expressed on the cell surface of the engineered immune cell.

[0033] **FIG. 11** provides exemplary data showing the activity of Human Jurkat T cells expressing an anti-CD19 CAR with 4-1BB costimulatory element (BBz) and either a CPG gene with no signal peptide (CPGnosigp) or a CPG transmembrane gene as described above. Human Jurkat T cells transduced with 19BBz/CPG retrovirus were co-cultured with a GFP+ Raji (human Burkitt's lymphoma) cancer target cell line for 24 hours (1:1 ratio) and flow cytometry analysis. Jurkat and Raji cells were differentiated by GFP expression (left panel). Activation of Jurkat cells was examined by assessing CD69 expression in GFP-negative cells (right panel).

[0034] **FIG. 12** provides exemplary data showing the activity of non-transduced primary human T cells and transduced human primary human T cells expressing an anti-CD19 CAR with 4-1BB costimulatory element (BBz) and either a CPG gene with no signal peptide (CPGnosigp) or a CPG transmembrane gene as described above. Primary non-transduced human T cells and transduced 19BBz and 19BBz/CPG CAR-T cells were co-cultured at a 1:1 ratio with GFP+ Raji cells. Following a 48-hour incubation cells were analyzed by flow cytometry (A) and %GFP+ cells was calculated (B).The increase in GFP negative cells (signifying the various CAR T cells prepared) to the same levels with both traditional CAR T and the 2 different SEAKER cells show the SEAKER cells are of comparable activity to traditional CARs

[0035] **FIG. 13** provides exemplary data showing expression of anti-CD19 CAR and a secreted CPG2. Primary human T cells were transduced with retrovirus encoding an anti-CD19 CAR with 4-1BB costimulatory element (19BBz) and either a CPG gene with no signal peptide (CPGnosigp) or a secreted form of the CPG gene with the signal peptide as

described above. Cell supernatants from human CAR-T cells encoding 19BBZ or 19BBz/CPG constructs were analyzed for CPG2 expression by immunoblot.

[0036] **FIG. 14** provides exemplary data showing that Human T cells, but not cancer cells, are resistant to Erlotinib. (A) Human Jurkat T cells (red circle) and human non-small cell lung carcinoma cell lines PC9 (black square) and HCC827 (blue triangle) were treated with increasing concentrations of the receptor tyrosine kinase inhibitor Erlotinib. (B) A glutamated Erlotinib prodrug (P-Erlotinib) displays no toxicity in PC9 cells (1μM) unless activated by CPG2. Cell viability was measured at 48hours by CellTitre-Glo® luminescence.

[0037] **FIG. 15** provides exemplary data showing the pro-drug activity of 293T cells expressing transmembrane CPG2. GFP+ $293t^{CPGTM}$ cells were co-cultured with lung adenocarcinoma cells (HCC827) at various ratios ($293t^{CPGTM}$: HCC827) and the erlotinib prodrug (p-erlotinib) was added. (A) Cells were analyzed at 120 hour by flow cytometry and % GFP was calculated. (B) Ratio of $293t^{CPGTM}$ cells to HCC827 cells was calculated for each cell ratio. This shows that the transmembrane CPG2 in the transduced cell is functional and converts the prodrug to active drug that kills the target cancer cell, but not the cell secreting the CPG2 enzyme.

[0038] **FIG. 16** provides exemplary data showing treatment of tumor bearing mice beginning day 7 (arrow) of SCID/Beige mice engrafted with WT1+, SET2 AML cells with 2E7 WT1 specific TCRm standard CAR T cells (black line) or armored with IL-12 secretion (black dots) improved survival relative to controls ($p<0.03$.) 4H11 is control CAR T cell with and without IL-12.

[0039] **FIG. 17** provides and exemplary schematic depiction of a CPG2 "kill-switch" construct. (A) In an exemplary embodiment, the CPG2 kill-switch CAR-T express a form of CPG2 that is sequestered within the cytosol and that will convert a cell-penetrant prodrug. (B) The CAR-T cell can also simultaneously express a transmembrane (or secreted) CPG2 enzyme for conversion of a prodrug at the target site.

[0040] **FIG. 18** illustrates expression of cytosol-sequestered CPG2 in mammalian cells. 293t cells transfected with constructs encoding a secreted form of CPG2 (CPG2) and a cytosol-sequestered form of CPG2 (CPG2-nosigp) were assayed for CPG2 expression by immunoblot of total cell lysates (Lanes 1-3) or cell supernatant (Lanes 4-6).

[0041] **FIG. 19** provides an exemplary structure of an analog prodrug designed to penetrate into SEAKER cells.

[0042] **FIG. 20A** provides a schematic of glutamate prodrug cleavage and linker decay leading to active drug synthesis following exposure to CPG2.

[0043] **FIG. 20B** provides a schematic of the 3 classes of prodrug compounds synthesized for use with the CPG2 enzyme platform.

[0044] **FIG. 20C** shows an Ampex Red assay demonstrating release of free glutamate from prodrug compounds upon incubation with recombinant CPG2 enzyme.

[0045] **FIG. 20D** shows a Cell Titer-Glo viability assay of SET2 cells incubated in 3μM AMS-glut with or without recombinant CPG2.

[0046] **FIG. 20E** shows a Cell Titer-Glo viability assay of SET2 cells incubated in 1μM Erlotinib-glut with or without recombinant CPG2.

[0047] **FIG. 20F** shows a cell toxicity assay of Jurkat cells incubated in ZD2767P with or without recombinant CPG2.

[0048] **FIG. 21A** shows Coomassie Blue staining of purified recombinant CPG2.

[0049] **FIG. 21B** shows a quantitative sandwich ELISA of purified recombinant CPG2.

[0050] **FIG. 21C** shows a Cell Titer-Glo viability assay of LNCap cells incubated with the AMS-glut prodrug or AMS.

[0051] **FIG. 21D** provides a schematic of the classes of prodrug compounds synthesized for use with the Beta-lactamase enzyme platform.

[0052] **FIG. 21E** shows Coomassie Blue staining of purified recombinant E. cloacae Beta-lactamase.

[0053] **FIG. 21F** shows a quantitative ELISA of purified recombinant Beta-lactamase.

[0054] **FIG. 22A** provides a schematic of three forms of CPG2 (internal, secreted, membrane-bound) generated for optimal expression in mammalian cells.

[0055] **FIG. 22B** provides immunoblots indicating expression of all three CPG2 isoforms in cell lysate (left panel) or secreted into the supernatant fluid (right panel) following transfection in Hek293t cells.

[0056] **FIG. 22C** shows a Cell Titer-Glo viability assay of SET2 cells incubated with AMS-glut prodrug in supernatant fluid from control Hek293t cells or supernatant from Hek293t cells expressing the secreted form of CPG2.

[0057] **FIG. 22D** demonstrated surface expression analysis of CPG2 proteins in Hek293t cells transduced to stably express the CPG2 gene.

[0058] **FIG. 22E** shows a Cell Titer-Glo viability assay of SET2 cells incubated with AMS-glut supernatant fluid from Hek293t cells engineered to stably express CPG2.

[0059] **FIG. 22F** shows a Cell Titer-Glo viability assay of Hek293t-CPG2 cells exposed to AMS-glut prodrug.

[0060] **FIG. 22G** provides a micrograph of Hek293t-CPG2 cells exposed to AMS-glut prodrug.

[0061] **FIG. 22H** shows an immunoblot analysis of supernatant fluid from Jurkat-CPG2 cells.

[0062] **FIG. 23A** shows an anti-CPG2 Immunoblot of lysate and supernatant fluid from Hek293t cells transduced with various forms of CPG2.

[0063] **FIG. 23B** shows a Cell Titer-Glo viability assay of SET2 cells incubated with AMS-glut prodrug in supernatant fluid from control Hek293t cells or supernatant from Hek293t cells expressing CPG2-int, CPG2WT-sec, or CPG2CD8-sec.

[0064] **FIG. 23C** shows a schematic of the pLGPW-CPG2 gene cassette.

[0065] **FIG. 24A** provides a cell viability analysis of co-cultures with increasing ratio of Hek293t-GFP+/CPG2 or Hek293t-GFP+ cells (Effector cells) to Hek293t WT (Bystander cells) in the presence of AMS-glut prodrug.

[0066] **FIG. 24B** shows GFP analysis of co-cultures with or without prodrug demonstrates equivalent depletion of both enzyme+ and enzyme – cell populations.

[0067] **FIG. 24C** shows quantification of total cell depletion in 1:1 E:T cocultures with or without prodrug.

[0068] **FIG. 24D** shows quantification of enzyme-negative (bystander cell) depletion in 1:1 E:T cocultures with or without prodrug.

[0069] **FIG. 25A** provides a schematic of SFG retroviral vectors engineered to express CPG2 or Beta-lactamase upstream of the CD19-BBz gene cassette.

[0070] **FIG. 25B** shows an ELISA analysis of CPG2 secretion from primary human T cells transduced with a vector encoding CPG2 either before or after the CAR gene cassette.

[0071] **FIG. 25C** shows a T cell activation analysis of 19BBz and CPG-19BBz Jurkat cells co-cultured with CD19+ (Raji) or CD19- (SET2) cells.

[0072] **FIG. 25D** shows enzyme secretion analysis of CPG2 Jurkats (left panel – ELISA), or Beta-lactamase Jurkats (right panel – nitrocefin).

[0073] **FIG. 25E** shows the transduction of primary human T cells with 19BBz, CPG-19BBz, and Beta-lac-BBz CARs.

[0074] **FIG. 25F** shows specific lysis of luc+/CD19+ Raji cells following coculture with non-transduced, 19BBz, CPG-BBz, or Beta-lac-BBz CAR-T cells.

[0075] **FIG. 25G** shows a Cell Titer-Glo viability assay of SET cells incubated with prodrug and supernatant fluid from human T cells expressing the SEAKER constructs.

[0076] **FIG. 25H** shows an analysis of enzyme accumulation in supernatant fluid of CPG2-19BBz (left panel) and Beta-lac-19BBz (right panel) T cells compared to 19BBz T cells.

[0077] **FIG. 25I** shows an analysis of enzyme accumulation in supernatant fluid of CPG2-19BBz (left panel) and Beta-lac-19BBz (right panel) T cells cocultured with CD19+ Raji cells and CD19- SET2 cells.

[0078] **FIG. 26A** provides a schematic of a 19BBz-CPG2 gene cassette in the SFG retroviral vector.

[0079] **FIG. 26B** shows the transduction of primary human T cells with 19BBz or 19BBz-CPG2 genes.

[0080] **FIG. 26C** shows the specific lysis of luc+/CD19+ Nalm6 cells following coculture with 19BBz or 19BBz-CPG2 CAR-T cells.

[0081] **FIG. 26D** shows an Anti-CPG2 immunoblot of anti-HA immunoprecipitation of supernatant fluid from 19BBz-CPG2 CAR-T cells.

[0082] **FIG. 26E** shows a Cell Titer-Glo viability assay of SET cells incubated with prodrug and supernatant fluid from human T cells expressing the 19BBz or 19BBz-CPG2 constructs.

[0083] **FIG. 27A** shows a schematic of two forms of Beta-lactamase generated for optimal expression in mammalian cells.

[0084] **FIG. 27B** shows an immunoblot indicating expression of only the truncated form of Beta-lactamase into the supernatant fluid of Hek293t cells transfected with the Beta-lactamase expression constructs.

[0085] **FIG. 27C** provides a Nitrocefin analysis of Beta-lactamase activity in supernatant fluid from cells expressing the Beta-lactamase constructs.

[0086] **FIG. 28A** shows a quantitative sandwich ELISA of purified recombinant CPG2 (left panel) and a quantitative nitrocefin assay of purified Beta-lactamase (right panel).

[0087] **FIG. 28B** shows a Cell Titer-Glo viability assay of SET cells incubated with prodrug and varying concentrations of purified recombinant CPG2 (left panel) or Beta-lactamase (right panel).

[0088] **FIG. 28C** shows a Cell Titer-Glo viability assay of SET cells incubated with prodrug and supernatant fluid from Hek293t cells transfected with CPG2 (left panel) or Beta-lactamase (right panel) expression constructs.

[0089] **FIG. 28D** shows a Cell Titer-Glo viability assay of Hek293t-CPG2 cells exposed to AMS-glut (left panel) or Hek293t-Beta-lac cells exposed to ceph-AMS (right panel).

[0090] **FIG. 28E** shows GFP analysis of Hek293t-CPG2 or Hek293t-Beta-lac co-cultures with or without prodrug demonstrates equivalent depletion of both enzyme+ and enzyme – cell populations.

[0091] **FIG. 29A** shows a representation of on-target (Raji: GFP-/CD19+) and off-target (SET2: GFP+/CD19-) coculture with CAR-T cells at time zero.

[0092] **FIG. 29B** shows a flow plot of 19BBz (left column) or CPG-19BBz (right column) T cells with SET2 and Raji cells with AMS-glut treatment (bottom row) or without AMS-glut treatment (top row), following 72hr of coculture.

[0093] **FIG. 29C** shows the quantitation of off-target (SET2) cells following respective cocultures.

[0094] **FIG. 30A** shows the quantitation of cell number following Raji/SET2/Beta-lac-19BBz T-cells coculture (72hr) with or without ceph-AMS prodrug. Respective cell populations are denoted by bar color.

[0095] **FIG. 30B** shows the specific lysis of luc+/CD19+ Raji cells following coculture with Beta-lac 19BBz with or without ceph-AMS prodrug.

[0096] **FIG. 31** shows pharmacokinetic and biodistribution studies related to the glut-AMS prodrug conducted in Nod mice.

DETAILED DESCRIPTION OF THE INVENTION

[0097] The present disclosure is not to be limited in terms of the particular embodiments described in this application, which are intended as single illustrations of individual aspects of the disclosure. All the various embodiments of the present disclosure will not be described herein. Many modifications and variations of the disclosure can be made without departing from its spirit and scope, as will be apparent to those skilled in the art. Functionally equivalent methods and apparatuses within the scope of the disclosure, in addition to those enumerated herein, will be apparent to those skilled in the art from the foregoing descriptions. Such modifications and variations are intended to fall within the scope of the appended claims. The present disclosure is to be limited only by the terms of the appended claims, along with the full scope of equivalents to which such claims are entitled.

[0098] It is to be understood that the present disclosure is not limited to particular uses, methods, reagents, compounds, compositions or biological systems, which can, of course, vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only, and is not intended to be limiting.

[0099] In addition, where features or aspects of the disclosure are described in terms of Markush groups, those skilled in the art will recognize that the disclosure is also thereby described in terms of any individual member or subgroup of members of the Markush group.

[0100] As will be understood by one skilled in the art, for any and all purposes, particularly in terms of providing a written description, all ranges disclosed herein also encompass any and all possible subranges and combinations of subranges thereof. Any listed range can be easily recognized as sufficiently describing and enabling the same range being broken down into at least equal halves, thirds, quarters, fifths, tenths, etc. As a non-limiting example, each range discussed herein can be readily broken down into a lower third, middle third and upper third, etc. As will also be understood by one skilled in the art all language such as “up to,” “at least,” “greater than,” “less than,” and the like, include the number recited and refer to ranges which can be subsequently broken down into subranges as discussed above. Finally, as will be understood by one skilled in the art, a range includes each individual member. Thus, for example, a group having 1-3 cells refers to groups having 1, 2, or 3 cells. Similarly, a group having 1-5 cells refers to groups having 1, 2, 3, 4, or 5 cells, and so forth.

Definitions

[0101] Unless defined otherwise, all technical and scientific terms used herein have the meaning commonly understood by a person skilled in the art to which this disclosure belongs. The following references provide one of skill with a general definition of many of the terms used in this invention: Singleton *et al.,* Dictionary of Microbiology and Molecular Biology (2nd ed. 1994); The Cambridge Dictionary of Science and Technology (Walker ed., 1988); The Glossary of Genetics, 5th Ed., R. Rieger *et al.* (eds.), Springer Verlag (1991); and Hale & Marham, The Harper Collins Dictionary of Biology (1991). As used herein, the following terms have the meanings ascribed to them below, unless specified otherwise. The terminology used herein is for the purpose of describing particular embodiments only and is not intended to be limiting of the disclosure.

[0102] As used herein, the singular forms "a", "an" and "the" are intended to include the plural forms as well, unless the context clearly indicates otherwise.

[0103] As used herein, the term "about" or "approximately" means within an acceptable error range for the particular value as determined by one of ordinary skill in the art, which will depend in part on how the value is measured or determined, *i.e.,* the limitations of the measurement system. For example, "about" can mean within 3 or more than 3 standard deviations, per the practice in the art. Alternatively, "about" can mean a range of up to 20%, preferably up to 10%, more preferably up to 5%, and more preferably still up to 1% of a given value. Alternatively, particularly with respect to biological systems or processes, the term can mean within an order of magnitude, preferably within 5-fold, and more preferably within 2-fold, of a value.

[0104] Generally, reference to a certain element such as hydrogen or H is meant to include all isotopes of that element. For example, if an R group is defined to include hydrogen or H, it also includes deuterium and tritium. Compounds comprising radioisotopes such as tritium, C^{14}, P^{32} and S^{35} are thus within the scope of the present technology. Procedures for inserting such labels into the compounds of the present technology will be readily apparent to those skilled in the art based on the disclosure herein.

[0105] In general, "substituted" refers to an organic group as defined below (*e.g.*, an alkyl group) in which one or more bonds to a hydrogen atom contained therein are replaced by a bond to non-hydrogen or non-carbon atoms. Substituted groups also include groups in which

one or more bonds to a carbon(s) or hydrogen(s) atom are replaced by one or more bonds, including double or triple bonds, to a heteroatom. Thus, a substituted group is substituted with one or more substituents, unless otherwise specified. In some embodiments, a substituted group is substituted with 1, 2, 3, 4, 5, or 6 substituents. Examples of substituent groups include: halogens (i.e., F, Cl, Br, and I); hydroxyls; alkoxy, alkenoxy, aryloxy, aralkyloxy, heterocyclyl, heterocyclylalkyl, heterocyclyloxy, and heterocyclylalkoxy groups; carbonyls (oxo); carboxylates; esters; urethanes; oximes; hydroxylamines; alkoxyamines; aralkoxyamines; thiols; sulfides; sulfoxides; sulfones; sulfonyls; pentafluorosulfanyl (i.e., SF_5), sulfonamides; amines; N-oxides; hydrazines; hydrazides; hydrazones; azides; amides; ureas; amidines; guanidines; enamines; imides; isocyanates; isothiocyanates; cyanates; thiocyanates; imines; nitro groups; nitriles (i.e., CN); and the like.

[0106] Substituted ring groups such as substituted cycloalkyl, aryl, heterocyclyl and heteroaryl groups also include rings and ring systems in which a bond to a hydrogen atom is replaced with a bond to a carbon atom. Therefore, substituted cycloalkyl, aryl, heterocyclyl and heteroaryl groups may also be substituted with substituted or unsubstituted alkyl, alkenyl, and alkynyl groups as defined below.

[0107] Alkyl groups include straight chain and branched chain alkyl groups having from 1 to 12 carbon atoms, and typically from 1 to 10 carbons or, in some embodiments, from 1 to 8, 1 to 6, or 1 to 4 carbon atoms. Alkyl groups may be substituted or unsubstituted. Examples of straight chain alkyl groups include groups such as methyl, ethyl, n-propyl, n-butyl, n-pentyl, n-hexyl, n-heptyl, and n-octyl groups. Examples of branched alkyl groups include, but are not limited to, isopropyl, iso-butyl, sec-butyl, tert-butyl, neopentyl, isopentyl, and 2,2-dimethylpropyl groups. Representative substituted alkyl groups may be substituted one or more times with substituents such as those listed above, and include without limitation haloalkyl (*e.g.*, trifluoromethyl), hydroxyalkyl, thioalkyl, aminoalkyl, alkylaminoalkyl, dialkylaminoalkyl, alkoxyalkyl, carboxyalkyl, and the like.

[0108] Cycloalkyl groups include mono-, bi- or tricyclic alkyl groups having from 3 to 12 carbon atoms in the ring(s), or, in some embodiments, 3 to 10, 3 to 8, or 3 to 4, 5, or 6 carbon atoms. Cycloalkyl groups may be substituted or unsubstituted. Exemplary monocyclic cycloalkyl groups include, but not limited to, cyclopropyl, cyclobutyl, cyclopentyl, cyclohexyl, cycloheptyl, and cyclooctyl groups. In some embodiments, the cycloalkyl group

has 3 to 8 ring members, whereas in other embodiments the number of ring carbon atoms range from 3 to 5, 3 to 6, or 3 to 7. Bi- and tricyclic ring systems include both bridged cycloalkyl groups and fused rings, such as, but not limited to, bicyclo[2.1.1]hexane, adamantyl, decalinyl, and the like. Substituted cycloalkyl groups may be substituted one or more times with, non-hydrogen and non-carbon groups as defined above. However, substituted cycloalkyl groups also include rings that are substituted with straight or branched chain alkyl groups as defined above. Representative substituted cycloalkyl groups may be mono-substituted or substituted more than once, such as, but not limited to, 2,2-, 2,3-, 2,4- 2,5- or 2,6-disubstituted cyclohexyl groups, which may be substituted with substituents such as those listed above.

[0109] Cycloalkylalkyl groups are alkyl groups as defined above in which a hydrogen or carbon bond of an alkyl group is replaced with a bond to a cycloalkyl group as defined above. Cycloalkylalkyl groups may be substituted or unsubstituted. In some embodiments, cycloalkylalkyl groups have from 4 to 16 carbon atoms, 4 to 12 carbon atoms, and typically 4 to 10 carbon atoms. Substituted cycloalkylalkyl groups may be substituted at the alkyl, the cycloalkyl or both the alkyl and cycloalkyl portions of the group. Representative substituted cycloalkylalkyl groups may be mono-substituted or substituted more than once, such as, but not limited to, mono-, di- or tri-substituted with substituents such as those listed above.

[0110] Alkenyl groups include straight and branched chain alkyl groups as defined above, except that at least one double bond exists between two carbon atoms. Alkenyl groups may be substituted or unsubstituted. Alkenyl groups have from 2 to 12 carbon atoms, and typically from 2 to 10 carbons or, in some embodiments, from 2 to 8, 2 to 6, or 2 to 4 carbon atoms. In some embodiments, the alkenyl group has one, two, or three carbon-carbon double bonds. Examples include, but are not limited to vinyl,
allyl, $-CH=CH(CH_3)$, $-CH=C(CH_3)_2$, $-C(CH_3)=CH_2$, $-C(CH_3)=CH(CH_3)$, $-C(CH_2CH_3)=CH_2$, among others. Representative substituted alkenyl groups may be mono-substituted or substituted more than once, such as, but not limited to, mono-, di- or tri-substituted with substituents such as those listed above.

[0111] Cycloalkenyl groups include cycloalkyl groups as defined above, having at least one double bond between two carbon atoms. Cycloalkenyl groups may be substituted or unsubstituted. In some embodiments the cycloalkenyl group may have one, two or three

double bonds but does not include aromatic compounds. Cycloalkenyl groups have from 4 to 14 carbon atoms, or, in some embodiments, 5 to 14 carbon atoms, 5 to 10 carbon atoms, or even 5, 6, 7, or 8 carbon atoms. Examples of cycloalkenyl groups include cyclohexenyl, cyclopentenyl, cyclohexadienyl, cyclobutadienyl, and cyclopentadienyl.

[0112] Alkynyl groups include straight and branched chain alkyl groups as defined above, except that at least one triple bond exists between two carbon atoms. Alkynyl groups may be substituted or unsubstituted. Alkynyl groups have from 2 to 12 carbon atoms, and typically from 2 to 10 carbons or, in some embodiments, from 2 to 8, 2 to 6, or 2 to 4 carbon atoms. In some embodiments, the alkynyl group has one, two, or three carbon-carbon triple bonds. Examples include, but are not limited to –
C≡CH, -C≡CCH_3, -CH_2C≡CCH_3, -C≡C$CH_2CH(CH_2CH_3)_2$, among others. Representative substituted alkynyl groups may be mono-substituted or substituted more than once, such as, but not limited to, mono-, di- or tri-substituted with substituents such as those listed above.

[0113] Aryl groups are cyclic aromatic hydrocarbons that do not contain heteroatoms. Aryl groups herein include monocyclic, bicyclic and tricyclic ring systems. Aryl groups may be substituted or unsubstituted. Thus, aryl groups include, but are not limited to, phenyl, azulenyl, heptalenyl, biphenyl, fluorenyl, phenanthrenyl, anthracenyl, indenyl, indanyl, pentalenyl, and naphthyl groups. In some embodiments, aryl groups contain 6-14 carbons, and in others from 6 to 12 or even 6-10 carbon atoms in the ring portions of the groups. In some embodiments, the aryl groups are phenyl or naphthyl. The phrase "aryl groups" includes groups containing fused rings, such as fused aromatic-aliphatic ring systems (*e.g.*, indanyl, tetrahydronaphthyl, and the like). Representative substituted aryl groups may be mono-substituted (*e.g.*, tolyl) or substituted more than once. For example, monosubstituted aryl groups include, but are not limited to, 2-, 3-, 4-, 5-, or 6-substituted phenyl or naphthyl groups, which may be substituted with substituents such as those listed above.

[0114] Aralkyl groups are alkyl groups as defined above in which a hydrogen or carbon bond of an alkyl group is replaced with a bond to an aryl group as defined above. Aralkyl groups may be substituted or unsubstituted. In some embodiments, aralkyl groups contain 7 to 16 carbon atoms, 7 to 14 carbon atoms, or 7 to 10 carbon atoms. Substituted aralkyl groups may be substituted at the alkyl, the aryl or both the alkyl and aryl portions of the group. Representative aralkyl groups include but are not limited to benzyl and phenethyl groups and

fused (cycloalkylaryl)alkyl groups such as 4-indanylethyl. Representative substituted aralkyl groups may be substituted one or more times with substituents such as those listed above.

[0115] Heterocyclyl groups include aromatic (also referred to as heteroaryl) and non-aromatic ring compounds containing 3 or more ring members, of which one or more is a heteroatom such as, but not limited to, N, O, and S. Heterocyclyl groups may be substituted or unsubstituted. In some embodiments, the heterocyclyl group contains 1, 2, 3 or 4 heteroatoms. In some embodiments, heterocyclyl groups include mono-, bi- and tricyclic rings having 3 to 16 ring members, whereas other such groups have 3 to 6, 3 to 10, 3 to 12, or 3 to 14 ring members. Heterocyclyl groups encompass aromatic, partially unsaturated and saturated ring systems, such as, for example, imidazolyl, imidazolinyl and imidazolidinyl groups. The phrase "heterocyclyl group" includes fused ring species including those comprising fused aromatic and non-aromatic groups, such as, for example, benzotriazolyl, 2,3-dihydrobenzo[1,4]dioxinyl, and benzo[1,3]dioxolyl. The phrase also includes bridged polycyclic ring systems containing a heteroatom such as, but not limited to, quinuclidyl. The phrase includes heterocyclyl groups that have other groups, such as alkyl, oxo or halo groups, bonded to one of the ring members, referred to as "substituted heterocyclyl groups". Heterocyclyl groups include, but are not limited to, aziridinyl, azetidinyl, pyrrolidinyl, imidazolidinyl, pyrazolidinyl, thiazolidinyl, tetrahydrothiophenyl, tetrahydrofuranyl, dioxolyl, furanyl, thiophenyl, pyrrolyl, pyrrolinyl, imidazolyl, imidazolinyl, pyrazolyl, pyrazolinyl, triazolyl, tetrazolyl, oxazolyl, isoxazolyl, thiazolyl, thiazolinyl, isothiazolyl, thiadiazolyl, oxadiazolyl, piperidyl, piperazinyl, morpholinyl, thiomorpholinyl, tetrahydropyranyl, tetrahydrothiopyranyl, oxathiane, dioxyl, dithianyl, pyranyl, pyridyl, pyrimidinyl, pyridazinyl, pyrazinyl, triazinyl, dihydropyridyl, dihydrodithiinyl, dihydrodithionyl, homopiperazinyl, quinuclidyl, indolyl, indolinyl, isoindolyl,azaindolyl (pyrrolopyridyl), indazolyl, indolizinyl, benzotriazolyl, benzimidazolyl, benzofuranyl, benzothiophenyl, benzthiazolyl, benzoxadiazolyl, benzoxazinyl, benzodithiinyl, benzoxathiinyl, benzothiazinyl, benzoxazolyl, benzothiazolyl, benzothiadiazolyl, benzo[1,3]dioxolyl, pyrazolopyridyl, imidazopyridyl (azabenzimidazolyl), triazolopyridyl, isoxazolopyridyl, purinyl, xanthinyl, adeninyl, guaninyl, quinolinyl, isoquinolinyl, quinolizinyl, quinoxalinyl, quinazolinyl, cinnolinyl, phthalazinyl, naphthyridinyl, pteridinyl, thianaphthyl, dihydrobenzothiazinyl, dihydrobenzofuranyl, dihydroindolyl,

dihydrobenzodioxinyl, tetrahydroindolyl, tetrahydroindazolyl, tetrahydrobenzimidazolyl, tetrahydrobenzotriazolyl, tetrahydropyrrolopyridyl, tetrahydropyrazolopyridyl, tetrahydroimidazopyridyl, tetrahydrotriazolopyridyl, and tetrahydroquinolinyl groups. Representative substituted heterocyclyl groups may be mono-substituted or substituted more than once, such as, but not limited to, pyridyl or morpholinyl groups, which are 2-, 3-, 4-, 5-, or 6-substituted, or disubstituted with various substituents such as those listed above.

[0116] Heteroaryl groups are aromatic ring compounds containing 5 or more ring members, of which, one or more is a heteroatom such as, but not limited to, N, O, and S. Heteroaryl groups may be substituted or unsubstituted. Heteroaryl groups include, but are not limited to, groups such as pyrrolyl, pyrazolyl, triazolyl, tetrazolyl, oxazolyl, isoxazolyl, thiazolyl, pyridinyl, pyridazinyl, pyrimidinyl, pyrazinyl, thiophenyl, benzothiophenyl, furanyl, benzofuranyl, indolyl, azaindolyl (pyrrolopyridinyl), indazolyl, benzimidazolyl, imidazopyridinyl (azabenzimidazolyl), pyrazolopyridinyl, triazolopyridinyl, benzotriazolyl, benzoxazolyl, benzothiazolyl, benzothiadiazolyl, imidazopyridinyl, isoxazolopyridinyl, thianaphthyl, purinyl, xanthinyl, adeninyl, guaninyl, quinolinyl, isoquinolinyl, tetrahydroquinolinyl, quinoxalinyl, and quinazolinyl groups. Heteroaryl groups include fused ring compounds in which all rings are aromatic such as indolyl groups and include fused ring compounds in which only one of the rings is aromatic, such as 2,3-dihydro indolyl groups. Representative substituted heteroaryl groups may be substituted one or more times with various substituents such as those listed above.

[0117] Heteroaralkyl groups are alkyl groups as defined above in which a hydrogen or carbon bond of an alkyl group is replaced with a bond to a heteroaryl group as defined above. Heteroaralkyl groups may be substituted or unsubstituted. Substituted heteroaralkyl groups may be substituted at the alkyl, the heteroaryl or both the alkyl and heteroaryl portions of the group. Representative substituted heteroaralkyl groups may be substituted one or more times with substituents such as those listed above.

[0118] Groups described herein having two or more points of attachment (i.e., divalent, trivalent, or polyvalent) within the compound of the present technology are designated by use of the suffix, “ene.” For example, divalent alkyl groups are alkylene groups, divalent aryl groups are arylene groups, divalent heteroaryl groups are heteroarylene groups, and so forth. Substituted groups having a single point of attachment to the compound of the present

technology are not referred to using the "ene" designation. Thus, *e.g.*, chloroethyl is not referred to herein as chloroethylene.

[0119] As used herein, the term "administration" of an agent to a subject includes any route of introducing or delivering the agent to a subject to perform its intended function. Administration can be carried out by any suitable route, including. but not limited to, intravenously, intramuscularly, intraperitoneally, subcutaneously, and other suitable routes as described herein. Administration includes self-administration and the administration by another.

[0120] As used herein, the term "cell population" refers to a group of at least two cells expressing similar or different phenotypes. In non-limiting examples, a cell population can include at least about 10, at least about 100, at least about 200, at least about 300, at least about 400, at least about 500, at least about 600, at least about 700, at least about 800, at least about 900, at least about 1000 cells, at least about 10,000 cells, at least about 100,000 cells, at least about 1×10^6 cells, at least about 1×10^7 cells, at least about 1×10^8 cells, at least about 1×10^9 cells, at least about 1×10^{10} cells, at least about 1×10^{11} cells, at least about 1×10^{12} cells, or more cells expressing similar or different phenotypes.

[0121] The term "amino acid" refers to naturally occurring and non-naturally occurring amino acids, as well as amino acid analogs and amino acid mimetics that function in a manner similar to the naturally occurring amino acids. Naturally encoded amino acids are the 20 common amino acids (alanine, arginine, asparagine, aspartic acid, cysteine, glutamine, glutamic acid, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, and valine) and pyrolysine and selenocysteine. Amino acid analogs refers to agents that have the same basic chemical structure as a naturally occurring amino acid, *i.e.,* an α carbon that is bound to a hydrogen, a carboxyl group, an amino group, and an R group, such as, homoserine, norleucine, methionine sulfoxide, methionine methyl sulfonium. Such analogs have modified R groups (such as, norleucine) or modified peptide backbones, but retain the same basic chemical structure as a naturally occurring amino acid. In some embodiments, amino acids forming a polypeptide are in the D form. In some embodiments, the amino acids forming a polypeptide are in the L form. In some embodiments, a first plurality of amino acids forming a polypeptide are in the D form and a second plurality are in the L form.

[0122] Amino acids are referred to herein by either their commonly known three letter symbols or by the one-letter symbols recommended by the IUPAC-IUB Biochemical Nomenclature Commission. Nucleotides, likewise, are referred to by their commonly accepted single-letter code.

[0123] The terms "polypeptide," "peptide," and "protein" are used interchangeably herein to refer to a polymer of amino acid residues. The terms apply to naturally occurring amino acid polymers as well as amino acid polymers in which one or more amino acid residues is a non-naturally occurring amino acid, *e.g.,* an amino acid analog. The terms encompass amino acid chains of any length, including full length proteins, wherein the amino acid residues are linked by covalent peptide bonds.

[0124] As used herein, a "control" is an alternative sample used in an experiment for comparison purpose. A control can be "positive" or "negative." For example, where the purpose of the experiment is to determine a correlation of the efficacy of a therapeutic agent for the treatment for a particular type of disease, a positive control (a composition known to exhibit the desired therapeutic effect) and a negative control (a subject or a sample that does not receive the therapy or receives a placebo) are typically employed.

[0125] As used herein, the term "effective amount" or "therapeutically effective amount" refers to a quantity of an agent sufficient to achieve a desired therapeutic effect. In the context of therapeutic applications, the amount of a therapeutic peptide administered to the subject can depend on the type and severity of the infection and on the characteristics of the individual, such as general health, age, sex, body weight and tolerance to drugs. It can also depend on the degree, severity and type of disease. The skilled artisan will be able to determine appropriate dosages depending on these and other factors.

[0126] As used herein, the term "expression" refers to the process by which polynucleotides are transcribed into mRNA and/or the process by which the transcribed mRNA is subsequently being translated into peptides, polypeptides, or proteins. If the polynucleotide is derived from genomic DNA, expression can include splicing of the mRNA in a eukaryotic cell. The expression level of a gene can be determined by measuring the amount of mRNA or protein in a cell or tissue sample. In one aspect, the expression level of a gene from one sample can be directly compared to the expression level of that gene from a control or reference sample. In another aspect, the expression level of a gene from one

sample can be directly compared to the expression level of that gene from the same sample following administration of the compositions disclosed herein. The term "expression" also refers to one or more of the following events: (1) production of an RNA template from a DNA sequence (*e.g.,* by transcription) within a cell; (2) processing of an RNA transcript (*e.g.,* by splicing, editing, 5' cap formation, and/or 3' end formation) within a cell; (3) translation of an RNA sequence into a polypeptide or protein within a cell; (4) post-translational modification of a polypeptide or protein within a cell; (5) presentation of a polypeptide or protein on the cell surface; and (6) secretion or presentation or release of a polypeptide or protein from a cell.

[0127] The term "linker" refers to synthetic sequences (*e.g.*, amino acid sequences) that connect or link two sequences, *e.g.*, that link two polypeptide domains. In some embodiments, the linker contains 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 of amino acid sequences.

[0128] As used herein the term "immune cell" refers to any cell that plays a role in the immune response. Immune cells are of hematopoietic origin, and include lymphocytes, such as B cells and T cells; natural killer cells; myeloid cells, such as monocytes, macrophages, dendritic cells, eosinophils, neutrophils, mast cells, basophils, and granulocytes.

[0129] As used herein, the term "native immune cell" refers to an immune cell that naturally occurs in the immune system.

[0130] As used herein, the term "engineered immune cell" refers to an immune cell that is genetically modified.

[0131] The term "lymphocyte" refers to all immature, mature, undifferentiated and differentiated white lymphocyte populations including tissue specific and specialized varieties. It encompasses, by way of non-limiting example, B cells, T cells, NKT cells, and NK cells. In some embodiments, lymphocytes include all B cell lineages including pre-B cells, progenitor B cells, early pro-B cells, late pro-B cells, large pre-B cells, small pre-B cells, immature B cells, mature B cells, plasma B cells, memory B cells, B-1 cells, B-2 cells and anergic AN1/T3 cell populations.

[0132] As used herein, the term "T-cell" includes naïve T cells, CD4+ T cells, CD8+ T cells, memory T cells, activated T cells, anergic T cells, tolerant T cells, chimeric B cells, and antigen-specific T cells.

[0133] As used herein "adoptive cell therapeutic composition" refers to any composition comprising cells suitable for adoptive cell transfer. In exemplary embodiments, the adoptive cell therapeutic composition comprises a cell type selected from a group consisting of a tumor infiltrating lymphocyte (TIL), TCR (*i.e.* heterologous T-cell receptor) modified lymphocytes and CAR (*i.e.* chimeric antigen receptor) modified lymphocytes. In another embodiment, the adoptive cell therapeutic composition comprises a cell type selected from a group consisting of T-cells, CD8+ cells, CD4+ cells, NK-cells, delta-gamma T-cells, regulatory T-cells and peripheral blood mononuclear cells. In another embodiment, TILs, T-cells, CD8+ cells, CD4+ cells, NK-cells, delta-gamma T-cells, regulatory T-cells or peripheral blood mononuclear cells form the adoptive cell therapeutic composition. In one embodiment, the adoptive cell therapeutic composition comprises T cells.

[0134] As used herein "tumor-infiltrating lymphocytes" or TILs refer to white blood cells that have left the bloodstream and migrated into a tumor.

[0135] As used herein, the term "antibody" means not only intact antibody molecules, but also fragments of antibody molecules that retain immunogen-binding ability. Such fragments are also well known in the art and are regularly employed both *in vitro* and *in vivo*. Accordingly, as used herein, the term "antibody" means not only intact immunoglobulin molecules but also the well-known active fragments $F(ab')_2$, and Fab. $F(ab')_2$, and Fab fragments that lack the Fc fragment of intact antibody, clear more rapidly from the circulation, and may have less non-specific tissue binding of an intact antibody (Wahl *et al., J. Nucl. Med.* 24:316-325 (1983)). The antibodies of the invention comprise whole native antibodies, monoclonal antibodies, human antibodies, humanized antibodies, camelised antibodies, multispecific antibodies, bispecific antibodies, chimeric antibodies, Fab, Fab', single chain V region fragments (scFv), single domain antibodies (*e.g.*, nanobodies and single domain camelid antibodies), V_{NAR} fragments, Bi-specific T-cell engager (BiTE) antibodies, minibodies, disulfide-linked Fvs (sdFv), and anti-idiotypic (anti-Id) antibodies, intrabodies, fusion polypeptides, unconventional antibodies and antigen-binding fragments of any of the above. In particular, antibodies include immunoglobulin molecules and immunologically active fragments of immunoglobulin molecules, i.e., molecules that contain an antigen-binding site. Immunoglobulin molecules can be of any type (*e.g.*, IgG, IgE, IgM, IgD, IgA and IgY), class (*e.g.*, IgGl, IgG2, IgG3, IgG4, IgAl and IgA2) or subclass.

[0136] In certain embodiments, an antibody is a glycoprotein comprising at least two heavy (H) chains and two light (L) chains inter-connected by disulfide bonds. Each heavy chain is comprised of a heavy chain variable region (abbreviated herein as V_H) and a heavy chain constant (C_H) region. The heavy chain constant region is comprised of three domains, CHI, CH2 and CH3. Each light chain is comprised of a light chain variable region (abbreviated herein as V_L) and a light chain constant C_L region. The light chain constant region is comprised of one domain, C_L. The V_H and V_L regions can be further subdivided into regions of hypervariability, termed complementarity determining regions (CDR), interspersed with regions that are more conserved, termed framework regions (FR). Each V_H and V_L is composed of three CDRs and four FRs arranged from amino-terminus to carboxy-terminus in the following order: FR1, CDR1, FR2, CDR2, FR3, CDR3, FR4. The variable regions of the heavy and light chains contain a binding domain that interacts with an antigen. The constant regions of the antibodies may mediate the binding of the immunoglobulin to host tissues or factors, including various cells of the immune system (*e.g.*, effector cells) and the first component (Cl q) of the classical complement system. As used herein interchangeably, the terms "antigen-binding portion", "antigen- binding fragment", or "antigen-binding region" of an antibody, refer to the region or portion of an antibody that binds to the antigen and which confers antigen specificity to the antibody; fragments of antigen-binding proteins, for example, antibodies includes one or more fragments of an antibody that retain the ability to specifically bind to an antigen (*e.g.*, an peptide/HLA complex). It has been shown that the antigen-binding function of an antibody can be performed by fragments of a full-length antibody. Examples of antigen-binding portions encompassed within the term "antibody fragments" of an antibody include a Fab fragment, a monovalent fragment consisting of the V_L, V_H, C_L and CHI domains; a $F(ab)_2$ fragment, a bivalent fragment comprising two Fab fragments linked by a disulfide bridge at the hinge region; a Fd fragment consisting of the V_H and CHI domains; a Fv fragment consisting of the V_L and V_H domains of a single arm of an antibody; a dAb fragment (Ward *et al.*, *Nature* 341 : 544-546 (1989)), which consists of a V_H domain; and an isolated complementarity determining region (CDR).

[0137] Antibodies and antibody fragments can be wholly or partially derived from mammals (*e.g.*, humans, non-human primates, goats, guinea pigs, hamsters, horses, mice, rats, rabbits and sheep) or non-mammalian antibody producing animals (*e.g.*, chickens,

ducks, geese, snakes, urodele amphibians). The antibodies and antibody fragments can be produced in animals or produced outside of animals, such as from yeast or phage (*e.g.*, as a single antibody or antibody fragment or as part of an antibody library).

[0138] Furthermore, although the two domains of the Fv fragment, V_L and V_H, are coded for by separate genes, they can be joined, using recombinant methods, by a synthetic linker that enables them to be made as a single protein chain in which the V_L and V_H regions pair to form monovalent molecules. These are known as single chain Fv (scFv); see *e.g.*, Bird *et al., Science* 242:423-426 (1988); and Huston *et al., Proc. Natl. Acad. Sci.* 85 : 5879-5883 (1988). These antibody fragments are obtained using conventional techniques known to those of ordinary skill in the art, and the fragments are screened for utility in the same manner as are intact antibodies.

[0139] An "isolated antibody" or "isolated antigen-binding protein" is one which has been identified and separated and/or recovered from a component of its natural environment. "Synthetic antibodies" or "recombinant antibodies" are generally generated using recombinant technology or using peptide synthetic techniques known to those of skill in the art.

[0140] As used herein, the term "single-chain variable fragment" or "scFv" is a fusion protein of the variable regions of the heavy (V_H) and light chains (V_L) of an immunoglobulin (*e.g.*, mouse or human) covalently linked to form a V_H: :V_L heterodimer. The heavy (V_H) and light chains (V_L) are either joined directly or joined by a peptide- encoding linker (*e.g.*, about 10, 15, 20, 25 amino acids), which connects the N-terminus of the V_H with the C-terminus of the V_L, or the C-terminus of the V_H with the N-terminus of the V_L. The linker is usually rich in glycine for flexibility, as well as serine or threonine for solubility. The linker can link the heavy chain variable region and the light chain variable region of the extracellular antigen-binding domain. In certain embodiments, the linker comprises amino acids having the sequence set forth in SEQ ID NO: 21 as provided below.

[0141] GGGGSGGGGSGGGGS (SEQ ID NO: 21)

[0142] In certain embodiments, the nucleic acid sequence encoding the amino acid sequence of SEQ ID NO: 21 is set forth in SEQ ID NO: 22, which is provided below:

[0143] ggcggcggcggatctggaggtggtggctcaggtggcggaggctcc (SEQ ID NO: 22)

[0144] Despite removal of the constant regions and the introduction of a linker, scFv proteins retain the specificity of the original immunoglobulin. Single chain Fv polypeptide antibodies can be expressed from a nucleic acid comprising V_H- and V_L-encoding sequences as described by Huston, *et al.* (*Proc. Nat. Acad. Sci. USA*, 85:5879-5883 (1988)). See, also, U.S. Patent Nos. 5,091,513, 5,132,405 and 4,956,778; and U.S. Patent Publication Nos. 20050196754 and 20050196754. Antagonistic scFvs having inhibitory activity have been described (see, *e.g.*, Zhao *et al., Hybridoma* (Larchmt) 27(6):455-51 (2008); Peter *et al., J Cachexia Sarcopenia Muscle* (2012); Shieh *et al., J Imunol* 183(4):2277-85 (2009); Giomarelli *et al., Thromb Haemost* 97(6):955-63 (2007); Fife eta., *J Clin Invst* 116(8):2252-61 (2006); Brocks *et al., Immunotechnology* 3(3): 173-84 (1997); Moosmayer *et al., Ther Immunol* 2(10):31- 40 (1995) Agonistic scFvs having stimulatory activity have been described (see, *e.g.*, Peter *et al., J Biol Chem* 25278(38):36740-7 (2003); Xie *et al., Nat Biotech* 15(8):768-71 (1997); Ledbetter *et al., Crit Rev Immunol* 17(5-6):427-55 (1997); Ho *et al., Bio Chim Biophys Acta* 1638(3):257-66 (2003)).

[0145] As used herein, "F(ab)" refers to a fragment of an antibody structure that binds to an antigen but is monovalent and does not have a Fc portion, for example, an antibody digested by the enzyme papain yields two F(ab) fragments and an Fc fragment (*e.g.*, a heavy (H) chain constant region; Fc region that does not bind to an antigen).

[0146] As used herein, "$F(ab')_2$" refers to an antibody fragment generated by pepsin digestion of whole IgG antibodies, wherein this fragment has two antigen binding (ab') (bivalent) regions, wherein each (ab^1) region comprises two separate amino acid chains, a part of a H chain and a light (L) chain linked by an S-S bond for binding an antigen and where the remaining H chain portions are linked together. A "$F(ab')_2$" fragment can be split into two individual Fab' fragments.

[0147] As used herein, "CDRs" are defined as the complementarity determining region amino acid sequences of an antibody which are the hypervariable regions of immunoglobulin heavy and light chains. See, *e.g.*, Kabat *et al.,* Sequences of Proteins of Immunological Interest, 4th U. S. Department of Health and Human Services, National Institutes of Health (1987). Generally, antibodies comprise three heavy chain and three light chain CDRs or CDR regions in the variable region. CDRs provide the majority of contact residues for the binding of the antibody to the antigen or epitope. In certain embodiments, the CDRs regions are

delineated using the Kabat system (Kabat, E. A., *et al.* Sequences of Proteins of Immunological Interest, Fifth Edition, U.S. Department of Health and Human Services, NIH Publication No. 91-3242(1991)).

[0148] As used herein, the term "affinity" is meant a measure of binding strength. Without being bound to theory, affinity depends on the closeness of stereochemical fit between antibody combining sites and antigen determinants, on the size of the area of contact between them, and on the distribution of charged and hydrophobic groups. Affinity also includes the term "avidity," which refers to the strength of the antigen- antibody bond after formation of reversible complexes (*e.g.*, either monovalent or multivalent). Methods for calculating the affinity of an antibody for an antigen are known in the art, comprising use of binding experiments to calculate affinity. Antibody activity in functional assays (*e.g.*, flow cytometry assay) is also reflective of antibody affinity. Antibodies and affinities can be phenotypically characterized and compared using functional assays (*e.g.*, flow cytometry assay). Nucleic acid molecules useful in the presently disclosed subject matter include any nucleic acid molecule that encodes a polypeptide or a fragment thereof. In certain embodiments, nucleic acid molecules useful in the presently disclosed subject matter include nucleic acid molecules that encode an antibody or an antigen-binding portion thereof. Such nucleic acid molecules need not be 100% identical with an endogenous nucleic acid sequence, but will typically exhibit substantial identity. Polynucleotides having "substantial homology" or "substantial identity" to an endogenous sequence are typically capable of hybridizing with at least one strand of a double-stranded nucleic acid molecule. By "hybridize" is meant pair to form a double-stranded molecule between complementary polynucleotide sequences (*e.g.*, a gene described herein), or portions thereof, under various conditions of stringency. (See, *e.g.*, Wahl, G. M. and S. L. Berger, *Methods Enzymol.* 152:399 (1987); Kimmel, A. R. *Methods Enzymol.* 152:507 (1987)).

[0149] For example, stringent salt concentration will ordinarily be less than about 750 mM NaCl and 75 mM trisodium citrate, preferably less than about 500 mM NaCl and 50 mM trisodium citrate, and more preferably less than about 250 mM NaCl and 25 mM trisodium citrate. Low stringency hybridization can be obtained in the absence of organic solvent, *e.g.*, formamide, while high stringency hybridization can be obtained in the presence of at least about 35% w/v formamide, and more preferably at least about 50% w/v formamide. Stringent

temperature conditions will ordinarily include temperatures of at least about 30°C, more preferably of at least about 37°C, and most preferably of at least about 42°C. Varying additional parameters, such as hybridization time, the concentration of detergent, *e.g.*, sodium dodecyl sulfate (SDS), and the inclusion or exclusion of carrier DNA, are well known to those skilled in the art. Various levels of stringency are accomplished by combining these various conditions as needed. In certain embodiments, hybridization will occur at 30°C in 750 mM NaCl, 75 mM trisodium citrate, and 1% w/v SDS. In certain embodiments, hybridization will occur at 37°C in 500 mM NaCl, 50 mM trisodium citrate, 1% w/v SDS, 35% w/v formamide, and 100 µg/ml denatured salmon sperm DNA (ssDNA). In certain embodiments, hybridization will occur at 42°C in 250 mM NaCl, 25 mM trisodium citrate, 1% w/v SDS, 50% w/v formamide, and 200 µg ssDNA. Useful variations on these conditions will be readily apparent to those skilled in the art.

[0150] For most applications, washing steps that follow hybridization will also vary in stringency. Wash stringency conditions can be defined by salt concentration and by temperature. As above, wash stringency can be increased by decreasing salt concentration or by increasing temperature. For example, stringent salt concentration for the wash steps will preferably be less than about 30 mM NaCl and 3 mM trisodium citrate, and most preferably less than about 15 mM NaCl and 1.5 mM trisodium citrate. Stringent temperature conditions for the wash steps will ordinarily include a temperature of at least about 25°C, more preferably of at least about 42°C, and even more preferably of at least about 68°C. In certain embodiments, wash steps will occur at 25°C in 30 mM NaCl, 3 mM trisodium citrate, and 0.1% w/v SDS. In certain embodiments, wash steps will occur at 42°C in 15 mM NaCl, 1.5 mM trisodium citrate, and 0.1% w/v SDS. In certain embodiments, wash steps will occur at 68°C in 15 mM NaCl, 1.5 mM trisodium citrate, and 0.1% w/v SDS. Additional variations on these conditions will be readily apparent to those skilled in the art. Hybridization techniques are well known to those skilled in the art and are described, for example, in Benton and Davis (*Science* 196: 180 (1977)); Grunstein and Rogness (*Proc. Natl. Acad. Sci., USA* 72:3961 (1975)); Ausubel *et al.* (Current Protocols in Molecular Biology, Wiley Interscience, New York, 2001); Berger and Kimmel (Guide to Molecular Cloning Techniques, 1987, Academic Press, New York); and Sambrook *et al.*, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, New York.

[0151] The terms "substantially homologous" or "substantially identical" mean a polypeptide or nucleic acid molecule that exhibits at least 50% or greater homology or identity to a reference amino acid sequence (for example, any one of the amino acid sequences described herein) or nucleic acid sequence (for example, any one of the nucleic acid sequences described herein). For example, such a sequence is at least about 60%, about 65%, about 70%, about 75%, about 80%, about 85%, about 90%, about 95% or about 99% homologous or identical at the amino acid level or nucleic acid to the sequence used for comparison (*e.g.*, a wild-type, or native, sequence). In some embodiments, a substantially homologous or substantially identical polypeptide contains one or more amino acid amino acid substitutions, insertions, or deletions relative to the sequence used for comparison. In some embodiments, a substantially homologous or substantially identical polypeptide contains one or more non-natural amino acids or amino acid analogs, including, D-amino acids and retroinverso amino, to replace homologous sequences.

[0152] Sequence homology or sequence identity is typically measured using sequence analysis software (for example, Sequence Analysis Software Package of the Genetics Computer Group, University of Wisconsin Biotechnology Center, 1710 University Avenue, Madison, Wis. 53705, BLAST, BESTFIT, GAP, or PILEUP/PRETTYBOX programs). Such software matches identical or similar sequences by assigning degrees of homology to various substitutions, deletions, and/or other modifications. In an exemplary approach to determining the degree of identity, a BLAST program may be used, with a probability score between e^{-3} and $^{e-100}$ indicating a closely related sequence.

[0153] As used herein, the percent homology between two amino acid sequences is equivalent to the percent identity between the two sequences. The percent identity between the two sequences is a function of the number of identical positions shared by the sequences (*i.e.*, % homology = # of identical positions/total # of positions x 100), taking into account the number of gaps, and the length of each gap, which need to be introduced for optimal alignment of the two sequences. The comparison of sequences and determination of percent identity between two sequences can be accomplished using a mathematical algorithm.

[0154] The percent homology between two amino acid sequences can be determined using the algorithm of E. Meyers and W. Miller (*Comput. Appl. Biosci.*, 4: 1 1-17 (1988)) which has been incorporated into the ALIGN program (version 2.0), using a PAM120 weight

residue table, a gap length penalty of 12 and a gap penalty of 4. In addition, the percent homology between two amino acid sequences can be determined using the Needleman and Wunsch (*J. Mol. Biol.* 48:444-453 (1970)) algorithm which has been incorporated into the GAP program in the GCG software package (available at www.gcg.com), using either a Blossum 62 matrix or a PAM250 matrix, and a gap weight of 16, 14, 12, 10, 8, 6, or 4 and a length weight of 1, 2, 3, 4, 5, or 6.

[0155] Additionally or alternatively, the amino acids sequences of the presently disclosed subject matter can further be used as a "query sequence" to perform a search against public databases to, for example, identify related sequences. Such searches can be performed using the XBLAST program (version 2.0) of Altschul, *et al.* (1990) *J. Mol. Biol.* 215 :403-10. BLAST protein searches can be performed with the XBLAST program, score = 50, wordlength = 3 to obtain amino acid sequences homologous to the specified sequences disclosed herein. To obtain gapped alignments for comparison purposes, Gapped BLAST can be utilized as described in Altschul *et al.,* (1997) *Nucleic Acids Res.* 25(17):3389-3402. When utilizing BLAST and Gapped BLAST programs, the default parameters of the respective programs (*e.g.*, XBLAST and NBLAST) can be used.

[0156] As used herein, the term "analog" refers to a structurally related polypeptide or nucleic acid molecule having the function of a reference polypeptide or nucleic acid molecule.

[0157] As used herein, the term "a conservative sequence modification" refers to an amino acid modification that does not significantly affect or alter the binding characteristics of the presently disclosed CAR (*e.g.*, the extracellular antigen-binding domain of the CAR) comprising the amino acid sequence. Conservative modifications can include amino acid substitutions, additions and deletions. Modifications can be introduced into the human scFv of the presently disclosed CAR by standard techniques known in the art, such as site-directed mutagenesis and PCR-mediated mutagenesis. Amino acids can be classified into groups according to their physicochemical properties such as charge and polarity. Conservative amino acid substitutions are ones in which the amino acid residue is replaced with an amino acid within the same group. For example, amino acids can be classified by charge: positively-charged amino acids include lysine, arginine, histidine, negatively-charged amino acids include aspartic acid, glutamic acid, neutral charge amino acids include alanine, asparagine,

cysteine, glutamine, glycine, isoleucine, leucine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, and valine. In addition, amino acids can be classified by polarity: polar amino acids include arginine (basic polar), asparagine, aspartic acid (acidic polar), glutamic acid (acidic polar), glutamine, histidine (basic polar), lysine (basic polar), serine, threonine, and tyrosine; non-polar amino acids include alanine, cysteine, glycine, isoleucine, leucine, methionine, phenylalanine, proline, tryptophan, and valine. Thus, one or more amino acid residues within a CDR region can be replaced with other amino acid residues from the same group and the altered antibody can be tested for retained function (*i.e.,* the functions set forth in (c) through (1) above) using the functional assays described herein. In certain embodiments, no more than one, no more than two, no more than three, no more than four, no more than five residues within a specified sequence or a CDR region are altered.

[0158] As used herein, the term "ligand" refers to a molecule that binds to a receptor. In particular, the ligand binds a receptor on another cell, allowing for cell-to-cell recognition and/or interaction.

[0159] As used herein, the term, "co-stimulatory signaling domain," or "co-stimulatory domain", refers to the portion of the CAR comprising the intracellular domain of a co-stimulatory molecule. Co-stimulatory molecules are cell surface molecules other than antigen receptors or Fc receptors that provide a second signal required for efficient activation and function of T lymphocytes upon binding to antigen. Examples of such co-stimulatory molecules include CD27, CD28, 4-1BB (CD137), OX40 (CD134), CD30, CD40, PD-1, ICOS (CD278), LFA-1, CD2, CD7, LIGHT, NKD2C, B7-H2 and a ligand that specifically binds CD83. Accordingly, while the present disclosure provides exemplary costimulatory domains derived from CD28 and 4-1BB, other costimulatory domains are contemplated for use with the CARs described herein. The inclusion of one or more co-stimulatory signaling domains can enhance the efficacy and expansion of T cells expressing CAR receptors. The intracellular signaling and co-stimulatory signaling domains can be linked in any order in tandem to the carboxyl terminus of the transmembrane domain.

[0160] As used herein, the term "chimeric co-stimulatory receptor" or "CCR" refers to a chimeric receptor that binds to an antigen and provides co-stimulatory signals, but does not provide a T-cell activation signal.

[0161] As used herein, regulatory region of a nucleic acid molecule means a cis- acting nucleotide sequence that influences expression, positively or negatively, of an operatively linked gene. Regulatory regions include sequences of nucleotides that confer inducible (i.e., require a substance or stimulus for increased transcription) expression of a gene. When an inducer is present or at increased concentration, gene expression can be increased. Regulatory regions also include sequences that confer repression of gene expression (i.e., a substance or stimulus decreases transcription). When a repressor is present or at increased concentration gene expression can be decreased. Regulatory regions are known to influence, modulate or control many in vivo biological activities including cell proliferation, cell growth and death, cell differentiation and immune modulation. Regulatory regions typically bind to one or more trans-acting proteins, which results in either increased or decreased transcription of the gene.

[0162] Particular examples of gene regulatory regions are promoters and enhancers. Promoters are sequences located around the transcription or translation start site, typically positioned 5' of the translation start site. Promoters usually are located within 1 Kb of the translation start site, but can be located further away, for example, 2 Kb, 3 Kb, 4 Kb, 5 Kb or more, up to and including 10 Kb. Enhancers are known to influence gene expression when positioned 5' or 3' of the gene, or when positioned in or a part of an exon or an intron. Enhancers also can function at a significant distance from the gene, for example, at a distance from about 3 Kb, 5 Kb, 7 Kb, 10 Kb, 15 Kb or more.

[0163] Regulatory regions also include, but are not limited to, in addition to promoter regions, sequences that facilitate translation, splicing signals for introns, maintenance of the correct reading frame of the gene to permit in-frame translation of mRNA and, stop codons, leader sequences and fusion partner sequences, internal ribosome binding site (IRES) elements for the creation of multigene, or polycistronic, messages, polyadenylation signals to provide proper polyadenylation of the transcript of a gene of interest and stop codons, and can be optionally included in an expression vector.

[0164] As used herein, "operably linked" with reference to nucleic acid sequences, regions, elements or domains means that the nucleic acid regions are functionally related to each other. For example, nucleic acid encoding a leader peptide can be operably linked to nucleic acid encoding a polypeptide, whereby the nucleic acids can be transcribed and translated to express a functional fusion protein, wherein the leader peptide effects secretion

of the fusion polypeptide. In some instances, the nucleic acid encoding a first polypeptide (*e.g.*, a leader peptide) is operably linked to nucleic acid encoding a second polypeptide and the nucleic acids are transcribed as a single mRNA transcript, but translation of the mRNA transcript can result in one of two polypeptides being expressed. For example, an amber stop codon can be located between the nucleic acid encoding the first polypeptide and the nucleic acid encoding the second polypeptide, such that, when introduced into a partial amber suppressor cell, the resulting single mRNA transcript can be translated to produce either a fusion protein containing the first and second polypeptides, or can be translated to produce only the first polypeptide. In another example, a promoter can be operably linked to nucleic acid encoding a polypeptide, whereby the promoter regulates or mediates the transcription of the nucleic acid.

[0165] As used herein, "synthetic," with reference to, for example, a synthetic nucleic acid molecule or a synthetic gene or a synthetic peptide refers to a nucleic acid molecule or polypeptide molecule that is produced by recombinant methods and/or by chemical synthesis methods. As used herein, production by recombinant means by using recombinant DNA methods means the use of the well-known methods of molecular biology for expressing proteins encoded by cloned DNA.

[0166] As used herein, "expression" refers to the process by which polypeptides are produced by transcription and translation of polynucleotides. The level of expression of a polypeptide can be assessed using any method known in art, including, for example, methods of determining the amount of the polypeptide produced from the host cell. Such methods can include, but are not limited to, quantitation of the polypeptide in the cell lysate by ELISA, Coomassie blue staining following gel electrophoresis, Lowry protein assay and Bradford protein assay.

[0167] As used herein, a "host cell" is a cell that is used in to receive, maintain, reproduce and amplify a vector. A host cell also can be used to express the polypeptide encoded by the vector. The nucleic acid contained in the vector is replicated when the host cell divides, thereby amplifying the nucleic acids.

[0168] As used herein, a "vector" is a replicable nucleic acid from which one or more heterologous proteins can be expressed when the vector is transformed into an appropriate host cell. Reference to a vector includes those vectors into which a nucleic acid encoding a

polypeptide or fragment thereof can be introduced, typically by restriction digest and ligation. Reference to a vector also includes those vectors that contain nucleic acid encoding a polypeptide. The vector is used to introduce the nucleic acid encoding the polypeptide into the host cell for amplification of the nucleic acid or for expression/display of the polypeptide encoded by the nucleic acid. The vectors typically remain episomal, but can be designed to effect integration of a gene or portion thereof into a chromosome of the genome. Also contemplated are vectors that are artificial chromosomes, such as yeast artificial chromosomes and mammalian artificial chromosomes. Selection and use of such vehicles are well known to those of skill in the art.

[0169] As used herein, a vector also includes "virus vectors" or "viral vectors." Viral vectors are engineered viruses that are operatively linked to exogenous genes to transfer (as vehicles or shuttles) the exogenous genes into cells.

[0170] As used herein, an "expression vector" includes vectors capable of expressing DNA that is operatively linked with regulatory sequences, such as promoter regions, that are capable of effecting expression of such DNA fragments. Such additional segments can include promoter and terminator sequences, and optionally can include one or more origins of replication, one or more selectable markers, an enhancer, a polyadenylation signal, and the like. Expression vectors are generally derived from plasmid or viral DNA, or can contain elements of both. Thus, an expression vector refers to a recombinant DNA or RNA construct, such as a plasmid, a phage, recombinant virus or other vector that, upon introduction into an appropriate host cell, results in expression of the cloned DNA. Appropriate expression vectors are well known to those of skill in the art and include those that are replicable in eukaryotic cells and/or prokaryotic cells and those that remain episomal or those which integrate into the host cell genome.

[0171] As used herein, the term “disease” refers to any condition or disorder that damages or interferes with the normal function of a cell, tissue, or organ. Examples of diseases include neoplasia or pathogen infection of cell.

[0172] An “effective amount” (or “therapeutically effective amount”) is an amount sufficient to affect a beneficial or desired clinical result upon treatment. An effective amount can be administered to a subject in one or more doses. In terms of treatment, an effective amount is an amount that is sufficient to palliate, ameliorate, stabilize, reverse or slow the

progression of the disease (*e.g.*, a neoplasia), or otherwise reduce the pathological consequences of the disease (*e.g.*, a neoplasia). The effective amount is generally determined by the physician on a case-by-case basis and is within the skill of one in the art. Several factors are typically taken into account when determining an appropriate dosage to achieve an effective amount. These factors include age, sex and weight of the subject, the condition being treated, the severity of the condition and the form and effective concentration of the engineered immune cells administered.

[0173] As used herein, the term "neoplasia" refers to a disease characterized by the pathological proliferation of a cell or tissue and its subsequent migration to or invasion of other tissues or organs. Neoplasia growth is typically uncontrolled and progressive, and occurs under conditions that would not elicit, or would cause cessation of, multiplication of normal cells. Neoplasias can affect a variety of cell types, tissues, or organs, including but not limited to an organ selected from the group consisting of bladder, colon, bone, brain, breast, cartilage, glia, esophagus, fallopian tube, gallbladder, heart, intestines, kidney, liver, lung, lymph node, nervous tissue, ovaries, pleura, pancreas, prostate, skeletal muscle, skin, spinal cord, spleen, stomach, testes, thymus, thyroid, trachea, urogenital tract, ureter, urethra, uterus, and vagina, or a tissue or cell type thereof. Neoplasias include cancers, such as sarcomas, carcinomas, or plasmacytomas (malignant tumor of the plasma cells).

[0174] As used herein, the term "heterologous nucleic acid molecule or polypeptide" refers to a nucleic acid molecule (*e.g.*, a cDNA, DNA or RNA molecule) or polypeptide that is not normally present in a cell or sample obtained from a cell. This nucleic acid may be from another organism, or it may be, for example, an mRNA molecule that is not normally expressed in a cell or sample.

[0175] As used herein, the term "immunoresponsive cell" refers to a cell that functions in an immune response or a progenitor, or progeny thereof.

[0176] As used herein, the term "modulate" refers positively or negatively alter. Exemplary modulations include an about 1%, about 2%, about 5%, about 10%, about 25%, about 50%, about 75%, or about 100% change.

[0177] As used herein, the term "increase" refers to alter positively by at least about 5%, including, but not limited to, alter positively by about 5%, by about 10%, by about 25%, by about 30%, by about 50%, by about 75%, or by about 100%.

[0178] As used herein, the term "reduce" refers to alter negatively by at least about 5% including, but not limited to, alter negatively by about 5%, by about 10%, by about 25%, by about 30%, by about 50%, by about 75%, or by about 100%.

[0179] As used herein, the term "isolated cell" refers to a cell that is separated from the molecular and/or cellular components that naturally accompany the cell.

[0180] As used herein, the term "isolated," "purified," or "biologically pure" refers to material that is free to varying degrees from components which normally accompany it as found in its native state. "Isolate" denotes a degree of separation from original source or surroundings. "Purify" denotes a degree of separation that is higher than isolation. A "purified" or "biologically pure" protein is sufficiently free of other materials such that any impurities do not materially affect the biological properties of the protein or cause other adverse consequences. That is, a nucleic acid or polypeptide of the presently disclosed subject matter is purified if it is substantially free of cellular material, viral material, or culture medium when produced by recombinant DNA techniques, or chemical precursors or other chemicals when chemically synthesized. Purity and homogeneity are typically determined using analytical chemistry techniques, for example, polyacrylamide gel electrophoresis or high performance liquid chromatography. The term "purified" can denote that a nucleic acid or protein gives rise to essentially one band in an electrophoretic gel. For a protein that can be subjected to modifications, for example, phosphorylation or glycosylation, different modifications may give rise to different isolated proteins, which can be separately purified.

[0181] As used herein, the term "secreted" is meant a polypeptide that is released from a cell via the secretory pathway through the endoplasmic reticulum, Golgi apparatus, and as a vesicle that transiently fuses at the cell plasma membrane, releasing the proteins outside of the cell. Small molecules, such as drugs, can also be secreted by diffusion through the membrane to the outside of cell.

[0182] As used herein, the term "specifically binds" or "specifically binds to" or "specifically target" is meant a polypeptide or fragment thereof that recognizes and binds a biological molecule of interest (*e.g.*, a polypeptide), but which does not substantially recognize and bind other molecules in a sample, for example, a biological sample, which includes or expresses a tumor antigen.

[0183] As used herein, the term "treating" or "treatment" refers to clinical intervention in an attempt to alter the disease course of the individual or cell being treated, and can be performed either for prophylaxis or during the course of clinical pathology. Therapeutic effects of treatment include, without limitation, preventing occurrence or recurrence of disease, alleviation of symptoms, diminishment of any direct or indirect pathological consequences of the disease, preventing metastases, decreasing the rate of disease progression, amelioration or palliation of the disease state, and remission or improved prognosis. By preventing progression of a disease or disorder, a treatment can prevent deterioration due to a disorder in an affected or diagnosed subject or a subject suspected of having the disorder, but also a treatment may prevent the onset of the disorder or a symptom of the disorder in a subject at risk for the disorder or suspected of having the disorder.

[0184] As used herein, the term "subject" refers to any animal (*e.g.*, a mammal), including, but not limited to, humans, non-human primates, rodents, and the like (*e.g.*, which is to be the recipient of a particular treatment, or from whom cells are harvested).

Overview

[0185] Adoptive transfer of chimeric antigen receptor (CAR) T cells has been shown to be an effective therapy for B-cell ALL and other hematopoietic cancers. However, primary failures, incomplete remissions, and relapse still occurs via multiple mechanisms including loss of the target antigen. Solid tumors have been more resistant to this form of therapy. Therefore, greater potency and mechanisms to defeat the immunosuppressive tumor microenvironment are needed for many cancer types. Resistance of tumors to the activity of CAR T cells, antigen loss variant escape, and relapse are still important hurdles to the success of CAR T cell therapy. Provided herein are engineered immune cells, including compositions comprising engineered immune cells and methods of use thereof, that address these issues. As described herein, immune cells can be engineered to constitutively or conditionally express an enzyme that synthesizes and releases a potent cytotoxic chemotherapy from a non-toxic prodrug at the cancer cell surface or into the tumor microenvironment (*i.e.*, a prodrug converting enzyme). (FIG. 1). In some embodiments, the engineered immune cells additionally express a chimeric antigen receptor for delivering the immune cell to the target site. These engineered immune cells are interchangeably called herein Synthetic Enzyme

Activated Killer (SEAKER) cells. Without intending to be bound by theory, the local release of potent anti-neoplastic drugs at the tumor site is expected to overcome the tumor micro-environmental immune resistance to the T cells, and the escape by antigen loss variants as the drug is antigen independent, not suppressed by other cells, and may diffuse locally. The methods provided herein allow for modular use of a wide range of drugs depending on the desired application. The drugs can also synergize with the direct immune-based cytotoxic effects of the engineered immune cells, *e.g.,* CAR T cells. Selective synthesis of the drug at the cancer cell by the engineered immune cells also reduces systemic toxicity of the administered drugs.

[0186] The engineered immune cells (*e.g.*, CAR T cells) provided herein that express an antigen receptor, *e.g.*, a chimeric antigen receptor, in combination with a wide variety of prodrugs. Methods for making prodrugs are provided herein and are known in art. In some embodiments, the active drug of the prodrug is a cytotoxic chemotherapeutic drug. Cytotoxic chemotherapeutic drugs can be non-targeted or targeted. Exemplary non-targeted cytotoxic cancer chemotherapies as well as prodrugs forms of the compounds are provided herein or known in the art and include, for example, mustards, methotrexate, doxorubicin, nucleosides, and AMS. Exemplary targeted cancer chemotherapies as well as prodrugs forms of the compounds are provided herein or known in the art and include, for example, erlotinib, dasatinib, and tyrosine kinase inhibitors. In addition, as described herein, prodrugs of regulatory drugs, heat shock protein (HSP) inhibitors, and multidrug resistance (MDR) inhibitors can be employed.

[0187] Prior methods to effect local distribution of prodrug converting enzymes have included antibody-directed enzyme prodrug therapy (ADEPT). ADEPT was conceived as a means to leverage enzyme catalysis to generate high local concentrations of a cytotoxic drug at tumor sites. ADEPT was an alternative to traditional antibody–drug conjugates (ADCs), which are constrained by the number of conjugation sites on the antibody. Typically, ADCs deliver only about one to four drug molecules per antibody. By comparison, ADEPT relies on monoclonal antibody (mAb)-mediated delivery of a chemically-conjugated activating enzyme (*i.e.,* a prodrug converting enzyme) to the tumor site, where the antibody binds to a tumor specific antigen. A non-toxic prodrug is then administered systemically, which is cleaved by the enzyme/antibody conjugate locally to synthesize the cytotoxic drug at the site of the

tumor. The ADEPT approach provided several advantages over the traditional ADC, namely scheduled control and stopping of the prodrug, rapid diffusion the small prodrug and drug versus a mAb, enzymatically-driven local increases in drug concentration, and possible killing of antigen loss cancer variants in the vicinity. However, toxicity is still an issue with ADEPT due to persistence of the enzyme/antibody conjugate in the blood. In addition, achieving a good therapeutic index is difficult due the poor tumor-to-blood ratio of the enzyme carried by the antibody. In fact, the ADEPT and ADC approaches have limited efficacy given that about 0.5% or less of the injected dose of antibody is typically found at the site of the solid tumor in a human, and the remaining 99.5% or more is elsewhere in the body.

[0188] In some embodiments, the engineered immune cells provided herein express a T-cell receptor (TCR) or other cell-surface ligand that binds to a target antigen, such as a tumor antigen and a prodrug converting enzyme. In some embodiments, the T cell receptor is a wild-type, or native, T-cell receptor. In some embodiments, the T cell receptor is a chimeric T-cell receptor (CAR).

[0189] In exemplary embodiments provided herein, the engineered immune cells provided herein express a T-cell receptor (TCR) (*e.g.*, a CAR) or other cell-surface ligand that binds to a CD19 tumor antigen. In some embodiments, the engineered immune cells provided herein express a T-cell receptor (TCR) (*e.g.*, a CAR) or other cell-surface ligand that binds to a CD19 tumor antigen presented in the context of an MHC molecule. In some embodiments, binds to a CD19 tumor antigen presented in the context of an HLA-A2 molecule. CD19 is a B cell lineage specific antigen that has been the target of many of the most effective CAR T cells in human trials. CD19 is a model antigen due to its well-characterized activity, pharmacology and toxicity.

[0190] In exemplary embodiments provided herein, the engineered immune cells provided herein express a T-cell receptor (TCR) (*e.g.*, a CAR) or other cell-surface ligand that binds to a "preferentially expressed antigen in melanoma" (PRAME) tumor antigen. In some embodiments, the engineered immune cells provided herein express a T-cell receptor (TCR) (*e.g.*, a CAR) or other cell-surface ligand that binds to a PRAME tumor antigen presented in the context of an MHC molecule. In some embodiments, the PRAME tumor antigen presented in the context of an HLA-A2 molecule. The PRAME protein is a currently

undruggable, retinoic acid receptor binding protein involved in differentiation, proliferation arrest, and apoptosis. PRAME is a cancer-testis antigen that has limited expression in healthy adult tissue restricted to the testes, ovaries, and endometrium. However, PRAME is over-expressed in multiple cancers including breast cancer, colon cancer, acute leukemias (50%), melanomas (90%), lymphomas, sarcomas among others, making it a highly attractive therapeutic target. After proteasomal processing the $PRAME^{300-309}$ peptide (ALYVDSLFFL (SEQ ID NO: 23) is presented on the cell surface in the context of an HLA-I haplotype HLA*A02:01 (HLA-A2).

[0191] In exemplary embodiments provided herein, the engineered immune cells provided herein express a T-cell receptor (TCR) (*e.g.*, a CAR) or other cell-surface ligand that binds to a Wilm's tumor protein 1 (WT1) tumor antigen. In some embodiments, the engineered immune cells provided herein express a T-cell receptor (TCR) (*e.g.*, a CAR) or other cell-surface ligand that binds to a WT1 tumor antigen presented in the context of an MHC molecule. In some embodiments, binds to a WT1 tumor antigen presented in the context of an HLA-A2 molecule. WT1 is an important, validated, and NCI-top ranked, cancer target antigen. WT1 is a zinc finger transcription factor essential to the embryonal development of the urogenital system. WT1 is highly expressed in most leukemias including AML, CML, ALL and MDS as well as in myeloma and several solid tumors, particularly ovarian carcinoma and mesothelioma. WT1 vaccines have advanced into clinical trials for patients with a variety of cancers. WT1 is distinguished by its importance to the survival of clonogenic leukemic cells, and the ability to treat tumors with T-cells specific for WT1 peptides in xenografted NOD/SCID mice, without adversely affecting normal hematopoiesis. WT1 peptide vaccination has been associated with complete or partial remissions of disease and prolonged survival.

[0192] Significant toxicities and even deaths after CAR T cell therapy, and TCR T cell therapy, and the high incidence of GVHD with its associated short- and long-term morbidity and mortality, strongly call for mechanisms to better control toxicities of the various T cell therapies. In some embodiments provided herein, the engineered immune cells provided herein express a prodrug converting enzyme intracellularly (*e.g.*, within the cytoplasm of the engineered immune cell) such that the engineered immune cells will die when a cell penetrating prodrug is administered, where the cell penetrating prodrug is converted to an

active drug by the intracellular prodrug converting enzyme. In some embodiments, the cell penetrating prodrug is specific for the cell type of the engineered immune cell or penetrates a particular cell type. In some embodiments, the cell penetrating prodrug is not specific for any cell type. Other normal cells that do not express the intracellular prodrug converting enzyme will not be affected. Thus, the technology described herein can be used as a "kill switch" or suicide vector to control cell therapies that are causing toxicities, or need to be terminated. An advantage of the present method is that traditional chemotherapeutic drugs can be used to achieve the kill switch effects.

[0193] The engineered immune cells (*e.g.*, CAR T cells) provided herein that express a chimeric antigen receptor in combination with a prodrug converting enzyme expressed on the surface of the cells or secreted provide numerous advantages over the existing ADEPT and ADC technologies. A non-exhaustive list of these advantages includes, for example: 1) The ability of the engineered immune cells (*e.g.*, CAR T cells) to significantly increase the quantity activating enzyme (*e.g.*, 100 times or more) at the tumor site. This is because the engineered immune cells contain nucleic acid encoding the prodrug converting enzyme for expression of numerous copies of the enzyme by the cell. In addition, the engineered immune cells will proliferate extensively (*e.g.*, 100 times or more) when it encounters the tumor specific antigen at the tumor site, thus significantly increasing production of the enzyme. 2) The prodrug can be administered after the engineered immune cells (*e.g.*, CAR T cells) are reaching peak numbers at the target site, which can lower the systemic exposure to the drug and ensure maximal localized conversion of the prodrug. 3) The engineered immune cells (*e.g.*, CAR T cells) can be easily generated by *in vitro* transduction of immune cells with nucleic acid encoding the chimeric antigen and the prodrug converting enzyme. Thus, in contrast to ADEPT or ADC, there is no difficult-to-manufacture chemical conjugate. 4) The engineered immune cells (*e.g.*, CAR T cells) can also have additive or synergistic anti-tumor activity of its own. Further, the activity of the engineered immune cells (*e.g.*, CAR T cells) can be adjusted by selection of co-stimulatory molecules include in the chimeric antigen receptor. 5) Gated conditional expression of the prodrug converting enzyme can be employed to allow better control of toxicity. 6) If the drug-mediated cancer killing only is needed and/or CAR T mediated killing is not desired, the engineered immune cells (*e.g.*, CAR T cells) can be further modified to engineer out the T cell-mediated inflammatory responses (*e.g.*,

cytokine release), which are responsible for much of the toxicity seen in humans. 7) Further, a "kill switch" via intracellular expression of a prodrug converting enzyme can allow selective destruction of the engineered immune cells.

Prodrugs and Prodrug Converting Enzymes

[0194] The engineered immune cells (*e.g.*, CAR T cells) provided herein express at least one prodrug converting enzyme that converts a prodrug into an active drug. In some embodiments, the prodrug converting enzyme is expressed on the surface of the engineered immune cell. In some embodiments, the prodrug converting enzyme is secreted by the engineered immune cell. The enzyme can be any enzyme which is capable of converting a prodrug into an active drug and which is not normally expressed on the surface of a cell (*e.g.*, a mammalian cell, such as a human cell) or released into the circulation. In some embodiments, the engineered immune cells provided herein express a intracellular prodrug converting enzyme (*e.g.* in the cytoplasm of the engineered immune cell). In some embodiments, the intracellular prodrug converting enzyme acts as a "kill switch" allow selective destruction of the engineered immune cells via intracellular conversion of a cell-penetrating prodrug.

[0195] In some embodiments, the engineered immune cells (*e.g.*, CAR T cells) provided herein express a prodrug converting enzyme that is expressed on the surface of the engineered immune cell and an intracellular prodrug converting enzyme. In some embodiments, the engineered immune cells (*e.g.*, CAR T cells) provided herein express a prodrug converting enzyme that is secreted by the engineered immune cell and an intracellular prodrug converting enzyme. In some embodiments, the cell surface or secreted prodrug converting enzyme and the intracellular prodrug converting enzyme have the same enzymatic activity (*e.g.*, the cell surface or secreted prodrug converting enzyme and the intracellular prodrug converting enzyme are the same enzyme). In some embodiments, the cell surface or secreted prodrug converting enzyme and the intracellular prodrug converting enzyme have different enzymatic activities (*e.g.*, the cell surface or secreted prodrug converting enzyme and the intracellular prodrug converting enzyme are different enzymes). Unless specifically distinguished, reference to a prodrug converting enzyme herein is intended to mean either the cell surface or secreted prodrug converting enzyme, the intracellular prodrug converting enzyme, or both.

[0196] In some embodiments, the prodrug converting enzyme will convert the prodrug into an active drug by removing a protecting group from the prodrug. In some embodiments, the protecting group will be cleaved as a whole from the prodrug. In some embodiments, the enzyme cleaves or alters part of the protecting group, resulting in a partially cleaved or altered protecting group which is unstable, resulting in spontaneous removal of the remainder of the group.

[0197] In some embodiments, the prodrug converting enzyme is a non-mammalian enzyme. Suitable non-mammalian enzymes include bacterial enzymes. Bacterial enzymes include carboxypeptidases, such as carboxypeptidase G2 (CPG2), which is a bacterial hydrolase enzyme isolated from *Pseudomonas sp.* RS-16 based on its ability to cleave N-linked glutamate from folate derivatives (*Pseudomonas* γ-glutamylhydrolase EC3.4.22.12, as disclosed in WO88/07378 and Levy and Goldstein, *J. Biol. Chem.* 242:2933 (1967)). CPG2 is specific for cleavage between an aromatic N-acyl moiety and glutamate, and has been used extensively in ADEPT strategies, including several systems that advanced to human clinical trials for cancer. In some embodiments, active drugs can be masked as glutamate prodrugs by coupling to amine or alcohol moieties in the drug (FIG. 2A), via various self-immolative linkers that undergo spontaneous decomposition to inert byproducts after glutamate hydrolysis (FIG. 2B). In certain embodiments, the prodrug converting enzyme is CPG2. In some embodiments, CPG2 comprises the sequence set forth in SEQ ID NO: 1, which is a secreted form of CPG2 including the leader sequence for secretion. In some embodiments, CPG2 comprises the sequence set forth in SEQ ID NO: 3, which is a CPG2 polypeptide without the leader sequence of SEQ ID NO:1 and optimized for human expression. In some embodiments, CPG2 comprises the sequence set forth in SEQ ID NO: 5, which is an exemplary secreted form of CPG2 including the leader sequence for secretion and optimized for human expression. In some embodiments, CPG2 comprises the sequence set forth in SEQ ID NOS: 7 or 40, which are exemplary transmembrane forms of CPG2 optimized for human expression with a CD8 leader sequence (*e.g.*, SEQ ID NO: 11), a transmembrane portion and a CD8 intracellular portion with a disrupted Lyk binding domain (*e.g.*, SEQ ID NO: 15).

[0198] In some embodiments, the prodrug converting enzyme is a β-lactamase, such as an *Enterobacter cloacae* β-lactamase. β-Lactamase (EC 3.5.2.6) is a serine protease produced by various bacteria; it catalyzes the hydrolysis of the β-lactam moiety in penicillin and other

similar β-lactam antibiotics to β-amino acid. β-lactamase is highly selective to the β-lactam containing compounds. Cephalosporin has been widely utilized as the core of β-lactamase-activated prodrugs due to its ability of releasing the drug from C3′ position following the cleavage of β-lactam ring by β-lactamase. In some embodiments, β-Lactamase comprises the sequence set forth in SEQ ID NO: 43, which is a secreted form of β-Lactamase including the leader sequence for secretion. In some embodiments, β-Lactamase comprises the sequence set forth in SEQ ID NO: 44, which is a β-Lactamase polypeptide without the leader sequence of SEQ ID NO:43 and optimized for human expression. In some embodiments, β-Lactamase comprises the sequence set forth in SEQ ID NO: 45, which is an exemplary secreted form of β-Lactamase including the leader sequence for secretion and optimized for human expression. In some embodiments, β-Lactamase comprises the sequence set forth in SEQ ID NOS: 46 or 47, which are exemplary transmembrane forms of β-Lactamase optimized for human expression with a CD8 leader sequence (*e.g.*, SEQ ID NO: 11), a transmembrane portion and optionally, a CD8 intracellular portion with a disrupted Lyk binding domain (*e.g.*, SEQ ID NO: 15).

[0199] Examples of other suitable non-mammalian enzymes include nitroreductases, such as an E.coli nitroreductase as disclosed in WO93/08288, thymidine kinase (tk), including viral tk such as VZV or HSV tk, β-glucosidase, β-glucoronidase, penicillin V amidase, penicillin G amidase and cytosine deaminase

[0200] In some embodiments, the enzyme is a mammalian enzyme which does not naturally occur in a human. In some embodiments, the enzyme is a human enzyme which is not normally accessible to the prodrug, is expressed in limited quantities outside of cells, is expressed in a compartment that is not reached by the prodrug, is expressed by a cell that can be killed by the prodrug activation but is not essential to the patient, and/or is expressed by a cell that is resistant to the active drug or not killed by the active drug. This includes, for example, enzymes from other species as well as mammalian enzymes which are altered in a manner which is selective for the prodrug. In some embodiment, the alteration means that the conversion of the prodrug to an active drug by the natural enzyme will be at a rate one or more orders of magnitude less than the rate at which the altered enzyme operates. Altered enzymes can be made by standard recombinant DNA techniques, *e.g.*, by cloning the enzyme, determining its gene sequence and altering the gene sequence by methods such as site-

directed mutagenesis. In some embodiments, the prodrug converting enzyme is carboxypeptidase A, or a mutant thereof, *e.g.* a T268G mutant of carboxypeptidase A.

[0201] For expression of the secreted prodrug converting enzymes, the transmembrane prodrug converting enzymes, or the intracellular prodrug converting enzymes, eukaryotic based expression systems are employed. For secretion or transmembrane expression, a signal peptide is included at the N-terminus of protein. The signal sequence or leader can be a peptide sequence (about 5, about 10, about 15, about 20, about 25, or about 30 amino acids long) present at the N-terminus of newly synthesized proteins that directs their entry to the secretory pathway. In certain embodiments, the signal peptide is covalently joined to the N-terminus of the prodrug converting enzyme (*e.g.*, a CPG2 enzyme or β-Lactamase). In certain embodiments, the signal peptide comprises a CPG2 signal sequence or a β-Lactamase signal sequence. In certain embodiments, the signal peptide comprises a CPG2 signal sequence comprising amino acids having the sequence set forth in SEQ ID NO: 9 as provided below.

[0202] MRPSIHRTAIAAVLATAFVAGT (SEQ ID NO: 9).

[0203] The nucleotide sequence encoding the amino acid sequence of SEQ ID NO: 9 is set forth in SEQ ID NO: 10, which is provided below:

[0204] atgcgaccgagtatccacagaacagcaatagctgctgtgcttgcaacagcgtttgtagcgggcacg (SEQ ID NO: 10)

[0205] In certain embodiments, the signal peptide comprises a CD8 signal polypeptide comprising amino acids having the sequence set forth in SEQ ID NO: 11 as provided below.

[0206] MALPVTALLLPLALLLHAARP (SEQ ID NO: 11)

[0207] The nucleotide sequence encoding the amino acid sequence of SEQ ID NO: 11 is set forth in SEQ ID NO: 12, which is provided below:

[0208] atggccctgccagtaacggctctgctgctgccacttgctctgctcctccatgcagccaggcct (SEQ ID NO: 12).

[0209] For intracellular expression the expressed prodrug converting enzyme does not comprise a signal peptide. Accordingly, the enzyme is not directed to the endoplasmic reticulum when translated and remains in the cytoplasm.

[0210] For cell surface expression of the prodrug converting enzyme, the expressed protein is anchored to the cell membrane. In some embodiments, the prodrug converting enzyme comprises a transmembrane domain. In some embodiments, the transmembrane

domain is covalently attached to the C-terminus of the CPG2 enzyme or β-lactamase. In some embodiments, the transmembrane domain is covalently attached to the N-terminus of the CPG2 enzyme or β-lactamase. In some embodiments, the transmembrane domain is CD8 transmembrane domain. In some embodiments, the transmembrane domain comprises a transmembrane spanning portion. In some embodiments, the membrane-spanning portion comprises the following sequence:

[0211] IYIWAPLAGTCGVLLLSLVIT (SEQ ID NO: 38)

[0212] In some embodiments, the CD8 transmembrane domain also comprise an intracellular portion having the amino acid sequence: LYCNHRNRRRVCKCPRPVVKSGDKPSLSARYV (SEQ ID NO: 13).

[0213] In some embodiments, the CD8 transmembrane domain comprise an intracellular portion in which the natural Lyk binding domain is disrupted to uncouple the endogenous function of CD8 from the CPG2 or β-lactamase molecule. For example, in some embodiments, the CD8 transmembrane domain portion with disrupted Lyk binding domain has the following sequence: LYCNHRNRRRVGGGRPVVKSGDKPSLSARYV (SEQ ID NO: 15)

[0214] In some embodiments, the transmembrane CPG2 enzyme has the following amino acid sequence, which includes a signal peptide that is removed during processing in the endoplasmic reticulum:

[0215] MALPVTALLLPLALLLHAARPALAQKRDNVLFQAATDEQPAVIKTLEKLV NIETGTGDAEGIAAAGNFLEAELKNLGFTVTRSKSAGLVVGDNIVGKIKGRGGKNLL LMSHMDTVYLKGILAKAPFRVEGDKAYGPGIADDKGGNAVILHTLKLLKEYGVRDY GTITVLFNTDEEKGSFGSRDLIQEEAKLADYVLSFEPTSAGDEKLSLGTSGIAYVQVQI TGKASHAGAAPELGVNALVEASDLVLRTMNIDDKAKNLRFQWTIAKAGQVSNIIPAS ATLNADVRYARNEDFDAAMKTLEERAQQKKLPEADVKVIVTRGRPAFNAGEGGKK LVDKAVAYYKEAGGTLGVEERTGGGTDAAYAALSGKPVIESLGLPGFGYHSDKAEY VDISAIPRRLYMAARLIMDLGAGKYPYDVPDYAGGGIYIWAPLAGTCGVLLLSLVIT LYCNHRNRRRVGGGRPVVKSGDKPSLSARYV (SEQ ID NO: 40).

[0216] In some alternative embodiments, a construct for expression of a CPG2 enzyme comprises a CPG2 without a signal peptide.

[0217] In some embodiments, the transmembrane β-lactamase enzyme has the following amino acid sequence, which includes a signal peptide that is removed during processing in the endoplasmic reticulum:

[0218] MALPVTALLLPLALLLHAARPTPVSEKQLAEVVANTITPLMAAQSVPGMA VAVIYQGKPHYYTFGKADIAANKPVTPQTLFELGSISKTFTGVLGGDAIARGEISLDD AVTRYWPQLTGKQWQGIRMLDLATYTAGGLPLQVPDEVTDNASLLRFYQNWQPQ WKPGTTRLYANASIGLFGALAVKPSGMPYEQAMTTRVLKPLKLDHTWINVPKAEEA HYAWGYRDGKAVRVSPGMLDAQAYGVKTNVQDMANWVMANMAPENVADASLK QGIALAQSRYWRIGSMYQGLGWEMLNWPVEANTVVEGSDSKVALAPLPVAEVNPP APPVKASWVHKTGSTGGFGAYVAFIPEKQIGIVMLANTSYPNPARVEAAYHILEALQ YPYDVPDYAGGGLYCNHRNRRRVCKCPRPVVKSGDKPSLSARYV (SEQ ID NO: 47).

[0219] In some alternative embodiments, a construct for expression of a β-lactamase enzyme comprises a β-lactamase without a signal peptide.

[0220] In some embodiments, the CPG2 or β-lactamase enzyme is attached to the cell surface via a glycosylphosphatidylinositol (GPI)-linker. Glypiated (GPI-linked) proteins contain a cleavable, hydrophobic amino-terminal signal sequence that targets the protein to the lumen of the endoplasmic reticulum (ER) and a cleavable, carboxy-terminal signal sequence that directs GPI anchoring. The GPI-anchoring signal consists of a hydrophobic region separated from the GPI-attachment site (ω-site) by a hydrophilic spacer region. (*See e.g.* Galian *et al.* (2012) *J Biol Chem.* 11; 287(20): 16399–16409).

[0221] Because the expressed secreted or transmembrane prodrug converting enzymes are processed through the Golgi apparatus and endoplasmic reticulum, they can become glycosylated, which may lead in a reduction in activity of the enzyme compared to its non-glycosylated form. Accordingly, in some embodiments, prodrug converting enzymes is altered from its native sequence by substitution, deletion or insertion at one or more (*e.g.*, two, three or four) glycosylation sites. For example, within the primary amino acid sequence of CPG2, there are three such consensus glycosylation motifs, located at residues Asn 222, Asn 264 and Asn 272 of SEQ ID NO: 1. In some embodiments, one or more of these glycosylation sites is altered to remove the glycosylation site. In some embodiments, one or more of Asn 222, Asn 264 and Asn 272 is removed. In some embodiments, one or more of

Asn 222, Asn 264 and Asn 272 is substituted with leucine or glutamine (*see*, *e.g.*, SEQ ID NOS: 3, 5, 7, and 40 which represent exemplary CPG2 polypeptides for expression).

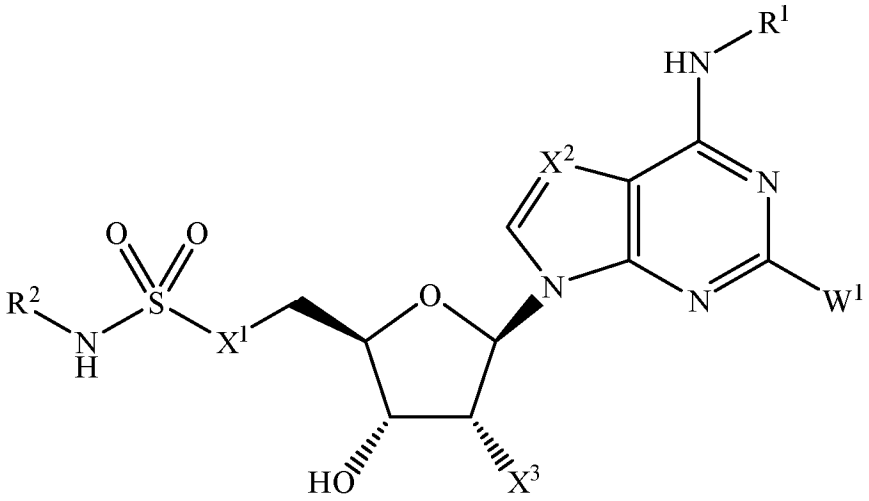
[0222] In some embodiments, at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more glycosylation sites in the prodrug converting enzyme are substituted. In some embodiments, 1, 2, 3, 4, 5 or more amino acids are deleted or inserted at or near the consensus glycosylation site. Typically, the alteration will be such that the enzyme retains its ability to convert a prodrug to an active drug at substantially the same rate as the unchanged, non-glycosylated enzyme. In this context, "substantially unchanged," is within 1 order of magnitude, such as from about 2-fold less activity to 2, 3, 4, 5, 6, 7, 8, 9, or 10 10-fold or more activity.

[0223] In some embodiments, the enzyme is altered by truncation, substitution, deletion or insertion relative to its native form so as long as the activity of the enzyme is substantially unchanged as defined above. For example, in some embodiments small truncations in the N- and/or C-terminal sequence (*e.g.*, about 1- to about 20 amino acids) relative to the native full-length sequence are employed. In some embodiments, such truncations are needed to link the polypeptide to the various other signal sequences or peptides as described herein. The activity of the altered enzyme can be measured in suitable model systems which can be prepared in routine ways known in the art.

[0224] A prodrug for use in the system is selected to be compatible with the prodrug converting enzyme, *i.e.*, such that the enzyme will be capable of converting the prodrug into an active drug. In some embodiments, the toxicity of the prodrug to the patient being treated will be at least one order of magnitude less toxic to the patient than the active drug. In some embodiments, the active drug is several, *e.g.*, 2, 3, 4, 5, or more orders of magnitude more toxic. Suitable prodrugs include prodrugs of cytotoxic cancer chemotherapeutic drugs including, but not limited to, nitrogen mustards, methotrexate, doxorubicin, nucleosides, AMS, targeted cytotoxic cancer therapies, such as erlotinib, dasatinib, tk inhibitors, and other compounds such as those described in WO88/07378, WO89/10140, WO90/02729, WO91/03460, EP-A-540 263, WO94/02450, WO95/02420 or WO95/03830, which are incorporated herein by reference. Additionally, prodrug forms can be made for regulatory drugs that affect cell function (*e.g.,* modulators of HDAC and methylation) to upregulate proteins and antigen presentation machinery (*e.g.,* HLA, Beta-2 microglobulin, TAP, peptide epitopes, proteasome) or to allow immune mediated drugs to improve cancer cell killing (*e.g.*,

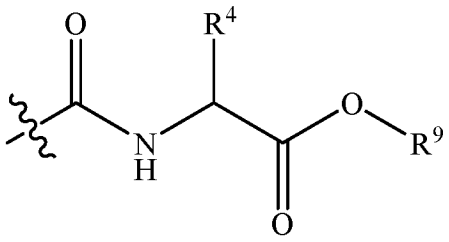
checkpoint blockade drug, CAR T cells or other adoptively administered cells, or antibodies), HSP inhibitors that would improve cancer cell killing by other cytotoxic drugs, and MDR inhibitors that would make a resistant cancer cell sensitive to a cancer therapeutic to which it was resistant. In some embodiments, where the prodrug converting enzyme is CPG2, the prodrug is a CPG2-labile glutamate prodrug. In some embodiments, where the prodrug converting enzyme is β-lactamase, the prodrug is a β-lactamase-labile β-lactam prodrug. In certain embodiments, where the prodrug converting enzyme is expressed intracellularly (*e.g.*, "kill switch" prodrug converting enzyme), the prodrug is able to be taken up by the cell (*e.g.*, is membrane permeable). Accordingly, in some embodiments, the prodrug is converted into a cytotoxic drug intracellularly.

[0225] Exemplary prodrugs include a compound of Formula I

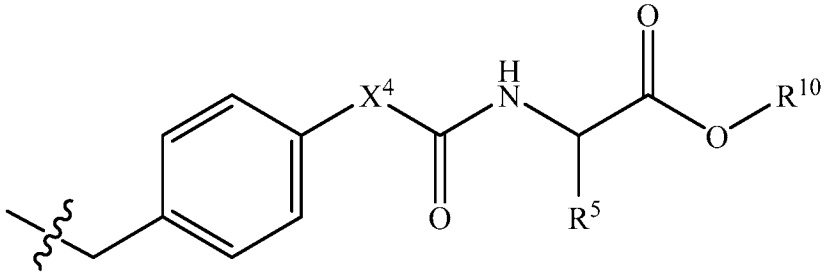


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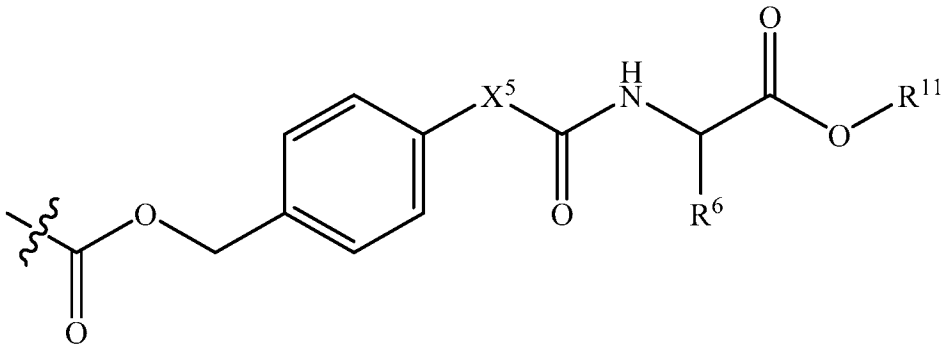
or a zwitterion, pharmaceutically acceptable salt, and/or solvate thereof, where X^1 is O or NH; X^2 is N or CH; X^3 is OH or H; W^1 is H or NH-R^3; two of R^1 ,R^2, and R^3 are H and the remaining R^1 ,R^2, and R^3 is



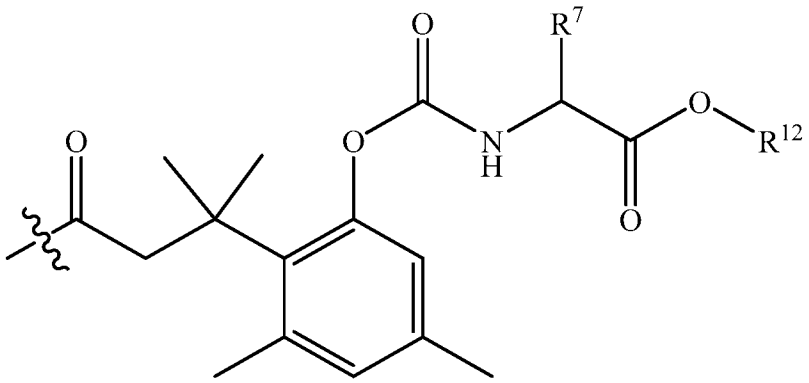
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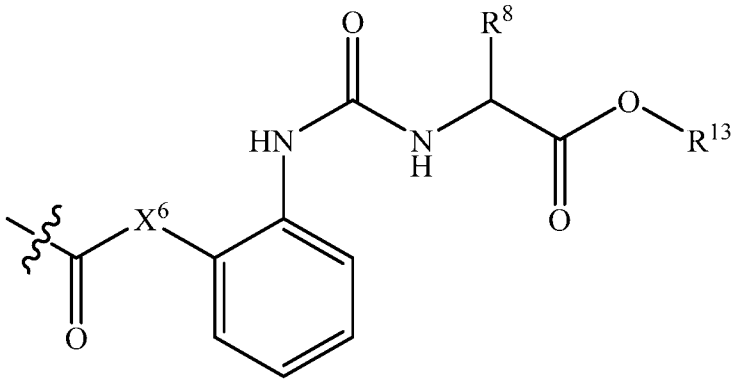
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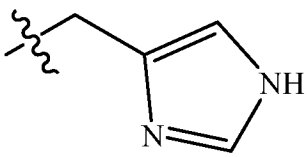
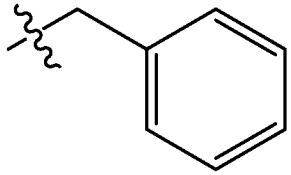


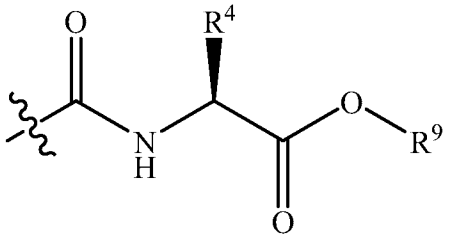
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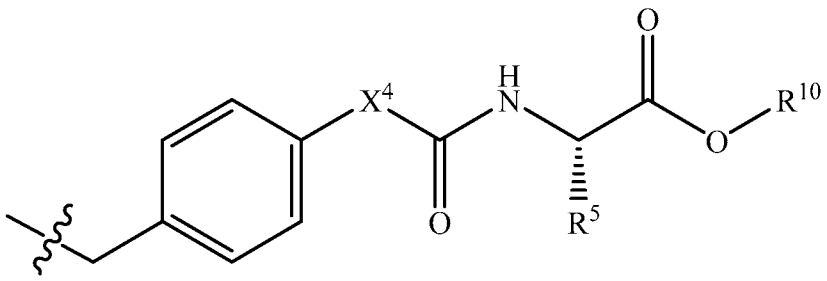


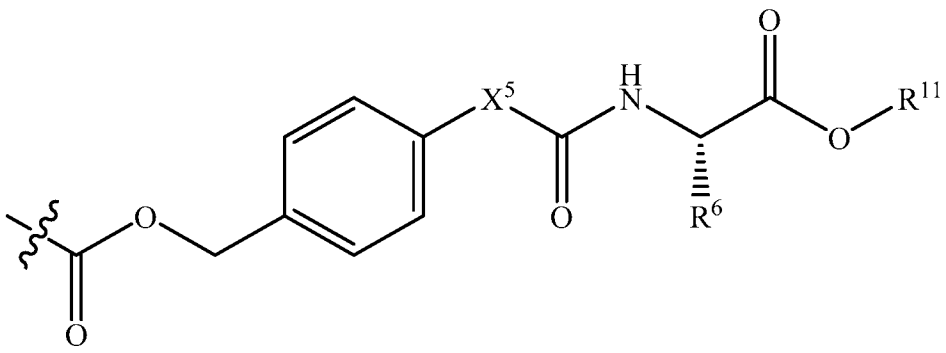
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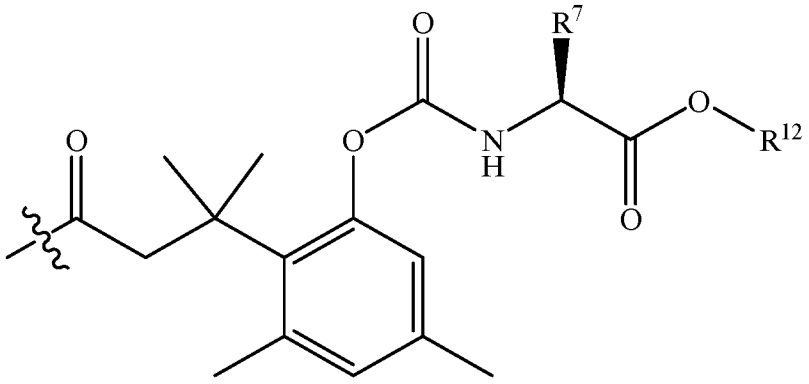
X^4 and X^5 are each independently O or NH; X^6 is O, NH, CH_2, or $C(Me)_2$; R^4, R^5, R^6, R^7, and R^8 are each independently H, $-(CH_2)_3-NH(NH)-NH_2$, $-(CH_2)_3-NH_2$, $-(CH_2)_4-NH_2$, $-CH_2-$

C(O)OR^{13a}, -(CH$_2$)$_2$-C(O)OR^{13b}, , or ; and R^9, R^{10}, R^{11}, R^{12}, R^{13}, R^{13a}, and R^{13b} are each independently H, alkyl, aryl, heteroaryl, aralkyl, heteroaralkyl, -CH$_2$-OC(O)-alkyl, -CH$_2$-OC(O)-aryl, -CH$_2$-OC(O)-heteroaryl, -CH$_2$-OC(O)-aralkyl, or -CH$_2$-OC(O)-heteroaralkyl. In particular, it may be that two of R^1 ,R^2, and R^3 are H and the remaining R^1 ,R^2, and R^3 is

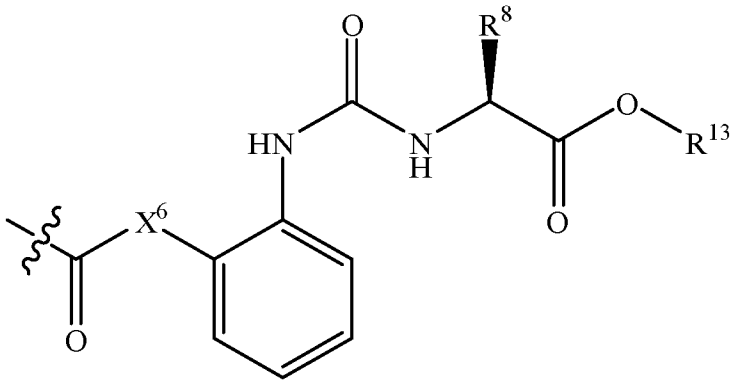
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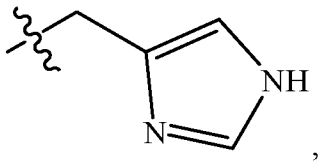
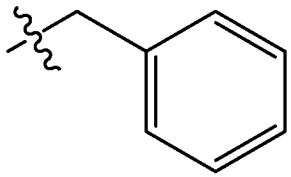
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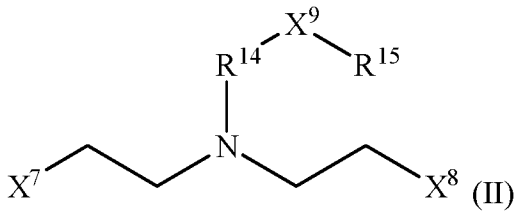


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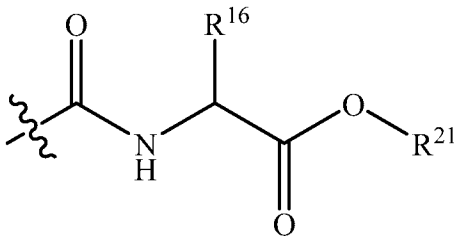
; and R^4, R^5, R^6, R^7, and R^8 are each independently -$(CH_2)_3$-NH(NH)-NH_2, -$(CH_2)_3$-NH_2, -$(CH_2)_4$-NH_2, -CH_2-C(O)OR^{13a}, -$(CH_2)_2$-C(O)OR^{13b}, , or . In any embodiment herein, it may be that R^9, R^{10}, R^{11}, R^{12}, R^{13}, R^{13a}, and R^{13b} are each independently neopentyl or –CH_2-OC(O)-*tert*-butyl.

[0226] Exemplary prodrugs useful in the present technology also include a compound of Formula II

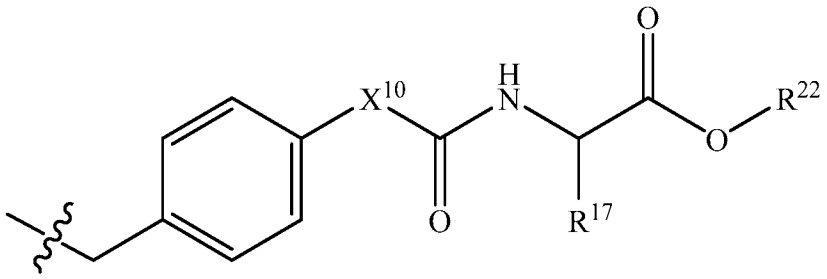


(II)

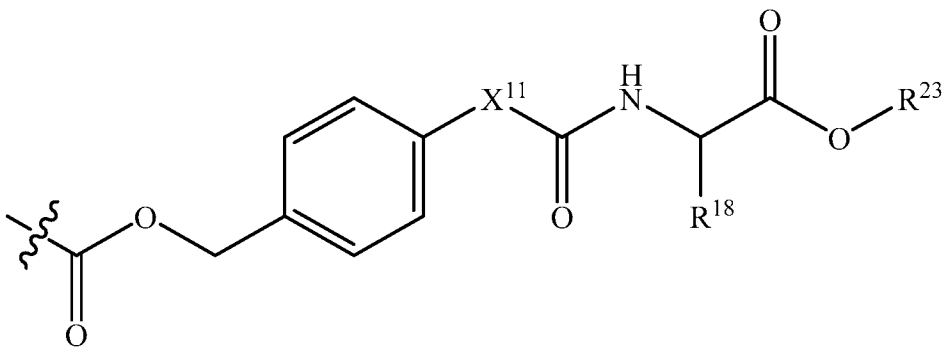
or a zwitterion, pharmaceutically acceptable salt, and/or solvate thereof, where X^7 and X^8 are each independently Cl, Br, I, O-S(O)$_2$$CH_3$, or O-S(O)$_2$-tolyl; X^9 is –C(O)-O, -C(O)-NH, O, or NH; R^{14} is a bond, alkylene, arylene, aralkylene, heteroarylene, or heteroaralkylene; R^{15} is



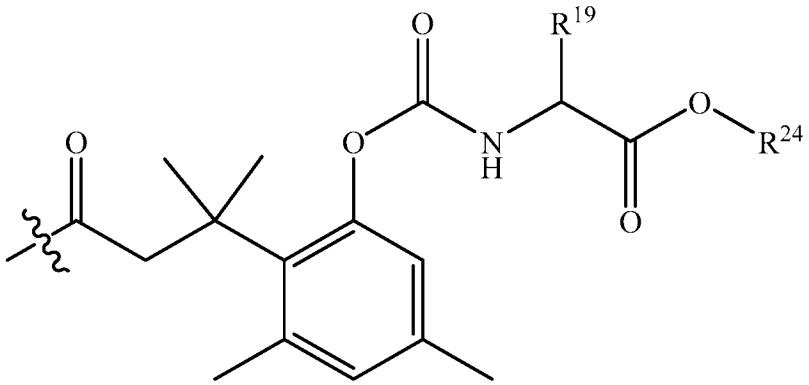
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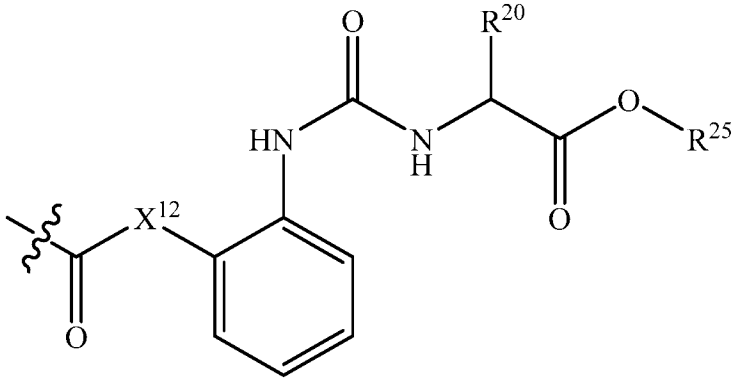
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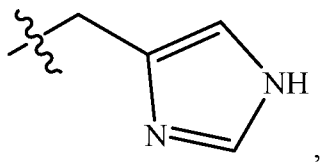


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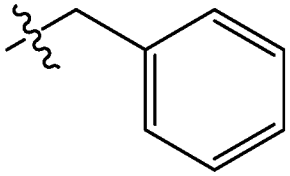


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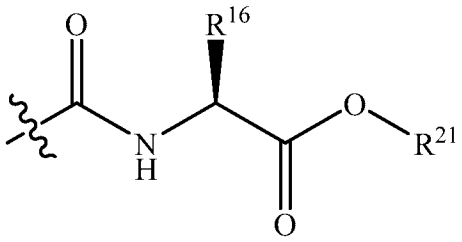
X^{10} and X^{11} are each independently O or NH; X^6 is O, NH, CH_2, or $C(Me)_2$; X^{12} is O, NH, CH_2, or $C(Me)_2$; R^{16}, R^{17}, R^{18}, R^{19}, and R^{20} are each independently H, $-(CH_2)_3-NH(NH)-NH_2$, $-(CH_2)_3-NH_2$, $-(CH_2)_4-NH_2$, $-CH_2-C(O)OR^{25a}$, $-(CH_2)_2-C(O)OR^{25b}$,



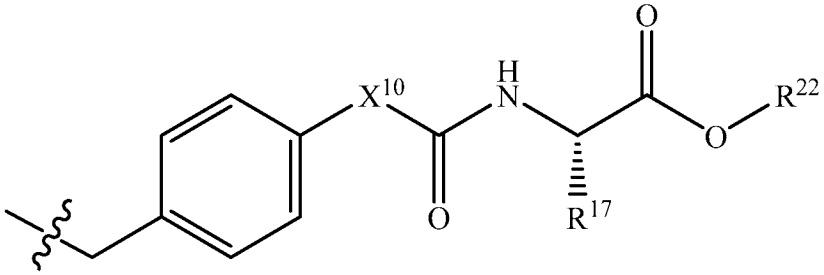
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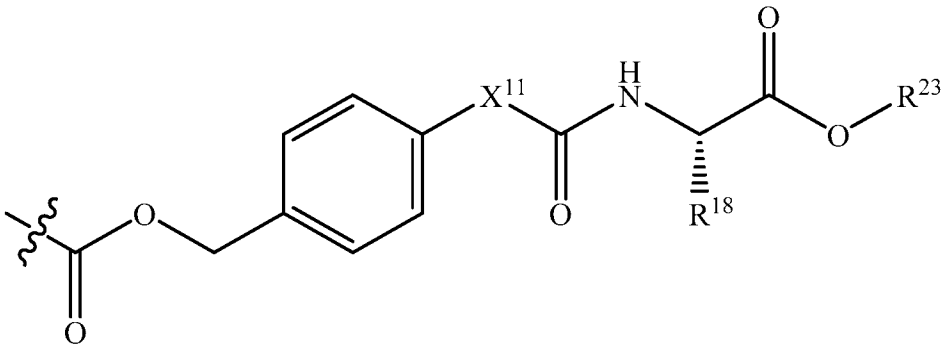
or ; and R^{21}, R^{22}, R^{23}, R^{24}, R^{25}, R^{25a}, and R^{25b} are each independently H, alkyl, aryl, heteroaryl, aralkyl, heteroaralkyl, $-CH_2-OC(O)$-alkyl, $-CH_2-OC(O)$-aryl, $-CH_2-OC(O)$-heteroaryl, $-CH_2-OC(O)$-aralkyl, or $-CH_2-OC(O)$-heteroaralkyl. In particular, R^{15} may be



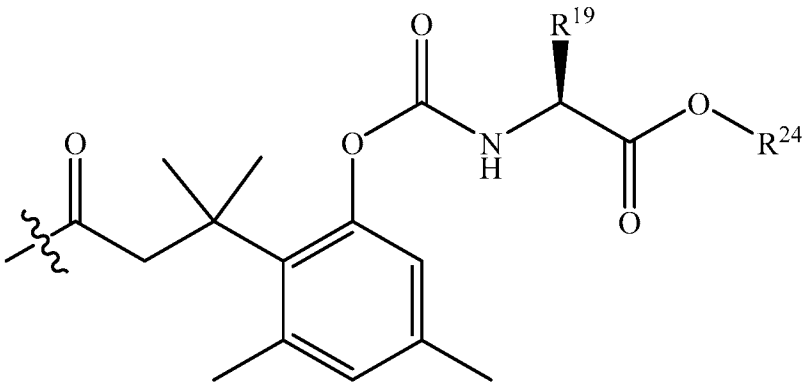
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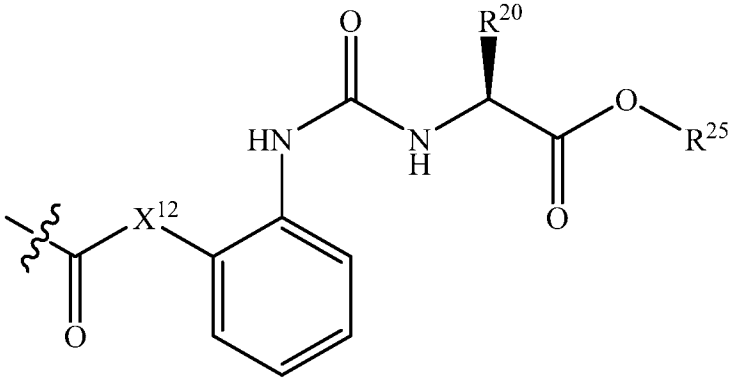
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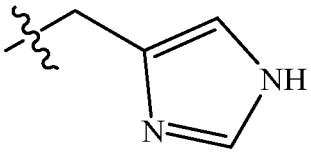
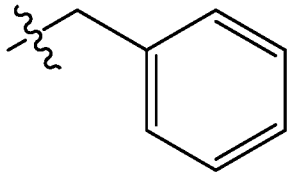
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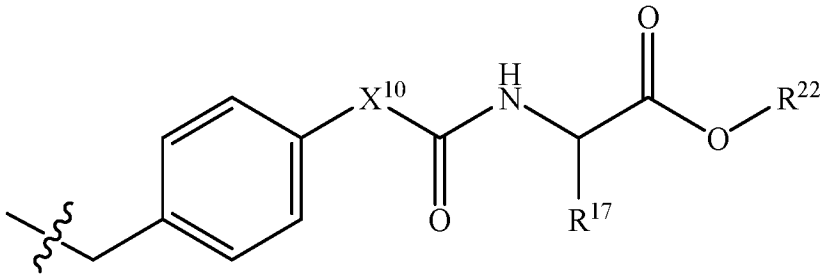


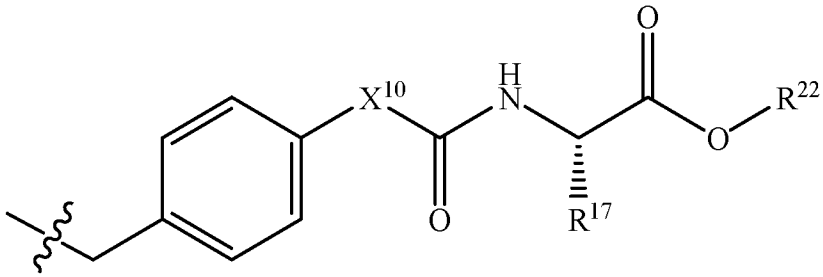
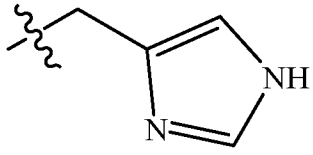
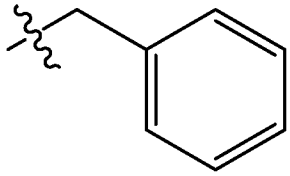
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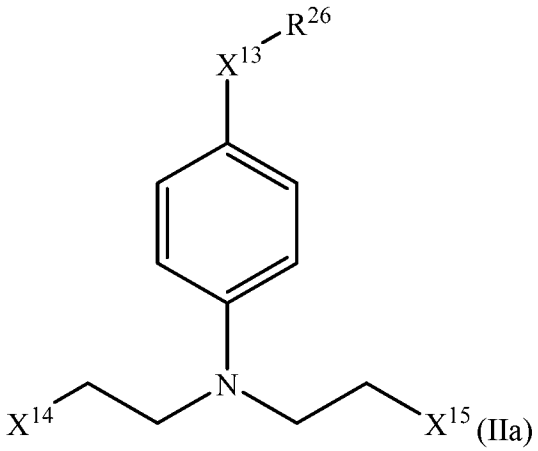
, where R^{16}, R^{17}, R^{18}, R^{19}, and R^{20} are each independently $-(CH_2)_3-NH(NH)-NH_2$, $-(CH_2)_3-NH_2$, $-(CH_2)_4-NH_2$, $-CH_2-C(O)OR^{25a}$,

-$(CH_2)_2$-C(O)OR^{25b},  , or  . In any embodiment herein, it may be that R^{21}, R^{22}, R^{23}, R^{24}, R^{25}, R^{25a}, and R^{25b} are each independently neopentyl or –CH_2-OC(O)-*tert*-butyl. In any embodiment herein, it may be that when X^9 is –C(O)-O, R^{15} is

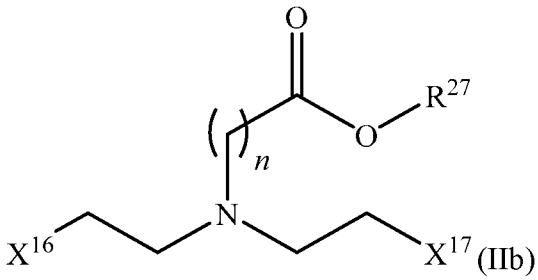
 . In any embodiment herein, it may be that when X^9 is –C(O)-O, R^{15} is

 , and R^{17} and R^{20} are each independently -$(CH_2)_3$-NH(NH)-NH_2, -$(CH_2)_3$-NH_2, -$(CH_2)_4$-NH_2, -CH_2-C(O)OR^{25a}, -$(CH_2)_2$-C(O)O R^{25b},  , or  .

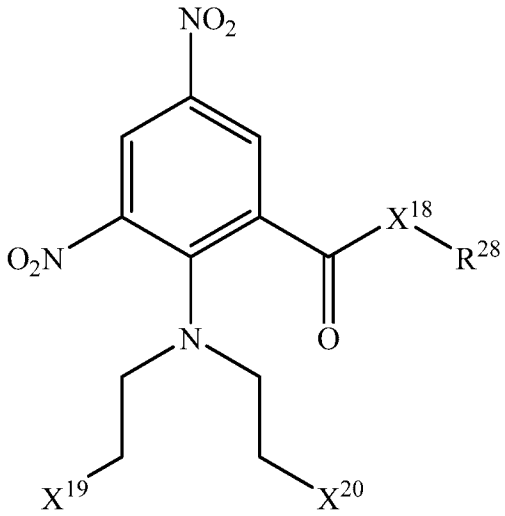
[0227] The prodrug compound of Formula II may be a compound of any one of Formulas IIa-IIe



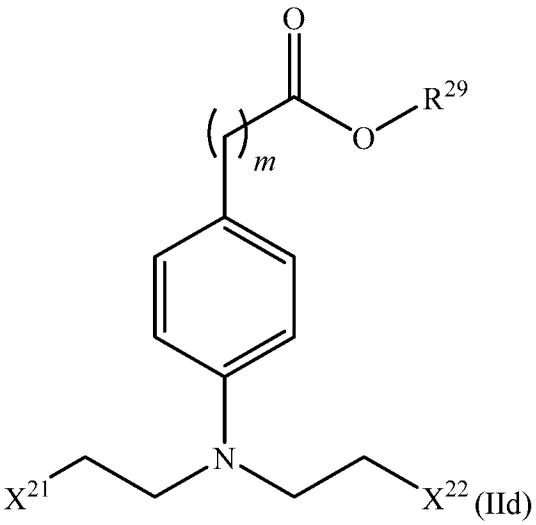
(IIa)



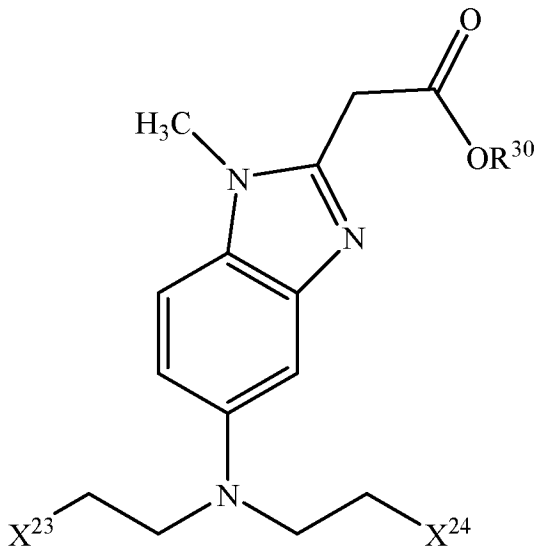
(IIb)



(IIc)

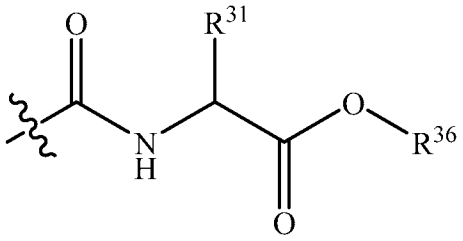


(IId)

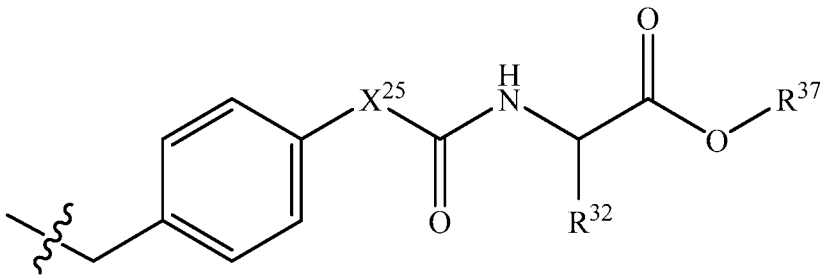


(IIe)

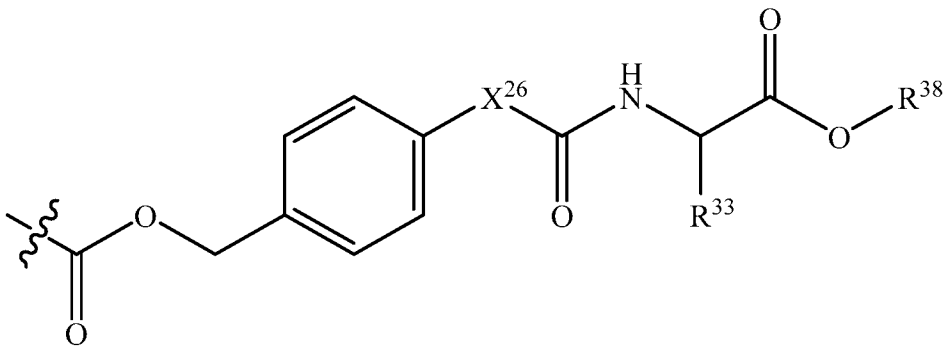
or a zwitterion, pharmaceutically acceptable salt, and/or solvate thereof, where X^{13} and X^{18} are each independently O or NH; X^{14}, X^{15}, X^{16}, X^{17}, X^{19}, X^{20}, X^{21}, X^{22}, X^{23} and X^{24} are each independently Cl, Br, I, O-$S(O)_2CH_3$, or O-$S(O)_2$-tolyl; *n* and *m* are each independently 0, 1, 2, 3, 4, or 5; R^{26}, R^{27}, R^{28}, R^{29}, and R^{30} are each independently



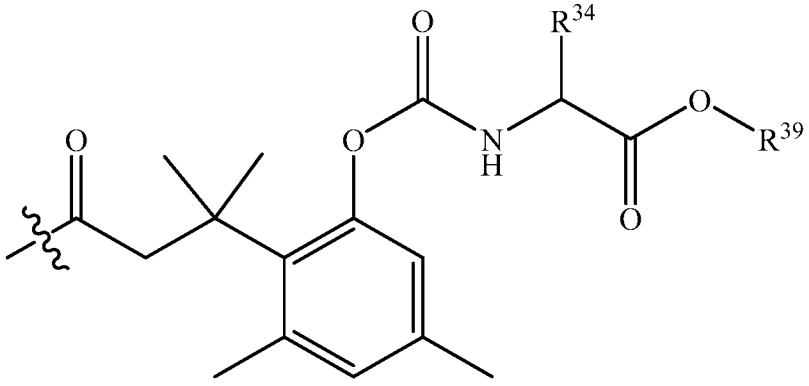
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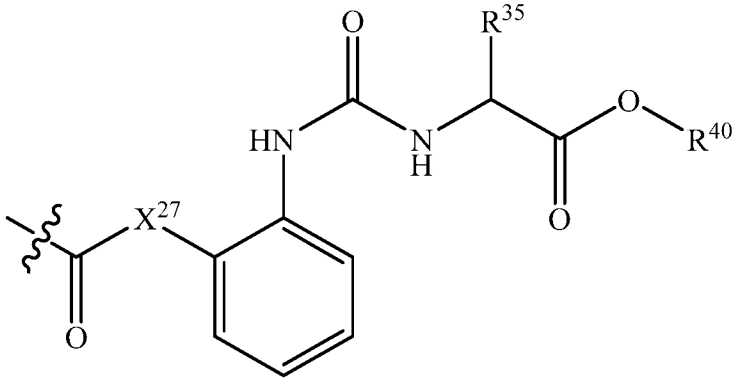
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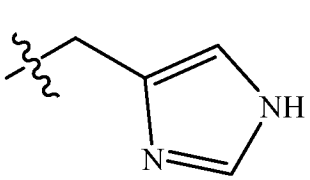
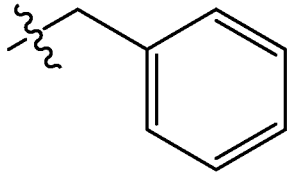


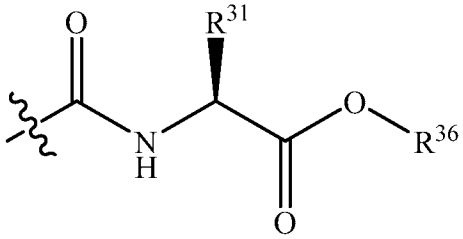
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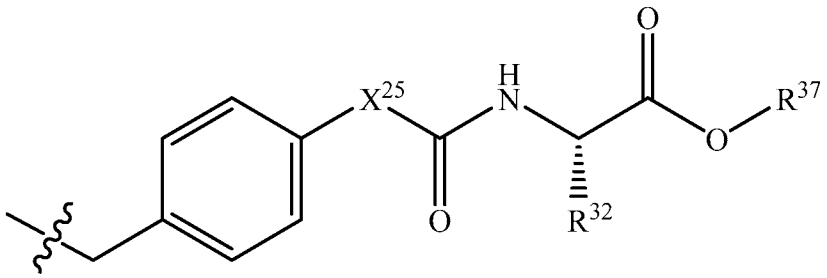
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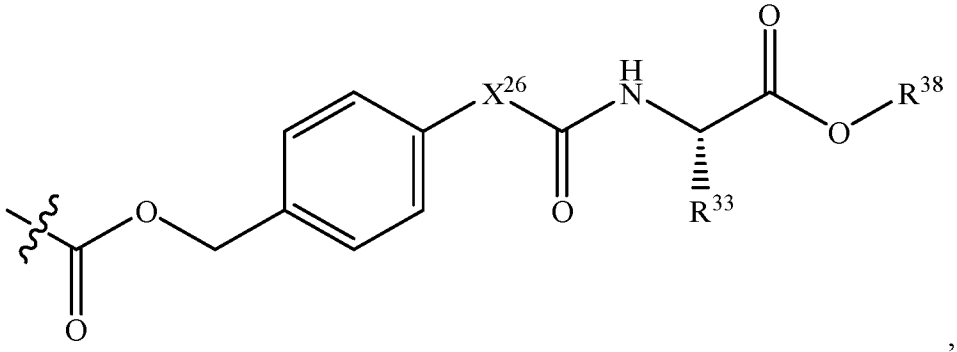
, X^{25} and X^{26} are each independently O or NH; X^{27} is O, NH, CH_2, or $C(Me)_2$; R^{31}, R^{32}, R^{33}, R^{34}, and R^{35} are each independently H, $-(CH_2)_3-NH(NH)-NH_2$, $-(CH_2)_3-NH_2$, $-(CH_2)_4-NH_2$, $-CH_2-C(O)OR^{40a}$, $-(CH_2)_2-C(O)OR^{40b}$, , or ; and R^{36}, R^{37}, R^{38}, R^{39}, R^{40}, R^{40a}, and R^{40b} are each independently H, alkyl, aryl, heteroaryl, aralkyl, heteroaralkyl, $-CH_2-OC(O)$-alkyl, $-CH_2-OC(O)$-aryl, $-CH_2-OC(O)$-heteroaryl, $-CH_2-OC(O)$-aralkyl, or $-CH_2-OC(O)$-heteroaralkyl. In any embodiment herein, R^{26}, R^{27}, R^{28}, R^{29}, and R^{30} may each independently be



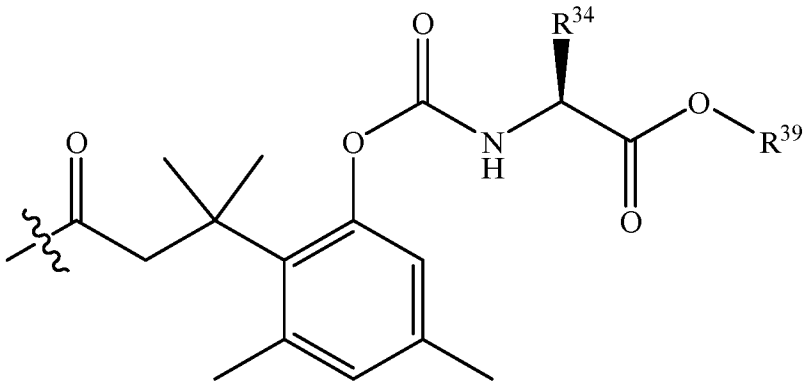
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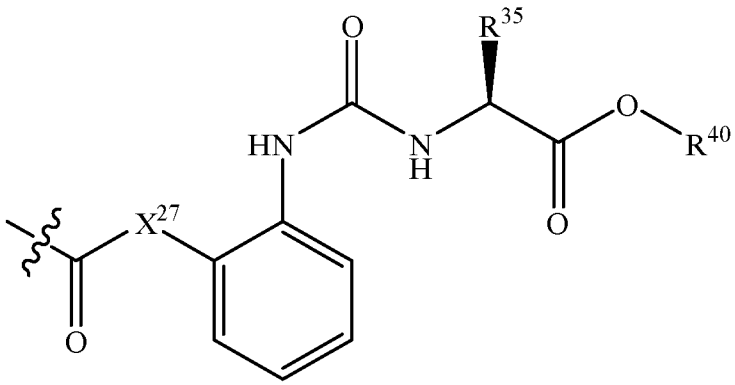
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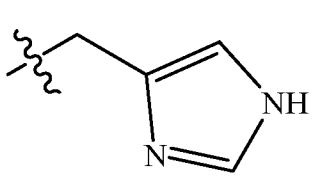
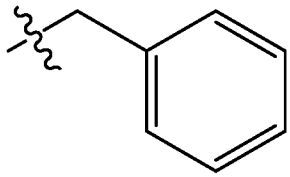


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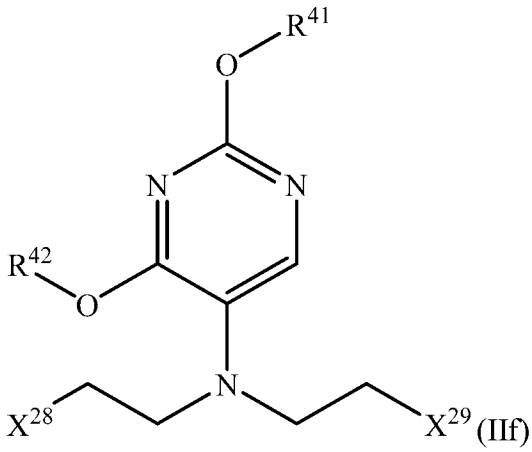
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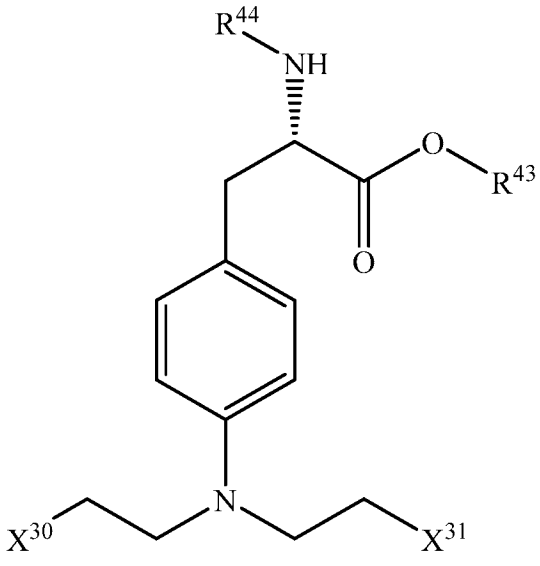
, where R^{31}, R^{32}, R^{33}, R^{34}, and R^{35} are each independently $-(CH_2)_3-NH(NH)-NH_2$, $-(CH_2)_3-NH_2$, $-(CH_2)_4-NH_2$, $-CH_2-C(O)OR^{40a}$, $-(CH_2)_2-C(O)O\,R^{40b}$, , or . In any embodiment herein, it may be that R^{36}, R^{37}, R^{38}, R^{39}, R^{40}, R^{40a}, and R^{40b} are each independently neopentyl or

$-CH_2-OC(O)$-*tert*-butyl.

[0228] The prodrug compound of Formula II may be a compound of Formula IIf or Formula IIg

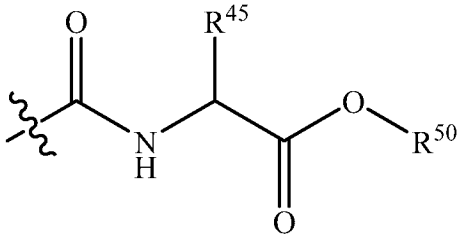


(IIf)

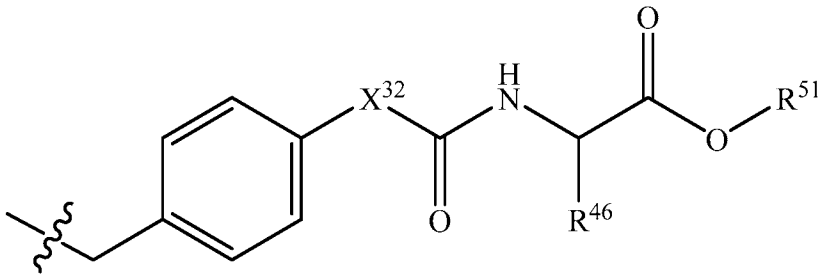


(IIg)

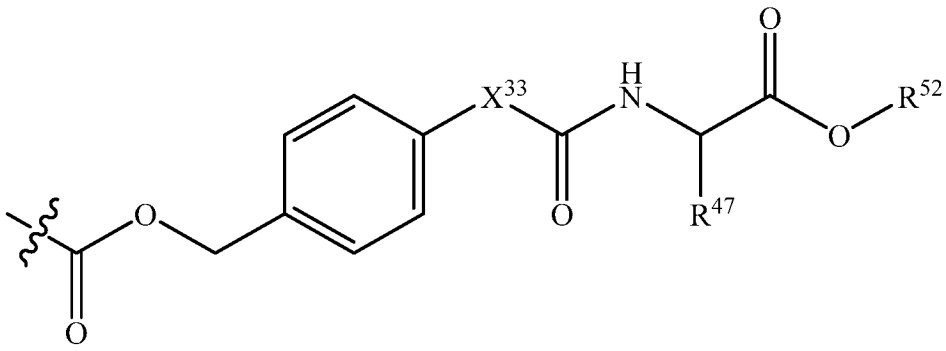
or a zwitterion, pharmaceutically acceptable salt, and/or solvate thereof, where; X^{28}, X^{29}, X^{30}, and X^{31} are each independently Cl, Br, I, O-S(O)$_2$CH$_3$, or O-S(O)$_2$-tolyl; one of R^{41} and R^{42} is H and one of R^{43} and R^{44} is H and the remaining R^{41} and R^{42} (for Formula IIf) and R^{43} and R^{44} (for Formula IIg) are each independently



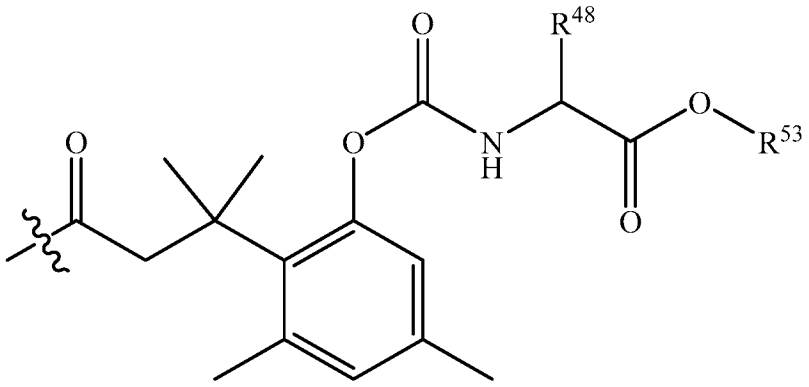
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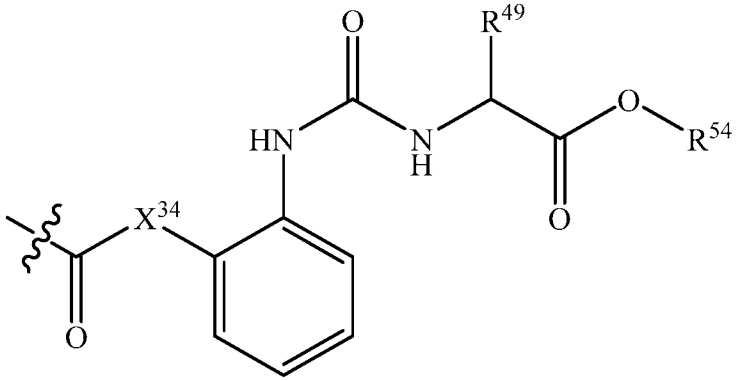
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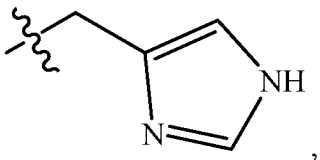
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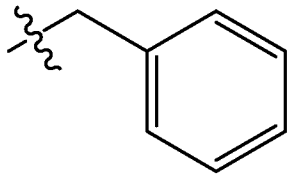
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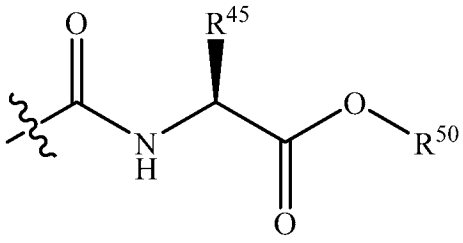
, where X^{32} and X^{33} are each independently O or NH; X^{34} is O, NH, CH_2, or $C(Me)_2$; R^{45}, R^{46}, R^{47}, R^{48}, and R^{49} are each independently H, $-(CH_2)_3-NH(NH)-NH_2$, $-(CH_2)_3-NH_2$, $-(CH_2)_4-NH_2$, $-CH_2-C(O)OR^{54a}$, $-(CH_2)_2-C(O)OR^{54b}$,



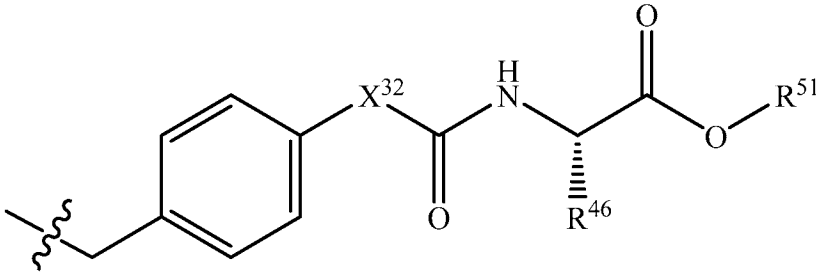
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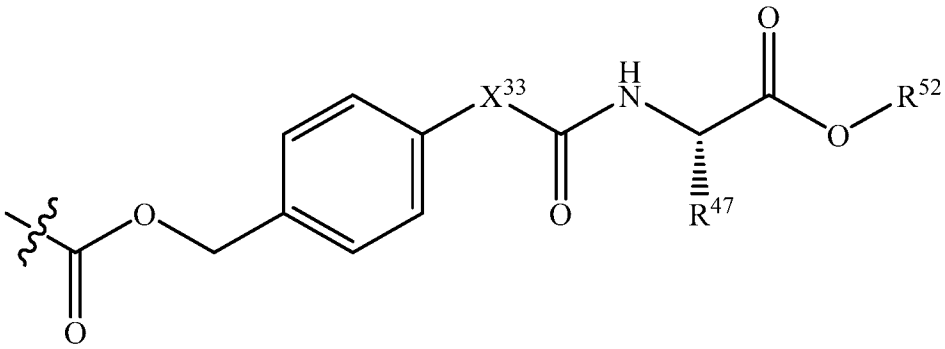
; and R^{50}, R^{51}, R^{52}, R^{53}, R^{54}, R^{54a}, and R^{54b} are each independently H, alkyl, aryl, heteroaryl, aralkyl, heteroaralkyl, $-CH_2-OC(O)$-alkyl, $-CH_2-OC(O)$-aryl, $-CH_2-OC(O)$-heteroaryl, $-CH_2-OC(O)$-aralkyl, or $-CH_2-OC(O)$-heteroaralkyl. In any embodiment herein, it may be that one of R^{41} and R^{42} is H and one of R^{43} and R^{44} is H and the remaining R^{41} and R^{42} (for Formula IIf) and R^{43} and R^{44} (for Formula IIg) are each independently



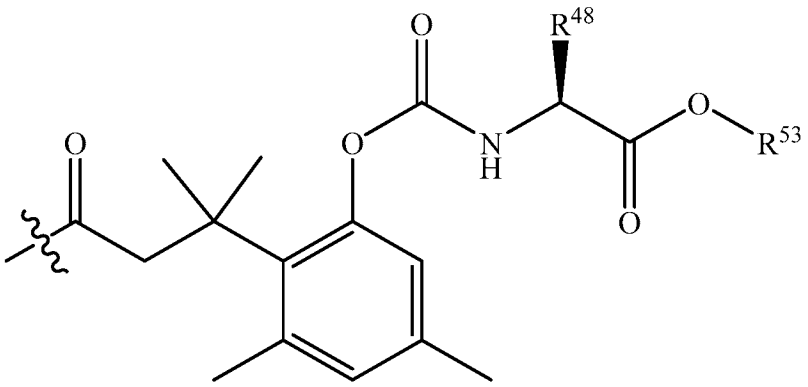
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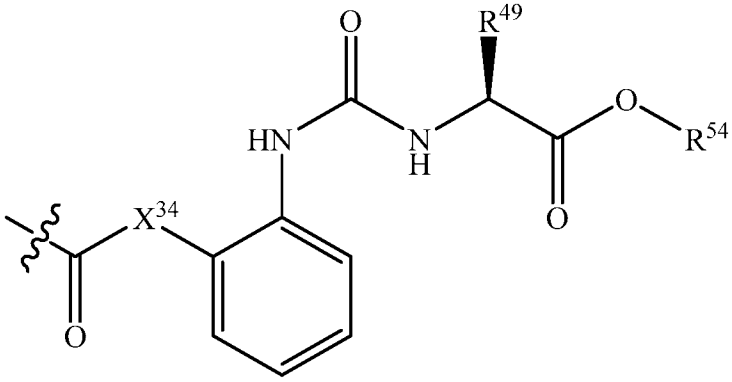
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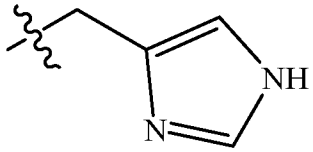
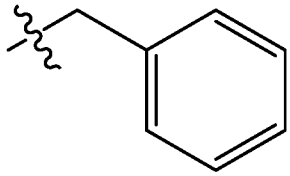
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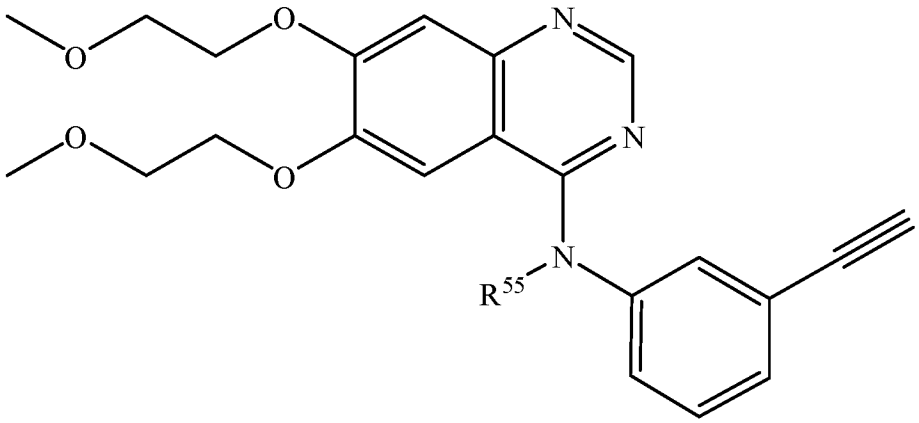


, where R^{45}, R^{46}, R^{47}, R^{48}, and R^{49} are each independently $-(CH_2)_3-NH(NH)-NH_2$, $-(CH_2)_3-NH_2$, $-(CH_2)_4-NH_2$, $-CH_2-C(O)OR^{54a}$,

-$(CH_2)_2$-C(O)O R^{54b}, , or . In any embodiment herein, it may be that R^{50}, R^{51}, R^{52}, R^{53}, R^{54}, R^{54a}, and R^{54b} are each independently neopentyl or

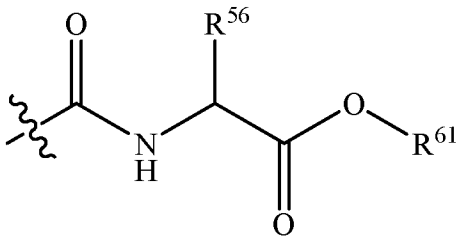
–CH_2-OC(O)-*tert*-butyl.

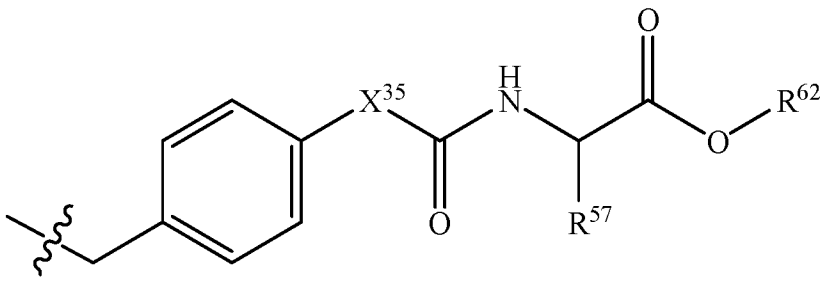
[0229] Exemplary prodrugs useful in the present technology further include prodrugs of erlotinib, such as compounds of Formula III

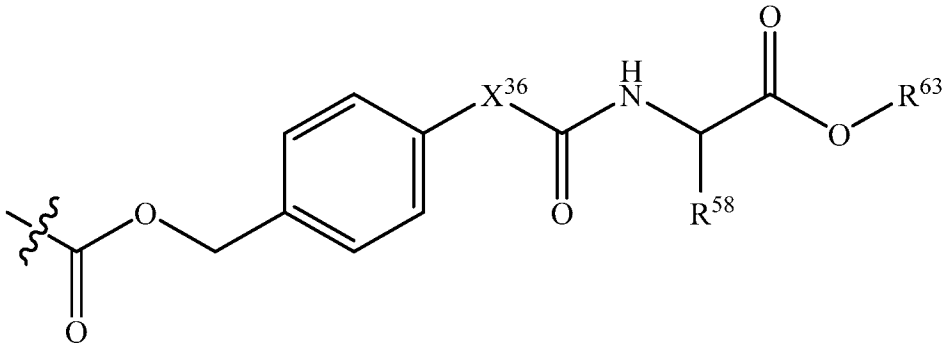


(III)

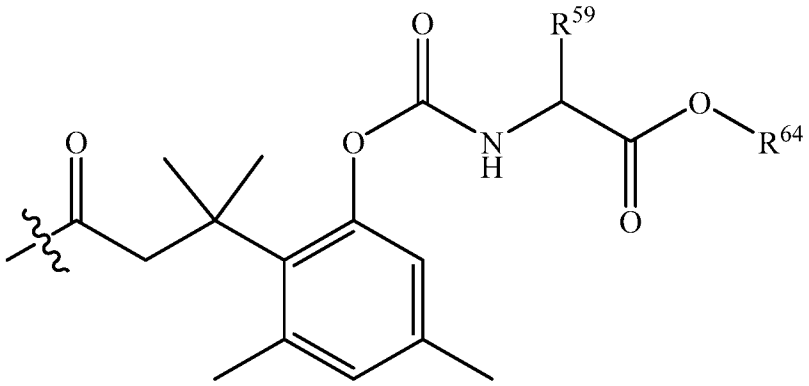
or a zwitterion, pharmaceutically acceptable salt, and/or solvate thereof, where R^{55} is

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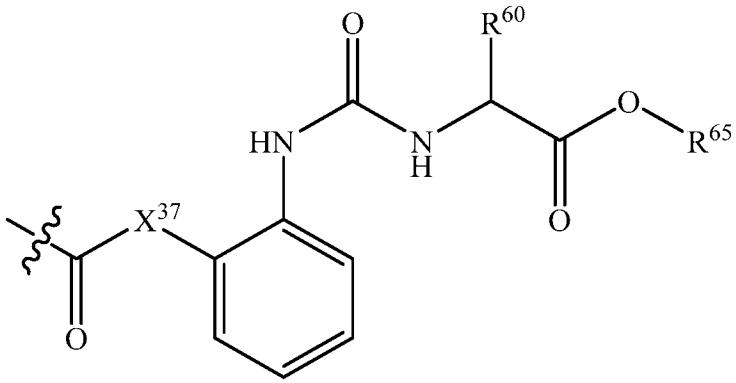
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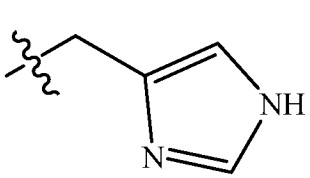
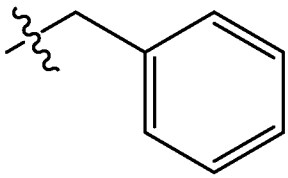


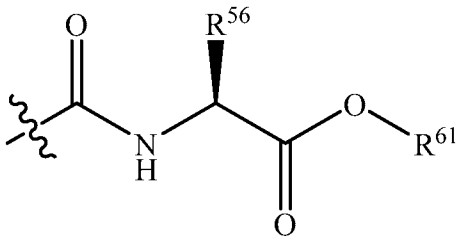
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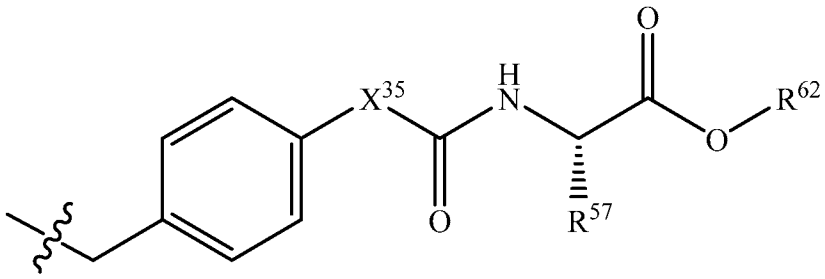
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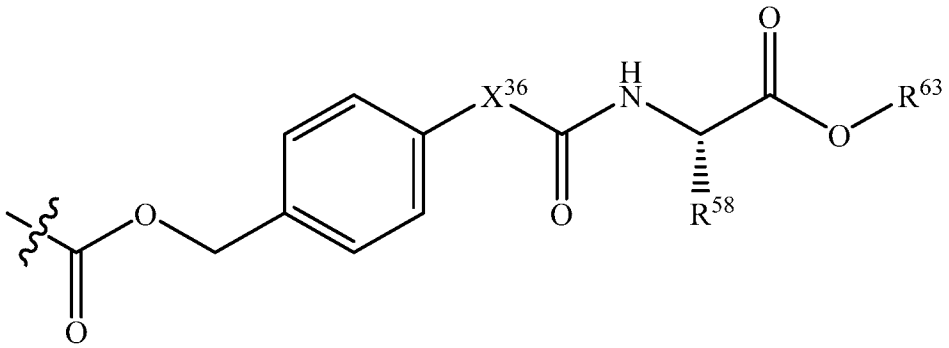
, where X^{35} and X^{36} are each independently O or NH; X^{37} is O, NH, CH_2, or $C(Me)_2$; R^{56}, R^{57}, R^{58}, R^{59}, and R^{60} are each independently H, $-(CH_2)_3-NH(NH)-NH_2$, $-(CH_2)_3-NH_2$, $-(CH_2)_4-NH_2$, $-CH_2-C(O)OR^{65a}$, $-(CH_2)_2-C(O)OR^{65b}$, , or ; and R^{61}, R^{62}, R^{63}, R^{64}, R^{65}, R^{65a}, and R^{65b} are each independently H, alkyl, aryl, heteroaryl, aralkyl, heteroaralkyl, $-CH_2-OC(O)-$alkyl, $-CH_2-OC(O)-$aryl, $-CH_2-OC(O)-$heteroaryl, $-CH_2-OC(O)-$aralkyl, or $-CH_2-OC(O)-$heteroaralkyl. In particular, R^{55} may be



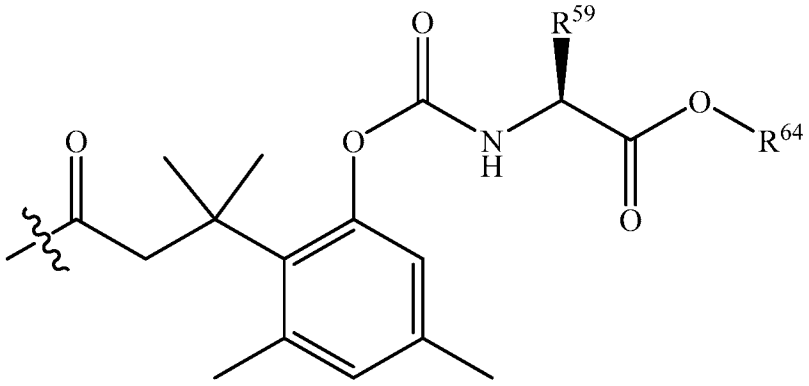
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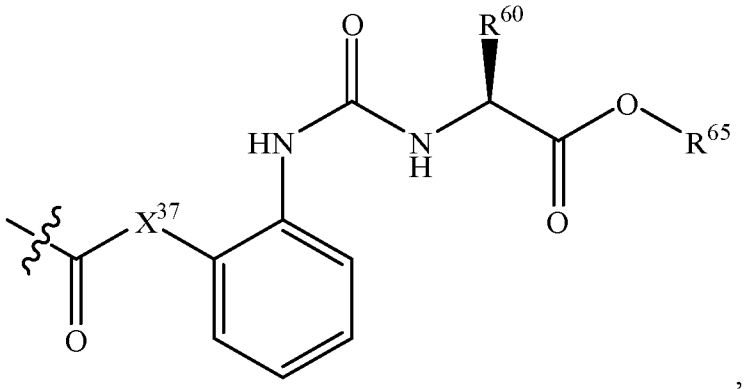
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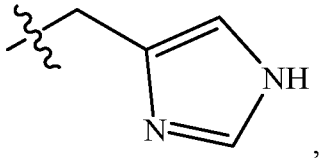
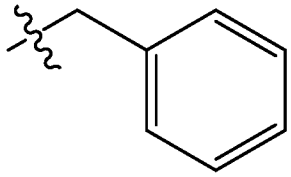


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, or



, where R^{56}, R^{57}, R^{58}, R^{59}, and R^{60} are each independently $-(CH_2)_3-NH(NH)-NH_2$, $-(CH_2)_3-NH_2$, $-(CH_2)_4-NH_2$, $-CH_2-C(O)OR^{65a}$, $-(CH_2)_2-C(O)OR^{65b}$, , or . In any embodiment herein, it may be that R^{61}, R^{62}, R^{63}, R^{64}, R^{65}, R^{65a}, and R^{65b} are each independently neopentyl or

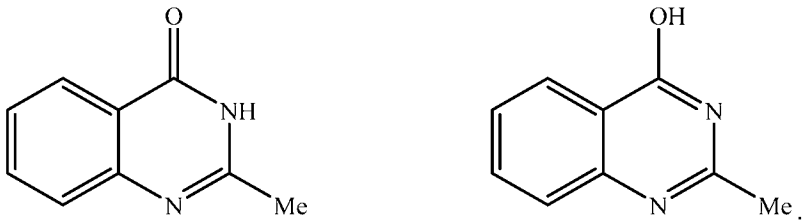
$-CH_2-OC(O)$-*tert*-butyl.

[0230] Pharmaceutically acceptable salts of compounds described herein include acid or base addition salts which retain the desired pharmacological activity and is not biologically undesirable (*e.g.*, the salt is not unduly toxic, allergenic, or irritating, and is bioavailable). When the compound of the present technology has a basic group, such as, for example, an amino group, pharmaceutically acceptable salts can be formed with inorganic acids (such as hydrochloric acid, hydroboric acid, nitric acid, sulfuric acid, and phosphoric acid), organic acids (*e.g.*, alginate, formic acid, acetic acid, benzoic acid, gluconic acid, fumaric acid, oxalic acid, tartaric acid, lactic acid, maleic acid, citric acid, succinic acid, malic acid, methanesulfonic acid, benzenesulfonic acid, naphthalene sulfonic acid, and p-toluenesulfonic acid) or acidic amino acids (such as aspartic acid and glutamic acid). When the compound of the present technology has an acidic group, such as for example, a carboxylic acid group, it can form salts with metals, such as alkali and earth alkali metals (*e.g.*, Na^+, Li^+, K^+, Ca^{2+}, Mg^{2+}, Zn^{2+}), ammonia or organic amines (*e.g.* dicyclohexylamine, trimethylamine, triethylamine, pyridine, picoline, ethanolamine, diethanolamine, triethanolamine) or basic amino acids (*e.g.*, arginine, lysine and ornithine). Such salts can be prepared in situ during isolation and purification of the compounds or by separately reacting the purified compound

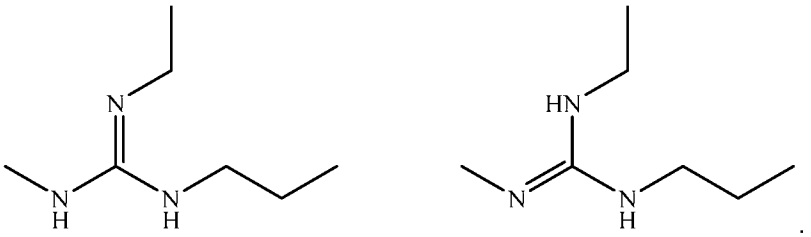
in its free base or free acid form with a suitable acid or base, respectively, and isolating the salt thus formed.

[0231] Those of skill in the art will appreciate that compounds of the present technology may exhibit the phenomena of tautomerism, conformational isomerism, geometric isomerism and/or stereoisomerism. As the formula drawings within the specification and claims can represent only one of the possible tautomeric, conformational isomeric, stereochemical or geometric isomeric forms, it should be understood that the present technology encompasses any tautomeric, conformational isomeric, stereochemical and/or geometric isomeric forms of the compounds having one or more of the utilities described herein, as well as mixtures of these various different forms.

[0232] "Tautomers" refers to isomeric forms of a compound that are in equilibrium with each other. The presence and concentrations of the isomeric forms will depend on the environment the compound is found in and may be different depending upon, for example, whether the compound is a solid or is in an organic or aqueous solution. For example, in aqueous solution, quinazolinones may exhibit the following isomeric forms, which are referred to as tautomers of each other:



[0233] As another example, guanidines may exhibit the following isomeric forms in protic organic solution, also referred to as tautomers of each other:



[0234] Because of the limits of representing compounds by structural formulas, it is to be understood that all chemical formulas of the compounds described herein represent all tautomeric forms of compounds and are within the scope of the present technology.

[0235] Stereoisomers of compounds (also known as optical isomers) include all chiral, diastereomeric, and racemic forms of a structure, unless the specific stereochemistry is expressly indicated. Thus, compounds used in the present technology include enriched or resolved optical isomers at any or all asymmetric atoms as are apparent from the depictions. Both racemic and diastereomeric mixtures, as well as the individual optical isomers can be isolated or synthesized so as to be substantially free of their enantiomeric or diastereomeric partners, and these stereoisomers are all within the scope of the present technology.

[0236] The compounds of the present technology may exist as solvates, especially hydrates. Hydrates may form during manufacture of the compounds or compositions comprising the compounds, or hydrates may form over time due to the hygroscopic nature of the compounds. Compounds of the present technology may exist as organic solvates as well, including DMF, ether, and alcohol solvates among others. The identification and preparation of any particular solvate is within the skill of the ordinary artisan of synthetic organic or medicinal chemistry.

[0237] The prodrug compounds taught herein can be modified based on the selection of the prodrug converting enzyme expressed. Non-limiting examples of other prodrug systems that can be used in combination with the present technology are described in the art for carboxypeptidase A (*e.g.*, prodrugs utilizing a phenylalanine mask, described *e.g.*, in Vitols *et al. Cancer Res.* 55(3):478–81 (1995) and Deckert *et al. Int J Oncol.* 24(5):1289–95 (2004)); glucosidases (*e.g.*, prodrugs utilizing a glycoside mask, described *e.g.,* in Tietze *et al. J Med Chem.* 52(2):537–43 (2009) and Tietze *et al. Chem Eur J.* 17(6):1922–9 (2001)); β-lactamase (*e.g.*, prodrugs utilizing a cephalosporin mask, described, *e.g.,* in Kerr *et al. Bioconjugate Chem.*9(2):255–9 (1998) and Zhou *et al. OncoTargets Ther.* 7:535–41 (2014)); and nitroreductases (*e.g.*, prodrugs utilizing a nitro→ amine mask, described, *e.g.*, in Williams *et al. Biochem J.* 471(2):131–53 (2015).

[0238] The prodrug compounds taught herein can be employed in any method or use requiring a prodrug compound. For example, any clinical application that employs an enzyme that can cleave the prodrugs provided herein to the active drug can use the provided prodrug compounds. Such methods and uses are not limited to the methods and uses described herein. For example, the prodrug compounds taught herein are not limited to use with the engineered immune cells (*e.g.*, SEAKER cells) and related technologies provided herein. In some

embodiments, the prodrug compounds provided herein are converted into an active drug by any suitable prodrug converting enzyme intracellularly (*e.g.* a suitable prodrug converting enzyme transduced into a cell or expressed by a cell). In some embodiments, the prodrug compounds provided herein are converted into an active drug by any suitable prodrug converting enzyme extracellularly. In some embodiments, the prodrug compounds are converted into an active drug by any suitable prodrug converting enzyme conjugated to ligand. In some embodiments, the ligand is an antibody. In some embodiments, the prodrug compounds are employed in combination with a directed enzyme prodrug therapy (DEPT). In some embodiments, the directed enzyme prodrug therapy is an antibody-directed enzyme prodrug therapy (ADEPT), a gene-directed enzyme prodrug therapy (GDEPT), a virus-directed enzyme prodrug therapy (VDEPT), a lectin-directed Enzyme-Activated Prodrug Therapy (LEAPT), polymer-directed enzyme prodrug therapy (PDEPT), clostridia-directed enzyme prodrug therapy (CDEPT) or any combination thereof.

Targeting Ligands and Target Antigens

[0239] In some embodiments, the engineered immune cells provided herein express a T-cell receptor (TCR) or other cell-surface ligand that binds to a target antigen, such as a tumor antigen. The cell-surface ligand can be any molecule that directs an immune cell to a target site (*e.g.*, a tumor site). Exemplary cell surface ligands include, for example endogenous receptors, engineered receptors, or other specific ligands to achieve targeting of the immune cell to a target site. In some embodiments, the receptor is a T cell receptor. In some embodiments, the T cell receptor is a wild-type, or native, T-cell receptor that binds to a target antigen. In some embodiments, the receptor, *e.g.* a T cell receptor, is non-native receptor (*e.g.,* not endogenous to the immune cells).In some embodiments, the receptor is a chimeric antigen receptor (CAR), for example, a T cell CAR, that binds to a target antigen.

[0240] In some embodiments, the target antigen expressed by a tumor cell. In some embodiments, the target antigen is expressed on the surface of a tumor cell. In some embodiments, the target antigen is a cell surface receptor. In some embodiments, the target antigen is a cell surface glycoprotein. In some embodiments, the target antigen is secreted by a tumor cell. In some embodiments, the target antigen is localized to the tumor microenvironment. In some embodiments, the target antigen is localized to the extracellular matrix or stroma of the tumor microenvironment. In some embodiments, the target antigen is

expressed by one or more cells located within the extracellular matrix or stroma of the tumor microenvironment.

[0241] In some embodiments, the target antigen is a tumor antigen selected from among 5T4, alpha 5β1-integrin, 707-AP, A33, AFP, ART-4, B7H4, BAGE, Bcl-2, β-catenin, Bcr-abl, MN/C IX antibody, CA125, CA19-9, CAMEL, CAP-1, CASP-8, CD4, CD5, CD19, CD20, CD21 , CD22, CD25, CDC27/m, CD33, CD37, CD45, CD52, CD56, CD80, CD123, CDK4/m, CEA, c-Met, CS-1, CT, Cyp-B, cyclin B1, DAGE, DAM, EBNA, EGFR, ErbB3, ELF2M, EMMPRIN, EpCam, ephrinB2, estrogen receptor, ETV6-AML1, FAP, ferritin, folate-binding protein, GAGE, G250, GD-2, GM2, GnT-V, gp75, gp100 (Pmel 17), HAGE, HER-2/neu, HLA-A*0201-R170I, HPV E6, HPV E7, Ki-67, HSP70-2M, HST-2, hTERT (or hTRT), iCE, IGF-1R, IL-2R, IL-5, KIAA0205, LAGE, LDLR/FUT, LRP, MAGE, MART, MART-1/melan-A, MART-2/Ski, MC1R, mesothelin, MUC, MUM-1 -B, myc, MUM-2, MUM-3, NA88-A, NYESO-1, NY-Eso-B, p53, , proteinase-3, p190 minor bcr-abl, Pml/RARα, PRAME, progesterone receptor, PSA, PSM, PSMA, ras, RAGE, RU1 or RU2, RORI, , SART-1 or SART-3, survivin, TEL/AML1, TGFβ, TPI/m, TRP-1, TRP-2, TRP-2/INT2, tenascin, TSTA tyrosinase, VEGF, and WT1. In certain embodiments, the target antigen is a tumor antigen selected from among CD19, WT1, PRAME.

[0242] Without limiting the foregoing, exemplary cancers can be treated by targeting the associated provided antigens include: leukemia/lymphoma (CD19, CD20, CD22, ROR1 , CD33); multiple myeloma (B-cell maturation antigen (BCMA)); prostate cancer (PSMA, WT1 , Prostate Stem Cell antigen (PSCA), SV40 T); breast cancer (HER2, ERBB2); stem cell cancer (CD133); ovarian cancer (L1-CAM, extracellular domain of MUC16 (MUC-CD), folate binding protein (folate receptor), Lewis Y); renal cell carcinoma (carboxy-anhydrase-IX (CAIX); melanoma (GD2); and pancreatic cancer (mesothelin, CEA, CD24).

[0243] Typical therapeutic anti-cancer mAb, like those that bind to CD19, recognize cell surface proteins, which constitute only a tiny fraction of the cellular protein content. Most mutated or oncogenic tumor associated proteins are typically nuclear or cytoplasmic. In certain instances, these intracellular proteins can be degraded in the proteasome, processed and presented on the cell surface by MHC class I molecules as T cell epitopes that are recognized by T cell receptors (TCRs). The development of mAb that mimic TCR function, "TCR mimic (TCRm)" or "TCR-like"; (*i.e.*, that recognize peptide antigens of key

intracellular proteins in the context of MHC on the cell surface) greatly extends the potential repertoire of tumor targets addressable by potent mAb. TCRm Fab, or scFv, and mouse IgG specific for the melanoma Ags, NY-ESO-1, hTERT, MART 1, gp100, and PR1, among others, have been developed. The antigen binding portions of such antibodies can be incorporated into the CARs provided herein. HLA-A2 is the most common HLA haplotype in the USA and EU (about 40% of the population). Therefore, potent TCRm mAb and native TCRs against tumor antigens presented in the context of HLA-A2 are useful in the treatment of a large populations.

[0244] Accordingly, in some embodiments, target antigen is a tumor antigen presented in the context of an MHC molecule. In some embodiments, the MHC protein is a MHC class I protein. In some embodiments, the MHC Class I protein is an HLA-A, HLA-B, or HLA-C molecules. In some embodiments, target antigen is a tumor antigen presented in the context of an HLA-A2 molecule. mAbs for intracellular WT1 and PRAME antigens presented in the context of surface HLA-A2 molecules have previously been developed. IgG1, afucosylated Fc forms, bispecific, BiTE, and CAR T cell formats have been made that exhibit potent therapeutic activity in multiple preclinical animal models. Such antibodies or portion thereof can be employed as described herein for the recognition of target antigens present on the surface of a target cell (*e.g.*, a tumor cell) in the context of an MHC molecule.

Chimeric Antigen Receptors

[0245] In some embodiments, the engineered immune cells provided herein express at least one chimeric antigen receptor (CAR). CARs are engineered receptors, which graft or confer a specificity of interest onto an immune effector cell. For example, CARs can be used to graft the specificity of a monoclonal antibody onto an immune cell, such as a T cell. In some embodiments, transfer of the coding sequence of the CAR is facilitated by nucleic acid vector, such as a retroviral vector.

[0246] There are currently three generations of CARs. In some embodiments, the engineered immune cells provided herein express a “first generation” CAR. “First generation” CARs are typically composed of an extracellular antigen binding domain (*e.g.*, a single-chain variable fragment (scFv)) fused to a transmembrane domain fused to cytoplasmic/intracellular domain of the T cell receptor (TCR) chain. “First generation” CARs typically have the intracellular domain from the CD3ζ chain, which is the primary transmitter

of signals from endogenous TCRs. "First generation" CARs can provide de novo antigen recognition and cause activation of both $CD4^+$ and $CD8^+$ T cells through their CD3ζ chain signaling domain in a single fusion molecule, independent of HLA-mediated antigen presentation.

[0247] In some embodiments, the engineered immune cells provided herein express a "second generation" CAR. "Second generation" CARs add intracellular domains from various co-stimulatory molecules (*e.g.*, CD28, 4-1BB, ICOS, OX40) to the cytoplasmic tail of the CAR to provide additional signals to the T cell. "Second generation" CARs comprise those that provide both co-stimulation (*e.g.*, CD28 or 4-IBB) and activation (*e.g.*, CD3ζ). Preclinical studies have indicated that "Second Generation" CARs can improve the antitumor activity of T cells. For example, robust efficacy of "Second Generation" CAR modified T cells was demonstrated in clinical trials targeting the CD19 molecule in patients with chronic lymphoblastic leukemia (CLL) and acute lymphoblastic leukemia (ALL).

[0248] In some embodiments, the engineered immune cells provided herein express a "third generation" CAR. "Third generation" CARs comprise those that provide multiple co-stimulation (*e.g.*, CD28 and 4-1BB) and activation (*e.g.*, CD3ζ).

[0249] In accordance with the presently disclosed subject matter, the CARs of the engineered immune cells provided herein comprise an extracellular antigen-binding domain, a transmembrane domain and an intracellular domain.

Extracellular Antigen-Binding Domain of a CAR

[0250] In certain embodiments, the extracellular antigen-binding domain of a CAR specifically binds a tumor antigen. In certain embodiments, the extracellular antigen-binding domain is derived from a monoclonal antibody (mAb) that binds to a tumor antigen. In some embodiments, the extracellular antigen-binding domain comprises an scFv. In some embodiments, the extracellular antigen-binding domain comprises a Fab, which is optionally crosslinked. In a some embodiments, the extracellular binding domain comprises a $F(ab)_2$. In some embodiments, any of the foregoing molecules are comprised in a fusion protein with a heterologous sequence to form the extracellular antigen-binding domain. In certain embodiments, the extracellular antigen-binding domain comprises a human scFv that binds specifically to a tumor antigen. In certain embodiments, the scFv is identified by screening scFv phage library with tumor antigen-Fc fusion protein.

[0251] In certain embodiments, the extracellular antigen-binding domain of a presently disclosed CAR has a high binding specificity and high binding affinity to a tumor antigen (*e.g.*, a mammalian tumor antigen, such as a human tumor antigen). For example, in some embodiments, the extracellular antigen-binding domain of the CAR (embodied, for example, in a human scFv or an analog thereof) binds to a particular tumor antigen with a dissociation constant (K_d) of about 1 x 10^{-5} M or less. In certain embodiments, the K_d is about 5 x 10^{-6} M or less, about 1 x 10^{-6} M or less, about 5 x 10^{-7} M or less, about 1 x 10^{-7} M or less, about 5 x 10^{-8} M or less, about 1 x 10^{-8} M or less, about 5 x 10^{-9} or less, about 4 x 10^{-9} or less, about 3 x 10^{-9} or less, about 2 x 10^{-9} or less, or about 1 x 10^{-9} M or less. In certain non-limiting embodiments, the K_d is from about 3 x 10^{-9} M or less. In certain non-limiting embodiments, the K_d is from about 3 x 10^{-9} to about 2 x 10^{-7}.

[0252] Binding of the extracellular antigen-binding domain (embodiment, for example, in a human scFv or an analog thereof) of a presently disclosed tumor antigen-targeted CAR can be confirmed by, for example, enzyme-linked immunosorbent assay (ELISA), radioimmunoassay (RIA), FACS analysis, bioassay (*e.g.*, growth inhibition), or Western Blot assay. Each of these assays generally detect the presence of protein-antibody complexes of particular interest by employing a labeled reagent (*e.g.*, an antibody, or a scFv) specific for the complex of interest. For example, the scFv can be radioactively labeled and used in a radioimmunoassay (RIA) (see, for example, Weintraub, B., Principles of Radioimmunoassays, Seventh Training Course on Radioligand Assay Techniques, The Endocrine Society, March, 1986, which is incorporated by reference herein). The radioactive isotope can be detected by such means as the use of a γ counter or a scintillation counter or by autoradiography. In certain embodiments, the extracellular antigen-binding domain of the tumor antigen-targeted CAR is labeled with a fluorescent marker. Non-limiting examples of fluorescent markers include green fluorescent protein (GFP), blue fluorescent protein (*e.g.*, EBFP, EBFP2, Azurite, and mKalamal), cyan fluorescent protein (*e.g.*, ECFP, Cerulean, and CyPet), and yellow fluorescent protein (*e.g.*, YFP, Citrine, Venus, and YPet). In certain embodiments, the human scFv of a presently disclosed tumor antigen-targeted CAR is labeled with GFP.

[0253] In some embodiments, the extracellular antigen-binding domain of the expressed CAR binds to tumor antigen that is expressed by a tumor cell. In some embodiments, the

extracellular antigen-binding domain of the expressed CAR binds to tumor antigen that is expressed on the surface of a tumor cell. In some embodiments, the extracellular antigen-binding domain of the expressed CAR binds to tumor antigen that is expressed on the surface of a tumor cell in combination with an MHC protein. In some embodiments, the MHC protein is a MHC class I protein. In some embodiments, the MHC Class I protein is an HLA-A, HLA-B, or HLA-C molecules. In some embodiments, the extracellular antigen-binding domain of the expressed CAR binds to tumor antigen that is expressed on the surface of a tumor cell not in combination with an MHC protein.

[0254] In some embodiments, the extracellular antigen-binding domain of the expressed CAR binds to tumor antigen selected from among 5T4, alpha 5β1-integrin, 707-AP, A33, AFP, ART-4, B7H4, BAGE, Bcl-2, β-catenin, Bcr-abl, MN/C IX antibody, CA125, CA19-9, CAMEL, CAP-1, CASP-8, CD4, CD5, CD19, CD20, CD21 , CD22, CD25, CDC27/m, CD33, CD37, CD45, CD52, CD56, CD80, CD123, CDK4/m, CEA, c-Met, CS-1, CT, Cyp-B, cyclin B1, DAGE, DAM, EBNA, EGFR, ErbB3, ELF2M, EMMPRIN, EpCam, ephrinB2, estrogen receptor, ETV6-AML1, FAP, ferritin, folate-binding protein, GAGE, G250, GD-2, GM2, GnT-V, gp75, gp100 (Pmel 17), HAGE, HER-2/neu, HLA-A*0201-R170I, HPV E6, HPV E7, Ki-67, HSP70-2M, HST-2, hTERT (or hTRT), iCE, IGF-1R, IL-2R, IL-5, KIAA0205, LAGE, LDLR/FUT, LRP, MAGE, MART, MART-1/melan-A, MART-2/Ski, MC1R, mesothelin, MUC, MUM-1 -B, myc, MUM-2, MUM-3, NA88-A, NYESO-1, NY-Eso-B, p53, , proteinase-3, p190 minor bcr-abl, Pml/RARα, PRAME, progesterone receptor, PSA, PSM, PSMA, ras, RAGE, RU1 or RU2, RORI, , SART-1 or SART-3, survivin, TEL/AML1, TGFβ, TPI/m, TRP-1, TRP-2, TRP-2/INT2, tenascin, TSTA tyrosinase, VEGF, and WT1. In certain embodiments, the extracellular antigen-binding domain of the expressed CAR binds to tumor antigen selected from among CD19, WT1, PRAME. Exemplary extracellular antigen-binding domains and methods of generating such domains and associated CARs are described in, *e.g.*, WO2016/191246, WO2017/023859, WO2015/188141, WO2015/070061, WO2012/135854, WO2014/055668, which are incorporated by reference in their entirety, including the sequence listings provided therein.

[0255] In some embodiments, the extracellular antigen-binding domain of the expressed CAR binds to a CD19 tumor antigen. In some embodiments, the extracellular antigen-binding domain of the expressed CAR binds to a CD19 tumor antigen presented in the context of an

MHC molecule. In some embodiments, binds to a CD19 tumor antigen presented in the context of an HLA-A2 molecule.

[0256] In some embodiments, the extracellular antigen-binding domain of the expressed CAR binds to a "preferentially expressed antigen in melanoma" (PRAME) tumor antigen. In some embodiments, the extracellular antigen-binding domain of the expressed CAR binds to a PRAME tumor antigen presented in the context of an MHC molecule. In some embodiments, binds to a PRAME tumor antigen presented in the context of an HLA-A2 molecule.

[0257] In some embodiments, extracellular antigen-binding domain of the expressed CAR binds to a WT1 (Wilm's tumor protein 1) tumor antigen. In some embodiments, the extracellular antigen-binding domain of the expressed CAR binds to a WT1 tumor antigen presented in the context of an MHC molecule. In some embodiments, the extracellular antigen-binding domain of the expressed CAR binds to a WT1 tumor antigen presented in the context of an HLA-A2 molecule.

[0258] In certain embodiments, the extracellular antigen-binding domain (*e.g.*, human scFv) comprises a heavy chain variable region and a light chain variable region, optionally linked with a linker sequence, for example a linker peptide (*e.g.*, SEQ NO:21), between the heavy chain variable region and the light chain variable region. In certain embodiments, the extracellular antigen-binding domain is a human scFv-Fc fusion protein or full length human IgG with V_H and V_L regions.

[0259] In certain embodiments, the extracellular antigen-binding domain comprises a human scFv that binds to a CD19 antigen. In some embodiments, the scFv comprises a polypeptide having an amino acid sequence of SEQ ID NO: 19.
MALPVTALLLPLALLLHAEVKLQQSGAELVRPGSSVKISCKASGYAFSSYWMNWVK
QRPGQGLEWIGQIYPGDGDTNYNGKFKGQATLTADKSSSTAYMQLSGLTSEDSAVY
FCARKTISSVVDFYFDYWGQGTTVTVSSGGGGSGGGGSGGGGSDIELTQSPKFMSTS
VGDRVSVTCKASQNVGTNVAWYQQKPGQSPKPLIYSATYRNSGVPDRFTGSGSGTD
FTLTITNVQSKDLADYFCQQYNRYPYTSGGGTKLEIKR (SEQ ID NO: 19)

[0260] In some embodiments, the scFv comprises a polypeptide having an amino acid sequence that is at least 80%, at least 85%, at least 90%, or at least 95% identical to SEQ ID

NO: 19. For example, the scFv comprises a polypeptide having an amino acid sequence that is about 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical to SEQ ID NO: 19.

[0261] In some embodiments, the scFv is encoded by a nucleic acid having a nucleic acid sequence of SEQ ID NO: 20.

ATGGCTCTCCCAGTGACTGCCCTACTGCTTCCCCTAGCGCTTCTCCTGCATGCAGA
GGTGAAGCTGCAGCAGTCTGGGGCTGAGCTGGTGAGGCCTGGGTCCTCAGTGAA
GATTTCCTGCAAGGCTTCTGGCTATGCATTCAGTAGCTACTGGATGAACTGGGTG
AAGCAGAGGCCTGGACAGGGTCTTGAGTGGATTGGACAGATTTATCCTGGAGAT
GGTGATACTAACTACAATGGAAAGTTCAAGGGTCAAGCCACACTGACTGCAGAC
AAATCCTCCAGCACAGCCTACATGCAGCTCAGCGGCCTAACATCTGAGGACTCTG
CGGTCTATTTCTGTGCAAGAAAGACCATTAGTTCGGTAGTAGATTTCTACTTTGA
CTACTGGGGCCAAGGGACCACGGTCACCGTCTCCTCAGGTGGAGGTGGATCAGG
TGGAGGTGGATCTGGTGGAGGTGGATCTGACATTGAGCTCACCCAGTCTCCAAA
ATTCATGTCCACATCAGTAGGAGACAGGGTCAGCGTCACCTGCAAGGCCAGTCA
GAATGTGGGTACTAATGTAGCCTGGTATCAACAGAAACCAGGACAATCTCCTAA
ACCACTGATTTACTCGGCAACCTACCGGAACAGTGGAGTCCCTGATCGCTTCACA
GGCAGTGGATCTGGGACAGATTTCACTCTCACCATCACTAACGTGCAGTCTAAAG
ACTTGGCAGACTATTTCTGTCAACAATATAACAGGTATCCGTACACGTCCGGAGG
GGGGACCAAGCTGGAGATCAAACGG (SEQ ID NO: 20)

[0262] In some embodiments, the scFv is encoded by a nucleic acid having a nucleic acid sequence that is at least 80%, at least 85%, at least 90%, or at least 95% identical to SEQ ID NO: 20. In some embodiments, the scFv is encoded by a nucleic acid having a nucleic acid sequence of SEQ ID NO: 20. In some embodiments, the scFv is encoded by a nucleic acid having a nucleic acid sequence that is about 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical to SEQ ID NO: 20.

[0263] In certain non-limiting embodiments, an extracellular antigen-binding domain of the presently disclosed CAR can comprise a linker connecting the heavy chain variable region and light chain variable region of the extracellular antigen-binding domain. As used herein, the term “linker” refers to a functional group (*e.g.*, chemical or polypeptide) that

covalently attaches two or more polypeptides or nucleic acids so that they are connected to one another. As used herein, a "peptide linker" refers to one or more amino acids used to couple two proteins together (*e.g.*, to couple V_H and V_L domains). In certain embodiments, the linker comprises amino acids having the sequence set forth in SEQ ID NO: 21. In certain embodiments, the nucleotide sequence encoding the amino acid sequence of SEQ ID NO: 21 is set forth in SEQ ID NO: 22.

[0264] In addition, the extracellular antigen-binding domain can comprise a leader or a signal peptide that directs the nascent protein into the endoplasmic reticulum. Signal peptide or leader can be essential if the CAR is to be glycosylated and anchored in the cell membrane. The signal sequence or leader can be a peptide sequence (about 5, about 10, about 15, about 20, about 25, or about 30 amino acids long) present at the N-terminus of newly synthesized proteins that directs their entry to the secretory pathway. In certain embodiments, the signal peptide is covalently joined to the N-terminus of the extracellular antigen-binding domain. In certain embodiments, the signal peptide comprises a CD8 signal polypeptide comprising amino acids having the sequence set forth in SEQ ID NO: 11 as provided below.

[0265] MALPVTALLLPLALLLHAARP (SEQ ID NO: 11)

[0266] The nucleotide sequence encoding the amino acid sequence of SEQ ID NO: 11 is set forth in SEQ ID NO: 12, which is provided below:

[0267] atggccctgccagtaacggctctgctgctgccacttgctctgctcctccatgcagccaggcct (SEQ ID NO: 12)

Transmembrane Domain of a CAR

[0268] In certain non-limiting embodiments, the transmembrane domain of the CAR comprises a hydrophobic alpha helix that spans at least a portion of the membrane. Different transmembrane domains result in different receptor stability. After antigen recognition, receptors cluster and a signal is transmitted to the cell. In accordance with the presently disclosed subject matter, the transmembrane domain of the CAR can comprise a CD8 polypeptide, a CD28 polypeptide, a CD3ζ polypeptide, a CD4 polypeptide, a 4-IBB polypeptide, an OX40 polypeptide, an ICOS polypeptide, a CTLA-4 polypeptide, a PD-1 polypeptide, a LAG-3 polypeptide, a 2B4 polypeptide, a BTLA polypeptide, a synthetic peptide (*e.g.*, a transmembrane peptide not based on a protein associated with the immune response), or a combination thereof.

[0269] In certain embodiments, the transmembrane domain of a presently disclosed CAR comprises a CD28 polypeptide. The CD28 polypeptide can have an amino acid sequence that is at least about 85%, about 90%, about 95%, about 96%, about 97%, about 98%, about 99% or 100% homologous to the sequence having a NCBI Reference No: PI0747 or NP006130 (SEQ ID NO: 24), or fragments thereof, and/or may optionally comprise up to one or up to two or up to three conservative amino acid substitutions. In certain embodiments, the CD28 polypeptide can have an amino acid sequence that is a consecutive portion of SEQ ID NO: 24 which is at least 20, or at least 30, or at least 40, or at least 50, and up to 220 amino acids in length. Alternatively or additionally, in non- limiting various embodiments, the CD28 polypeptide has an amino acid sequence of amino acids 1 to 220, 1 to 50, 50 to 100, 100 to 150, 114 to 220, 150 to 200, or 200 to 220 of SEQ ID NO: 24. In certain embodiments, the CAR of the presently disclosed comprises a transmembrane domain comprising a CD28 polypeptide, and an intracellular domain comprising a co-stimulatory signaling region that comprises a CD28 polypeptide. In certain embodiments, the CD28 polypeptide comprised in the transmembrane domain and the intracellular domain has an amino acid sequence of amino acids 114 to 220 of SEQ ID NO: 24.

[0270] SEQ ID NO: 24 is provided below:

[0271] MLRLLLALNLFPSIQVTGNKILVKQSPMLVAYDNALSCKYSYNLFSREFR ASLHKGLDSAVEVCWYGNYSQQLQVYSKTGFNCDGKLGNESVTFYLQNLYQTDIYF CKIEVMYPPPYLDNEKSNGTIIHVKGKHLCPSPLFPGPSKPFWVLVWGGVLACYSLL VTVAFIIFWVRSKRSRLLHSDYMNMTPRRPGPTRKHYQPYAPPRDFAAYRS (SEQ ID NO: 24)

[0272] In accordance with the presently disclosed subject matter, a "CD28 nucleic acid molecule" refers to a polynucleotide encoding a CD28 polypeptide. In certain embodiments, the CD28 nucleic acid molecule encoding the CD28 polypeptide comprised in the transmembrane domain and the intracellular domain (*e.g.*, the co- stimulatory signaling region) of the presently disclosed CAR (amino acids 114 to 220 of SEQ ID NO: 24) comprises nucleic acids having the sequence set forth in SEQ ID NO: 25 as provided below.

[0273] attgaagttatgtatcctcctccttacctagacaatgagaagagcaatggaaccattatccatgtgaaagggaaacacct ttgtccaagtcccctatttcccggaccttctaagccctttgggtgctggtggtggttggtggagtcctggcttgctatagcttgctagtaac

agtggcctttattattttctgggtgaggagtaagaggagcaggctcctgcacagtgactacatgaacatgactccccgccgccccgggcccacccgcaagcattaccagccctatgccccaccacgcgacttcgcagcctatcgctcc (SEQ ID NO: 25)

[0274] In certain embodiments, the transmembrane domain comprises a CD8 polypeptide. The CD8 polypeptide can have an amino acid sequence that is at least about 85%, about 90%, about 95%, about 96%, about 97%, about 98%, about 99% or about 100%) homologous to SEQ ID NO: 26 (homology herein may be determined using standard software such as BLAST or FASTA) as provided below, or fragments thereof, and/or may optionally comprise up to one or up to two or up to three conservative amino acid substitutions. In certain embodiments, the CD8 polypeptide can have an amino acid sequence that is a consecutive portion of SEQ ID NO: 26 which is at least 20, or at least 30, or at least 40, or at least 50, and up to 235 amino acids in length. Alternatively or additionally, in non-limiting various embodiments, the CD8 polypeptide has an amino acid sequence of amino acids 1 to 235, 1 to 50, 50 to 100, 100 to 150, 150 to 200, or 200 to 235 of SEQ ID NO: 26.

[0275] MALPVTALLLPLALLLHAARPSQFRVSPLDRTWNLGETVELKCQVLLSNPTSGCSWLFQPRGAAASPTFLLYLSQNKPKAAEGLDTQRFSGKRLGDTFVLTLSDFRRENEGYYFCSALSNSIMYFSHFVPVFLPAKPTTTPAPRPPTPAPTIASQPLSLRPEACRPAAGGAVHTRGLDFACDIYIWAPLAGTCGVLLLSLVITLYCNHRNRRRVCKCPRPWKSGDKPSLSARYV (SEQ ID NO: 26)

[0276] In accordance with the presently disclosed subject matter, a "CD8 nucleic acid molecule" refers to a polynucleotide encoding a CD8 polypeptide.

[0277] In certain non-limiting embodiments, a CAR can also comprise a spacer region that links the extracellular antigen-binding domain to the transmembrane domain. The spacer region can be flexible enough to allow the antigen- binding domain to orient in different directions to facilitate antigen recognition while preserving the activating activity of the CAR. In certain non-limiting embodiments, the spacer region can be the hinge region from IgGl, the CH_2CH_3 region of immunoglobulin and portions of CD3, a portion of a CD28 polypeptide (*e.g.*, SEQ ID NO: 24), a portion of a CD8 polypeptide (*e.g.*, SEQ ID NO: 26), a variation of any of the foregoing which is at least about 80%, at least about 85%>, at least about 90%, or at least about 95% homologous thereto, or a synthetic spacer sequence. In certain non-limiting embodiments, the spacer region may have a length between about 1-50 (*e.g.*, 5-25, 10-30, or 30-50) amino acids.

Intracellular Domain of a CAR

[0278] In certain non-limiting embodiments, an intracellular domain of the CAR can comprise a CD3ζ polypeptide, which can activate or stimulate a cell (*e.g.*, a cell of the lymphoid lineage, *e.g.*, a T cell). CD3ζ comprises 3 ITAMs, and transmits an activation signal to the cell (*e.g.*, a cell of the lymphoid lineage, *e.g.*, a T cell) after antigen is bound. The CD3ζ polypeptide can have an amino acid sequence that is at least about 85%, about 90%, about 95%, about 96%, about 97%, about 98%, about 99% or about 100% homologous to the sequence having a NCBI Reference No: NP_932170 (SEQ ID NO: 48), or fragments thereof, and/or may optionally comprise up to one or up to two or up to three conservative amino acid substitutions. In non-limiting certain embodiments, the CD3ζ polypeptide can have an amino acid sequence that is a consecutive portion of SEQ ID NO: 27 which is at least 20, or at least 30, or at least 40, or at least 50, and up to 164 amino acids in length. Alternatively or additionally, in non-limiting various embodiments, the CD3ζ polypeptide has an amino acid sequence of amino acids 1 to 164, 1 to 50, 50 to 100, 100 to 150, or 150 to 164 of SEQ ID NO: 27. In certain embodiments, the CD3ζ polypeptide has an amino acid sequence of amino acids 52 to 164 of SEQ ID NO: 27.

[0279] SEQ ID NO: 27 is provided below:

[0280] MKWKALFTAAILQAQLPITEAQSFGLLDPKLCYLLDGILFIYGVILTALFLR VKFSRSADAPAYQQGQNQLYNELNLGRREEYDVLDKRRGRDPEMGGKPQRRKNPQ EGLYNELQKDKMAEAYSEIGMKGERRRGKGHDGLYQGLSTATKDTYDALHMQALP PR (SEQ ID NO: 27)

[0281] In certain embodiments, the CD3ζ polypeptide has the amino acid sequence set forth in SEQ ID NO: 28, which is provided below:

[0282] RVKFSRSAEPPAYQQGQNQLYNELNLGRREEYDVLDKRRGRDPEMGGKP RRKNPQEGLYNELQKDKMAEAYSEIGMKGERRRGKGHDGLYQGLSTATKDTYDAL HMQALPPR (SEQ ID NO: 28)

[0283] In accordance with the presently disclosed subject matter, a "CD3ζ nucleic acid molecule" refers to a polynucleotide encoding a CD3ζ polypeptide. In certain embodiments, the CD3ζ nucleic acid molecule encoding the CD3ζ polypeptide (SEQ ID NO: 28) comprised in the intracellular domain of the presently disclosed CAR comprises a nucleotide sequence as set forth in SEQ ID NO: 29 as provided below.

[0284] agagtgaagttcagcaggagcgcagagccccccgcgtaccagcagggccagaaccagctctataacgagctcaat ctaggacgaagagaggagtacgatgttttggacaagagacgtggccgggaccctgagatgggggggaaagccgagaaggaagaac cctcaggaaggcctgtacaatgaactgcagaaagataagatggcggaggcctacagtgagattgggatgaaaggcgagcgccgga ggggcaaggggcacgatggcctttaccagggtctcagtacagccaccaaggacacctacgacgcccttcacatgcaggccctgccc cctcgcg (SEQ ID NO: 29)

[0285] In certain non-limiting embodiments, an intracellular domain of the CAR further comprises at least one signaling region. The at least one signaling region can include a CD28 polypeptide, a 4- IBB polypeptide, an OX40 polypeptide, an ICOS polypeptide, a DAP- 10 polypeptide, a PD-1 polypeptide, a CTLA-4 polypeptide, a LAG-3 polypeptide, a 2B4 polypeptide, a BTLA polypeptide, a synthetic peptide (not based on a protein associated with the immune response), or a combination thereof.

[0286] In certain embodiments, the signaling region is a co-stimulatory signaling region.

[0287] In certain embodiments, the co- stimulatory signaling region comprises at least one co- stimulatory molecule, which can provide optimal lymphocyte activation. As used herein, "co-stimulatory molecules" refer to cell surface molecules other than antigen receptors or their ligands that are required for an efficient response of lymphocytes to antigen. The at least one co-stimulatory signaling region can include a CD28 polypeptide, a 4-IBB polypeptide, an OX40 polypeptide, an ICOS polypeptide, a DAP-10 polypeptide, or a combination thereof. The co-stimulatory molecule can bind to a co-stimulatory ligand, which is a protein expressed on cell surface that upon binding to its receptor produces a co-stimulatory response, *i.e.,* an intracellular response that effects the stimulation provided when an antigen binds to its CAR molecule. Co-stimulatory ligands, include, but are not limited to CD80, CD86, CD70, OX40L, 4-1BBL, CD48, TNFRSF14, and PD- Ll . As one example, a 4-1BB ligand (*i.e.,* 4-1BBL) may bind to 4-1BB (also known as "CD 137") for providing an intracellular signal that in combination with a CAR signal induces an effector cell function of the CAR^+ T cell. CARs comprising an intracellular domain that comprises a co-stimulatory signaling region comprising 4-1BB, ICOS or DAP-10 are disclosed in U.S. 7,446,190, which is herein incorporated by reference in its entirety. In certain embodiments, the intracellular domain of the CAR comprises a co-stimulatory signaling region that comprises a CD28 polypeptide. In certain embodiments, the intracellular domain of the CAR comprises a co-

stimulatory signaling region that comprises two co- stimulatory molecules: CD28 and 4-1BB or CD28 and OX40.

[0288] 4-IBB can act as a tumor necrosis factor (TNF) ligand and have stimulatory activity. The 4-IBB polypeptide can have an amino acid sequence that is at least about 85%, about 90%, about 95%, about 96%, about 97%, about 98%, about 99% or 100% homologous to the sequence having a NCBI Reference No: P41273 or NP_001552 (SEQ ID NO: 30) or fragments thereof, and/or may optionally comprise up to one or up to two or up to three conservative amino acid substitutions.

[0289] SEQ ID NO: 30 is provided below:

[0290] MGNSCYNIVATLLLVLNFERTRSLQDPCSNCPAGTFCDNNRNQICSPCPPN SFSSAGGQRTCDICRQCKGVFRTRKECSSTSNAECDCTPGFHCLGAGCSMCEQDCKQ GQELTKKGCKDCCFGTFNDQKRGICRPWTNCSLDGKSVLGTKERDWCGPSPADLSP GASSVTPPAPAREPGHSPQIISFFLALTSTALLFLLFFLTLRFSWKRGRKKLLYIFKQPF MRPVQTTQEEDGCSCRFPEEEEGGCEL (SEQ ID NO: 30)

[0291] In accordance with the presently disclosed subject matter, a "4- IBB nucleic acid molecule" refers to a polynucleotide encoding a 4- IBB polypeptide.

[0292] An OX40 polypeptide can have an amino acid sequence that is at least about 85%, about 90%, about 95%, about 96%, about 97%, about 98%, about 99% or 100% homologous to the sequence having a NCBI Reference No: P43489 or NP 003318 (SEQ ID NO: 31), or fragments thereof, and/or may optionally comprise up to one or up to two or up to three conservative amino acid substitutions.

[0293] SEQ ID NO: 31 is provided below:

[0294] MCVGARRLGRGPCAALLLLGLGLSTVTGLHCVGDTYPSNDRCCHECRPG NGMVSRCSRSQNTVCRPCGPGFYNDWSSKPCKPCTWCNLRSGSERKQLCTATQDTV CRCRAGTQPLDSYKPGVDCAPCPPGHFSPGDNQACKPWTNCTLAGKHTLQPASNSS DAICEDRDPPATQPQETQGPPARPITVQPTEAWPRTSQGPSTRPVEVPGGRAVAAILG LGLVLGLLGPLAILLALYLLRRDQRLPPDAHKPPGGGSFRTPIQEEQADAHSTLAKI (SEQ ID NO: 31)

[0295] In accordance with the presently disclosed subject matter, an "OX40 nucleic acid molecule" refers to a polynucleotide encoding an OX40 polypeptide.

[0296] An ICOS polypeptide can have an amino acid sequence that is at least about 85%, about 90%, about 95%, about 96%, about 97%, about 98%, about 99% or 100% homologous to the sequence having a NCBI Reference No: NP_036224 (SEQ ID NO: 32) or fragments thereof, and/or may optionally comprise up to one or up to two or up to three conservative amino acid substitutions.

[0297] SEQ ID NO: 32 is provided below:

[0298] MKSGLWYFFLFCLRIKVLTGEINGSANYEMFIFHNGGVQILCKYPDIVQQF KMQLLKGGQILCDLTKTKGSGNTVSIKSLKFCHSQLSNNSVSFFLYNLDHSHANYYF CNLSIFDPPPFKVTLTGGYLHIYESQLCCQLKFWLPIGCAAFVWCILGCILICWLTKKK YSSSVHDPNGEYMFMRATAKKSRLTDVTL (SEQ ID NO: 32)

[0299] In accordance with the presently disclosed subject matter, an "ICOS nucleic acid molecule" refers to a polynucleotide encoding an ICOS polypeptide.

[0300] CTLA-4 is an inhibitory receptor expressed by activated T cells, which when engaged by its corresponding ligands (CD80 and CD86; B7-1 and B7-2, respectively), mediates activated T cell inhibition or anergy. In both preclinical and clinical studies, CTLA-4 blockade by systemic antibody infusion, enhanced the endogenous anti-tumor response albeit, in the clinical setting, with significant unforeseen toxicities.

[0301] CTLA-4 contains an extracellular V domain, a transmembrane domain, and a cytoplasmic tail. Alternate splice variants, encoding different isoforms, have been characterized. The membrane-bound isoform functions as a homodimer interconnected by a disulfide bond, while the soluble isoform functions as a monomer. The intracellular domain is similar to that of CD28, in that it has no intrinsic catalytic activity and contains one YVKM motif able to bind PI3K, PP2A and SHP-2 and one proline-rich motif able to bind SH3 containing proteins. One role of CTLA-4 in inhibiting T cell responses seem to be directly via SHP-2 and PP2A dephosphorylation of TCR-proximal signaling proteins such as CD3 and LAT. CTLA-4 can also affect signaling indirectly via competing with CD28 for CD80/86 binding. CTLA-4 has also been shown to bind and/or interact with PI3K, CD80, AP2M1, and PPP2R5A.

[0302] In accordance with the presently disclosed subject matter, a CTLA-4 polypeptide can have an amino acid sequence that is at least about 85%, about 90%, about 95%, about 96%, about 97%, about 98%, about 99% or about 100% homologous to UniProtKB/Swiss-

Prot Ref. No.: P16410.3 (SEQ ID NO: 33) (homology herein may be determined using standard software such as BLAST or FASTA) or fragments thereof, and/or may optionally comprise up to one or up to two or up to three conservative amino acid substitutions.

[0303] SEQ ID NO: 33 is provided below:

[0304] MACLGFQRHKAQLNLATRTWPCTLLFFLLFIPVFCKAMHVAQPAWLASS RGIASFVCEYASPGKATEVRVTVLRQADSQVTEVCAATYMMGNELTFLDDSICTGTS SGNQLTIQGLRAMDTGLYICKVELMYPPPYYLGIGNGTQIYVIDPEPCPDSDFLLWIL AAVSSGLFFYSFLLTAVSLSKMLKKRSPLTTGVYVKMPPTEPECEKQFQPYFIPIN (SEQ ID NO: 33)

[0305] In accordance with the presently disclosed subject matter, a "CTLA-4 nucleic acid molecule" refers to a polynucleotide encoding a CTLA-4 polypeptide.

[0306] PD-1 is a negative immune regulator of activated T cells upon engagement with its corresponding ligands PD-L1 and PD-L2 expressed on endogenous macrophages and dendritic cells. PD-1 is a type I membrane protein of 268 amino acids. PD-1 has two ligands, PD-L1 and PD-L2, which are members of the B7 family. The protein's structure comprises an extracellular IgV domain followed by a transmembrane region and an intracellular tail. The intracellular tail contains two phosphorylation sites located in an immunoreceptor tyrosine-based inhibitory motif and an immunoreceptor tyrosine- based switch motif, that PD-1 negatively regulates TCR signals. SHP- I and SHP-2 phosphatases bind to the cytoplasmic tail of PD-1 upon ligand binding. Upregulation of PD-L1 is one mechanism tumor cells may evade the host immune system. In pre- clinical and clinical trials, PD-1 blockade by antagonistic antibodies induced anti -tumor responses mediated through the host endogenous immune system. In accordance with the presently disclosed subject matter, a PD-1 polypeptide can have an amino acid sequence that is at least about 85%, about 90%, about 95%, about 96%, about 97%, about 98%, about 99% or about 100% homologous to NCBI Reference No: NP_005009.2 (SEQ ID NO: 34) or fragments thereof, and/or may optionally comprise up to one or up to two or up to three conservative amino acid substitutions.

[0307] SEQ ID NO: 34 is provided below:

[0308] MQIPQAPWPVVWAVLQLGWRPGWFLDSPDRPWNPPTFSPALLWTEGDN ATFTCSFSNTSESFVLNWYRMSPSNQTDKLAAFPEDRSQPGQDCRFRVTQLPNGRDF HMSVVRARRNDSGTYLCGAISLAPKAQIKESLRAELRVTERRAEVPTAHPSPSPRPAG

QFQTLVVGWGGLLGSLVLLVWVLAVICSRAARGTIGARRTGQPLKEDPSAVPVFSV DYGELDFQWREKTPEPPVPCVPEQTEYATIVFPSGMGTSSPARRGSADGPRSAQPLRP EDGHCSWPL (SEQ ID NO: 34)

[0309] In accordance with the presently disclosed subject matter, a "PD-1 nucleic acid molecule" refers to a polynucleotide encoding a PD-1 polypeptide.

[0310] Lymphocyte-activation protein 3 (LAG-3) is a negative immune regulator of immune cells. LAG-3 belongs to the immunoglobulin (Ig) superfamily and contains 4 extracellular Ig-like domains. The LAG3 gene contains 8 exons. The sequence data, exon/intron organization, and chromosomal localization all indicate a close relationship of LAG3 to CD4. LAG3 has also been designated CD223 (cluster of differentiation 223).

[0311] In accordance with the presently disclosed subject matter, a LAG-3 polypeptide can have an amino acid sequence that is at least about 85%, about 90%, about 95%, about 96%, about 97%, about 98%, about 99% or about 100% homologous to UniProtKB/Swiss-Prot Ref. No. : P18627.5 (SEQ ID NO: 35) or fragments thereof, and/or may optionally comprise up to one or up to two or up to three conservative amino acid substitutions.

[0312] SEQ ID NO: 35 is provided below:

[0313] MWEAQFLGLLFLQPLWVAPVKPLQPGAEVPWWAQEGAPAQLPCSPTIPL QDLSLLRRAGVTWQHQPDSGPPAAAPGHPLAPGPHPAAPSSWGPRPRRYTVLSVGP GGLRSGRLPLQPRVQLDERGRQRGDFSLWLRPARRADAGEYRAAVHLRDRALSCRL RLRLGQASMTASPPGSLRASDWVILNCSFSRPDRPASVHWFRNRGQGRVPVRESPHH HLAESFLFLPQVSPMDSGPWGCILTYRDGFNVSIMYNLTVLGLEPPTPLTVYAGAGS RVGLPCRLPAGVGTRSFLTAKWTPPGGGPDLLVTGDNGDFTLRLEDVSQAQAGTYT CHIHLQEQQLNATVTLAIITVTPKSFGSPGSLGKLLCEVTPVSGQERFVWSSLDTPSQR SFSGPWLEAQEAQLLSQPWQCQLYQGERLLGAAVYFTELSSPGAQRSGRAPGALPA GHLLLFLILGVLSLLLLVTGAFGFHLWRRQWRPRRFSALEQGIHPPQAQSKIEELEQE PEPEPEPEPEPEPEPEPEQL (SEQ ID NO: 35)

[0314] In accordance with the presently disclosed subject matter, a "LAG-3 nucleic acid molecule" refers to a polynucleotide encoding a LAG-3 polypeptide.

[0315] Natural Killer Cell Receptor 2B4 (2B4) mediates non-MHC restricted cell killing on NK cells and subsets of T cells. To date, the function of 2B4 is still under investigation, with the 2B4-S isoform believed to be an activating receptor, and the 2B4- L isoform

believed to be a negative immune regulator of immune cells. 2B4 becomes engaged upon binding its high-affinity ligand, CD48. 2B4 contains a tyrosine-based switch motif, a molecular switch that allows the protein to associate with various phosphatases. 2B4 has also been designated CD244 (cluster of differentiation 244).

[0316] In accordance with the presently disclosed subject matter, a 2B4 polypeptide can have an amino acid sequence that is at least about 85%, about 90%, about 95%, about 96%, about 97%, about 98%, about 99% or about 100% homologous to UniProtKB/Swiss-Prot Ref. No.: Q9BZW8.2 (SEQ ID NO: 36) or fragments thereof, and/or may optionally comprise up to one or up to two or up to three conservative amino acid substitutions.

[0317] SEQ ID NO: 36 is provided below:

[0318] MLGQWTLILLLLLKVYQGKGCQGSADHWSISGVPLQLQPNSIQTKVDSIA WKKLLPSQNGFHHILKWENGSLPSNTSNDRFSFIVKNLSLLIKAAQQQDSGLYCLEVT SISGKVQTATFQVFVFESLLPDKVEKPRLQGQGKILDRGRCQVALSCLVSRDGNVSY AWYRGSKLIQTAGNLTYLDEEVDINGTHTYTCNVSNPVSWESHTLNLTQDCQNAHQ EFRFWPFLVIIVILSALFLGTLACFCVWRRKRKEKQSETSPKEFLTIYEDVKDLKTRRN HEQEQTFPGGGSTIYSMIQSQSSAPTSQEPAYTLYSLIQPSRKSGSRKRNHSPSFNSTIY EVIGKSQPKAQNPARLSRKELENFDVYS (SEQ ID NO: 36)

[0319] In accordance with the presently disclosed subject matter, a "2B4 nucleic acid molecule" refers to a polynucleotide encoding a 2B4 polypeptide.

[0320] B- and T-lymphocyte attenuator (BTLA) expression is induced during activation of T cells, and BTLA remains expressed on Thl cells but not Th2 cells. Like PD1 and CTLA4, BTLA interacts with a B7 homolog, B7H4. However, unlike PD-1 and CTLA-4, BTLA displays T-Cell inhibition via interaction with tumor necrosis family receptors (TNF-R), not just the B7 family of cell surface receptors. BTLA is a ligand for tumor necrosis factor (receptor) superfamily, member 14 (TNFRSF14), also known as herpes virus entry mediator (HVEM). BTLA-HVEM complexes negatively regulate T-cell immune responses. BTLA activation has been shown to inhibit the function of human $CD8^+$ cancer-specific T cells. BTLA has also been designated as CD272 (cluster of differentiation 272).

[0321] In accordance with the presently disclosed subject matter, a BTLA polypeptide can have an amino acid sequence that is at least about 85%, about 90%, about 95%, about 96%, about 97%, about 98%, about 99% or about 100% homologous to UniProtKB/Swiss-

Prot Ref. No. : Q7Z6A9.3 (SEQ ID NO: 37) or fragments thereof, and/or may optionally comprise up to one or up to two or up to three conservative amino acid substitutions.

[0322] SEQ ID NO: 37 is provided below:

[0323] MKTLPAMLGTGKLFWVFFLIPYLDIWNIHGKESCDVQLYIKRQSEHSILAG DPFELECPVKYCANRPHVTWCKLNGTTCVKLEDRQTSWKEEKNISFFILHFEPVLPN DNGSYRCSANFQSNLIESHSTTLYVTDVKSASERPSKDEMASRPWLLYRLLPLGGLP LLITTCFCLFCCLRRHQGKQNELSDTAGREINLVDAHLKSEQTEASTRQNSQVLLSET GIYDNDPDLCFRMQEGSEVYSNPCLEENKPGIVYASLNHSVIGPNSRLARNVKEAPTE YASICVRS (SEQ ID NO: 37)

[0324] In accordance with the presently disclosed subject matter, a "BTLA nucleic acid molecule" refers to a polynucleotide encoding a BTLA polypeptide.

Exemplary CAR and Prodrug converting enzyme Constructs

[0325] In certain embodiments, the CAR and prodrug converting enzyme are expressed as single polypeptide linked by a self-cleaving linker, such as a P2A linker. In certain embodiments, the CAR and prodrug converting enzyme are expressed as two separate polypeptides.

[0326] In certain embodiments, the CAR comprises an extracellular antigen-binding region that comprises a human scFv that specifically binds to a human tumor antigen, a transmembrane domain comprising a CD28 polypeptide, and an intracellular domain comprising a CD3ζ polypeptide and a co-stimulatory signaling region that comprises a 4-1BB polypeptide, as shown in FIG. 3. As shown in FIG. 3, the CAR also comprises a signal peptide or a leader covalently joined to the N-terminus of the extracellular antigen-binding domain. The signal peptide comprises amino acids having the sequence set forth in SEQ ID NO: 11. In certain embodiments, the human scFv is selected from the group consisting of an anti-CD19 scFv, and anti-WT1 scFv, and anti-PRAME scFv.

[0327] In some embodiments, the nucleic acid encoding the CAR and the prodrug converting enzyme (*e.g.*, CPG2 or β-lactamase) is operably linked an inducible promoter. In some embodiments, the nucleic acid encoding the CAR and the prodrug converting enzyme (*e.g.*, CPG2 or β-lactamase) is operably linked a constitutive promoter. In some embodiments, the nucleic acid encoding the CAR and the nucleic acid encoding prodrug converting enzyme (*e.g.*, CPG2 or β-lactamase) are operably linked to two separate

promoters. In some embodiments, the nucleic acid encoding the CAR is operably linked a constitutive promoter and the prodrug converting enzyme (*e.g.*, CPG2 or β-lactamase) is operably linked an constitutive promoter. In some embodiments, the nucleic acid encoding the CAR is operably linked a constitutive promoter and the prodrug converting enzyme (*e.g.*, CPG2 or β-lactamase) is operably linked an inducible promoter.

[0328] In some embodiments, the inducible promoter is a synthetic Notch promoter that is activatable in a CAR T cell, where the intracellular domain of the CAR contains a transcriptional regulator that is released from the membrane when engagement of the CAR with the tumor antigen induces intramembrane proteolysis (*see*, *e.g.* Morsut *et al.*, *Cell* 164(4): 780–791 (2016). Accordingly, transcription of the prodrug converting enzyme is induce upon binding of the engineered immune cell with the tumor antigen.

[0329] The presently disclosed subject matter also provides isolated nucleic acid molecules encoding the CAR/prodrug converting enzyme constructs described herein or a functional portion thereof. In certain embodiments, the isolated nucleic acid molecule encodes an anti-CD19-targeted CAR comprising a human scFv that specifically binds to a human CD19 polypeptide, a transmembrane domain comprising a CD8 polypeptide, and an intracellular domain comprising a CD3ζ polypeptide and a co-stimulatory signaling region comprising a 4-1BB polypeptide, a P2A self-cleaving peptide, and a CPG2 or β-lactamase polypeptide fused to a signal peptide and a transmembrane domain comprising a CD8 polypeptide (*see, e.g.*, FIG. 3A and exemplary constructs set forth in SEQ ID NOS: 48-51).

[0330] In certain embodiments, the isolated nucleic acid molecule encodes an anti-CD19-targeted CAR comprising a human scFv that specifically binds to a human CD19 polypeptide, a transmembrane domain comprising a CD8 polypeptide, and an intracellular domain comprising a CD3ζ polypeptide and a co-stimulatory signaling region comprising a 4-1BB polypeptide, a P2A self-cleaving peptide, and a CPG2 or β-lactamase polypeptide fused to a signal peptide (*see, e.g.*, FIG. 3B).

[0331] In certain embodiments, the isolated nucleic acid molecule encodes an anti-CD19-targeted CAR comprising a human scFv that specifically binds to a human CD19 polypeptide fused to a synthetic Notch transmembrane domain and an intracellular cleavable transcription factor (*see, e.g.*, FIG. 3C) In certain embodiments, the isolated nucleic acid molecule encodes

a CPG2 or β-lactamase enzyme inducible by release of the transcription factor of a synthetic Notch system.

[0332] In certain embodiments, the isolated nucleic acid molecule encodes a functional portion of a presently disclosed CAR constructs. As used herein, the term "functional portion" refers to any portion, part or fragment of a CAR, which portion, part or fragment retains the biological activity of the targeted CAR (the parent CAR). For example, functional portions encompass the portions, parts or fragments of a tumor antigen-targeted CAR that retains the ability to recognize a target cell, to treat a disease, *e.g.*, solid tumor, to a similar, same, or even a higher extent as the parent CAR. In certain embodiments, an isolated nucleic acid molecule encoding a functional portion of a tumor antigen- targeted CAR can encode a protein comprising, *e.g.*, about 10%, about 20%, about 25%, about 30%, about 35%, about 40%, about 45%, about 50%, about 55%, about 60%, about 65%, about 70%, about 75%, about 80%, about 85%, about 90%, and about 95%, or more of the parent CAR.

Immune Cells

[0333] The presently disclosed subject matter provides engineered immune cells expressing a prodrug converting enzyme and a T-cell receptor (*e.g.*, a CAR) or other ligand that comprises an extracellular antigen-binding domain, a transmembrane domain and an intracellular domain, where the extracellular antigen- binding domain specifically binds tumor antigen, including a tumor receptor or ligand, as described above. In certain embodiments immune cells can be transduced with a presently disclosed CAR/prodrug converting enzyme constructs such that the cells express the CAR and the prodrug converting enzyme. The presently disclosed subject matter also provides methods of using such cells for the treatment of a tumor. The engineered immune cells of the presently disclosed subject matter can be cells of the lymphoid lineage or myeloid lineage. The lymphoid lineage, comprising B, T, and natural killer (NK) cells, provides for the production of antibodies, regulation of the cellular immune system, detection of foreign agents in the blood, detection of cells foreign to the host, and the like. Non-limiting examples of immune cells of the lymphoid lineage include T cells, Natural Killer (NK) cells, embryonic stem cells, and pluripotent stem cells (*e.g.*, those from which lymphoid cells may be differentiated). T cells can be lymphocytes that mature in the thymus and are chiefly responsible for cell-mediated immunity. T cells are involved in the adaptive immune system. The T cells of the presently

disclosed subject matter can be any type of T cells, including, but not limited to, T helper cells, cytotoxic T cells, memory T cells (including central memory T cells, stem-cell-like memory T cells (or stem-like memory T cells), and two types of effector memory T cells: *e.g.*, T_{EM} cells and TEMRA cells, Regulatory T cells (also known as suppressor T cells), Natural killer T cells, Mucosal associated invariant T cells, and γδ T cells. Cytotoxic T cells (CTL or killer T cells) are a subset of T lymphocytes capable of inducing the death of infected somatic or tumor cells. In certain embodiments, the CAR-expressing T cells express Foxp3 to achieve and maintain a T regulatory phenotype.

[0334] Natural killer (NK) cells can be lymphocytes that are part of cell-mediated immunity and act during the innate immune response. NK cells do not require prior activation in order to perform their cytotoxic effect on target cells.

[0335] The engineered immune cells of the presently disclosed subject matter can express an extracellular antigen-binding domain (*e.g.*, a human scFv, a Fab that is optionally crosslinked, or a $F(ab)_2$) that specifically binds to a tumor antigen, for the treatment of cancer, *e.g.*, for treatment of solid tumor. Such engineered immune cells can be administered to a subject (*e.g.*, a human subject) in need thereof for the treatment of cancer. In some embodiments, the immune cell is a lymphocyte, such as a T cell, a B cell or a natural killer (NK) cell. In certain embodiments, the engineered immune cell is a T cell. The T cell can be a $CD4^+$ T cell or a CD8 T cell. In certain embodiments, the T cell is a $CD4^+$ T cell. In certain embodiments, the T cell is a $CD8^+$ T cell.

[0336] A presently disclosed engineered immune cells can further include at least one recombinant or exogenous co-stimulatory ligand. For example, a presently disclosed engineered immune cells can be further transduced with at least one co- stimulatory ligand, such that the engineered immune cells co-expresses or is induced to co-express the tumor antigen-targeted CAR and the at least one co-stimulatory ligand. The interaction between the tumor antigen-targeted CAR and at least one co-stimulatory ligand provides a non-antigen-specific signal important for full activation of an immune cell (*e.g.*, T cell). Co-stimulatory ligands include, but are not limited to, members of the tumor necrosis factor (TNF) superfamily, and immunoglobulin (Ig) superfamily ligands. TNF is a cytokine involved in systemic inflammation and stimulates the acute phase reaction. Its primary role is in the regulation of immune cells. Members of TNF superfamily share a number of common

features. The majority of TNF superfamily members are synthesized as type II transmembrane proteins (extracellular C-terminus) containing a short cytoplasmic segment and a relatively long extracellular region. TNF superfamily members include, without limitation, nerve growth factor (NGF), CD40L (CD40L)/CD 154, CD137L/4-1BBL, TNF-a, CD134L/OX40L/CD252, CD27L/CD70, Fas ligand (FasL), CD30L/CD153, tumor necrosis factor beta (TNFP)/lymphotoxin-alpha (LTa), lymphotoxin-beta O-Tβ), CD257/B cell-activating factor (B AFF)/Bly s/THANK/Tall- 1 , glucocorticoid-induced TNF Receptor ligand (GITRL), and T F-related apoptosis-inducing ligand (TRAIL), LIGHT (TNFSF14). The immunoglobulin (Ig) superfamily is a large group of cell surface and soluble proteins that are involved in the recognition, binding, or adhesion processes of cells. These proteins share structural features with immunoglobulins — they possess an immunoglobulin domain (fold). Immunoglobulin superfamily ligands include, but are not limited to, CD80 and CD86, both ligands for CD28, PD-L1/(B7-H1) that ligands for PD-1. In certain embodiments, the at least one co-stimulatory ligand is selected from the group consisting of 4-1BBL, CD80, CD86, CD70, OX40L, CD48, TNFRSF14, PD-L1, and combinations thereof. In certain embodiments, the engineered immune cell comprises one recombinant co-stimulatory ligand that is 4-1BBL. In certain embodiments, the engineered immune cell comprises two recombinant co-stimulatory ligands that are 4-1BBL and CD80. CARs comprising at least one co-stimulatory ligand are described in U.S. Patent No. 8,389,282, which is incorporated by reference in its entirety.

[0337] Furthermore, a presently disclosed engineered immune cells can further comprise at least one exogenous cytokine. For example, a presently disclosed engineered immune cell can be further transduced with at least one cytokine, such that the engineered immune cells secretes the at least one cytokine as well as expresses the tumor antigen-targeted CAR. In certain embodiments, the at least one cytokine is selected from the group consisting of IL-2, IL- 3, IL-6, IL-7, IL-11, IL-12, IL-15, IL-17, and IL-21. In certain embodiments, the cytokine is IL-12.

[0338] The engineered immune cells can be generated from peripheral donor lymphocytes, *e.g.*, those disclosed in Sadelain, M., *et al., Nat Rev Cancer* 3 :35-45 (2003) (disclosing peripheral donor lymphocytes genetically modified to express CARs), in Morgan, R.A. *et al., Science* 314: 126-129 (2006) (disclosing peripheral donor lymphocytes

genetically modified to express a full-length tumor antigen-recognizing T cell receptor complex comprising the α and β heterodimer), in Panelli *et al. J Immunol* 164:495-504 (2000); Panelli *et al. J Immunol* 164:4382-4392 (2000) (disclosing lymphocyte cultures derived from tumor infiltrating lymphocytes (TILs) in tumor biopsies), and in Dupont *et al. Cancer Res* 65:5417-5427 (2005); Papanicolaou *et al. Blood* 102:2498-2505 (2003) (disclosing selectively in v/Yro-expanded antigen-specific peripheral blood leukocytes employing artificial antigen-presenting cells (AAPCs) or pulsed dendritic cells). The engineered immune cells (*e.g.*, T cells) can be autologous, non-autologous (*e.g.*, allogeneic), or derived *in vitro* from engineered progenitor or stem cells.

[0339] In certain embodiments, a presently disclosed engineered immune cells (*e.g.*, T cells) expresses from about 1 to about 5, from about 1 to about 4, from about 2 to about 5, from about 2 to about 4, from about 3 to about 5, from about 3 to about 4, from about 4 to about 5, from about 1 to about 2, from about 2 to about 3, from about 3 to about 4, or from about 4 to about 5 vector copy numbers per cell of a presently disclosed tumor antigen-targeted CAR and/or prodrug converting enzyme.

[0340] For example, the higher the CAR expression level in an engineered immune cell, the greater cytotoxicity and cytokine production the engineered immune cell exhibits. An engineered immune cell (*e.g.*, T cell) having a high tumor antigen-targeted CAR expression level can induce antigen-specific cytokine production or secretion and/or exhibit cytotoxicity to a tissue or a cell having a low expression level of tumor antigen-targeted CAR, *e.g.*, about 2,000 or less, about 1,000 or less, about 900 or less, about 800 or less, about 700 or less, about 600 or less, about 500 or less, about 400 or less, about 300 or less, about 200 or less, about 100 or less of tumor antigen binding sites/cell. Additionally or alternatively, the cytotoxicity and cytokine production of a presently disclosed engineered immune cell (*e.g.*, T cell) are proportional to the expression level of tumor antigen in a target tissue or a target cell. For example, the higher the expression level of human tumor antigen in the target, the greater cytotoxicity and cytokine production the engineered immune cell exhibits.

[0341] As described herein, the co-expression of prodrug converting enzyme increases the cytotoxic effect in the CAR T cells by converting a prodrug into an active drug at the target site. In certain embodiments, an engineered immune cells of the present disclosure exhibits a cytotoxic effect against tumor antigen-expressing cells that is at least about 2-

times, about 3- times, about 4-times, about 5-times, about 6-times, about 7-times, about 8-times, about 9- times, about 10-times, about 20-times, about 30-times, about 40-times, about 50-times, about 60-times, about 70-times, about 80-times, about 90-times, or about 100-times, the cytotoxic effect in the absence of the prodrug converting enzyme.

[0342] The unpurified source of immune cells may be any known in the art, such as the bone marrow, fetal, neonate or adult or other hematopoietic cell source, *e.g.*, fetal liver, peripheral blood or umbilical cord blood. Various techniques can be employed to separate the cells. For instance, negative selection methods can remove non-immune cell initially. Monoclonal antibodies are particularly useful for identifying markers associated with particular cell lineages and/or stages of differentiation for both positive and negative selections.

[0343] A large proportion of terminally differentiated cells can be initially removed by a relatively crude separation. For example, magnetic bead separations can be used initially to remove large numbers of irrelevant cells. Preferably, at least about 80%, usually at least 70% of the total hematopoietic cells will be removed prior to cell isolation.

[0344] Procedures for separation include, but are not limited to, density gradient centrifugation; resetting; coupling to particles that modify cell density; magnetic separation with antibody-coated magnetic beads; affinity chromatography; cytotoxic agents joined to or used in conjunction with a mAb, including, but not limited to, complement and cytotoxins; and panning with antibody attached to a solid matrix, *e.g.,* plate, chip, elutriation or any other convenient technique.

[0345] Techniques for separation and analysis include, but are not limited to, flow cytometry, which can have varying degrees of sophistication, *e.g.*, a plurality of color channels, low angle and obtuse light scattering detecting channels, impedance channels.

[0346] The cells can be selected against dead cells, by employing dyes associated with dead cells such as propidium iodide (PI). Preferably, the cells are collected in a medium comprising 2% fetal calf serum (FCS) or 0.2% bovine serum albumin (BSA) or any other suitable, preferably sterile, isotonic medium.

[0347] In some embodiments, the engineered immune cells comprise one or more additional modifications. For example, in some embodiments, the engineered immune cells comprise and express (is transduced to express) an antigen recognizing receptor that binds to

a second antigen that is different than selected tumor antigen. The inclusion of an antigen recognizing receptor in addition to a presently disclosed CAR on the engineered immune cell can increase the avidity of the CAR or the engineered immune cell comprising thereof on a targeted cell, especially, the CAR is one that has a low binding affinity to a particular tumor antigen, *e.g.*, a K_d of about 2 x 10^{-8} M or more, about 5 x 10^{-8} M or more, about 8 x 10^{-8} M or more, about 9 x 10^{-8} M or more, about 1 x 10^{-7} M or more, about 2 x 10^{-7} M or more, or about 5 x 10^{-7} M or more.

[0348] In certain embodiments, the antigen recognizing receptor is a chimeric co-stimulatory receptor (CCR). CCR is described in Krause, *et al., J. Exp. Med.* 188(4):619-626(1998), and US20020018783, the contents of which are incorporated by reference in their entireties. CCRs mimic co-stimulatory signals, but unlike, CARs, do not provide a T-cell activation signal, *e.g.*, CCRs lack a CD3ζ polypeptide. CCRs provide co-stimulation, *e.g.*, a CD28-like signal, in the absence of the natural co-stimulatory ligand on the antigen-presenting cell. A combinatorial antigen recognition, *i.e.,* use of a CCR in combination with a CAR, can augment T-cell reactivity against the dual-antigen expressing T cells, thereby improving selective tumor targeting. Kloss *et al.,* describe a strategy that integrates combinatorial antigen recognition, split signaling, and, critically, balanced strength of T-cell activation and costimulation to generate T cells that eliminate target cells that express a combination of antigens while sparing cells that express each antigen individually (Kloss *et al., Nature Biotechnology* 31(l):71-75 (2013)). With this approach, T-cell activation requires CAR-mediated recognition of one antigen, whereas costimulation is independently mediated by a CCR specific for a second antigen. To achieve tumor selectivity, the combinatorial antigen recognition approach diminishes the efficiency of T-cell activation to a level where it is ineffective without rescue provided by simultaneous CCR recognition of the second antigen. In certain embodiments, the CCR comprises an extracellular antigen-binding domain that binds to an antigen different than selected tumor antigen, a transmembrane domain, and a co-stimulatory signaling region that comprises at least one co-stimulatory molecule, including, but not limited to, CD28, 4-1BB, OX40, ICOS, PD-1, CTLA-4, LAG-3, 2B4, and BTLA. In certain embodiments, the co- stimulatory signaling region of the CCR comprises one co-stimulatory signaling molecule. In certain embodiments, the one co-stimulatory signaling molecule is CD28. In certain embodiments, the one co-stimulatory signaling

molecule is 4-IBB. In certain embodiments, the co-stimulatory signaling region of the CCR comprises two co- stimulatory signaling molecules. In certain embodiments, the two co-stimulatory signaling molecules are CD28 and 4-IBB. A second antigen is selected so that expression of both selected tumor antigen and the second antigen is restricted to the targeted cells (*e.g.*, cancerous tissue or cancerous cells). Similar to a CAR, the extracellular antigen-binding domain can be a scFv, a Fab, a $F(ab)_2$; or a fusion protein with a heterologous sequence to form the extracellular antigen-binding domain. In certain embodiments, the CCR comprises a scFv that binds to CD 138, transmembrane domain comprising a CD28 polypeptide, and a co-stimulatory signaling region comprising two co-stimulatory signaling molecules that are CD28 and 4-IBB.

[0349] In certain embodiments, the antigen recognizing receptor is a truncated CAR. A "truncated CAR" is different from a CAR by lacking an intracellular signaling domain. For example, a truncated CAR comprises an extracellular antigen-binding domain and a transmembrane domain, and lacks an intracellular signaling domain. In accordance with the presently disclosed subject matter, the truncated CAR has a high binding affinity to the second antigen expressed on the targeted cells, *e.g.*, myeloma cells. The truncated CAR functions as an adhesion molecule that enhances the avidity of a presently disclosed CAR, especially, one that has a low binding affinity to tumor antigen, thereby improving the efficacy of the presently disclosed CAR or engineered immune cell (*e.g.*, T cell) comprising thereof. In certain embodiments, the truncated CAR comprises an extracellular antigen-binding domain that binds to CD 138, a transmembrane domain comprising a CD8 polypeptide. A presently disclosed T cell comprises or is transduced to express a presently disclosed CAR targeting tumor antigen and a truncated CAR targeting CD138. In certain embodiments, the targeted cells are solid tumor cells.

In some embodiments, the engineered immune cells are further modified to suppress expression of one or more genes. In some embodiments, the engineered immune cells are further modified via genome editing. Various methods and compositions for targeted cleavage of genomic DNA have been described. Such targeted cleavage events can be used, for example, to induce targeted mutagenesis, induce targeted deletions of cellular DNA sequences, and facilitate targeted recombination at a predetermined chromosomal locus. *See*, for example, U.S. Patent Nos. 7,888,121 ; 7,972,854; 7,914,796; 7,951,925; 8,110,379;

8,409,861 ; 8,586,526; U.S. Patent Publications 20030232410; 20050208489; 20050026157; 20050064474; 20060063231 ; 201000218264; 20120017290; 20110265198; 20130137104; 20130122591; 20130177983 and 20130177960, the disclosures of which are incorporated by reference in their entireties. These methods often involve the use of engineered cleavage systems to induce a double strand break (DSB) or a nick in a target DNA sequence such that repair of the break by an error born process such as non-homologous end joining (NHEJ) or repair using a repair template (homology directed repair or HDR) can result in the knock out of a gene or the insertion of a sequence of interest (targeted integration). Cleavage can occur through the use of specific nucleases such as engineered zinc finger nucleases (ZFN), transcription-activator like effector nucleases (TALENs), or using the CRISPR/Cas system with an engineered crRNA/tracr RNA ('single guide RNA') to guide specific cleavage. In some embodiments, the engineered immune cells are modified to disrupt or reduce expression of an endogenous T-cell receptor gene (*see*, *e.g.* WO 2014153470, which is incorporated by reference in its entirety). In some embodiments, the engineered immune cells are modified to result in disruption or inhibition of PD1, PDL-1 or CTLA-4 (see, *e.g.* U.S. Patent Publication 20140120622), or other immunosuppressive factors known in the art (Wu et al. (2015) Oncoimmunology 4(7): e1016700, Mahoney et al. (2015) Nature Reviews Drug Discovery 14, 561–584).

Vectors

[0350] Many expression vectors are available and known to those of skill in the art and can be used for expression of polypeptides provided herein, for example, for expression of a chimeric antigen receptor, a first prodrug converting enzyme, and/or a second prodrug converting enzyme. The choice of expression vector will be influenced by the choice of host expression system. Such selection is well within the level of skill of the skilled artisan. In general, expression vectors can include transcriptional promoters and optionally enhancers, translational signals, and transcriptional and translational termination signals. Expression vectors that are used for stable transformation typically have a selectable marker which allows selection and maintenance of the transformed cells. In some cases, an origin of replication can be used to amplify the copy number of the vector in the cells.

[0351] Vectors also can contain additional nucleotide sequences operably linked to the ligated nucleic acid molecule, such as, for example, an epitope tag such as for localization,

e.g. a hexa-his tag or a myc tag, hemagglutinin tag or a tag for purification, for example, a GST fusion, and a sequence for directing protein secretion and/or membrane association.

[0352] Expression of the antibodies or antigen-binding fragments thereof can be controlled by any promoter/enhancer known in the art. Suitable bacterial promoters are well known in the art and described herein below. Other suitable promoters for mammalian cells, yeast cells and insect cells are well known in the art and some are exemplified below. Selection of the promoter used to direct expression of a heterologous nucleic acid depends on the particular application and is within the level of skill of the skilled artisan. Promoters which can be used include but are not limited to eukaryotic expression vectors containing the SV40 early promoter (Bernoist and Chambon, *Nature* 290:304-310(1981)), the promoter contained in the 3' long terminal repeat of Rous sarcoma virus (Yamamoto *et al.*, *Cell* 22:787-797(1980)), the herpes thymidine kinase promoter (Wagner *et al.*, *Proc. Natl. Acad. Sci. USA* 75: 1441-1445 (1981)), the regulatory sequences of the metallothionein gene (Brinster *et al.*, *Nature* 296:39-42 (1982)); prokaryotic expression vectors such as the β-lactamase promoter (Jay *et al.*, *Proc. Natl. Acad. Sci. USA* 75:5543 (1981)) or the tac promoter (DeBoer *et al.*, *Proc. Natl. Acad. Sci. USA* 50:21-25(1983)); see also "Useful Proteins from Recombinant Bacteria": in *Scientific American* 242:79-94 (1980)); plant expression vectors containing the nopaline synthetase promoter (Herrera- Estrella *et al.*, *Nature* 505:209-213(1984)) or the cauliflower mosaic virus 35S RNA promoter (Gardner *et al.*, *Nucleic Acids Res.* 9:2871(1981)), and the promoter of the photosynthetic enzyme ribulose bisphosphate carboxylase (Herrera-Estrella *et al.*, *Nature* 510: 1 15-120(1984)); promoter elements from yeast and other fungi such as the Gal4 promoter, the alcohol dehydrogenase promoter, the phosphoglycerol kinase promoter, the alkaline phosphatase promoter, and the following animal transcriptional control regions that exhibit tissue specificity and have been used in transgenic animals: elastase I gene control region which is active in pancreatic acinar cells (Swift *et al.*, *Cell* 55:639-646 (1984); Ornitz *et al.*, *Cold Spring Harbor Symp. Quant. Biol.* 50:399-409 (1986); MacDonald, *Hepatology* 7:425-515 (1987)); insulin gene control region which is active in pancreatic beta cells (Hanahan *et al.*, *Nature* 515: 115-122 (1985)), immunoglobulin gene control region which is active in lymphoid cells (Grosschedl *et al.*, *Cell* 55:647-658 (1984); Adams *et al.*, *Nature* 515:533-538 (1985); Alexander *et al.*, *Mol. Cell Biol.* 7: 1436-1444 (1987)), mouse mammary tumor virus

control region which is active in testicular, breast, lymphoid and mast cells (Leder *et al.*, *Cell* 15:485-495 (1986)), albumin gene control region which is active in liver (Pinckert et al., *Genes and Devel.* 1:268-276 (1987)), alpha-fetoprotein gene control region which is active in liver (Krumlauf *et al.*, *Mol. Cell. Biol.* 5:1639-403 (1985)); Hammer *et al.*, *Science* 255:53-58 (1987)), alpha-1 antitrypsin gene control region which is active in liver (Kelsey *et al.*, *Genes and Devel.* 7:161-171 (1987)), beta globin gene control region which is active in myeloid cells (Magram *et al.*, *Nature* 515:338-340 (1985)); Kollias *et al.*, *Cell* 5:89-94 (1986)), myelin basic protein gene control region which is active in oligodendrocyte cells of the brain (Readhead *et al.*, *Cell* 15:703-712 (1987)), myosin light chain-2 gene control region which is active in skeletal muscle (Shani, *Nature* 514:283-286 (1985)), and gonadotrophic releasing hormone gene control region which is active in gonadotrophs of the hypothalamus (Mason *et al.*, *Science* 254: 1372- 1378 (1986)).

[0353] In addition to the promoter, the expression vector typically contains a transcription unit or expression cassette that contains all the additional elements required for the expression of the antibody, or portion thereof, in host cells. A typical expression cassette contains a promoter operably linked to the nucleic acid sequence encoding the antibody chain and signals required for efficient polyadenylation of the transcript, ribosome binding sites and translation termination. Additional elements of the cassette can include enhancers. In addition, the cassette typically contains a transcription termination region downstream of the structural gene to provide for efficient termination. The termination region can be obtained from the same gene as the promoter sequence or can be obtained from different genes.

[0354] Some expression systems have markers that provide gene amplification such as thymidine kinase and dihydrofolate reductase. Alternatively, high yield expression systems not involving gene amplification are also suitable, such as using a baculovirus vector in insect cells, with a nucleic acid sequence encoding a germline antibody chain under the direction of the polyhedron promoter or other strong baculovirus promoter.

[0355] Any methods known to those of skill in the art for the insertion of DNA fragments into a vector can be used to construct expression vectors containing a nucleic acid encoding any of the polypeptides provided herein. These methods can include *in vitro* recombinant DNA and synthetic techniques and in vivo recombinants (genetic recombination). The insertion into a cloning vector can, for example, be accomplished by

ligating the DNA fragment into a cloning vector which has complementary cohesive termini. If the complementary restriction sites used to fragment the DNA are not present in the cloning vector, the ends of the DNA molecules can be enzymatically modified. Alternatively, any site desired can be produced by ligating nucleotide sequences (linkers) onto the DNA termini; these ligated linkers can contain specific chemically synthesized nucleic acids encoding restriction endonuclease recognition sequences.

[0356] Exemplary plasmid vectors useful to produce the polypeptides provided herein contain a strong promoter, such as the HCMV immediate early enhancer/promoter or the MHC class I promoter, an intron to enhance processing of the transcript, such as the HCMV immediate early gene intron A, and a polyadenylation (poly A) signal, such as the late SV40 polyA signal.

[0357] Genetic modification of engineered immune cells (*e.g.*, T cells, NK cells) can be accomplished by transducing a substantially homogeneous cell composition with a recombinant DNA or RNA construct. The vector can be a retroviral vector (*e.g.*, gamma retroviral), which is employed for the introduction of the DNA or RNA construct into the host cell genome. For example, a polynucleotide encoding the tumor antigen-targeted CAR and the prodrug converting enzyme can be cloned into a retroviral vector and expression can be driven from its endogenous promoter, from the retroviral long terminal repeat, or from an alternative internal promoter.

[0358] Non-viral vectors or RNA may be used as well. Random chromosomal integration, or targeted integration (*e.g.*, using a nuclease, transcription activator-like effector nucleases (TALENs), Zinc-finger nucleases (ZFNs), and/or clustered regularly interspaced short palindromic repeats (CRISPRs), or transgene expression (*e.g.*, using a natural or chemically modified RNA) can be used.

[0359] For initial genetic modification of the cells to provide tumor antigen-targeted CAR and the prodrug converting enzyme expressing cells, a retroviral vector is generally employed for transduction, however any other suitable viral vector or non-viral delivery system can be used. For subsequent genetic modification of the cells to provide cells comprising an antigen presenting complex comprising at least two co-stimulatory ligands, retroviral gene transfer (transduction) likewise proves effective. Combinations of retroviral vector and an appropriate packaging line are also suitable, where the capsid proteins will be

functional for infecting human cells. Various amphotropic virus-producing cell lines are known, including, but not limited to, PA12 (Miller, *et al. Mol. Cell. Biol.* 5:431-437(1985)); PA317 (Miller, *et al. Mol. Cell. Biol.* 6:2895-2902(1986)); and CRIP (Danos, *et al. Proc. Natl. Acad. Sci. USA* 85:6460-6464(1988)). Non -amphotropic particles are suitable too, *e.g.*, particles pseudotyped with VSVG, RD114 or GALV envelope and any other known in the art.

[0360] Possible methods of transduction also include direct co-culture of the cells with producer cells, *e.g.*, by the method of Bregni, *et al. Blood* 80: 1418-1422(1992), or culturing with viral supernatant alone or concentrated vector stocks with or without appropriate growth factors and polycations, *e.g.*, by the method of Xu, *et al. Exp. Hemat.* 22:223-230(1994); and Hughes, *et al. J. Clin. Invest.* 89: 1817(1992).

[0361] Transducing viral vectors can be used to express a co-stimulatory ligand and/or secretes a cytokine (*e.g.*, 4-1BBL and/or IL-12) in an engineered immune cell. Preferably, the chosen vector exhibits high efficiency of infection and stable integration and expression (see, *e.g.*, Cayouette *et al., Human Gene Therapy* 8:423-430 (1997); Kido *et al., Current Eye Research* 15:833-844 (1996); Bloomer *et al., Journal of Virology* 71 :6641-6649, 1997; Naldini *et al., Science* 272:263 267 (1996); and Miyoshi *et al., Proc. Natl. Acad. Sci. U.S.A.* 94: 10319, (1997)). Other viral vectors that can be used include, for example, adenoviral, lentiviral, and adeno-associated viral vectors, vaccinia virus, a bovine papilloma virus, or a herpes virus, such as Epstein-Barr Virus (also see, for example, the vectors of Miller, *Human Gene Therapy* 15-14, (1990); Friedman, *Science* 244: 1275-1281 (1989); Eglitis *et al., BioTechniques* 6:608-614, (1988); Tolstoshev *et al., Current Opinion in Biotechnology* 1:55-61 (1990); Sharp, *The Lancet* 337: 1277-1278 (1991); Cornetta *et al., Nucleic Acid Research and Molecular Biology* 36:311-322 (1987); Anderson, *Science* 226:401-409 (1984); Moen, *Blood Cells* 17:407-416 (1991); Miller *et al., Biotechnology* 7:980-990 (1989); Le Gal La Salle *et al., Science* 259:988-990 (1993); and Johnson, *Chest* 107:77S-83S (1995)). Retroviral vectors are particularly well developed and have been used in clinical settings (Rosenberg *et al., N. Engl. J. Med* 323:370 (1990); Anderson *et al.,* U.S. Pat. No. 5,399,346).

[0362] In certain non-limiting embodiments, the vector expressing a presently disclosed tumor antigen-targeted CAR is a retroviral vector, *e.g.*, an oncoretroviral vector.

[0363] Non-viral approaches can also be employed for the expression of a protein in cell. For example, a nucleic acid molecule can be introduced into a cell by administering the nucleic acid in the presence of lipofection (Feigner *et al., Proc. Nat'l. Acad. Sci. U.S.A.* 84:7413, (1987); Ono *et al., Neuroscience Letters* 17:259 (1990); Brigham *et al., Am. J. Med. Sci.* 298:278, (1989); Staubinger *et al., Methods in Enzymology* 101 :512 (1983)), asialoorosomucoid-polylysine conjugation (Wu *et al., Journal of Biological Chemistry* 263 : 14621 (1988); Wu *et al., Journal of Biological Chemistry* 264: 16985 (1989)), or by micro-injection under surgical conditions (Wolff *et al., Science* 247: 1465 (1990)). Other non-viral means for gene transfer include transfection *in vitro* using calcium phosphate, DEAE dextran, electroporation, and protoplast fusion. Liposomes can also be potentially beneficial for delivery of DNA into a cell. Transplantation of normal genes into the affected tissues of a subject can also be accomplished by transferring a normal nucleic acid into a cultivatable cell type ex vivo (*e.g.*, an autologous or heterologous primary cell or progeny thereof), after which the cell (or its descendants) are injected into a targeted tissue or are injected systemically. Recombinant receptors can also be derived or obtained using transposases or targeted nucleases (*e.g.*, Zinc finger nucleases, meganucleases, or TALE nucleases). Transient expression may be obtained by RNA electroporation.

[0364] cDNA expression for use in polynucleotide therapy methods can be directed from any suitable promoter (*e.g.*, the human cytomegalovirus (CMV), simian virus 40 (SV40), or metallothionein promoters), and regulated by any appropriate mammalian regulatory element or intron (*e.g.*, the elongation factor la enhancer/promoter/intron structure). For example, if desired, enhancers known to preferentially direct gene expression in specific cell types can be used to direct the expression of a nucleic acid. The enhancers used can include, without limitation, those that are characterized as tissue- or cell-specific enhancers. Alternatively, if a genomic clone is used as a therapeutic construct, regulation can be mediated by the cognate regulatory sequences or, if desired, by regulatory sequences derived from a heterologous source, including any of the promoters or regulatory elements described above.

[0365] The resulting cells can be grown under conditions similar to those for unmodified cells, whereby the modified cells can be expanded and used for a variety of purposes. VI. Polypeptides and Analogs and Polynucleotides

[0366] Also included in the presently disclosed subject matter are extracellular antigen-binding domains that specifically binds to a tumor antigen (*e.g.*, human tumor antigen) (*e.g.*, an scFv (*e.g.*, a human scFv), a Fab, or a $(Fab)_2$), CD3ζ, CD8, CD28, etc. polypeptides or fragments thereof, and polynucleotides encoding thereof that are modified in ways that enhance their anti -tumor activity when expressed in an engineered immune cell. The presently disclosed subject matter provides methods for optimizing an amino acid sequence or a nucleic acid sequence by producing an alteration in the sequence. Such alterations may comprise certain mutations, deletions, insertions, or post-translational modifications. The presently disclosed subject matter further comprises analogs of any naturally-occurring polypeptide of the presently disclosed subject matter. Analogs can differ from a naturally-occurring polypeptide of the presently disclosed subject matter by amino acid sequence differences, by post-translational modifications, or by both. Analogs of the presently disclosed subject matter can generally exhibit at least about 85%, about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%), about 98%, about 99% or more identity or homology with all or part of a naturally-occurring amino, acid sequence of the presently disclosed subject matter. The length of sequence comparison is at least about 5, about 10, about 15, about 20, about 25, about 50, about 75, about 100 or more amino acid residues. Again, in an exemplary approach to determining the degree of identity, a BLAST program may be used, with a probability score between e^{-3} and e^{-100} indicating a closely related sequence. Modifications comprise *in vivo* and *in vitro* chemical derivatization of polypeptides, *e.g.*, acetylation, carboxylation, phosphorylation, or glycosylation; such modifications may occur during polypeptide synthesis or processing or following treatment with isolated modifying enzymes. Analogs can also differ from the naturally-occurring polypeptides of the presently disclosed subject matter by alterations in primary sequence. These include genetic variants, both natural and induced (for example, resulting from random mutagenesis by irradiation or exposure to ethanemethyl sulfate or by site-specific mutagenesis as described in Sambrook, Fritsch and Maniatis, Molecular Cloning: A Laboratory Manual (2nd ed.), CSH Press, 1989, or Ausubel *et al.,* supra). Also included are cyclized peptides, molecules, and analogs which contain residues other than L-amino acids, *e.g.*, D-amino acids or non-naturally occurring or synthetic amino acids, *e.g.*, beta (β) or gamma (γ) amino acids.

[0367] In addition to full-length polypeptides, the presently disclosed subject matter also provides fragments of any one of the polypeptides or peptide domains of the presently disclosed subject matter. A fragment can be at least about 5, about 10, about 13, or about 15 amino acids. In some embodiments, a fragment is at least about 20 contiguous amino acids, at least about 30 contiguous amino acids, or at least about 50 contiguous amino acids. In some embodiments, a fragment is at least about 60 to about 80, about 100, about 200, about 300 or more contiguous amino acids. Fragments of the presently disclosed subject matter can be generated by methods known to those of ordinary skill in the art or may result from normal protein processing (*e.g.*, removal of amino acids from the nascent polypeptide that are not required for biological activity or removal of amino acids by alternative mRNA splicing or alternative protein processing events).

[0368] Non-protein analogs have a chemical structure designed to mimic the functional activity of a protein of the invention. Such analogs are administered according to methods of the presently disclosed subject matter. Such analogs may exceed the physiological activity of the original polypeptide. Methods of analog design are well known in the art, and synthesis of analogs can be carried out according to such methods by modifying the chemical structures such that the resultant analogs increase the antineoplastic activity of the original polypeptide when expressed in an engineered immune cell. These chemical modifications include, but are not limited to, substituting alternative R groups and varying the degree of saturation at specific carbon atoms of a reference polypeptide. The protein analogs can be relatively resistant to *in vivo* degradation, resulting in a more prolonged therapeutic effect upon administration. Assays for measuring functional activity include, but are not limited to, those described in the Examples below.

[0369] In accordance with the presently disclosed subject matter, the polynucleotides encoding an extracellular antigen-binding domain that specifically binds to tumor antigen (*e.g.*, human tumor antigen) (*e.g.,* an scFv (*e.g.,* a human scFv), a Fab, or a $(Fab)_2$), CD3 , CD8, CD28) can be modified by codon optimization. Codon optimization can alter both naturally occurring and recombinant gene sequences to achieve the highest possible levels of productivity in any given expression system. Factors that are involved in different stages of protein expression include codon adaptability, mRNA structure, and various cis- elements in transcription and translation. Any suitable codon optimization methods or technologies that

are known to ones skilled in the art can be used to modify the polynucleotides of the presently disclosed subject matter, including, but not limited to, OptimumGene™, Encor optimization, and Blue Heron.

Administration

[0370] Engineered immune cells expressing the tumor antigen-targeted CAR and a prodrug converting enzyme of the presently disclosed subject matter can be provided systemically or directly to a subject for treating or preventing a neoplasia. In certain embodiments, engineered immune cells are directly injected into an organ of interest (*e.g.*, an organ affected by a neoplasia). Alternatively or additionally, the engineered immune cells are provided indirectly to the organ of interest, for example, by administration into the circulatory system (*e.g.*, the tumor vasculature). Expansion and differentiation agents can be provided prior to, during or after administration of cells and compositions to increase production of T cells *in vitro* or *in vivo*.

[0371] Engineered immune cells of the presently disclosed subject matter can be administered in any physiologically acceptable vehicle, systemically or regionally, normally intravascularly, intraperitoneally, intrathecally, or intrapleurally, although they may also be introduced into bone or other convenient site where the cells may find an appropriate site for regeneration and differentiation (*e.g.*, thymus). In certain embodiments, at least 1 x 10^5 cells can be administered, eventually reaching 1 x 10^{10} or more. In certain embodiments, at least 1 x 10^6 cells can be administered. A cell population comprising engineered immune cells can comprise a purified population of cells. Those skilled in the art can readily determine the percentage of engineered immune cells in a cell population using various well-known methods, such as fluorescence activated cell sorting (FACS). The ranges of purity in cell populations comprising engineered immune cells can be from about 50% to about 55%, from about 55% to about 60%, from about 65% to about 70%, from about 70% to about 75%, from about 75% to about 80%, from about 80% to about 85%; from about 85% to about 90%, from about 90% to about 95%, or from about 95 to about 100%. Dosages can be readily adjusted by those skilled in the art (*e.g.*, a decrease in purity may require an increase in dosage). The engineered immune cells can be introduced by injection, catheter, or the like. If desired, factors can also be included, including, but not limited to, interleukins, *e.g.*, IL-2, IL-3, IL 6,

IL-11, IL-7, IL-12, IL-15, IL-21, as well as the other interleukins, the colony stimulating factors, such as G-, M- and GM-CSF, interferons, *e.g.*, γ- interferon.

[0372] In certain embodiments, compositions of the presently disclosed subject matter comprise pharmaceutical compositions comprising engineered immune cells expressing a tumor antigen-targeted CAR and a prodrug converting enzyme with a pharmaceutically acceptable carrier. Administration can be autologous or non-autologous. For example, engineered immune cells expressing a tumor antigen-targeted CAR and a prodrug converting enzyme and compositions comprising thereof can be obtained from one subject, and administered to the same subject or a different, compatible subject. Peripheral blood derived T cells of the presently disclosed subject matter or their progeny (*e.g.*, *in vivo*, ex vivo or *in vitro* derived) can be administered via localized injection, including catheter administration, systemic injection, localized injection, intravenous injection, or parenteral administration. When administering a pharmaceutical composition of the presently disclosed subject matter (*e.g.*, a pharmaceutical composition comprising engineered immune cells expressing a tumor antigen-targeted CAR), it can be formulated in a unit dosage injectable form (solution, suspension, emulsion).

Formulations

[0373] Engineered immune cells expressing a tumor antigen-targeted CAR and prodrug converting enzyme and compositions comprising thereof can be conveniently provided as sterile liquid preparations, *e.g.*, isotonic aqueous solutions, suspensions, emulsions, dispersions, or viscous compositions, which may be buffered to a selected pH. Liquid preparations are normally easier to prepare than gels, other viscous compositions, and solid compositions. Additionally, liquid compositions are somewhat more convenient to administer, especially by injection. Viscous compositions, on the other hand, can be formulated within the appropriate viscosity range to provide longer contact periods with specific tissues. Liquid or viscous compositions can comprise carriers, which can be a solvent or dispersing medium containing, for example, water, saline, phosphate buffered saline, polyol (for example, glycerol, propylene glycol, liquid polyethylene glycol, and the like) and suitable mixtures thereof.

[0374] Sterile injectable solutions can be prepared by incorporating the compositions of the presently disclosed subject matter, *e.g.*, a composition comprising engineered immune

cells, in the required amount of the appropriate solvent with various amounts of the other ingredients, as desired. Such compositions may be in admixture with a suitable carrier, diluent, or excipient such as sterile water, physiological saline, glucose, dextrose, or the like. The compositions can also be lyophilized. The compositions can contain auxiliary substances such as wetting, dispersing, or emulsifying agents (*e.g.*, methylcellulose), pH buffering agents, gelling or viscosity enhancing additives, preservatives, flavoring agents, colors, and the like, depending upon the route of administration and the preparation desired. Standard texts, such as "REMINGTON' S PHARMACEUTICAL SCIENCE", 17th edition, 1985, incorporated herein by reference, may be consulted to prepare suitable preparations, without undue experimentation.

[0375] Various additives which enhance the stability and sterility of the compositions, including antimicrobial preservatives, antioxidants, chelating agents, and buffers, can be added. Prevention of the action of microorganisms can be ensured by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, and the like. Prolonged absorption of the injectable pharmaceutical form can be brought about by the use of agents delaying absorption, for example, aluminum monostearate and gelatin. According to the presently disclosed subject matter, however, any vehicle, diluent, or additive used would have to be compatible with the engineered immune cells of the presently disclosed subject matter.

[0376] The compositions can be isotonic, *i.e.*, they can have the same osmotic pressure as blood and lacrimal fluid. The desired isotonicity of the compositions of the presently disclosed subject matter may be accomplished using sodium chloride, or other pharmaceutically acceptable agents such as dextrose, boric acid, sodium tartrate, propylene glycol or other inorganic or organic solutes. Sodium chloride is preferred particularly for buffers containing sodium ions.

[0377] Viscosity of the compositions, if desired, can be maintained at the selected level using a pharmaceutically acceptable thickening agent. Methylcellulose can be used because it is readily and economically available and is easy to work with. Other suitable thickening agents include, for example, xanthan gum, carboxymethyl cellulose, hydroxypropyl cellulose, carbomer, and the like. The concentration of the thickener can depend upon the agent selected. The important point is to use an amount that will achieve the selected viscosity.

Obviously, the choice of suitable carriers and other additives will depend on the exact route of administration and the nature of the particular dosage form, *e.g.*, liquid dosage form (*e.g.*, whether the composition is to be formulated into a solution, a suspension, gel or another liquid form, such as a time release form or liquid-filled form).

[0378] Those skilled in the art will recognize that the components of the compositions should be selected to be chemically inert and will not affect the viability or efficacy of the engineered immune cells as described in the presently disclosed subject matter. This will present no problem to those skilled in chemical and pharmaceutical principles, or problems can be readily avoided by reference to standard texts or by simple experiments (not involving undue experimentation), from this disclosure and the documents cited herein.

[0379] One consideration concerning the therapeutic use of the engineered immune cells of the presently disclosed subject matter is the quantity of cells necessary to achieve an optimal effect. The quantity of cells to be administered will vary for the subject being treated. In certain embodiments, from about 10^2 to about 10^{12}, from about 10^3 to about 10^{11}, from about 10^4 to about 10^{10}, from about 10^5 to about 10^9, or from about 10^6 to about 10^8 engineered immune cells of the presently disclosed subject matter are administered to a subject. More effective cells may be administered in even smaller numbers. In some embodiments, at least about 1×10^8, about 2×10^8, about 3×10^8, about 4×10^8, about 5×10^8, about 1×10^9, about 5×10^9, about 1×10^{10}, about 5×10^{10}, about 1×10^{11}, about 5×10^{11}, about 1×10^{12} or more engineered immune cells of the presently disclosed subject matter are administered to a human subject. The precise determination of what would be considered an effective dose may be based on factors individual to each subject, including their size, age, sex, weight, and condition of the particular subject. Dosages can be readily ascertained by those skilled in the art from this disclosure and the knowledge in the art. Generally, prodrugs are administered at doses that are nontoxic or tolerable to the patient. In some embodiments, the prodrug is administered at an amount that is about 10 to about 1000 times higher than that possible for the active drug. A suitable dose of prodrug can be from about 0.1 to 200 mg/Kg, such as about from 10 to 100 mg/Kg per patient per day or from 5 to 2000 mg/m^2 (*e.g.*, 200 mg/m^2).

[0380] The skilled artisan can readily determine the amount of cells and optional additives, vehicles, and/or carrier in compositions and to be administered in methods of the

presently disclosed subject matter. Typically, any additives (in addition to the active cell(s) and/or agent(s)) are present in an amount of from about 0.001% to about 50% by weight) solution in phosphate buffered saline, and the active ingredient is present in the order of micrograms to milligrams, such as from about 0.0001 wt % to about 5 wt %, from about 0.0001 wt% to about 1 wt %, from about 0.0001 wt% to about 0.05 wt%, from about 0.001 wt% to about 20 wt %, from about 0.01 wt% to about 10 wt %, or from about 0.05 wt% to about 5 wt %. For any composition to be administered to an animal or human, and for any particular method of administration, toxicity should be determined, such as by determining the lethal dose (LD) and LD50 in a suitable animal model *e.g.*, rodent such as mouse; and, the dosage of the composition(s), concentration of components therein and timing of administering the composition(s), which elicit a suitable response. Such determinations do not require undue experimentation from the knowledge of the skilled artisan, this disclosure and the documents cited herein. And, the time for sequential administrations can be ascertained without undue experimentation

Methods for Therapy

[0381] For treatment, the amount of the engineered immune cells provided herein administered is an amount effective in producing the desired effect, for example, treatment of a cancer or one or more symptoms of a cancer. An effective amount can be provided in one or a series of administrations of the engineered immune cells provided herein. An effective amount can be provided in a bolus or by continuous perfusion. For adoptive immunotherapy using antigen-specific T cells, cell doses in the range of about 10^6 to about 10^{10} are typically infused. Co-expression of the prodrug converting enzyme as disclosed herein, may permit lower doses of the engineered immune cells to be administered, *e.g.*, about 10^4 to about 10^8. Upon administration of the engineered immune cells into the subject, the engineered immune cells are induced that are specifically directed against one tumor antigen. "Induction" of T cells can include inactivation of antigen-specific T cells such as by deletion or anergy. Inactivation is particularly useful to establish or reestablish tolerance such as in autoimmune disorders. The engineered immune cells of the presently disclosed subject matter can be administered by any methods known in the art, including, but not limited to, pleural administration, intravenous administration, subcutaneous administration, intranodal administration, intratumoral administration, intrathecal administration, intrapleural

administration, intraperitoneal administration, and direct administration to the thymus. In certain embodiments, the engineered immune cells and the compositions comprising thereof are intravenously administered to the subject in need. Methods for administering cells for adoptive cell therapies, including, for example, donor lymphocyte infusion and CAR T cell therapies, and regimens for administration are known in the art and can be employed for administration of the engineered immune cells provided herein.

[0382] The presently disclosed subject matter provides various methods of using the engineered immune cells (*e.g.*, T cells) provided herein, expressing a tumor antigen-targeted receptor (*e.g.*, a CAR) and a prodrug converting enzyme. For example, the presently disclosed subject matter provides methods of reducing tumor burden in a subject. In one non-limiting example, the method of reducing tumor burden comprises administering an effective amount of the presently disclosed engineered immune cells to the subject and administering a suit prodrug for conversion by the expressed prodrug converting enzyme, thereby inducing tumor cell death in the subject. In some embodiments, the engineered immune cells and the prodrug are administered at different times. For example, in some embodiments, the engineered immune cells are administered and then the prodrug is administered. In some embodiments, the prodrug is administered 1 hour, 2 hours, 3 hours, 4 hours, 5 hours, 6 hours, 7 hours, 8 hours, 9 hours, 10 hours, 11 hours, 12 hours, 18 hours, 24 hours, 30 hours, 26 hours, 48 hours or longer after the administration of the engineered immune cells.

[0383] The presently disclosed engineered immune cells either alone or in combination with the prodrug can reduce the number of tumor cells, reduce tumor size, and/or eradicate the tumor in the subject. In certain embodiments, the method of reducing tumor burden comprises administering an effective amount of engineered immune cells to the subject, thereby inducing tumor cell death in the subject. Non-limiting examples of suitable tumors include adrenal cancers, bladder cancers, blood cancers, bone cancers, brain cancers, breast cancers, carcinoma, cervical cancers, colon cancers, colorectal cancers, corpus uterine cancers, ear, nose and throat (ENT) cancers, endometrial cancers, esophageal cancers, gastrointestinal cancers, head and neck cancers, Hodgkin's disease, intestinal cancers, kidney cancers, larynx cancers, acute and chronic leukemias, liver cancers, lymph node cancers, lymphomas, lung cancers, melanomas, mesothelioma, myelomas, nasopharynx cancers, neuroblastomas, non-Hodgkin's lymphoma, oral cancers, ovarian cancers, pancreatic cancers,

penile cancers, pharynx cancers, prostate cancers, rectal cancers, sarcoma, seminomas, skin cancers, stomach cancers, teratomas, testicular cancers, thyroid cancers, uterine cancers, vaginal cancers, vascular tumors, and metastases thereof. In some embodiments, the cancer is a relapsed or refractory cancer. In some embodiments, the cancer is resistant to one or more cancer therapies, *e.g.*, one or more chemotherapeutic drugs.

[0384] The presently disclosed subject matter also provides methods of increasing or lengthening survival of a subject having a neoplasia (*e.g.*, a tumor). In one non-limiting example, the method of increasing or lengthening survival of a subject having neoplasia (*e.g.*, a tumor) comprises administering an effective amount of the presently disclosed engineered immune cell to the subject, thereby increasing or lengthening survival of the subject. The presently disclosed subject matter further provides methods for treating or preventing a neoplasia (*e.g.*, a tumor) in a subject, comprising administering the presently disclosed engineered immune cells to the subject.

[0385] Cancers whose growth may be inhibited using the engineered immune cells of the presently disclosed subject matter comprise cancers typically responsive to immunotherapy. Non-limiting examples of cancers for treatment include multiple myeloma, neuroblastoma, glioma, acute myeloid leukemia, colon cancer, pancreatic cancer, thyroid cancer, small cell lung cancer, and NK cell lymphoma. In certain embodiments, the cancer is multiple myeloma.

[0386] Additionally, the presently disclosed subject matter provides methods of increasing immune-activating cytokine production in response to a cancer cell in a subject. In one non-limiting example, the method comprises administering the presently disclosed engineered immune cell to the subject. The immune-activating cytokine can be granulocyte macrophage colony stimulating factor (GM-CSF), IFN-α, IFN-β, IFN-γ, TNF-a, IL-2, IL-3, IL-6, IL-1 1, IL-7, IL-12, IL-15, IL-21, interferon regulatory factor 7 (IRF7), and combinations thereof. In certain embodiments, the engineered immune cells including a tumor antigen-specific CAR of the presently disclosed subject matter increase the production of GM-CSF, IFN-γ, and/or TNF-a.

[0387] Suitable human subjects for therapy typically comprise two treatment groups that can be distinguished by clinical criteria. Subjects with “advanced disease” or “high tumor burden” are those who bear a clinically measurable tumor (*e.g.*, multiple myeloma). A

clinically measurable tumor is one that can be detected on the basis of tumor mass (*e.g.*, by palpation, CAT scan, sonogram, mammogram or X-ray; positive biochemical or histopathologic markers on their own are insufficient to identify this population). A pharmaceutical composition embodied in the presently disclosed subject matter is administered to these subjects to elicit an anti -tumor response, with the objective of palliating their condition. Ideally, reduction in tumor mass occurs as a result, but any clinical improvement constitutes a benefit. Clinical improvement comprises decreased risk or rate of progression or reduction in pathological consequences of the tumor (*e.g.*, multiple myeloma).

[0388] A second group of suitable subjects is known in the art as the "adjuvant group." These are individuals who have had a history of neoplasia (*e.g.*, multiple myeloma), but have been responsive to another mode of therapy. The prior therapy can have included, but is not restricted to, surgical resection, radiotherapy, and traditional chemotherapy. As a result, these individuals have no clinically measurable tumor. However, they are suspected of being at risk for progression of the disease, either near the original tumor site, or by metastases. This group can be further subdivided into high-risk and low-risk individuals. The subdivision is made on the basis of features observed before or after the initial treatment. These features are known in the clinical arts, and are suitably defined for each different neoplasia. Features typical of high-risk subgroups are those in which the tumor (*e.g.*, multiple myeloma) has invaded neighboring tissues, or who show involvement of lymph nodes. Another group has a genetic predisposition to neoplasia (*e.g.*, multiple myeloma) but has not yet evidenced clinical signs of neoplasia (*e.g.*, multiple myeloma). For instance, women testing positive for a genetic mutation associated with breast cancer, but still of childbearing age, can wish to receive one or more of the antigen-binding fragments described herein in treatment prophylactically to prevent the occurrence of neoplasia until it is suitable to perform preventive surgery.

[0389] The subjects can have an advanced form of disease (*e.g.*, multiple myeloma), in which case the treatment objective can include mitigation or reversal of disease progression, and /or amelioration of side effects. The subjects can have a history of the condition, for which they have already been treated, in which case the therapeutic objective will typically include a decrease or delay in the risk of recurrence.

[0390] Further modification can be introduced to the tumor antigen-targeted CAR-expressing engineered immune cells (*e.g.*, T cells) to avert or minimize the risks of

immunological complications (known as "malignant T-cell transformation"), *e.g.*, graft versus-host disease (GvHD), or when healthy tissues express the same target antigens as the tumor cells, leading to outcomes similar to GvFID.

[0391] In some embodiments provided herein , the engineered immune cells express a prodrug converting enzyme intracellularly. In some embodiments, the intracellular prodrug converting enzyme is constitutively expressed. In some embodiments, the intracellular prodrug converting enzyme is conditionally expressed. In some embodiments, the patient is administered a prodrug that is a cell-penetrating prodrug which is converted into an active drug by the intracellular prodrug converting enzyme within the cytoplasm of the engineered immune cells. In some embodiments, the converted active drug causes the death of the engineered immune cells. The prodrug can be administered at a time point following administration of the engineered immune cells. In some embodiments, the time point is dependent on one or more factors such as the appearance of one or more signs of toxicity (*e.g.*, fever, hypotension, edema, swelling, redness, rash, pruritis, neurologic changes, stupor, confusion, nausea, diarrhea, hemoptysis, hematuria, blood in the stool), the concentration of immune cells at the tumor site, progression of disease, and/or desire to end treatment in the subject as determined by a physician.

[0392] Further modification of the engineered immune cells can also include engineering a suicide gene into the tumor antigen-targeted CAR-expressing T cells. Suitable suicide genes include, but are not limited to, Herpes simplex virus thymidine kinase (hsv- tk), inducible Caspase 9 Suicide gene (iCasp-9), and a truncated human epidermal growth factor receptor (EGFRt) polypeptide. In certain embodiments, the suicide gene is an EGFRt polypeptide. The EGFRt polypeptide can enable T cell elimination by administering anti-EGFR monoclonal antibody (*e.g.*, cetuximab). EGFRt can be covalently joined to the C-terminus of the intracellular domain of the tumor antigen-targeted CAR. The suicide gene can be included within the vector comprising nucleic acids encoding the presently disclosed tumor antigen-targeted CARs. In this way, administration of a prodrug designed to activate the suicide gene (*e.g.*, a prodrug (*e.g.*, API 903 that can activates iCasp-9) during malignant T-cell transformation (*e.g.*, GVHD) triggers apoptosis in the suicide gene-activated CAR-expressing T cells. The incorporation of a suicide gene into the a presently disclosed tumor antigen-targeted CAR gives an added level of safety with the ability to eliminate the majority of CAR

T cells within a very short time period. A presently disclosed engineered immune cell (*e.g.*, a T cell) incorporated with a suicide gene can be pre-emptively eliminated at a given time point post CAR T cell infusion, or eradicated at the earliest signs of toxicity.

Articles of Manufacture and Kits

[0393] The presently disclosed subject matter provides kits for the treatment or prevention of a neoplasia (*e.g.*, solid tumor). In certain embodiments, the kit comprises a therapeutic or prophylactic composition containing an effective amount of an engineered immune cell that expresses a tumor antigen-targeted receptor (*e.g.*, a CAR), a first prodrug converting enzyme intracellularly, and a second prodrug converting enzyme on the surface of the engineered immune cell or secreted, in unit dosage form. In some embodiments, the engineered immune cells further express at least one co-stimulatory ligand. In some embodiments, the kit comprises a sterile container which contains a therapeutic or prophylactic vaccine; such containers can be boxes, ampules, bottles, vials, tubes, bags, pouches, blister-packs, or other suitable container forms known in the art. Such containers can be made of plastic, glass, laminated paper, metal foil, or other materials suitable for holding medicaments.

[0394] If desired, the engineered immune cell can be provided together with instructions for administering the engineered immune cell to a subject having or at risk of developing a neoplasia (*e.g.*, solid tumor). The instructions will generally include information about the use of the composition for the treatment or prevention of a neoplasia (*e.g.*, solid tumor). In other embodiments, the instructions include at least one of the following: description of the therapeutic agent; dosage schedule and administration for treatment or prevention of a neoplasia (*e.g.*, solid tumor) or symptoms thereof; precautions; warnings; indications; counter-indications; overdose information; adverse reactions; animal pharmacology; clinical studies; and/or references. The instructions may be printed directly on the container (when present), or as a label applied to the container, or as a separate sheet, pamphlet, card, or folder supplied in or with the container.

EXAMPLES

[0395] The practice of the present invention employs, unless otherwise indicated, conventional techniques of molecular biology (including recombinant techniques), microbiology, cell biology, biochemistry, and immunology, which are well within the purview of the skilled artisan. Such techniques are explained fully in the literature, such as, "Molecular Cloning: A Laboratory Manual", second edition (Sambrook, 1989); "Oligonucleotide Synthesis" (Gait, 1984); "Animal Cell Culture" (Freshney, 1987); "Methods in Enzymology" "Handbook of Experimental Immunology" (Weir, 1996); "Gene Transfer Vectors for Mammalian Cells" (Miller and Calos, 1987); "Current Protocols in Molecular Biology" (Ausubel, 1987); "PCR: The Polymerase Chain Reaction", (Mullis, 1994); "Current Protocols in Immunology" (Coligan, 1991). These techniques are applicable to the production of the polynucleotides and polypeptides of the invention, and, as such, may be considered in making and practicing the invention. Particularly useful techniques for particular embodiments will be discussed in the sections that follow.

[0396] The following examples are put forth so as to provide those of ordinary skill in the art with a complete disclosure and description of how to make and use the compositions, and assay, screening, and therapeutic methods of the invention, and are not intended to limit the scope of what the inventors regard as their invention.

Example 1. Synthesis of prodrugs of cytotoxic and targeted small molecules for use with SEAKER cells.

[0397] This example describes the synthesis of exemplary prodrugs based on sulfamoyladenosine (AMS) (structure 1c) (FIG. 2C) for cleavage by the bacterial hydrolase enzyme CPG2. AMS is a close analog of nucleocidin and a potent cytotoxic molecule (HepG2 IC_{50} = 9 nM). A detailed structure–activity relationship (SAR) study was performed, which indicated that cytotoxicity of AMS is dramatically reduced or eliminated by substitution at the 6-amino (Me_2, >500 μM) *C*2 (Ph, ≈500 μM), *C*8 (*n*-Pr, >250 μM), or sulfamate positions (salicyl, >500 μM). Based on these SAR data, AMS prodrug 1a ("P-AMS"; FIG. 2C) was synthesized from 2′,3′-*O*-isopropylidene-5′-*O*-TBS-adenosine (structure 3, FIG. 2D) by 6-*N*-acylation with CDI-activated bis-*O*-*t*-Bu-glutamate (30%), desilylation (TBAF), sulfamoylation (H_2NSO_2Cl, DMA), and acetonide hydrolysis (TFA, H_2O, 15% over 3 steps). Two alternative *N*-sulfamate-linked prodrugs (not shown) attached

via CO-PAB-CO and TML-CO linkers were synthesized (FIG. 2B). A more detailed discussion of the P-AMS synthesis and the synthesis of the *N*-sulfamate-linked prodrug attached via a TML-CO linker ("Trimethyllock-AMS") is provided below in this example. As an alternative cytotoxic using a known anticancer therapeutic, the mustard prodrug ZD2767P (structure 2a, FIG. 2C) was also synthesized as previously reported (Springer *et al.*, *J Med Chem.* 37(15):2361–70(1994) and Niculescu-Duvaz *et al.*, *Tetrahedron Lett.* 46(40):6919–22 (2005)).

[0398] As an example of a targeted therapy approach, an erlotinib prodrug (structure 6a, FIG. 2E) was also designed. To avoid potential hydrolytic instability of an *N,N*-diarylamide (*cf.* CO or CO-PAB-CO linkers), a *N*-benzyl linker (PAB-CO) that undergo spontaneous elimination after glutamate cleavage was synthesized. Similar 1,6-elimination of a *p*-aminobenzyl quaternary ammonium salt has been demonstrated (Staben *et al. Nat Chem.* 8(12):1112–9 (2016). The synthesis was achieved by conversion of the known benzyl alcohol (structure 4, FIG. 2D) to the corresponding mesylate (MsCl)(structure 5, FIG. 2D), *N*-alkylation of erlotinib (25% over 2 steps), and deprotection of the *t*-Bu esters (TFA, CH_2Cl_2, 3%, unoptimized). A more detailed discussion and characterization of this erlotinib prodrug synthesis is provided below in this example.

[0399] The *in vitro* cytotoxicity of AMS (structure 1c) and its prodrug variant (structure 1a) was tested against a variety of cell lines using a CellTitre Glo® assay (Promega, Madison, WI). As expected, AMS is highly cytotoxic to HEK293T (kidney, IC50 = 666 nM), HL 60 (leukemia; 70 nM), Jurkat (T cell; 18 nM), and primary T-cells (66 nM), while prodrug 1a is non-toxic (IC50 > 1,000 nM; SI selectivity index up to > 56). Moreover, co-treatment with the prodrug and supernatant from CPG2-transfected HEK293T cells (*see* Example 3) led to cytotoxicity approaching that of the free drug (50 nM), consistent with CPG2-mediated cleavage of the prodrug to release the active parent drug. Similar results were obtained with the mustard prodrug ZD2767P (structure 2a) (IC50 = 1.7 μM with CPG2 supernatant). In contrast, AMS prodrug 1a was not activated by HEK293T cells expressing CPG intracellularly, which likely attributed to poor cell-penetrance of the prodrug due to the free bis-anionic gluatamate moiety.

[0400] *In vitro* cytotoxicity of AMS-related structures was also assessed to determine the modifications of AMS that alter cytotoxicity. The *in vitro* cytotoxicity of each of these AMS-

related structures was tested against both hepatocellular carcinoma HepG2 and normal human fibroblast MRC5 cell lines using a CellTitre Glo® assay (Promega, Madison, WI). The modifications and results are provided in Table 1 below.

Table 1.

	Shorthand ref (MSK ref)	X	R^1	R^2	IC_{50}, HepG2 (μM)	IC_{50}, MRC5 (μM)
	AMS (*IcsTam1*)	O	-OH	-OH	0.009	0.030
	AMSN (*IcsTam12*)	NH	-OH	-OH	0.36	0.39
	2'-deoxy-AMS (*IcsTam13*)	O	-OH	-H	<1	<1
					0.55	0.48
	3'-deoxy-AMS (*IcsTam14*)	O	-H	-OH	>5	>5
					~500	~100
		R^1	**R^2**	**R^3**		
	6-desN-AMS (*IcsTam18*)	-H	-H	-H	<1	<1
					0.16	0.13
	6-NHMe-AMS (*IcsTam4*)	-NHMe	-H	-H	1.8	0.9

6-NMe_2-AMS (*testTam22*)	$-NMe_2$	-H	-H	>500	>500	
6-NCyp-AMS (*testTam5*)	-NHCyp	-H	-H	10.9	7.2	
6-OMe-AMS (*testTam21*)	$-OCH_3$	-H	-H	141	129	
2-NH_2-AMS (*testTam19*)	$-NH_2$	$-NH_2$	-H	<1	<1	
				1.75	1.17	
2-phenyl-AMS (*testTam6*)	$-NH_2$	-Ph	-H	~500	>500	
2-CCPh-AMS (*testTam7*)	$-NH_2$	- CCPh	-H	20.7	13.4	
2-NHPh-AMS (*testTam8*)	$-NH_2$	- NHP h	-H	>500	123.5	
8-Cl-AMS (*testTam17*)	$-NH_2$	-H	-Cl	<1	3.2	
				4.51	3.51	
8-propyl-AMS (*testTam20*)	$-NH_2$	-H	- propyl	>250	>250	
				>500	>500	

		B	R			
	IMS (IcsTam2)		-OH		>500	>500
	GMS (IcsTam3)		-OH		>500	>500
	Tubericidin-MS (IcsTam23)		-OH		1.7	<0.015
					0.018	0.005
	1'-homo-AMS, n = 1 (IcsTam15)		-OH		>500	>500
	1'-homo-AMS, n = 2 (IcsTam16)		-OH		>500	>500

CMS (lcsTam11)		-OH		>500	>500
UMS (lcsTam10)		-OH		>500	>500
TMS (lcsTam9)		-H		>500	>500

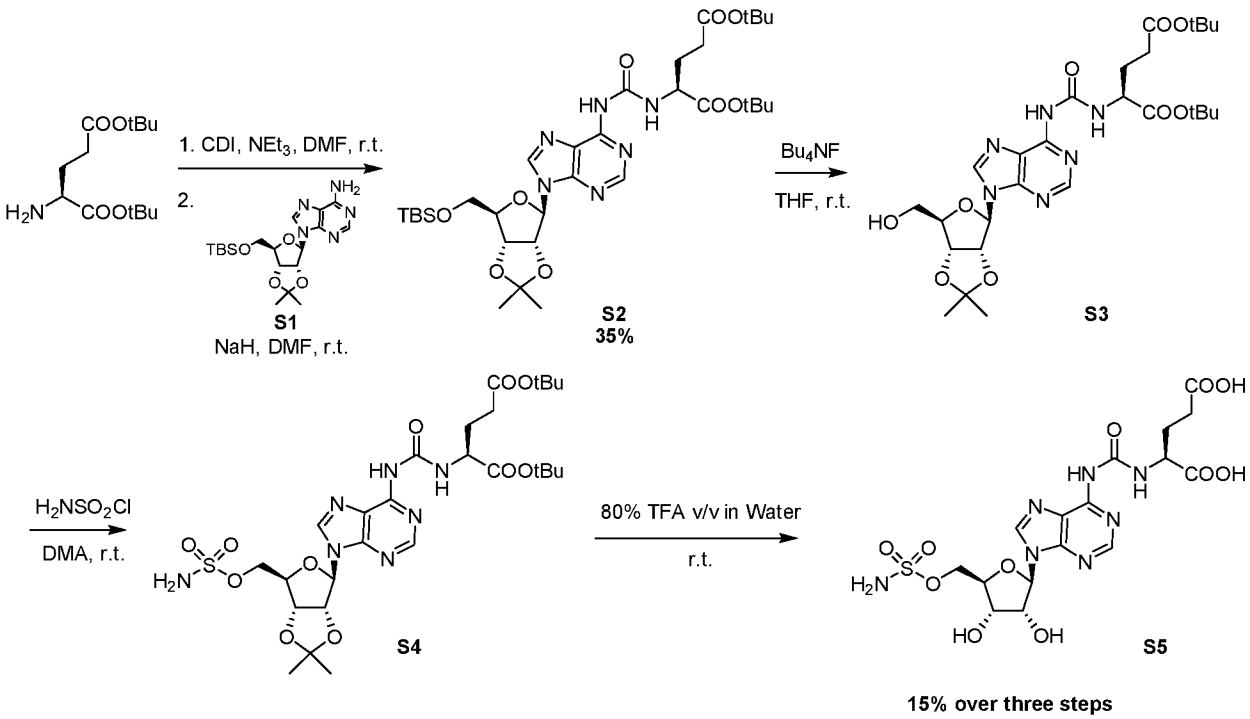
Surprisingly, while most modifications resulted in a marked decrease in cytotoxicity, several compounds continued to possess sufficient cytotoxic activity for utility in the present technology. For example, AMSN, 2'-deoxy-AMS, 6-desN-AMS, 2-NH_2-AMS, and Tubericidin-MS are each well-disposed for generation of prodrugs and use in the present technology.

[0401] Representative Synthesis and Examples of Compounds of Formula I

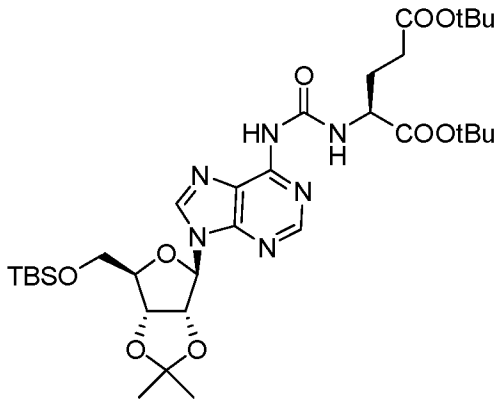
[0402] *Synthesis and Characterization of P-AMS*

[0403] Synthesis of the prodrug compound P-AMS is summarized in Scheme 1 below (where P-AMS is also referred to as **S5**) and further detailed thereafter.

Scheme 1.



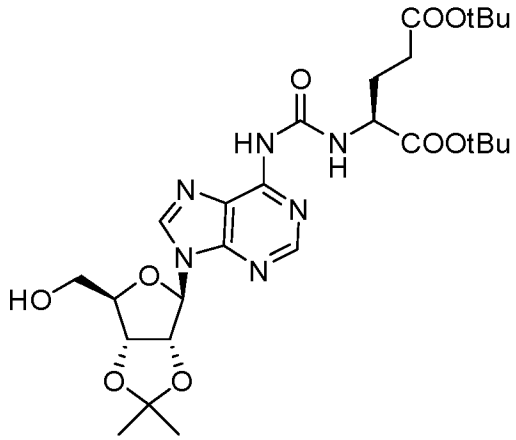
[0404] di-tert-butyl ((9-((3aR,4R,6R,6aR)–6-(((tert-butyldimethylsilyl)oxy) methyl)-2,2-dimethyltetrahydrofuro[3,4-d][1,3]dioxol-4-yl)-9H-purin-6-yl)carbamoyl)-L-glutamate) (S2).



S1 was prepared as described in Somu, R. V.; Boshof, H.; Qiao, C.; Bennett, E. M.; Barry, C. E.; Aldrich, C. C. *J. Med. Chem.* **2006,** *49* (1), 31-34. In a 25 mL round bottom flask at 25 °C, (S)-di-tertbutyl glutamate (300 mg, 1.16 mmol, 1.0 equiv) was suspended in 8 mL anhyd DMF. 1,1'-Carbonyldiimidazole (CDI, 187 mg, 1.16 mmol, 1 equiv) was added in one portion, and the mixture was stirred for 1 h. Separately, a solution of **S1** (828 mg, 1.96 mmol, 1.7 equiv) in anhyd DMF (4 mL) was added 60% NaH in mineral oil (117.8 mg, 2.95 mmol, 2.6 equiv) for 15 min. This separate adenosine mixture was added dropwise to the CDI activated glutamate mixture at 25 °C. The reaction was stirred at 25 °C overnight for an additional 16-20 h, until complete conversion had occurred as judged by LC-MS. The mixture was then quenched with satd aq NaCl. The aqueous mixture was extracted with ethyl acetate (3 × 20 mL), and the combined organic extracts were dried over $MgSO_4$, filtered, and concentrated by rotary evaporation to afford the crude product as a yellow oil. Purification by silica flash chromatography (3:1 hexanes/EtOAc to 1:1 hexanes/EtOAc) yielded product **S2** (286 mg, 35%) as a light yellow oil. **TLC**: R_f 0.35 (1:1 hexanes/EtOAc). **^{1}H-NMR** (500 MHz, $CDCl_3$): δ 9.91 (d, 1H, J = 8.0), 8.61 (s, 1H), 8.19 (s, 1H), 7.94 (s, 1H), 6.20 (s, 1H), 5.23 (dd, 1H, J = 10, 5.2), 4.94 (dd, 1H, J = 10, 4.9), 4.61 (q, 1H, 4.6), 3.89 (dd, 1H, J = 8.8, 4.0), 3.77 (dd, 1H, J = 8.7, 3.9), 3.33 (dd, 1H, J = 7.9, 5.2), 2.42 (m, 2H), 2.26 (m, 1H), 2.09 (m, 1H), 1.64 (s, 3H), 1.50 (s, 9H), 1.43 (s, 9H), 1.41 (s, 3H), 0.83 (s, 9H), 0.01 (dd, 6H, J = 1.6). **^{13}C-NMR** (126 MHz, $CDCl_3$): δ 206.8, 172.0, 171.0, 153.3, 151.3, 150.0, 120.8,

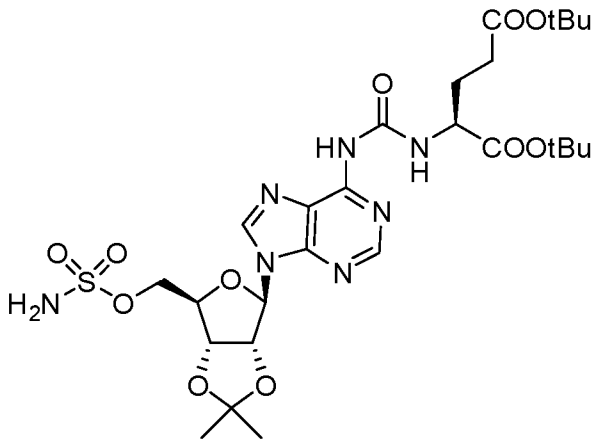
114.1, 91.9, 87.5, 85.1, 82.1, 81.5, 80.5, 77.2, 63.6, 60.3, 53.4, 31.6, 30.9, 28.3, 28.0, 27.2, 25.8, 25.3, 21.0, 18.3, 14.2, -5.5. **ESI-MS** *m/z* (rel int): (pos) 707.4 ($[M+H]^+$, 100).

[0405] di-tert-butyl ((9-((3aR,4R,6R,6aR)-6-(hydroxymethyl)-2,2-dimethyltetrahydrofuro[3,4-d][1,3]dioxol-4-yl)-9H-purin-6-yl)carbamoyl)-L-glutamate (S3).



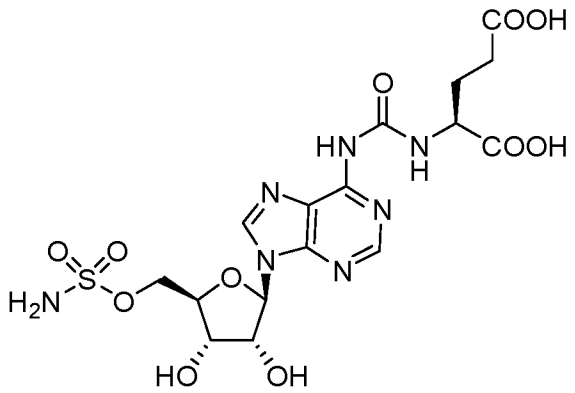
In a 50 mL round bottom flask at 25 °C, **S2** (286 mg, 404 µmol, 1.0 equiv) was suspended in 15 mL THF. 1M TBAF in THF (0.88 mL, 0.88 mmol, 2.2 equiv) was added in one portion, and the mixture was stirred for 1 h at 25 °C. The mixture was then quenched with satd aq NaCl. The aqueous mixture was extracted with ethyl acetate (3 × 20 mL), dried over $MgSO_4$, filtered, and concentrated by rotary evaporation to afford the crude product (274 mg) as a yellow oil. Crude product was carried over to the next step without further purification. **Crude ^{1}H-NMR** (500 MHz, $CDCl_3$) δ 8.57 (s, 1H), 8.41 (s, 1H), 6.01 (d, J = 4.0 Hz, 1H), 5.43 (s, 1H), 5.18 (dd, J = 5.9, 4.1 Hz, 1H), 5.05 (d, J = 5.9 Hz, 1H), 4.56 (dt, J = 7.9, 3.9 Hz, 1H), 4.53 (s, 1H), 3.95 (d, J = 1.8 Hz, 0H), 3.94 – 3.91 (m, 1H), 3.77 (d, J = 12.6 Hz, 1H), 3.42 (s, 2H), 3.34 – 3.27 (m, 1H), 2.43 – 2.27 (m, 2H), 2.27 – 2.17 (m, 1H), 2.10 – 2.00 (m, 1H), 1.61 (s, 3H), 1.60 (s, 3H), 1.48 (s, 9H), 1.38 (s, 9H). **ESI-MS** *m/z* (rel int): (pos) 593.3 ($[M+H]^+$, 100).

[0406] di-tert-butyl ((9-((3aR,4R,6R,6aR)-2,2–dimethyl–6-((sulfamoyloxy) methyl) tetrahydrofuro[3,4-d][1,3]dioxol-4-yl)-9H-purin-6-yl)carbamoyl)-L-glutamate (S4).



In a 25 mL round bottom flask at 0 °C, **S3** (274.4 mg, 462 µmol, 1.0 equiv) was suspended in 10 mL anhyd DMA. Sulfamoyl chloride (133.4 mg, 1.156 mmol, 2.5 equiv) was added in one portion, and the mixture was stirred for 1 h warming to 25°C. The mixture was then quenched with satd aq $NaHCO_3$. The aqueous mixture was extracted with ethyl acetate (3 x 15 mL), dried over $MgSO_4$, filtered, and concentrated by rotary evaporation to afford the crude product (310 mg) as a yellow oil. Crude product was carried to next step without further purification. **Crude ^{1}H-NMR** (500 MHz, $CDCl_3$) δ 8.24 (s, 1H), 8.20 (s, 1H), 5.94 (d, J = 2.8 Hz, 1H), 5.04 (dd, J = 6.1, 2.8 Hz, 1H), 4.77 (dd, J = 6.2, 2.6 Hz, 1H), 4.29 – 4.18 (m, 2H), 4.02 (dd, J = 10.9, 4.4 Hz, 1H), 3.97 (dd, J = 10.8, 4.6 Hz, 1H), 3.72 (q, J = 7.1 Hz, 2H), 2.11 – 1.84 (m, 3H), 1.65 (s, 6H), 1.14 (s, 9H), 1.05 (s, 9H). **ESI-MS** *m/z* (rel int): (pos) 672.3 ($[M+H]^+$, 100).

[0407] ((9-((2R,3R,4S,5R)-3,4-dihydroxy-5-((sulfamoyloxy)methyl)tetrahydrofuran-2-yl)-9H-purin-6-yl)carbamoyl)-L-glutamic acid (S5).

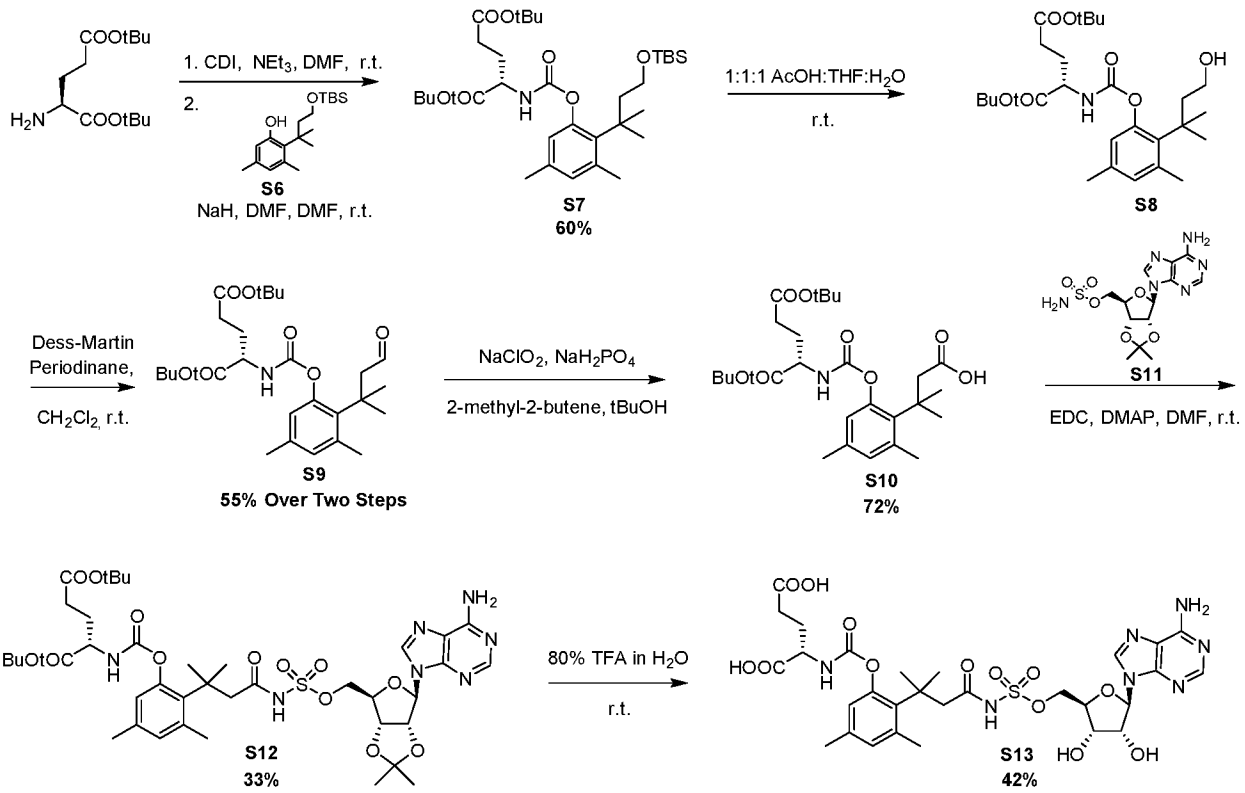


In a 25 mL round bottom flask at 25 °C, **S4** (310.3 mg, 462 μmol, 1.0 equiv) was suspended in 22.5 mL 80% aq TFA solution. The reaction was stirred at 25 °C for an additional overnight for 5-6 h, until complete conversion had occurred as judged by LC-MS. The mixture was then quenched with 10mL MeOH, and azeotroped through for a total of three times. The crude mixture was then purified through prep-HPLC and lyophilized overnight to yield **S4** (36.7mg, 70.6 □mol, 15% over three steps) as a white powder. **^{1}H-NMR** (500 MHz, Methanol-d_4): δ 8.67 (s, 1H), 8.56 (s, 1H), 6.20 (d, J = 4.9 Hz, 1H), 4.75 (t, J = 5.0 Hz, 1H), 4.63 (dd, J = 7.9, 5.2 Hz, 1H), 4.46 (dd, J = 8.0, 3.0 Hz, 2H), 4.43 – 4.35 (m, 2H), 2.59 – 2.44 (m, 2H), 2.37 (dq, J = 13.6, 7.5, 7.0 Hz, 1H), 2.16 (dq, J = 14.4, 7.6 Hz, 1H). 7 exchangeable H not observed. **^{13}C-NMR** (126 MHz, Methanol-d_4): 176.3, 174.6, 173.5, 155.5, 151.9, 150.3, 144.0, 121.3, 90.5, 83.8, 75.7, 71.8, 69.6, 54.0, 49.5, 31.0, 28.4. **ESI-MS** *m/z* (rel int): (pos) 520.2 ($[M+H]^+$, 100).

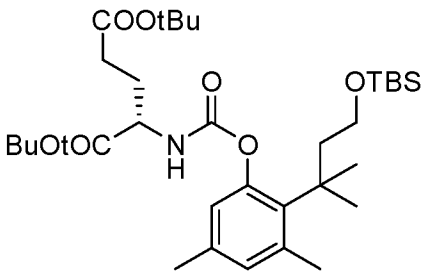
[0408] *Synthesis and Characterization of Trimethyllock-AMS*

[0409] Synthesis of the prodrug compound Trimethyllock-AMS is summarized in Scheme 2 below (where Trimethyllock-AMS is also referred to as **S13**) and further detailed thereafter.

Scheme 2.



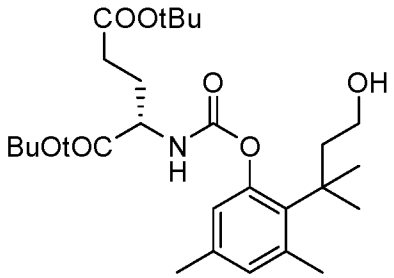
[0410] di-tert-butyl ((2-(4-((tert-butyldimethylsilyl)oxy)-2-methylbutan-2-yl)-3,5-dimethylphenoxy)carbonyl)-L-glutamate (S7).



S6 was synthesized as described in Wang, B.; Gangwar, S.; Pauletti, G. M.; Siahaan, T. J.; Borchardt, R. T.; *J. Org. Chem.* **1997,** *62*, 1363-1367. In a 50 mL round bottom flask at 25 °C, L-di-tertbutyl glutamate (1.12 g, 4.32 mmol, 1.0 equiv) was suspended in 10.79 mL anhyd DMF. 1,1'-Carbonyldiimidazole (CDI, 770 mg, 4.75 mmol, 1.1 equiv) and triethylamine (662 µL, 4.75 mmol, 1.1 equiv) was added in one portion, and the mixture was stirred for 1 h. Separately, **S6** (1.04 g, 3.24 mmol, 0.75 equiv) was suspended in anhyd DMF (10.8 mL) and added 60% NaH in mineral oil (173 mg, 4.32 mmol, 1.0 equiv) for 15 min.

This separate **S6** mixture was added dropwise to the CDI activated glutamate mixture at 25 °C. The reaction was stirred at 25 °C for overnight for an additional 17-20 h, until complete conversion had occurred as judged by TLC. The mixture was then quenched with satd aq NaCl, diluted with 50 mL ethyl acetate. The organic layer was then extracted, washed with satd aq NaCl (1 × 30mL) followed by water (2 × 30mL). The aqueous layers were then combined and extracted with ethyl acetate (2 × 30mL). All organic layers were then combined, dried over $MgSO_4$, filtered, and concentrated by rotary evaporation to afford the crude product as a yellow oil. Purification by silica flash chromatography (9:1 hexanes/EtOAc to 3:1 hexanes/EtOAc) yielded product **S7** (1.16 g, 60%) as a yellow oil. **TLC**: R_f 0.46 (3:1 hexanes/EtOAc). **^{1}H-NMR** (500 MHz, $CDCl_3$): δ 6.77 (s, 1H), 6.63 (s, 1H), 5.67 (d, J = 8.2 Hz, 1H), 3.50 (t, J = 7.5 Hz, 2H), 2.50 (s, 3H), 2.46 – 2.25 (m, 2H), 2.21 (s, 3H), 2.04 (t, J = 7.5 Hz, 2H), 2.01-1.92 (m, 1H), 1.49 (s, 13H), 1.45 (s, 9H), 0.84 (s, 9H), -0.03 (s, 6H). **^{13}C NMR** (126 MHz, $CDCl_3$) δ 155.6, 137.5, 135.7, 128.9, 126.6, 116.5, 61.8, 45.0, 39.4, 32.0, 28.0, 25.5, 20.2, 18.3, -5.3. **ESI-MS** *m/z* (rel int): (pos) 608.5 ($[M+H]^+$, 100).

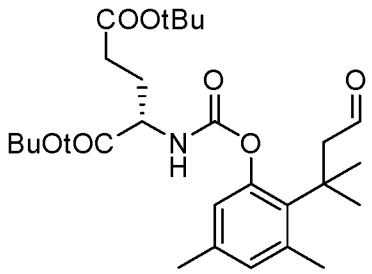
[0411] di-tert-butyl ((2-(4-hydroxy-2-methylbutan-2-yl)-3,5-dimethylphenoxy)carbonyl)-L-glutamate (S8).



In a 50 mL round bottom flask at 25 °C, **S7** (1.159 g, 1.906 mmol, 1.0 equiv) was suspended in 12 mL 1:1 v:v THF:H_2O solution. 12 mL of acetic acid was added, and the reaction was stirred at 25 °C for an additional 3 h, until complete conversion had occurred as judged by LC-MS. The mixture was then concentrated by rotary evaporator, and toluene (10 mL) was added to the mixture and concentrated by rotary evaporator. Azeotroping with toluene was repeated for a total of three times. Crude product **S8** (941 mg) was carried to next step without further purification. **Crude ^{1}H-NMR** (500 MHz, $CDCl_3$) δ 6.73 (s, 1H), 6.59 (s, 1H), 6.23 (d, J = 8.1 Hz, 1H), 4.23 (m, 1H), 3.47 (td, J = 7.3, 6.4, 3.3 Hz, 2H), 2.45 (s, 3H), 2.38 –

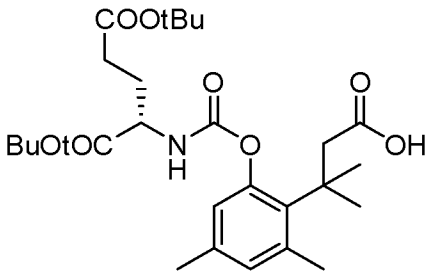
2.27 (m, 2H), 2.16 (s, 3H), 2.07 – 2.00 (m, 1H), 1.99 (s, 3H), 1.96 – 1.91 (m, 1H), 1.90 (s, 3H), 1.44 (s, 9H), 1.42 (s, 9H). **Crude ^{13}C NMR** (126 MHz, $CDCl_3$) δ 172.5, 171.2, 155.6, 137.6, 135.9, 129.0, 128.5, 126.6, 125.2, 116.2, 82.7, 81.2, 61.3, 54.3, 44.9, 39.4, 31.8, 28.0, 25.9, 25.5, 20.2. **ESI-MS** *m/z* (rel int): (pos) 494.4 ($[M+H]^+$, 100).

[0412] di-tert-butyl ((2-(4-hydroxy-2-methylbutan-2-yl)-3,5-dimethylphenoxy)carbonyl)-L-glutamate (S9).



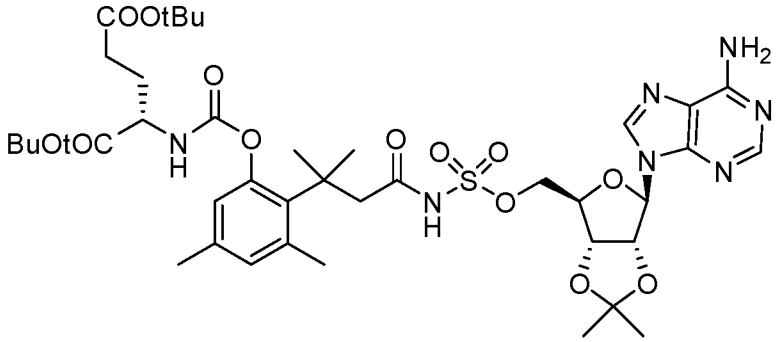
In a 50 mL round bottom flask at 0 °C, Crude **S8** (941 mg, 1.906 mmol, 1.0 equiv) was suspended in 19 mL CH_2Cl_2. Dess-Martin periodinane (1.78 g, 4.19 mmol, 2.2 equiv) was added and stirred warming to 25 °C for 17 h. The mixture was then concentrated by rotary evaporator, and purified directly by s and toluene (10 mL) was added to the mixture and concentrated by rotary evaporator. Purification by silica flash chromatography (3:1 hexanes/EtOAc) yielded product **S10** (513 mg, 55 % over two steps) as a light yellow oil. **TLC**: R_f 0.27 (3:1 hexanes/EtOAc). **^{1}H-NMR** (500 MHz, $CDCl_3$): δ 9.52 (t, J = 2.6 Hz, 1H), 6.78 (s, 1H), 6.64 (s, 1H), 5.85 (d, J = 8.1 Hz, 1H), 4.27 (dt, J = 8.1, 4.0 Hz, 2H), 2.80 (s, 2H), 2.48 (s, 3H), 2.42 – 2.27 (m, 2H), 2.19 (s, 3H), 2.17 – 2.08 (m, 1H), 1.98 – 1.88 (m, 1H), 1.55 (s, 6H), 1.54 (s, 3H), 1.45 (s, 9H), 1.42 (s, 9H). **^{13}C-NMR** (126 MHz, $CDCl_3$): δ 203.7, 172.2, 170.9, 154.4, 153.2, 152.8, 149.3, 137.7, 136.8, 135.0, 133.0, 132.5, 94.3, 82.6, 81.0, 68.4, 64.7, 56.9, 54.2, 49.2, 38.9, 38.8, 38.2, 31.6, 28.1, 27.5, 27.2, 25.7, 25.5, 22.6, 20.8, 20.3, 15.2. **ESI-MS** *m/z* (rel int): (pos) 493.2 ($[M+H]^+$, 100).

[0413] (S)-3-(2-(((1,5-di-tert-butoxy-1,5-dioxopentan-2-yl)carbamoyl)oxy)-4,6-dimethylphenyl)-3-methylbutanoic acid (S10).



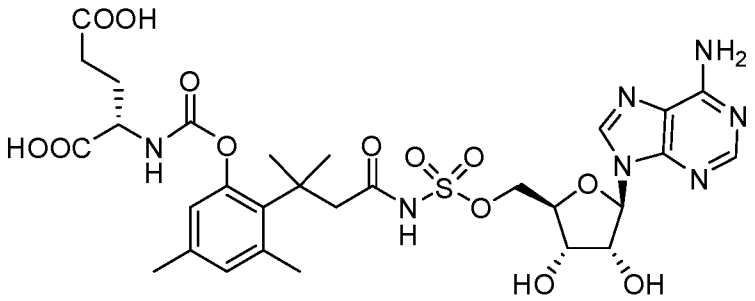
In a 50 mL round bottom flask at 0 °C, Crude **S9** (513 mg, 1.04 mmol, 1.0 equiv) was suspended in 12 mL 1:1 H_2O:2-methyl-2-butene. NaH_2PO_4 (264 mg, 2.2 mmol, 2.1 equiv), tBuOH (48.7 mg, 0.657 mmol, 0.63 equiv), and $NaClO_2$ (416 mg, 3.685 mmol, 3.5 equiv) was added sequentially and stirred warming to 25 °C for 22 h. The mixture was then quenched with sat aq NH_4Cl, and the pH of the mixture was then adjusted to ~3 with 1M aq HCl. The mixture was then washed with ethyl acetate (3 × 10mL), and the combined organic layers were then washed with brine (2 × 20 mL). The organic layers were then concentrated by rotary evaporator. Purification by silica flash chromatography (3:1 hexanes/EtOAc to 1:1 hexanes/EtOAc) yielded product **S10** (384 mg, 72 %) as a light yellow oil. **TLC**: R_f 0.15 (1:1 hexanes/EtOAc). **^{1}H-NMR** (500 MHz, $CDCl_3$) δ 6.76 (s, 1H), 6.63 (s, 1H), 5.96 (d, J = 8.2 Hz, 1H), 4.28 (td, J = 8.3, 4.9 Hz, 2H), 2.90 – 2.74 (m, 2H), 2.49 (s, 3H), 2.45 – 2.26 (m, 2H), 2.19 (s, 3H), 2.15 (m 1H), 1.99 – 1.91 (m, 1H), 1.57 (s, 3H), 1.56 (s, 3H), 1.46 (s, 9H), 1.43 (s, 9H). **^{13}C-NMR** (126 MHz, $CDCl_3$) δ 176.4, 172.0, 170.9, 154.6, 149.3, 137.7, 136.0, 133.6, 132.1, 123.0, 82.3, 80.7, 60.3, 54.1, 48.0, 38.6, 31.4, 31.2, 27.9, 27.4, 25.1, 20.9, 20.1, 14.0. **ESI-MS** *m/z* (rel int): (pos) 508.3 ($[M+H]^+$, 100).

[0414] di-tert-butyl ((2-(4-(((((3aR, 4R, 6R, 6aR)–6-(6–amino-9H-purin-9-yl)-2,2-dimethyltetrahydrofuro[3,4-d][1,3]dioxol-4-yl)methoxy)sulfonyl)amino)-2-methyl-4-oxobutan-2-yl)-3,5-dimethylphenoxy)carbonyl)-L-glutamate (S12).



S11 was synthesized as discussed in Moreau, C.; et al. *J. Med. Chem.* **2013,** *56*, 10079-10102. In a 25 mL round bottom flask at 25 °C, **S10** (307 mg, 0.605 mmol, 1.0 equiv) was suspended in 6 mL anhyd DMF. To the solution was then added EDCI (140 mg, 0.904 mmol, 3.2 equiv), and DMAP (110 mg, 0.904 mmol, 3.1 equiv) for 20 min. Separately, **S11** (111 mg, 0.287 mmol, 1 equiv) was then suspended in 6 mL anyd DMF and then added dropwise to the **S10** mixture. The reaction was then stirred overnight under 25 °C for 18 h. The mixture was then diluted with 9 mL ethyl acetate, washed with satd aq NH_4Cl (3 × 10 mL) and satd aq $NaHCO_3$ (3 × 10 mL), and the organic layer was then dried with $MgSO_4$. Purification by silica flash chromatography (5% MeOH in CH_2Cl_2 to 10% MeOH in CH_2Cl_2) yielded semi-pure product **S12** (83 mg, 33 %) as a transparent oil. **TLC**: R_f 0.44 (10% MeOH in CH_2Cl_2). **Crude ^{1}H-NMR** (500 MHz, $CDCl_3$): δ 7.38–7.28 (m, 5H), 5.60 (m, 2H), 4.63 (m, 1H), 4.58 (s, 2H), 4.36 (t, 1H, J = 5.6), 3.49 (dd, 1H, J = 9.5, 3.7), 3.44 (dd, 1H, J = 9.6, 7.9), 3.34 (s, 3H), 3.32 (s, 3H), 2.68 (d, 1H, J = 2.5), 2.52 (m, 1H), 2.38 (m, 1H). **Crude ^{13}C-NMR** (125 MHz, $CDCl_3$): δ 137.9, 131.0, 128.5–127.5 (Ar), 103.8, 73.6, 73.4, 66.7, 53.5, 53.2, 31.8. **ESI-MS** *m/z* (rel int): (pos) 876.1 ($[M+H]^+$, 100).

[0415] ((2-(4-(((((2R,3S,4R,5R)-5-(6-amino-9H-purin-9-yl)-3,4-dihydroxytetrahydrofuran-2-yl)methoxy)sulfonyl)amino)-2-methyl-4-oxobutan-2-yl)-3,5-dimethylphenoxy)carbonyl)-L-glutamic acid (S13).

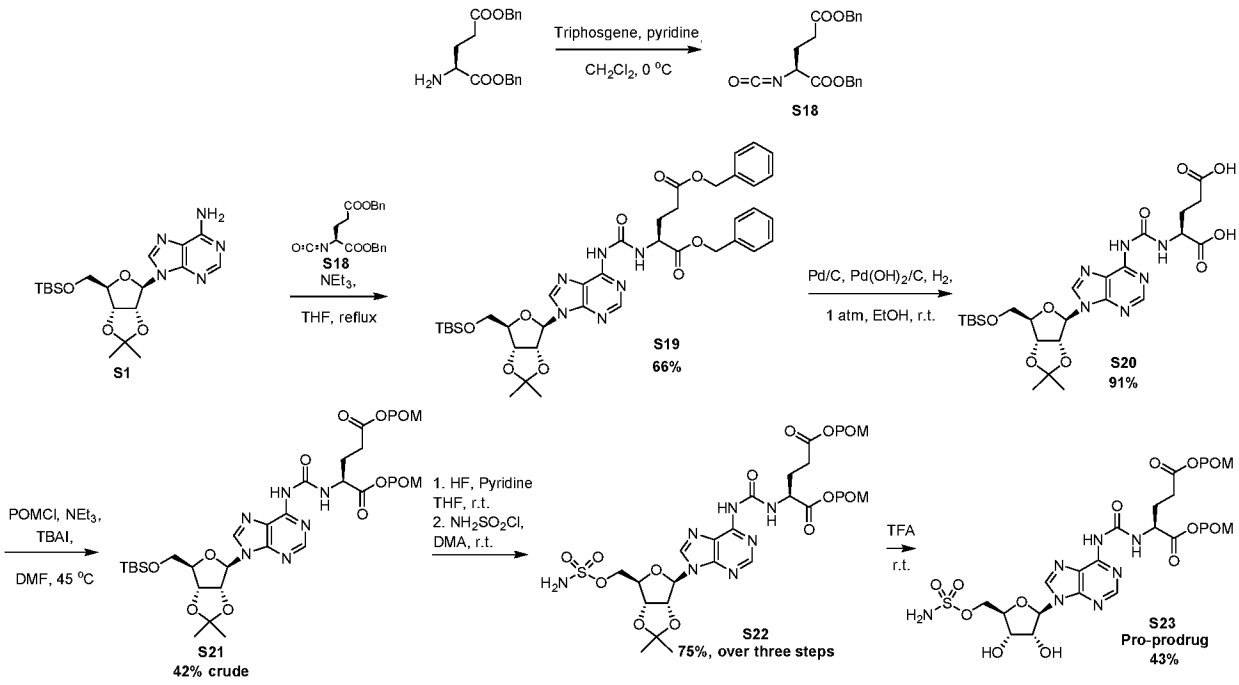


In a 10 mL glass vial at 25 °C, **S12** (20 mg, 22.8 □mol, 1.0 equiv) was suspended in 456 µL 80% aq TFA. The reaction was then stirred under 25 °C for 2 h. The mixture was then diluted with 5 mL H_2O, azeotroped TFA with toluene (3 x 5 mL) through concentration by the rotary evaporator. Purification by prep-HPLC and lyophilization yielded product **S13** (6.95 mg, 42 %) as a white powder. **^{1}H-NMR** (500 MHz, Methanol-d_4) δ 8.41 (s, 1H), 8.29 (s, 1H), 6.73 (d, J = 2.0 Hz, 1H), 6.63 (s, 1H), 6.07 (s, 1H), 4.57 (t, J = 5.1 Hz, 1H), 4.31 (dd, J = 9.4, 4.9 Hz, 1H), 4.22 (dt, J = 9.7, 3.7 Hz, 4H), 2.85 (d, J = 15.3 Hz, 1H), 2.77 (d, J = 15.3 Hz, 1H), 2.52 (t, J = 7.4 Hz, 3H), 2.48 (s, 3H), 2.37 – 2.24 (m, 1H), 2.15 (s, 3H), 2.09 – 1.97 (m, 1H), 1.60 (d, J = 3.0 Hz, 6H). 7 exchangeable H not observed. **ESI-MS** *m/z* (rel int): (pos) 724.3 ($[M+H]^+$, 100).

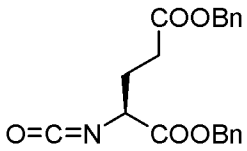
[0416] *Synthesis and Characterization of an AMS-related Pro-Prodrug of the Present Technology*

[0417] Synthesis of a representative AMS-related pro-prodrug compound (**S23**) is summarized in Scheme 3 below and further detailed thereafter.

Scheme 3.

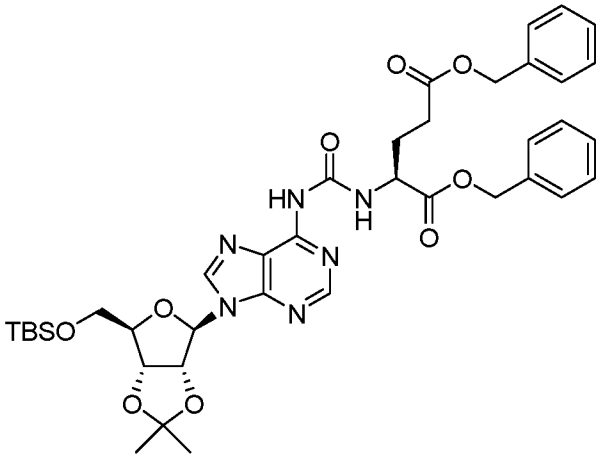


[0418] Dibenzyl (S)-2-isocyanatopentanedioate (S18).



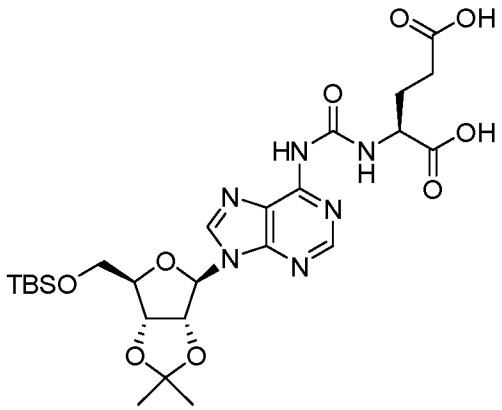
In a 25 mL round bottom flask at 0 °C, dibenzyl glutamate (693 mg, 2.1 mmol, 1.0 equiv) was suspended in 7 mL CH_2Cl_2, added pyridine (681 µL, 8.46 mmol), and stirred for 15 min. Triphosgene (313 mg, 1.06 mmol, 0.5 equiv) was added and the resulting yellow solution was stirred at 0 °C for 2 h. The reaction mixture was extracted two times with 10 mL of cold 0.5 M aqueous HCl and 10 mL of crushed ice. Each aqueous layer was re-extracted with 10 mL of CH_2Cl_2. The combined organic phases were extracted with a mixture of 10 mL of cold satd aq NaCl solution and 10 mL of crushed ice, dried over $MgSO_4$, filtered, and concentrated by rotary evaporation to afford the crude isocyanate (733mg isolated, 99%) yield as a light brown oil (during workup, the isocyanate is only exposed to water for a total of 5-10 min.). Characterization of compound **S18** was consistent as previously reported in Kozikowski, A.P.; et al. *J. Med. Chem.*, **2004**, *47*, 1729–1738. Crude product was used without further purification.

[0419] 9-((3aR,4R,6R,6aR)-6-(((tert-butyldimethylsilyl)oxy)methyl)-2,2-dimethyltetrahydrofuro [3,4-d][1,3]dioxol-4-yl)-9H-purin-6-amine (S19).



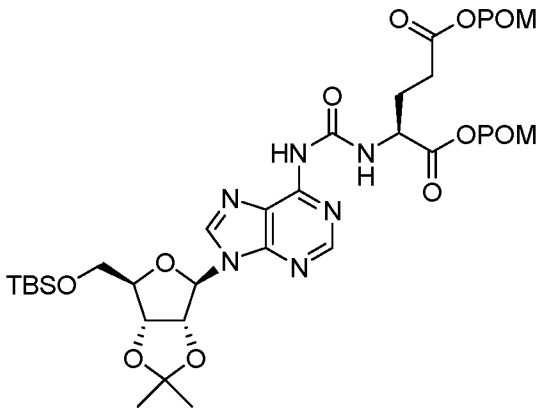
In a 50 mL round bottom flask, **S18** (747 mg, 2.11 mmol, 1.75 equiv) was suspended in 12 mL anhyd THF and added triethylamine (186 μL, 1.33 mmol, 1.1 equiv). Separately, **S1** (509 mg, 1.21 mmol, 1.0 equiv) was suspended in 12 mL anhyd THF and was then added to the **S18** mixture dropwise. The reaction was stirred under reflux for an additional 18 h, until an additional **S18** (426 mg, 1.21mmol, 1 equiv) and triethylamine (186 μL, 1.33 mmol, 1.1 equiv) were added and stirred for an additional 6 h. The reaction was then diluted with 40 mL CH_2Cl_2, and the organic layers were then washed with satd aq NaCl (15mL) and water (15 mL). The organic layers were then concentrated by rotary evaporator, and purification by silica flash chromatography (9:1 hexanes:EtOAc to 3:1 hexanes:EtOAc to 3:1 EtOAc:hexanes) yielded product **S19** (618 mg, 66 %) as a light yellow oil. **^{1}H-NMR** (500 MHz, $CDCl_3$) δ 10.05 (d, J = 7.9 Hz, 1H), 8.53 (s, 1H), 8.22 (s, 1H), 8.07 (s, 1H), 7.33 (m, 10H), 6.21 (d, J = 2.5 Hz, 1H), 5.21 (s, 3H), 5.18 (s, 1H), 5.13 (s, 1H), 5.09 (s, 3H), 4.80 (td, J = 7.8, 5.2 Hz, 1H), 4.59 (td, J = 8.1, 4.8 Hz, 1H), 4.47 (d, J = 2.9 Hz, 1H), 3.89 (dd, J = 11.3, 3.6 Hz, 1H), 3.77 (dd, J = 11.3, 3.9 Hz, 1H), 2.45 – 2.37 (m, 2H), 2.27 – 2.15 (m, 1H), 2.04 (m, 1H), 1.65 (s, 3H), 1.42 (s, 3H), 0.82 (s, 9H), 0.01 (d, J = 2.2 Hz, 6H). **^{13}C-NMR** (126 MHz, $CDCl_3$): □ 172.3, 171.5, 153.4, 151.3, 149.9, 141.2, 135.8, 135.3, 128.2, 120.8, 114.2, 91.9, 87.5, 85.2, 81.4, 67.2, 66.4, 63.6, 52.8, 41.2, 30.4, 27.8, 27.2, 25.8, 18.3, 13.8, -5.5. **ESI-MS** *m/z* (rel int): (pos) 775.6 ($[M+H]^+$, 100).

[0420] ((9-((3aR,4R,6R,6aR)-6-(((tert-butyldimethylsilyl)oxy)methyl)-2,2-dimethyltetrahydrofuro[3,4-d][1,3]dioxol-4-yl)-9H-purin-6-yl)carbamoyl)-L-glutamic acid (S20)



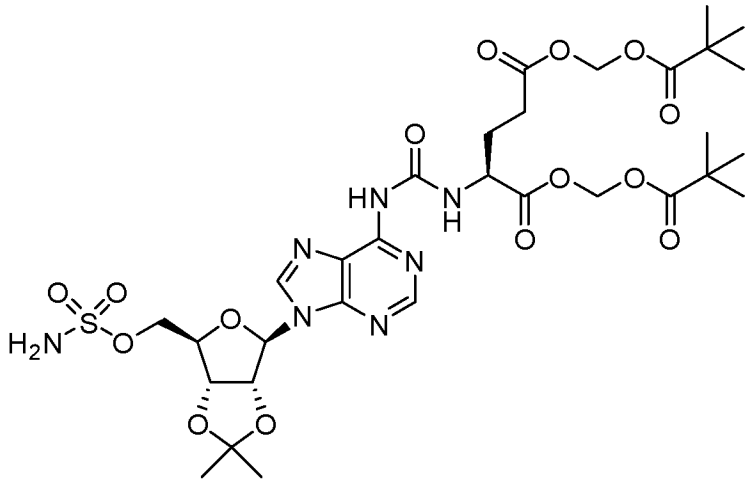
In a 100 mL round bottom flask, **S19** (618 mg, 0.797 mmol, 1.0 equiv) was suspended in 40 mL anhyd EtOH, added 10% Pd/C (170 mg, 0.159 mmol, 0.2 equiv) and 10% $Pd(OH_2)/C$ (112 mg, 0.159 mmol, 0.2 equiv), and sealed. The resulting mixture was then vacuum evacuated and subjected to hydrogen atmosphere for a total of three times. A hydrogen balloon was then set up, and the reaction proceeded for 2 h and judged through completion by LC/MS. The resulting mixture was then filtered with a celite pad, and the celite pad was subsequently washed with MeOH (2 x 60mL). The resulting filtrate was then concentrated by rotary evaporator and subjected to high vacuum to yield product **S20** (430 mg, 91%) as a transparent oil. The product was used without further purification. **Crude ^{1}H-NMR** (500 MHz, Methanol-d_4): δ 8.62 (s, 1H), 8.45 (s, 1H), 6.25 (s, 1H), 5.42 (d, J = 6.1 Hz, 1H), 5.02 (d, J = 6.1 Hz, 1H), 4.50 (d, J = 6.7 Hz, 1H), 4.39 (s, 1H), 4.27 (s, 1H), 3.85 (d, J = 4.2 Hz, 1H), 3.82 – 3.72 (m, 1H), 2.59 – 2.30 (m, 2H), 2.15-2.10 (m, 2H), 1.60 (s, 3H), 1.39 (s, 3H), 1.15 (s, 2H), 0.82 (s, 9H), -0.01 (s, 6H). **Crude ^{13}C-NMR** (126 MHz, Methanol-d_4) δ 177.2, 155.7, 152.7, 151.8, 151.4, 143.6, 121.8, 115.2, 92.9, 89.3, 86.1, 83.2, 64.9, 42.4, 32.1, 31.7, 29.6, 27.6, 26.4, 25.7, 19.3, 14.2, -5.2. **ESI-MS** *m/z* (rel int): (pos) 595.4 ($[M+H]^+$, 100).

[0421] bis((pivaloyloxy)methyl) ((9-((3aR,4R,6R,6aR)-6-(((tert-butyldimethylsilyl)oxy)methyl)-2,2-dimethyltetrahydrofuro[3,4-d][1,3]dioxol-4-yl)-9H-purin-6-yl)carbamoyl)-L-glutamate (S21).



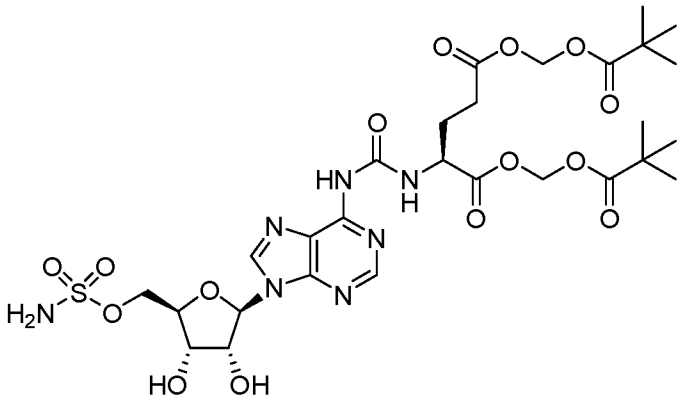
In a 25 mL round bottom flask at 25 °C, **S20** (260 mg, 0.437 mmol, 1.0 equiv) was suspended in 2.1 mL anhyd DMF. Triethylamine (184 µL, 1.31 mmol, 3 equiv), chloromethyl pivalate (630 µL, 4.37 mmol, 10 equiv), and tetrabutylammonium iodide (484 mg, 1.31 mmol, 3 equiv) was added and stirred 5 h. The mixture was then raised to 45 °C and stirred for an additional 23 h. The resulting mixture was then diluted with ethyl acetate (10mL), washed with satd aq NH_4Cl (2 x 10 mL) and satd aq NaCl (2 x 10 mL) and dried over $MgSO_4$. The organic layer was then concentrated by rotary evaporator, and was subsequently purified by silica flash chromatography (5% MeOH in CH_2Cl_2) yielded semi-crude product **S20** (152 mg, 42 % crude yield) as a yellow oil. Observed three co-spots on the TLC that could not be separated through chromatography. **TLC**: R_f 0.45 (5% MeOH in CH_2Cl_2). **Crude ^{1}H-NMR** (500 MHz, $CDCl_3$): δ 7.38–7.28 (m, 5H), 5.60 (m, 2H), 4.63 (m, 1H), 4.58 (s, 2H), 4.36 (t, 1H, J = 5.6), 3.49 (dd, 1H, J = 9.5, 3.7), 3.44 (dd, 1H, J = 9.6, 7.9), 3.34 (s, 3H), 3.32 (s, 3H), 2.68 (d, 1H, J = 2.5), 2.52 (m, 1H), 2.38 (m, 1H). **Crude ^{13}C-NMR** (125 MHz, $CDCl_3$): δ 137.9, 131.0, 128.5–127.5 (Ar), 103.8, 73.6, 73.4, 66.7, 53.5, 53.2, 31.8. **ESI-MS** *m/z* (rel int): (pos) 823.7 ($[M+H]^+$, 100).

[0422] bis((pivaloyloxy)methyl) ((9 - ((3aR,4R,6R,6aR) - 2, 2 – dimethyl – 6 - ((sulfamoyloxy) methyl)tetrahydrofuro[3,4-d][1,3]dioxol-4-yl)-9H-purin-6-yl)carbamoyl)-L-glutamate (S22).



In a 25 mL plastic falcon tube at 25 °C, **S21** (76 mg, 0.128 mmol, 1.0 equiv) was suspended in 1.3 mL anhyd THF. Hydrogen fluoride pyridine (609 µL, 4.65 mmol, 50 equiv) was added dropwise and stirred for 25 min. The reaction was then quenched by addition with cold satd aq $NaHCO_3$ to pH 7. The aqueous mixture was then extracted with CH_2Cl_2 (2 x 10 mL), and the combined organic extracts were washed with water (2 x 5 mL), dried over $MgSO_4$, and concentrated by rotary evaporator to give crude intermediate**.** In a 15 mL round bottom flask at 25 °C, the crude intermediate was then azeotroped with benzene (5mL) and was suspended in 1.9 mL anhyd DMA. Sulfamoyl chloride (54 mg, 465 µmol, 5 equiv) was added in one portion and stirred for 30 min. The reaction was then diluted with ethyl acetate (5 mL), and the organic layers were washed with satd aq NaCl (2 x 5 mL) and water (2 x 5 mL). The combined organic extracts were then concentrated by rotary evaporator subsequently purified by prep-HPLC and lyophilized to yield **S22** (55 mg, 75% over two steps) as a white powder. **TLC**: R_f 0.45 (5% MeOH in CH_2Cl_2). **^{1}H NMR** (500 MHz, Chloroform-*d*) δ 8.72 (s, 1H), 8.63 (s, 1H), 6.30 (s, 1H), 5.87 (d, J = 5.7 Hz, 1H), 5.80 (d, J = 5.5 Hz, 1H), 5.72 (s, 1H), 5.49 – 5.38 (m, 1H), 5.01 (d, J = 6.0 Hz, 1H), 4.71 (s, 1H), 4.32 (q, J = 10.8 Hz, 2H), 2.52 (t, J = 7.1 Hz, 2H), 2.33 (dd, J = 14.2, 6.9 Hz, 1H), 2.18 – 2.06 (m, 1H), 1.62 (s, 3H), 1.40 (s, 3H), 1.20 (s, 9H), 1.19 (s, 9H). **ESI-MS** *m/z* (rel int): (pos) 788.6 ($[M+H]^+$, 100).

[0423] bis((pivaloyloxy)methyl) ((9-((2R,3R,4S,5R)-3,4-dihydroxy-5-((sulfamoyloxy) methyl) tetrahydrofuran-2-yl)-9H-purin-6-yl)carbamoyl)-L-glutamate.



In a 10 mL glass vial at 25 °C, **S22** (12.5 mg, 15.9 μmol, 1.0 equiv) was suspended in 1.58 mL TFA. The reaction mixture was stirred for 6 h. The reaction was quenched with 1 mL MeOH and was then concentrated by rotary evaporator. The crude mixture was then azeotroped with MeOH(3 x 1 mL). The extracts were then purified by prep-HPLC and lyophilized to yield **S23** (3.91 mg, 33%) as a white powder. **TLC**: R_f 0.45 (5% MeOH in CH_2Cl_2). **^{1}H NMR (500 MHz, Methanol-d_4)** δ 8.67 (d, J = 1.6 Hz, 1H), 8.53 (s, 1H), 8.47 (s, 1H), 6.20 (d, J = 4.9 Hz, 1H), 5.94 (d, J = 5.6 Hz, 1H), 5.82 (d, J = 5.7 Hz, 1H), 5.76 (t, J = 4.7 Hz, 2H), 4.77 (t, J = 5.0 Hz, 1H), 4.69 (td, J = 5.5, 2.7 Hz, 1H), 4.48 (dd, J = 9.6, 4.4 Hz, 1H), 4.39 (ddd, J = 11.3, 9.3, 3.9 Hz, 1H), 2.61 (h, J = 9.9 Hz, 2H), 2.35 (dt, J = 11.8, 6.0 Hz, 1H), 2.26 – 2.13 (m, 1H), 1.23 (d, J = 3.1 Hz, 9H), 1.21 (s, 9H). **ESI-MS** *m/z* (rel int): (pos) 748.5 ($[M+H]^+$, 55); 537.3 ($[M\text{-Ribose}+H]^+$, 100).

[0424] Representative Synthesis and Examples of Compounds of Formula II

[0425] As noted above, the mustard prodrug ZD2767P (structure 2a, FIG. 2C) was synthesized as previously reported (Springer *et al.*, *J Med Chem.* 37(15):2361–70(1994) and Niculescu-Duvaz *et al.*, *Tetrahedron Lett.* 46(40):6919–22 (2005)). Based on the present disclosure, including in regard to the representative syntheses of prodrug compounds of Formulas I & III and pro-prodrug compounds of Formula I, a person of ordinary skill in the art is well-enabled to generate compounds of Formula II. Further guidance towards synthesis of compounds of Formula II may be found in Niculescu-Duvaz *et al.*, *J. Med. Chem.* 47(10):2651-58 (2004), Niculescu-Duvaz *et al.*, *J. Med. Chem.* 46(9):1690-705 (2003), Springer *et al.*, *J. Med. Chem.* 38(26):5051-65, and Springer *et al.*, *Eur J Cancer*

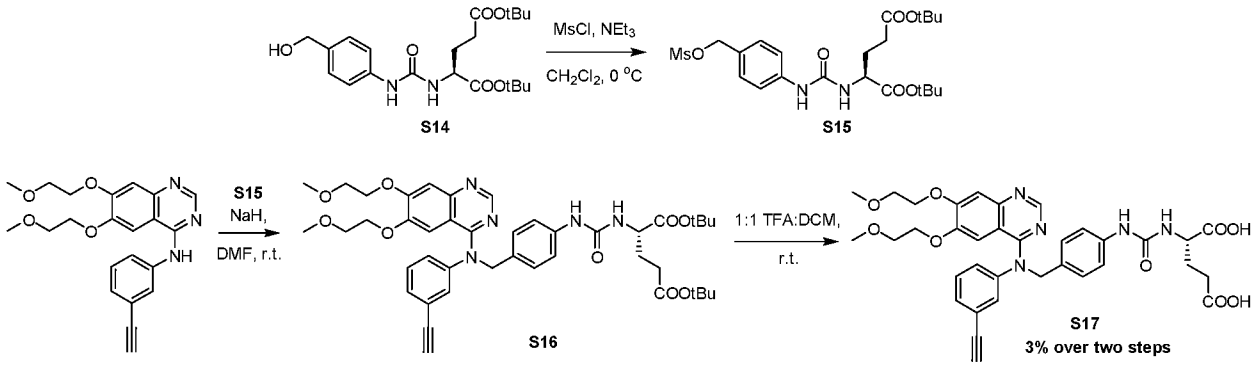
27(11):1361-66(1991) (each of which is incorporated herein by reference), as well as references cited in each therein.

[0426] Representative Synthesis and Examples of Compounds of Formula III

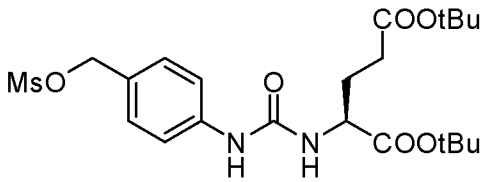
[0427] *Synthesis and Characterization of Glu-PABA-erlotinib*

[0428] Synthesis of a representative prodrug of erlotinib, Glu-PABA-erlotinib (**S17**), is summarized in Scheme 4 below and further detailed thereafter.

Scheme 4.



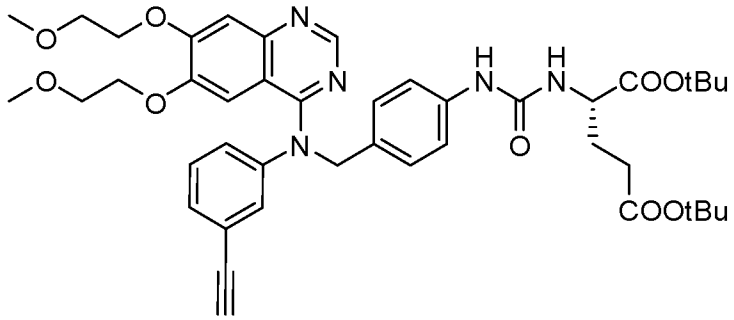
[0429] **di-tert-butyl ((4-(((methylsulfonyl)oxy)methyl)phenyl)carbamoyl)-L-glutamate (S15).**



S14 was synthesized as discussed in Niculescu-Duvaz, D.; et al. *J. Med. Chem.* **1998**, 41, 5297–5309. In a 15 mL round bottom flask at 0 °C, **S14** (90 mg, 220 µmol, 1.0 equiv) was suspended in 2.2 mL anhyd CH_2Cl_2. Triethylamine (61 µL, 441 µmol, 2 equiv) and methanesulfonyl chloride (34 µL, 441 µmol, 2 equiv) were added and the reaction was stirred at 0 °C for an additional 2 h until judged by completion through TLC. The mixture was then diluted by 5 mL CH_2Cl_2, and the organic layers was washed with water (1 × 10 mL). The aqueous layer was then washed with CH_2Cl_2 (3 × 10 mL), and the combined organic extracts were then concentrated by rotary evaporator. The crude product was azeotroped (3 × 5mL) and placed on high vacuum for 1 h. Crude product **S15** (107 mg) was unstable and carried to next step without further purification. **TLC:** R_f 0.80 (1:1 hexanes/EtOAc). **Crude ^{1}H NMR:**

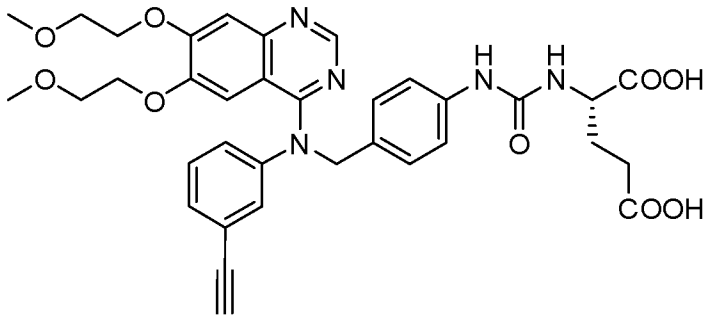
(500 MHz, $CDCl_3$) δ 7.34 (m, Hz, 2H), 7.24 – 6.85 (m, 2H), 3.66 (s, 3H), 2.42 – 2.27 (m, 2H), 2.13 – 2.03 (m, 1H), 1.89 (m, 1H), 1.40 (s, 9H).

[0430] di-tert-butyl ((4-(((6,7-bis(2-methoxyethoxy)quinazolin-4-yl)(3-ethynylphenyl)amino)methyl)phenyl)carbamoyl)-L-glutamate (S16).



In a 15 mL round bottom flask at 0 °C, erlotinib (72 mg, 184 μmol, 1.0 equiv) was suspended in 1.8 mL anhyd DMF and added 60% NaH in mineral oil (11 mg, 275 μmol, 1.5 equiv) for 5 min. Separately, **S15** (107 mg, 220 μmol, 1.2 equiv, assumed 100% pure) was suspended in 1.8 mL anhyd DMF, cooled to 0 °C, and added dropwise to erlotinib mixture at 0 °C. The resulting was stirred for an additional 30 min at 0 °C, until complete conversion had occurred as judged by LC-MS. The mixture was then quenched with 1mL water, and the aqueous layers were then washed with ethyl acetate (3 × 5 mL). The combined organic extracts were then concentrated by rotary evaporator, placed on high vacuum, and carried to the next step as a crude product **S16** (11.1 mg) without further purification. **ESI-MS** *m/z* (rel int): (pos) 784.7 ($[M+H]^+$, 100).

[0431] ((4-(((6,7-bis(2-methoxyethoxy)quinazolin-4-yl)(3-ethynylphenyl)amino)methyl)phenyl)carbamoyl)-L-glutamic acid (S17).



In a 10 mL glass vial at 25 °C, crude compound **S16** (11.1 mg, 14.1 μmol, 1.0 equiv, assumed 100% pure) was suspended in 950 μL 1:1 TFA:CH_2Cl_2 solution. The reaction was stirred for 1 h until complete conversion had occurred as judged by LC-MS. The mixture was then

azeotroped with MeOH (3 × 3 mL), concentrated by rotary evaporator, and purified through prep-HPLC to yield product **S17** (0.43 mg, 3.5% yield over two steps) as a white powder. **^{1}H NMR** (500 MHz, Methanol-d_4) δ 8.79 (s, 1H), 7.58 – 7.40 (m, 4H), 7.32 – 7.21 (m, 8H), 7.17 (s, 1H), 6.42 (s, 1H), 5.52 (s, 2H), 4.37 (dd, J = 8.4, 5.1 Hz, 1H), 4.32 – 4.27 (m, 3H), 3.84 – 3.77 (m, 3H), 3.65 (s, 1H), 3.52 (d, J = 5.2 Hz, 8H), 3.41 (s, 3H), 3.35 (s, 3H), 2.42 (m, 6.7 Hz, 2H), 2.25 – 2.13 (m, 1H), 2.02 – 1.89 (m, 1H). **ESI-MS** *m/z* (rel int): (pos) 672.4 ($[M+H]^+$, 100).

[0432] Based on the present disclosure, including in regard to the representative syntheses of pro-prodrug compounds of Formula I, a person of ordinary skill in the art is well-enabled to generate compounds of Formula III.

Example 2. Methods of Testing Prodrug Candidates

[0433] Candidate prodrugs for use with selected prodrug converting enzymes can be tested through *in vitro* assays with filtering at each step to achieve a Target Product Profile (Table 2).

Table 2

Parameter	Accept	Ideal
CPG2 kinetics (k_{cat}/K_m, $s^{-1}\mu M^{-1}$)	≥ 1	≥ 10
drug activity (IC_{50}, nM)	≤ 1,000	≤ 100
prodrug Selectivity Index (SI, fold)	≥ 10	≥ 100
prodrug activation (IC_{50} pro/free, fold)	≥ 20	≥ 5
aqueous solubility pH 7.4 (logS, M)	≥ −5	≥ −4
plasma stability ($t_{1/2}$)	≥ 2 h	≥ 4 h
microsomal stability ($t_{1/2}$)	≥ 0.5 h	≥ 4 h
plasma protein binding	≤ 95%	≤ 75%

[0434] These results can be used to design improved prodrugs. The kinetics (Km, kcat) of CPG2-mediated prodrug cleavage is determined using recombinant, purified CPG2 (Jeyaharan *et al. Protein Expr Purif.* 127:44-52 (2016)). The reaction is monitored based on decreased UV absorbance, as described previously for ZD2767P (Springer *et al.*, *J Med Chem.* 37(15):2361–70 (1994) and Springer *et al.*, *J Med Chem.* 38(26):5051–65 (1995)) and methotrexate (Sherwood *et al.*, *Eur J Biochem.* 148(3):447–53(1985)). Prodrug stability is confirmed in physiologic conditions. Prodrugs with kcat/Km ≥ 1 s–1μM–1 (ideally ≥ 10) are advanced.

[0435] The cytotoxicity of each prodrug/drug pair can be tested in antigen-positive tumor cell lines, including HL 60 (leukemia), Jurkat (T cell), MDA 231 (breast), OVCAR (ovarian),

SW480 (colon), SET2 (AML), as well as MDR variants and primary cancer cells. Pairs with drug activity ≤ 1 μM (*e.g.*, ≤ 100 nM) and SI ≥ 10 (*e.g.*, ≥ 100) are advanced.

[0436] The prodrug cytotoxicity in the presence of purified CPG2 and supernatant from CPG2-secreting HEK293T cells is also tested. The prodrug and unmasked dependent drug are quantified by LC MS/MS. Prodrugs with IC50 within 20-fold of free drug (*e.g.*, 5-fold) are advanced.

[0437] The drug and prodrug activity in HEK293T cells expressing secreted, surface-associated, and intracellular CPG2 are also tested. Prodrugs with IC50 within 20-fold of free drug (*e.g.*, 5-fold) are advanced.

[0438] The prodrugs are also tested for *in vitro* ADME (absorption, distribution, metabolism, and excretion) properties: *e.g.,* solubility at pH 7.4, mouse/human plasma/whole blood stability, mouse/human microsomal stability (±NADPH, ±UDPGA), mouse/human plasma protein binding. Prodrugs with acceptable *in vitro* properties are advanced to *in vivo* evaluation.

Example 3. Construction of Cells Expressing a Chimeric Antigen Receptor

[0439] This Example describes the construction of a cell that comprises a CAR (*e.g.*, with an antigen-binding domain specific for CD19, WT1, or PRAME). In this exemplary embodiment, CPG2 is engineered to have a transmembrane domain and localizes to the extracellular side of the cell membrane, where it can hydrolyze and activate prodrugs that come in contact with the cell into active drugs (*e.g.*, prodrugs of Example 1, which are hydrolyzed into active cytotoxic drugs). The activation of the drug enhances cytolytic action locally within the tumor at the site where the CAR T cell is targeted via the antigen binding domain.

[0440] The CD19 CAR T cells and WT1 CAR T cells have been made and initial publications on the methods to create them and to characterize their activity are described (Brentjens et al., *Sci. Trans. Med.* 5(177):177ra38 (2013), Pegram *et al. Leukemia* 29(2):415-22 (2015)). The PRAME-reactive CAR T cell will be made and characterized similarly.

[0441] The mammalian optimized CD19, WT1 and PR20 ($(PRAME^{300-309})$ scFv sequences were utilized to generate the antigen binding domain of the CARs. The variable heavy and light chains of the scFv are connected via a $(Gly4Ser)_3$ linker. A leader peptide

(*e.g.*, a CD8 signal sequence) was added to the N-terminus of the scFv for localization. In alternative embodiments, a c-Myc tag can be added to allow detection of the CAR by flow cytometry. A CD8 transmembrane domain follows the antigen binding domain. On the cytoplasmic side of the CAR, 4-1BB was used as a costimulatory element due to the increased persistence of 4-1BB CAR T cells (Oka *et al. PNAS.* 101:13885-90 (2004)). The CAR can be optimized to include a spacer domain upstream of the CD8 transmembrane domain if desired. The nucleic acid encoding the CAR was cloned into an SFG retroviral vector containing the 4-1BB signaling and CD3ζ activation domains, forming a second generation CAR (Brentjens *et al. Clin Cancer Res.* 13(18 Pt 1):5426-35 (2007)). A clinical grade construct, without the c-Myc tag, can also be generated, for which an anti-idiotype mAb to allow detection of the CAR can be generated.

[0442] Stable HEK293T viral producing cell lines were generated, subcloned and used to transduce primary human T cells as described previously (Curran et al. *American Society of Gene Therapy* 23(4):769-78 (2015)). Following transduction, CAR expression was verified by flow cytometry, using an anti-idiotype antibody to detect the anti-CD19 ScFv.

[0443] For co-expression of the CPG2 enzyme, nucleic acid encoding the carboxypeptidase CPG2 enzyme was cloned into the CAR vector as described in Example 5.

Example 4. Generation of Transmembrane and Secreted CPG2 constructs and Characterization of Expressed CPG2

[0444] This example describes the generation of transmembrane and secreted forms of CPG and their characterization when expressed in bacteria or mammalian cells. The transmembrane form of CPG2 encodes a CD8 signal sequence (MALPVTALLLPLALLLHAARP (SEQ ID NO: 11) at the 5′ terminus of the CPG2 encoding sequence and at its 3′ terminus, the sequence derived from the transmembrane domain of CD8 to permit membrane anchoring and extracellular exposure of the enzyme (FIG. 3A).

[0445] The gene encoding the CPG2 enzyme was initially cloned into the pET151 vector using the pET151 Directional TOPO® expression system, which incorporates an N-terminal 6x His tag (Invitrogen). For bacterial expression, the endogenous signal sequence was removed to facilitate protein isolation (*see* Jeyaharan *et al.*, *Protein Expression and*

Purification (2016)). Removal of signal sequence allowed expression of a soluble CPG2 species.

[0446] The amino acid sequence used for CPG2 (without start Met and leader signal sequence) is as follows:

ALAQKRDNVLFQAATDEQPAVIKTLEKLVNIETGTGDAEGIAAAGNFLEAELKNLGF
TVTRSKSAGLVVGDNIVGKIKGRGGKNLLLMSHMDTVYLKGILAKAPFRVEGDKAY
GPGIADDKGGNAVILHTLKLLKEYGVRDYGTITVLFNTDEEKGSFGSRDLIQEEAKLA
DYVLSFEPTSAGDEKLSLGTSGIAYVQVQITGKASHAGAAPELGVNALVEASDLVLR
TMNIDDKAKNLRFQWTIAKAGQVSNIIPASATLNADVRYARNEDFDAAMKTLEERA
QQKKLPEADVKVIVTRGRPAFNAGEGGKKLVDKAVAYYKEAGGTLGVEERTGGGT
DAAYAALSGKPVIESLGLPGFGYHSDKAEYVDISAIPRRLYMAARLIMDLGAGK
(SEQ ID NO: 3, CPG2 without leader sequence and optimized for human expression)

[0447] Two variants of the of the transmembrane CPG2 construct were generated. One construct includes a CD8 signal sequence, the CPG2 sequence, an optional HA tag, a CD8-membrane-spanning portion and a CD8 intracellular portion.

[0448] CD8 signal sequence:

[0449] MALPVTALLLPLALLLHAARP (SEQ ID NO: 11)

[0450] CD8-membrane-spanning portion:

[0451] IYIWAPLAGTCGVLLLSLVIT (SEQ ID NO: 38)

[0452] CD8 intracellular domain amino acid sequence:

LYCNHRNRRRVCKCPRPVVKSGDKPSLSARYV (SEQ ID NO: 13)

[0453] The full CPG-CD8 TM sequence is as follows:

[0454] MALPVTALLLPLALLLHAARPALAQKRDNVLFQAATDEQPAVIKTLEKLV
NIETGTGDAEGIAAAGNFLEAELKNLGFTVTRSKSAGLVVGDNIVGKIKGRGGKNLL
LMSHMDTVYLKGILAKAPFRVEGDKAYGPGIADDKGGNAVILHTLKLLKEYGVRDY
GTITVLFNTDEEKGSFGSRDLIQEEAKLADYVLSFEPTSAGDEKLSLGTSGIAYVQVQI
TGKASHAGAAPELGVNALVEASDLVLRTMNIDDKAKNLRFQWTIAKAGQVSNIIPAS
ATLNADVRYARNEDFDAAMKTLEERAQQKKLPEADVKVIVTRGRPAFNAGEGGKK
LVDKAVAYYKEAGGTLGVEERTGGGTDAAYAALSGKPVIESLGLPGFGYHSDKAEY
VDISAIPRRLYMAARLIMDLGAGKYPYDVPDYAGGGIYIWAPLAGTCGVLLLSLVIT
LYCNHRNRRRVGGGRPVVKSGDKPSLSARYV (SEQ ID NO: 40)

[0455] The second construct is identical to the sequence above except that the natural Lyk binding domain is disrupted to uncouple the endogenous function of CD8 from the CPG molecule. CD8 transmembrane domain portion with disrupted Lyk binding domain : LYCNHRNRRRVGGGRPVVKSGDKPSLSARYV (SEQ ID NO: 15)

[0456] For the secreted form of CPG2, a CPG2 signal sequence (MRPSIHRTAIAAVLATAFVAGT (SEQ ID NO: 9) was inserted at the 5′ terminus of the CPG2 encoding sequence (FIG. 3B) to facilitate secretion.

[0457] The CPG2-pET151 vector was transformed into BL21 star *E.coli* cells and induced with IPTG. Bacteria were homogenized in a French Press and purified by affinity chromatography over Ni++ beads using standard protocols. Imidazole was exchanged via PBS dialysis and protein was purified via desalting spin columns. The expressed and purified CPG2 was assayed to confirm that the active enzyme was a potent converter of glutamated prodrugs. FIG. 4A shows that the initial bacterially produced CPG2 is highly active. Pro-AMS was activated by recombinant CPG2 *in vitro*. Human Jurkat T cells were incubated with or without P-AMS in the presence of increasing concentration of purified CPG2. Cell viability of the Jurkat cells was measured at 48 hours by CellTitre-Glo® luminescence. Studies to detect free glutamate in solution indicated that most glutamate cleavage occurs within one hour *in vitro*. (FIG. 4B) Similar enzyme kinetics will be used to assess the characteristics of the enzyme activity for each of the prodrugs similarly. This PK is done in order to optimize expression and if necessary, optimize the prodrugs. These studies also allow assessment of susceptibility of target cells and normal cells *in vivo*.

[0458] Transient expression studies in eukaryotic cells were carried out by cloning the CPG2 variants the pcDNA3.1+ vector (Invitrogen) which utilizes the CMV promoter for eukaryotic expression. Expression studies were performed using Lipofectamine to transfect in HEK293T cells.

[0459] The CPG2 expression vector was then transduced into HEK293T cells to characterize the appropriate expression and activity of the transmembrane form. FIG. 4 demonstrates surface expression of a membrane-anchored CPG2. HEK293T cells transfected with a membrane-tethered CPG2 ($CPG2_{TM}$) expressing a hemagglutinin (HA) tag. The cells were exposed to surface staining with an anti-HA antibody and compared to cells stained with isotype control antibody (FIG. 5).

[0460] FIG. 6 demonstrates localization of the various CPG constructs, either transmembrane or secreted form, by comparing HEK293T total cell lysates (FIG. 6, left) to detect the transmembrane form versus the supernatant (FIG. 6, right) for the secreted form.

[0461] FIG. 7 demonstrates that the secreted form of the CPG2 expressed in HEK293T cells has enzymatic activity *in vitro*. Jurkat T cells were exposed to increasing concentrations of AMS (circle) or Pro-AMS with media alone (triangle) or supernatant from CPG2-secreting HEK293T cells (square). Cell viability was measured at 48 hours by CellTitre-Glo® luminescence assay. As shown in FIG. 7, increasing amount of the prodrug with the CPG2 supernatant resulted in a significant decrease in cell viability that was comparable to the AMS prodrug at around 100 nM, whereas control media did not.

[0462] FIG. 8 further demonstrates that the secreted form of the CPG2 expressed in HEK293T cells has enzymatic activity *in vitro*. Jurkat T cells were exposed to increasing concentrations of ZD2767P with media alone (triangle) or supernatant from CPG2-secreting HEK293T cells (square). Cell viability was measured at 48 hours by CellTitre-Glo® luminescence assay. As shown in FIG. 8, increasing amounts of the prodrug with the CPG2 supernatant resulted in a significant decrease in cell viability, whereas control media did not.

[0463] HEK293T cell lines expressing stably-integrated CPG2 variants were also generated using the retroviral pLGPW vector (a modified vector derived from pLHCX (Addgene) which drives GFP expression from the 5'LTR promoter, and CPG2 expression from the CMV promoter. Retrovirus was generated by transfection of the retroviral vector and VSVg glycoprotein for pseudotyping in gp293 packaging cells (Clontech).

[0464] FIG. 9 demonstrates cell-secreted and transmembrane CPG2 expressed in stably transfected HEK293T cells activates an AMS prodrug. Expression of CPG2 without a signal peptide (CPG2nosigp) was employed as a control. HEK293t cells stably transfected with CPG2 variants were exposed to P-AMS (1μM) for 48 hours and analyzed by bright field microscopy (FIG. 9A). Cells were exposed to varying concentrations of P-AMS and cell viability was analyzed at 48hr post treatment (FIG. 9B). The cell-secreted and transmembrane CPG2 each induced significant cell death whereas the control construct did not.

Example 5. Construction of SEAKER cells that comprise a CD19, WT1, or PRAME CAR T in combination with a CPG2 enzyme.

[0465] To generate the CPG2-secreting and CPG2-transmembrane SEAKER variants of the CAR T cells described in Example 3, the nucleic acid encoding CPG2 enzyme, either secreted or transmembrane form, respectively, was cloned and inserted downstream of the CD3-ζ chain of the CAR, separated by a self-cleaving P2A peptide sequence which generates an independent CPG2 protein (FIGS. 3A, B).

[0466] Constructs were generated using the Gibson Assembly strategy (NEB) to insert the CPG constructs into existing SFG retroviral vectors expressing the CD19 ScFv. In brief, anti-CD19 SFG vectors (19-1BBz and 19-28z) were linearized downstream of the CD3ζ sequence and a fragment containing the P2A sequence followed by the CPG variant were inserted (*see*, *e.g.* SEQ ID NOS: 48 and 49).

[0467] The CAR-CPG2 constructs were transduced into HEK293T cells to characterize the expression and secretion of the secretory form in the cell supernatant and the enzymatic activity *in vitro*. The secreted forms showed significant activity in activating the cytotoxic drug of P-AMS and ZD2767P prodrugs as demonstrated in Example 4. The transmembrane form is also active.

[0468] In addition to the transmembrane and secreted form of CPG2, a conditionally expressed form of the CPG enzyme is also constructed using the synthetic Notch inducible system (FIG. 3C) (see *e.g.*, Kloss *et al.*, *Nature biotechnology* 31(1):71-5 (2013); Lim *et al.* *Cell* 168(4):724-40 (2017); and Roybal *et al. Cell* 164(4):770-9 (2016)). This format gates expression of CPG2 (secreted or in the membrane) only upon engagement of the target of the primary CAR. Thus, the CPG2 enzyme selectively activates the prodrug only upon contact with target cells, which further enhances specificity and is expected to reduce potential toxicity, both locally and systemically. The kinetics of drug formation from prodrug, the potency of the SEAKER construct, and the off-target effects on antigen negative cells are assessed.

Example 6. Characterization of Primary T cells and T cell lines transduced with SEAKER cell retroviral constructs encoding anti-CD19 CAR and CPG2

[0469] The retroviral constructs encoding the anti-CD19 CAR and CPG2 enzymes described above were then introduced into T cell lines (*e.g.*, Jurkat) and primary T cells to measure expression of the chimeric antigen receptor (CAR) and CPG2 and characterize their

activity. Jurkat cells and primary human T cells were transduced with retrovirus encoding the anti-CD19 CAR with the 4-1BB costimulatory element alone (19BBz) or with either CPGnosigp (no signal peptide control) or CPGTM (transmembrane) genes. Transduction levels were assessed by staining for anti-CD19 ScFv expression on the cell surface and flow cytometry. FIG. 10 demonstrates that the CAR protein is expressed on the cell surface of the engineered immune cells that express the CPG2 enzyme.

[0470] The activity of Human Jurkat T cells expressing an anti-CD19 CAR with 4-1BB costimulatory element (BBz) and either a CPG gene with no signal peptide (CPGnosigp) or a CPG transmembrane gene was also assessed. Human Jurkat T cells transduced with 19BBz/CPG retrovirus were co-cultured with a GFP+ Raji (human Burkitt's lymphoma) cancer target cell line for 24 hours (1:1 ratio) and flow cytometry analysis. Jurkat and Raji cells were differentiated by GFP expression (left panel). Activation of Jurkat cells was examined by assessing CD69 expression in GFP-negative cells (right panel). FIG. 11 shows that the CAR T cells recognize and are activated by the target cancer cells, as evidenced by increased CD69 expression, demonstrating that the pathways are functional in the SEAKER cell.

[0471] In a separate experiment, the activity of primary human T cells expressing an anti-CD19 CAR with 4-1BB costimulatory element (BBz) and either a CPG gene with no signal peptide (CPGnosigp) or a CPG transmembrane gene was also assessed. Primary human T cells and human 19BBz and 19BBz/CPG CAR-T cells were co-cultured at a 1:1 ratio with GFP+ Raji cells. Following a 48-hour incubation cells were analyzed by flow cytometry (FIG. 12A) and %GFP+ cells was calculated (FIG. 12B).The increase in GFP negative cells (signifying the various CAR T cells prepared) to the same levels with both traditional CAR T and the 2 different SEAKER cells show the SEAKER cells are of comparable activity to traditional CARs.

[0472] Human T cells transduced with a retroviral construct for secreted CPG2 were also analyzed. Cell supernatant from human CAR-T cells encoding 19BBZ, 19BBz/CPG or control 19BBz/CPGnosigp constructs were analyzed for CPG2 expression by immunoblot. As shown in FIG. 13, the CPG2-secreting SEAKER cell immunoblot showed that the enzyme is found in the supernatant fluid showing that the cell is actively secreting the CPG2 enzyme.

The SEAKER cell that has CPG2 without a signal sequence (CPG3nosigp) does not secrete the enzyme.

Example 7. Human T cell resistance to Erlotinib and conversion of a glutamated Erlotinib prodrug (P-Erlotinib) by transmembrane CPG2

[0473] The efficacy of the engineered immune cells can be improved by employing s prodrug that is converted by the engineered immune cell into a cytotoxic drug that causes cell death in cells at the target site (*e.g.*, tumor cells) but does not cause death of the engineered immune cell itself, thus providing that the engineered immune cells remain viable and active in the patient. One example of a drug that is not toxic to T cells is Erlotinib. A prodrug-form of Erlotinib was generated as described in Example 1.

[0474] FIG. 14 provides exemplary data showing that Human T cells, but not cancer cells, are resistant to Erlotinib. Human Jurkat T cells, primary T cells and human non-small cell lung carcinoma cell lines PC9 and HCC827 were treated with increasing concentrations of the receptor tyrosine kinase inhibitor Erlotinib. As shown in the figure, primary T cells and T cell lines exhibited resistance to increasing concentrations of Erlotinib as compared to PC9 and HCC827, which showed significant cell death in the presence of the drug (FIG. 14A). A glutamated Erlotinib prodrug (P-Erlotinib), however, displays no toxicity in PC9 cells (1μM) unless activated by CPG2 (FIG. 14B). PC9 cells were incubated in the presence of Erlotinib, P-Erlotinib, or P-Erlotinib in the presence of purified CPG2. Cell viability was measured at 48 hours by CellTitre-Glo® luminescence. FIG. 14B shows that the cleaved prodrug is of comparable activity to the active drug.

[0475] The retroviral constructs encoding the anti-CD19 CAR and transmembrane CPG2 enzyme described above were transduced into 293T cells to test the conversion activity of transmembrane CPG2 for an Erlotinib prodrug. GFP+ $293t^{CPGTM}$ cells were co-cultured with lung adenocarcinoma cells (HCC827) at various ratios ($293t^{CPGTM}$: HCC827) and the erlotinib prodrug (p-erlotinib) was added. Cells were analyzed at 120 hr. by flow cytometry (FIG. 15A) and the percentage of GFP cells and the ratio of 293tCPGTM cells to HCC827 cells was calculated for each cell ratio (FIG. 15B). FIG. 15 shows that the transmembrane CPG2 in the transduced cell is functional and converts the prodrug to active drug that kills the target cancer cell, but not the cell secreting the enzyme.

Example 8. Efficacy of each SEAKER cell *in vitro* vs various cancer targets.

[0476] Once each of the SEAKER formats are produced and stable, CAR T cells using different donors are generated and tested for activity against cancer cell lines, both positive and negative, fresh cancer cells, and normal PBMCs, using previously published methods and cells (Brentjens *et al.*, *Sci Trans Med* 5(177):177ra38 (2013); Dao *et al.*, *Sci Trans Med* 13(5):ra33 (2013)). The ability of the various SEAKERs to redirect T cell function are analyzed *in vitro* by detecting the cancer cell line specific cytotoxicity, cytokine secretion and proliferative function of SEAKER cells, as previously described for CAR T cells (Curran *et al.*, *Molecular therapy : J Am Soc Gene Ther*. 23(4):769-78 (2015)).

[0477] Converted drug-mediated cytotoxicity are measured using standard assays (*see, e.g.,* FIGS. 5- 7). Specific T cell cytotoxicity are measured using a standard Chromium release assays against a panel of CD19+, WT1+, $PRAME^+$ or negative tumor cells (*see, e.g.*, Lasek et al. *Cancer immunology, immunotherapy: CII* 63(5):419-35 (2014); Dao *et al.*, *Sci Trans Med* 13(5):ra33 (2013); Pinilla-Ibarz *et al.*, *Leukemia* 20(11):2025-33(2006)).

[0478] Specific cytokine secretion are measured by collecting supernatant from 24 hour co-cultures of SEAKER cells and $PRAME^+$ or $PRAME^-$ tumor cells. The presence of cytokines are analyzed using Luminex technology.

[0479] The ability of the CD3-28z CAR to stimulate T cell proliferation are analyzed by co-culturing transduced T cells with antigen + or - tumor cells and monitoring T cell expansion with flow cytometry using enumeration beads, as published previously (Koneru *et al.*, *Oncoimmunology* 4(3):e994446(2015)). T cells transduced to express a CAR targeted to an irrelevant antigen are used as a control.

[0480] The CAR T cells are also assayed to determine whether there is additive or synergistic killing with the drug action plus the CAR T cytotoxicity versus the CAR T alone. Antigen positive and negative target cells are mixed for measuring differential killing. A CAR T that does not activate upon binding ('null" or weaker activating first generation CARs) to target by deleting the 4-1BB and CD3 signaling is created. The constructs are assayed and compared *in vitro*, where proliferation is needed to assess amplification of the signal at the cancer cells. T cell cytotoxicity are also separated from the drug action by use of trans well experiments and separation of media for "cis vs trans" killing of antigen-positive

and negative cell targets in the well or in wells into which media from the SEAKER cell is transferred in order to assess action of the drug alone in the various constructs and, for example, to compare membrane bound versus secreted forms.

Example 9. Efficacy and PK of SEAKER cell systems in animal models.

[0481] Following *in vitro* assessment of the SEAKER cell activities, efficacy of the SEAKER cells *in vivo* in animal models of various cancers is assessed. The pharmacokinetics (PK) of prodrug and drug in normal and neoplastic tissues is also assessed. The efficacy, toxicity, PK, and biodistribution of each prodrug/drug combination is assessed in relevant animal models. The efficacy, toxicity, PK, therapeutic index, and biodistribution of each SEAKER cell type and drug system in models is also assessed.

[0482] The ability of SEAKER cells to eradicate tumor *in vivo* is assessed using preclinical xenogeneic murine models. For example, SCID-Beige or NSG mice are inoculated, either IV for hematological malignancies, or IP or SC for solid tumors, with tumor cells modified to express Firefly Luciferase to allow bioluminescent imaging (FIG. 16)(see, *e.g.*, Koneru *et al. Oncoimmunology* 4(3):e994446 (2015); Lasek *et al.*, *Cancer immunology, immunotherapy : CII.* 63(5):419-35 (2014); and Pegram *et al.*, *Blood* 119(18):4133-41(2012)). Exemplary antigen positive tumor cell lines to be assayed, depending on the SEAKER cell specificity, include SET2 AML, BV173 ALL, AML14, MAC1 T lymphoma, breast cancer MDA-231, or ovarian cancer OVCAR3 lines, colon SW480. These cells have already propagated in mice.

[0483] Following tumor cell inoculation, mice are subsequently treated with systemic infusion of SEAKER or control cells (*e.g.*, expression a CAR targeted to an irrelevant antigen). Disease progression is monitored both clinically and with bioluminescent imaging as described previously (Santos *et al.*, *Nature medicine* 15(3):338-44(2009)). Persistence of SEAKER cells is determined by collecting peripheral blood from treated mice and flow cytometry to detect SEAKER cells. SEAKER function over time is determined by detection of cytokines in the serum of treated mice using Luminex technology. Drug and prodrug levels in the serum, target and off target normal tissues are measured by LC-MS/MS assay. Comparisons of drug levels in on- and off-target tissues with each SEAKER are also compared.

[0484] PK analysis and biodistribution of SEAKER cells and drugs in normal and in tumor bearing mice. Timing of the administration of the prodrug is expected to be optimal when the SEAKER cells are reaching peak concentrations at the tumor cell site. The SEAKER cells are tagged with a luminescent probe to allow real time imaging of their kinetics and biodistribution in mice. Prodrugs alone are initially quantified in plasma by LC-MS/MS at 0.08, 0.25, 0.5, 1, 2, 4, 8, and 24-h post-administration to determine appropriate doses and schedules (based on previous determination of prodrug plasma protein binding [C_{free}] and drug *in vitro* cytotoxicity [IC_{50}]). Subsequently, prodrug and drug concentrations in SEAKER-pretreated mice are quantitated in plasma and tumor to correlate drug release and clearance with observed antitumor efficacy

[0485] Toxicity in mice. Healthy and xenografted mice are followed daily and scored for 5 clinical signs of toxicity (per IACUC protocols) and weekly for weight gain or loss. Peripheral blood cell counts are assessed in selected mice and bone marrow, spleen, kidney and liver pathology are analyzed at sacrifice. Comparisons of anticancer activity versus clinical toxicity are conducted.

Example 10. Design and Assays for "Kill Switch" Prodrug System for use in SEAKER cell Technologies

[0486] This example describes the construction of a SEAKER cell that comprises a CAR in combination with an engineered cytoplasmic CPG2 enzyme that functions as a "kill switch" to activate a cell-penetrating prodrug which selectively destroys the engineered immune cell, but not cells that do not express the cytoplasmic CPG2 enzyme. (FIG. 17)

[0487] The great potency of adoptive T cell approaches, including T cells and CAR T cells, coupled with their ability to rapidly proliferate *in vivo*, has been accompanied by severe toxicities and deaths in patients. The strategy described herein allows administered engineered immune cells to be turned off safely should toxicity arise or the cells need to be reduced *in vivo*. The CPG2 enzyme is expressed in the cytoplasm of the engineered immune cell. At times after infusion of the cells, the non-toxic cell-penetrating prodrug can be administered to the patient, wherein it will be converted into cytotoxic drug only in the engineered immune cell, but nowhere else in the subject. In addition, because the prodrug can be administered in various doses and schedules, tuning of the shutdown effects and re-

emergence of the effector cells can be modulated as needed. In some examples, the engineered immune cells express the CPG2 intracellularly (*e.g.*, in the cytoplasm), and also secrete or expressed the CPG2 on the surface. Following administration of these engineered immune cells to a subject, the cells will localize to the tumor site via expression a tumor-antigen specific receptor, such as a chimeric antigen receptor (*see* FIG. 17B). A non-penetrant prodrug is then administered to activate the killing of cancer targets. Then, when needed, a cell-penetrant prodrug is administered to kill the engineered immune cells.

[0488] An initial construct was made (*see* FIG. 17A) which expresses a chimeric antigen receptor and an intracellular form of the CPG2 enzyme. As shown in FIG. 18, the CPG2 expressed from this construct is expressed inside the cell and is not secreted.

[0489] A dose-response relationship over several logs for killing of the 293 T cells and SEAKER or CAR T cells from multiple donors are assayed. Initial studies are carried out with AMS pro-prodrug 1d (FIG. 1), in which the glutamate moiety is masked as the corresponding bis-POM ester to enable cell penetration, after which cellular esterases are removed the POM groups to unveil the glutamate moiety (1a) for cleavage by intracellular CPG2, releasing the active drug (1a) selectively in the cell. Pro-prodrug, prodrug, and drug concentrations in supernatant and cell lysates are quantitated by LC-MS/MS to assess cell penetration and prodrug cleavage rates. The dose range for killing the engineered immune cells with each of several prodrugs is expected to be in the achievable range for models *in vivo*. The extent and timing of the killing over 1-4 days is also evaluated *in vitro*.

[0490] Mouse models are first tested without tumors to determine if the cells are eradicated or reduced and over what time frame. Real time imaging is used to quantitate the effects of the administer prodrugs.

[0491] Tumor bearing models are then assessed for alteration the growth of the engineered immune cell (*e.g.*, due to proliferation) and the PK of the prodrug. Toxicity to animals is monitored and assayed for optimizing timing for administration of the cell-penetrating prodrug.

Example 11

[0492] This example describes the generation of transmembrane and secreted forms of CPG and/or Beta-lactamase and their characterization when expressed in mammalian cells.

In this exemplary embodiment, several cytotoxic drugs were synthesized carrying a terminal glutamate masking residue linked via a self-immolating moiety that decays following glutamate cleavage to result in formation of the active cytotoxin (FIG. 20A). These prodrugs included the masked forms of the nucleoside analog AMS (glut-AMS), the alkylating nitrogen mustard (ZDP275), and the targeted kinase inhibitor Erlotinib (glut-erlotinib) (FIG. 20B). IC_{50} values were generated for each pair of drugs against a panel of cancer cell lines and primary cell lines to determine the effective therapeutic indices that could be achieved with the new prodrug platforms.

[0493] Recombinant CPG2 protein was initially synthesized, purified (FIG. 21A and 21B), and incubated with increasing concentrations of prodrug in a glutamate release assay (FIG. 20C). Mass spectrometry was used to determine that the glutamate moiety was released from all of the glutamate prodrugs. The *in vitro* cytotoxicity of the prodrugs (FIG. 20B) were tested against the myeloid leukemia cell line SET2 using a CellTitre Glo® assay (Promega, Madison, WI). As expected, the prodrugs were not cytotoxic to the SET2 cells, however, co-incubation of the prodrugs with the purified, recombinant CPG2 resulted in the subsequent death of SET2 cells, consistent with CPG2-mediated cleavage of the prodrug to release the active parent drug (FIGS. 20D-20F).

[0494] It was determined that the AMS prodrug expressed high (>100) selective indices for all cell types tested, and remained stable in the presence of the endogenous glutamate carboxypeptidase expressed in human prostate cells, confirming its specificity for the CPG2 carboxypeptidase (FIG. 21C). This prodrug/drug combination was chosen for further development of the SEAKER systems.

[0495] This example describes the generation of transmembrane and secreted forms of CPG and their characterization when expressed in bacteria or mammalian cells. Next, it was demonstrated that CPG2 could be genetically encoded and expressed in eukaryotic cells while maintaining enzymatic function and without harming the host cells. Transient transfection of HEK293t cells demonstrated that CPG2 tolerates various structural modifications, including the addition of an HA epitope tag, restriction to the cytoplasm, tethering to the cell surface, or routing through the secretory system to permit secretion into the extracellular milieu (FIG. 22A and 22B). Immunoblot analysis determined that the addition of the CD8 cytosolic tail to the c-terminus of CPG2 increased secretion (FIG. 23A).

[0496] FIGS. 22C and 23B-23C demonstrate that the secreted form of CPG2 expressed in HEK 293t cells has enzymatic activity *in vitro*. SET2 cells were exposed to increasing concentrations of AMS (square) or glut-AMS with media alone (- control, triangle) or supernatant from CPG2-secreting HEK293t cells (circle). Cell viability was measured by CellTitre-Glo® luminescence assay. As shown in FIG. 22C, increasing amount of the prodrug with the CPG2 supernatant resulted in a significant decrease in cell viability that was comparable to the AMS prodrug at around 100 nM, whereas control media did not.

[0497] FIG. 23A demonstrates an immunoblot analysis of the stable expression of CPG2 and Beta-lactamase in HEK293t cells transduced with retrovirus, which were subsequently analyzed for enzyme expression and function. The HA epitope tag permitted staining of surface-level CPG2 in cells expressing either the secreted or transmembrane forms of the enzyme (FIG. 22D). Enzyme was detected in cell supernatant fluid and transfer of supernatant fluid from $293t^{CPG2\text{-}sec}$, but not the other forms of CPG2 or a GFP expressing control cell line in the presence of prodrug, resulted in death of SET2 cells (FIG. 22E). Cell viability was measured at 48 hours by CellTitre-Glo® luminescence. FIGS. 22F and 22G demonstrate that direct treatment of $293t^{CPG2}$ cell lines with prodrug resulted in cell death, indicating that cells expressing CPG2 are sensitized to prodrug treatment.

[0498] Retroviral constructs encoding the CPG2 enzyme described above were then introduced into T cell lines (*e.g.*, Jurkat) to measure expression of CPG2 and characterize the activity. FIG. 22H shows an immunoblot analysis which demonstrates stable expression of CPG2 enzyme via retroviral transduction.

[0499] A second eukaryotic-adapted prodrug-activating enzyme was developed based on the *E. cloacae*-derived Beta-lactamase (B-lac), as well as a series of lactam prodrugs which were activated by recombinant B-lac (FIG. 21D). As with CPG2, B-lac was produced and secreted from stable 293t cells ($293t^{Beta\text{-}lac}$) and Jurkat cells (FIG. 21E). The secreted form of Beta lactamase displayed enzymatic activity in stable cell lines, and successfully activated various lactam prodrugs (FIG. 28C), which possessed high selective indices in various cell types (Table 3).

Table 3. Cell selectivity data for AMS prodrugs.

	AMS (ng/mL)	glut-AMS (ng/mL)	glut-AMS Selective Index	ceph-AMS	ceph-AMS Selective Index
HEK293t	666	>5000	>7.5	2000	~3
Skov-3	350	>5000	>14.3	>1250	.3.6
Jurkat (T-cell)	17.7	>1250	>162.3		
Human T cell	227.4	>3000	>13.2	>1250	>6
Nalm6	36.9	>3000	>81.3		
Raji	17.6	>3000	>170.5		
Set2	34.4	>3000	>87.2	~2200	~64

[0500] Cell permeability studies in human epithelial CACO-2 cells revealed that the glut-AMS prodrug exhibited no cell permeability and would thus not diffuse into CPG2-expressing cells prior to enzyme release (Table 4), however, the active drug could still kill the secreting cell after unmasking. To further investigate this effect, bystander toxicity assays were conducted in which enzyme-positive cells (effector cells) were cocultured with enzyme-negative cells (target cells), in the presence of prodrug. Cell counts following prodrug treatment demonstrated that increased effector:target ratios resulted in higher total cell death, with depletion of ~80% of cells when $293t^{CPG2\text{-}sec/GFP}$ or $293t^{Beta\text{-}lac/GFP}$ were cocultured at a 1:1 ratio with WT 293t cells (FIG. 24A). Analysis of cell fluorescence revealed equivalent depletion of both GFP+ (effector) and GFP- (target) cells, indicating that both cell populations (effector cells and target cells) are destroyed by the activated prodrug (FIG. 24B-24D).

Table 4. Cell permeability studies of prodrugs in human epithelial CACO-2 cells.

S.No	Compounds	Papp (10^{-6} cm/sec)	Efflux Ratio (B-A/A-B)
1	Loperamide (A-B)	2.2 ± 0.3	7.7
	Loperamide (B-A)	16.9 ± 1.1	
	Loperamide + Ver (A-B)	10.5 ± 1.5	0.7
	Loperamide + Ver (B-A)	7.8 ± 0.5	
2	Glutamate-AMS (A-B)	0.0 ± 0.0	NC
	Glutamate-AMS (B-A)	0.0 ± 0.0	
	Glutamate-AMS + Ver (A-B)	0.0 ± 0.0	NC
	Glutamate-AMS + Ver (B-A)	0.0 ± 0.0	

[0501] To integrate the enzyme prodrug systems with existing CAR-T cell platforms, a construct was created that positioned CPG2 or B-lac upstream of the 19BBz CAR cassette (FIG. 25A). A quantitative ELISA was also developed to determine the respective enzyme output of each construct (**FIG. 21F**). As a comparison, the secreted CPG2 construct was also cloned by introducing a P2A self-cleaving peptide sequence followed by CPG2 directly downstream of a prototypical anti CD-19 CAR containing the 4-1BB costimulatory element (19BBz) in the previously published SFG retroviral vector backbone (FIG. 26A). Supernatant fluid isolated from 19BBz-CPG2 CAR-T cells did not potently activate prodrug, as supernatant transfer experiments with prodrug did not cause death of SET2 cells (FIG. 26), despite high transduction levels of CPG2-sec-19BBz in primary human T cells, effective T-cell killing activity compared to a standard 19BBz CAR, and the detection of secreted CPG2 in the cellular supernatant fluid (FIG. 26). Unexpectedly, primary T cells expressing the CPG-19BBz construct expressed nearly 20-fold higher levels of CPG2 than cells transduced with the 19BBz-CPG2 construct (FIG. 25B). These data suggested that gene sequence was

critical to the level of enzyme produced and allows for a second level of control of the amplitude of the system if needed. The higher amplitude configuration was chosen for all subsequent CAR-T cell studies, including a Beta-lac-19BBz CAR.

[0502] CPG-19BBz and Beta-lac-19BBz constructs were transduced at high efficiency in various T cell donors (FIG. 25C), which maintained equivalent antigen-dependent cytotoxic effect observed in WT 19BBz cells (FIG. 25D) and secreted robust levels of each respective enzyme (FIG. 25E). Further, supernatant fluid from the SEAKER CAR-T cells (19BBz, CPG-19BBz, and (Beta-lac-19BBz) converted prodrug and killed SET2 cells in a *trans*-toxicity assay, indicating that the SEAKER cells produce and secrete high enough levels of functional enzyme to achieve the aims (FIG. 25F). Time course analysis *via* CellTitre Glo® of CPG-19BBz and Beta-lac-19BBz T cells demonstrated accumulation of CPG2 in the supernatant of primary cells over 96 hours (FIG. 25G). To determine whether antigen-induced stimulation would cause expansion and increased secretion of CPG2 or Beta-lactamase enzymes, SEAKER T cells were co-cultured with CD19+ Raji cells, and the amount of enzyme secreted was significantly increased following 24 hours at a 1:1 coculture (FIG. 25H). Thus, antigen-dependent expansion of SEAKER T cells results in increased output of enzyme into the extracellular milieu.

[0503] To determine if a CAR-T cell functionalized to produce a small molecule drug will eliminate antigen-low or antigen-negative cells (antigen loss variants) as well as cell that the CAR T cell does not directly engage, the ability of CPG2 or Beta-lactamase expression by SEAKER T cells to eliminate antigen-negative cells in a coculture experiment was examined (FIG. 25H). SEAKER T cells were cocultured with CD19+ Raji cells and CD19- SET2 cells. Following 24 hours of coculture, glut-AMS was added to the wells, and total cell viability was measured following an additional 24-hr incubation period (FIG. 25I). While 19BBz CAR-T cells eliminated only the CD19+ Raji cells, SEAKER cells were able to eliminate both antigen+ and antigen- cell populations. The data demonstrate that functionalized CAR-T cells can eliminate antigen-low or antigen-negative cells.

[0504] This exemplary embodiment will describe the generation of SEAKER CAR-T cells capable of eliminating tumor populations in an antigen-loss variant tumor model *in-vivo*. An “on target” SKOV-3 human ovarian cancer cell line will be developed which will express

the human CD19 ectodomain and the mCherry reporter (SKOV-3$^{CD19\text{-}ecto/mCherry}$), and an "off target" line expressing firefly luciferase and the GFP reporter (SKOV-3$^{luc/GFP}$). Cell viability studies will demonstrate that SKOV-3 cells will then be susceptible to killing by the active AMS drug, but not the glut-AMS prodrug. Co-culture experiments of the SKOV-3 cell lines and SEAKER T cells shall demonstrate that while standard 19BBz CAR T cells, or SEAKER T cells in the absence of prodrug will only deplete SKOV-3$^{CD19\text{-}ecto/mCherry}$ cells, both SKOV-3 cell types will be eliminated in cocultures with SEAKER T cells containing the glut-AMS prodrug.

[0505] The SKOV-3 cells will then be adapted for an *in vivo* mixed tumor model, where the IP cavity of NSG mice will be engrafted IP with a 1:1 ratio of SKOV-3$^{CD19\text{-}ecto/mCherry}$ and SKOV-3$^{luc/GFP}$. At 5 days post engraftment, the 19BBz CAR T cells or SEAKER CAR T cells will be administered *via* IP injection. Previously conducted pharmacokinetic and biodistribution studies conducted in Nod mice indicated that the glut-AMS prodrug is non-toxic and cleared rapidly with a half-life of 30-40 minutes (FIG. 31). Without wishing to be bound by theory, it is believed that daily IP dosing of high prodrug concentrations would be adequately converted into AMS. Three days post T cell injection, glut-AMS prodrug will be administered *via* IP injection daily for 3 consecutive days. Mice in the SEAKER CAR-T + glut-AMS arm should demonstrate significantly enhanced survival.

[0506] Bioluminescent imaging studies should demonstrate the elimination of luc+ off-target tumor in mice that receive SEAKER CAR-T cells plus prodrug, but not in mice that receive no prodrug, or standard 19BBz CAR-T cells with prodrug. Flow cytometry analysis of tumors harvested from mice shall demonstrate the depletion of both tumor types in mice that receive SEAKER- T cells + prodrug, but not in mice from the other experimental groups. ELISA assays will be used to confirm high levels of CPG2 in fluid from the IP cavity of mice that received SEAKER CAR T cells. Tandem mass spectrometry will be used to identify the presence of active AMS in IP fluid from SEAKER-T cell + prodrug mice, which will confirm that secreted CPG2 successfully activated the prodrug. This data will demonstrate that SEAKER T cells possess increased functionality by eliminating both on and off target tumor cells in a solid tumor model of antigen escape.

Example 12

[0507] This example describes the generation of transmembrane and secreted forms of Beta-lactamase and the characterization when expressed in mammalian cells. In this exemplary embodiment, transient transfection of HEK293t cells demonstrated that Beta-lactamase tolerates various structural modifications, including the routing of the enzyme through the secretory system to permit secretion into the extracellular milieu (FIG. 27A and 27B). An immunoblot analysis of the supernatant of HEK293t cells expressing the various constructs (CD8-β-lac or β-lac-trunc) indicated the expression of only the truncated form of Beta-lactamase (β-lac-trunc), and not the membrane-tethered form of Beta-lactamase(CD8-β-lac) (FIG. 27B). Nitrocefin analysis of Beta-lactamase activity in the supernatant fluid from HEK293t cells expressing the various constructs (CD8-β-lac or β-lac-trunc) confirmed the expression and secretion of Beta-lactamase from Hek293t cells compared to supernatant fluid from control HEK293t cells or purified recombinant Beta-lactamase (FIG. 27C).

[0508] To determine to respective enzyme output of the Beta-lactamase constructs relative to the previous CPG2 constructs, a quantitative sandwich ELISA was used to determine the concentration (ng/mL) of purified recombinant CPG2, and a quantitative nitrocefin assay was used to determine the concentration (ng/mL) of purified recombinant Beta-lactamase (FIG. 28A). Cell viability was measured by CellTitre-Glo® luminescence in the presence of prodrug (glut-AMS or ceph-AMS) and varying concentrations of purified recombinant CPG2 or Beta-lactamase (FIG. 28B). To demonstrate that the secreted form of CPG2 or Beta-lactamase expressed in HEK 293t cells has enzymatic activity *in vitro*, SET2 cells were exposed to increasing concentrations of AMS (square) or glut-AMS/ceph-AMS (appropriate prodrug) with media alone (- control, triangle) or supernatant from CPG2-secreting HEK293t cells (circle). As shown in FIG. 28C, increasing amounts of the ceph-AMS prodrug with the Beta-lactamase supernatant resulted in a significant decrease in cell viability that was comparable to the AMS prodrug at around 100 nM, whereas control media did not.

[0509] To assess the *cis*-toxicity of the HEK293t expressing the various constructs of CPG2 and Beta-lactamase, a CellTitre Glo® luminescence assay was carried out in which HEK293t, HEK293t-CPG2-int, HEK293t-CPG2-sec, or HEK293t-CPG2-trn cells were exposed to glut-AMS and HEK293t or HEK293t-Beta-lac cells were exposed to ceph-AMS.

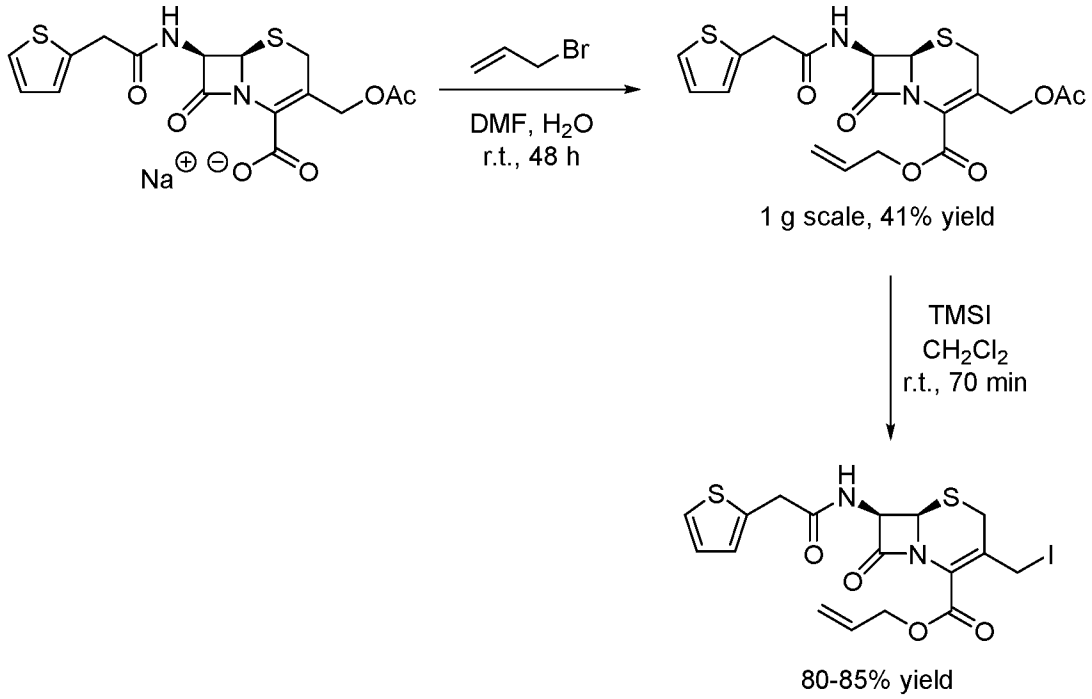
As shown in FIG. 28D, HEK293t-CPG2-sec, or HEK293t-CPG2-trn, and HEK293t-Beta-lac cells produce and secrete high enough levels of functional enzyme to induce cell death in bystander cells. To investigate this effect further, bystander toxicity assays were conducted in which enzyme-positive cells (effector cells) were cocultured with enzyme-negative cells (target cells), in the presence of the appropriate prodrug. Analysis of cell fluorescence demonstrated equivalent depletion of both GFP+ (effector) and GFP- (target) cells, indicating that both cell populations (effector cells and target cells) are destroyed by the activated prodrug (FIG. 28E).

The enzyme prodrug systems were incorporated into existing CAR-T cell platforms using a construct that positioned CPG2 or B-lac upstream of a 19BBz CAR cassette.

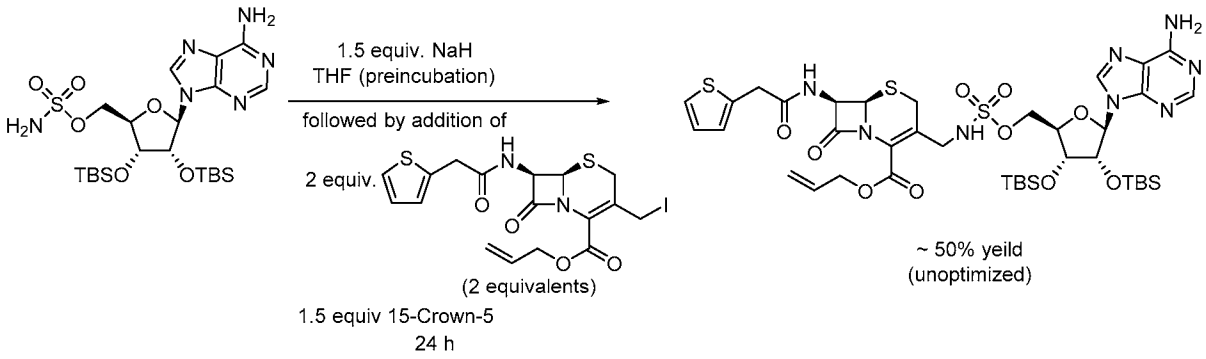
[0510] To determine if a CAR-T cell functionalized to produce a small molecule drug will eliminate antigen-low or antigen-negative cells (antigen loss variants) as well as cells that the CAR T cell does not directly engage, the ability of CPG2 expression by SEAKER T cells to eliminate antigen-negative cells in a coculture experiment was examined (FIGS. 29A-29C). SEAKER T cells (CPG-19BBz) were cocultured with (GFP-/CD19+) Raji cells and (GFP+/CD19-) SET2 cells. As shown in FIG. 29B, cocultures were treated with or without the glut-AMS prodrug following 72 hours of coculture. Flow cytometry was utilized to analyze the off target hits (SET2), which are quantified in FIG. 29C. FIG. 30A demonstrates the total cell viability of Raji (CD19+), SET2 (CD19-), and β-lac-19BBz T cells following a 72 hour coculture of the β-lac-19BBz T cells with either the Raji (CD19+) or SET2 (CD19-) cells with or without the ceph-AMS prodrug. FIG. 30B demonstrates that β-lac-19BBz T cells in the presence of the ceph-AMS prodrug were able to induce cell lysis in a luciferase assay in Raji cells expressing luciferase (Raji/Luc; CD19+/luc+) at a ratio of about 0.1:1.

Example 13

Scheme 5. Generation of Iodinated Cephalothin Derivative



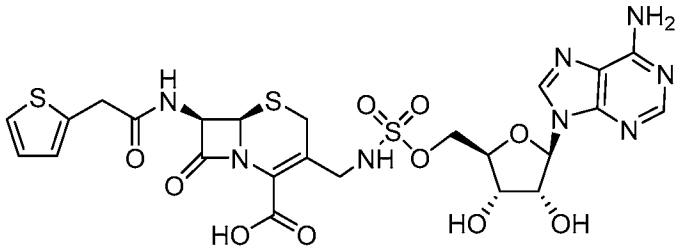
Scheme 6. Unoptimized Generation of an Exemplary β-Lactamase Prodrug Precursor of the Present Technology



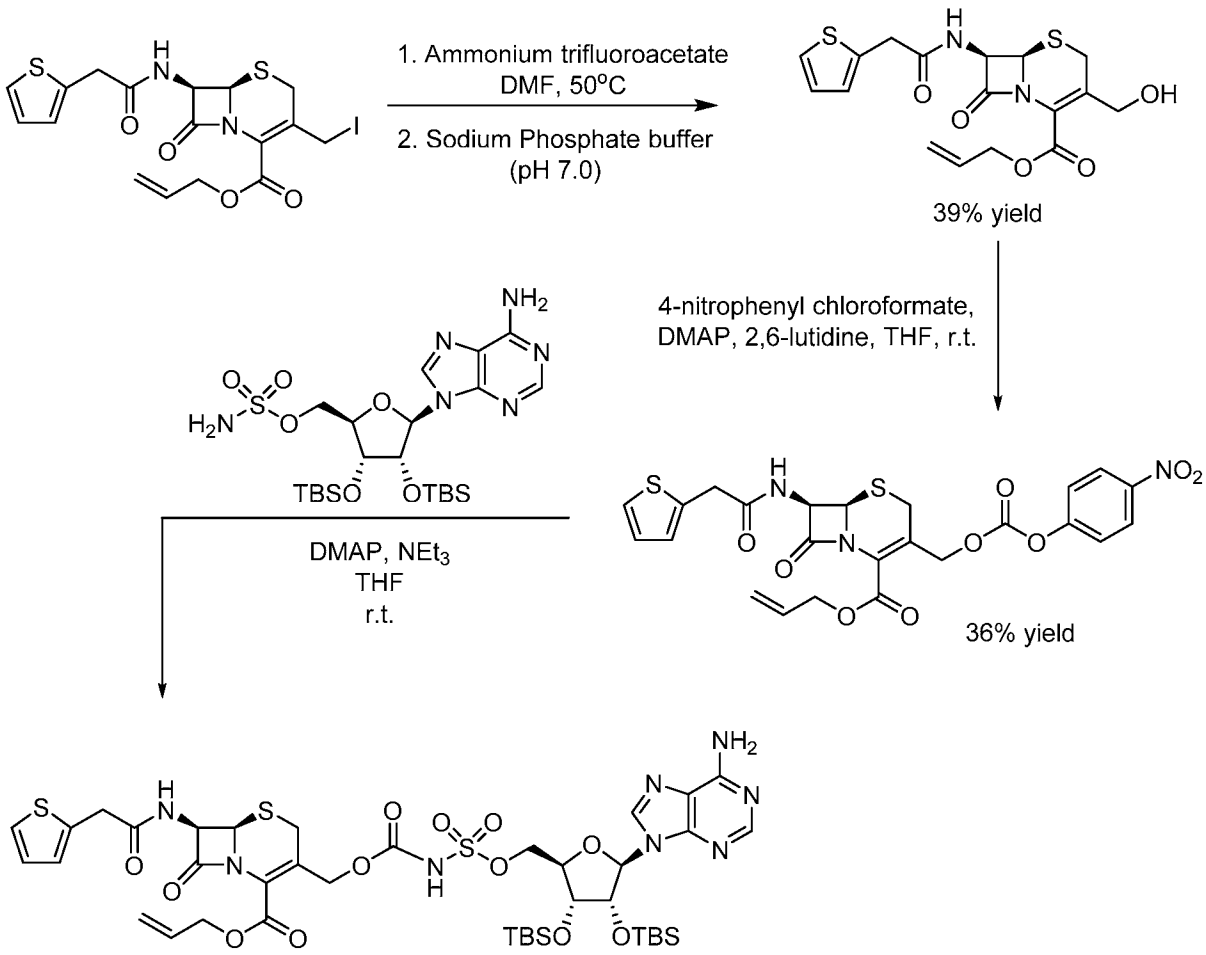
The exemplary β-lactamase prodrug precursor of Scheme 6 may then be deprotected (such as by HF·pyridine and appropriate allyl deprotection conditions such as Pd(0)-catalyzed deprotection) to provide the exemplary β-lactamase prodrug illustrated below in Scheme 7. Greene *et al.* (1991) *Protective Groups in Organic Synthesis,* 3rd Ed. (John Wiley & Sons,

Inc., New York), incorporated herein by reference, provides exemplary deprotection procedures.

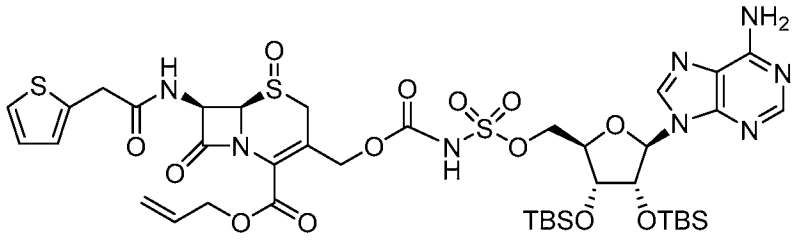
Scheme 7.



Scheme 8. Unoptimized Generation of an Exemplary Carbamate-Containing β-Lactamase Prodrug Precursor of the Present Technology

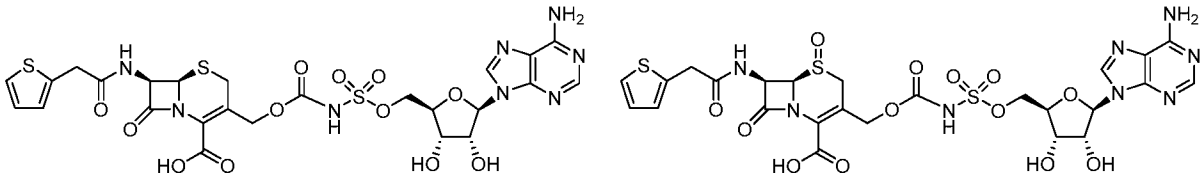


The sulfoxide variant illustrated below was also generated, where a similar synthesis as depicted in Scheme 8 may be utilized to generate this sulfoxide variant.

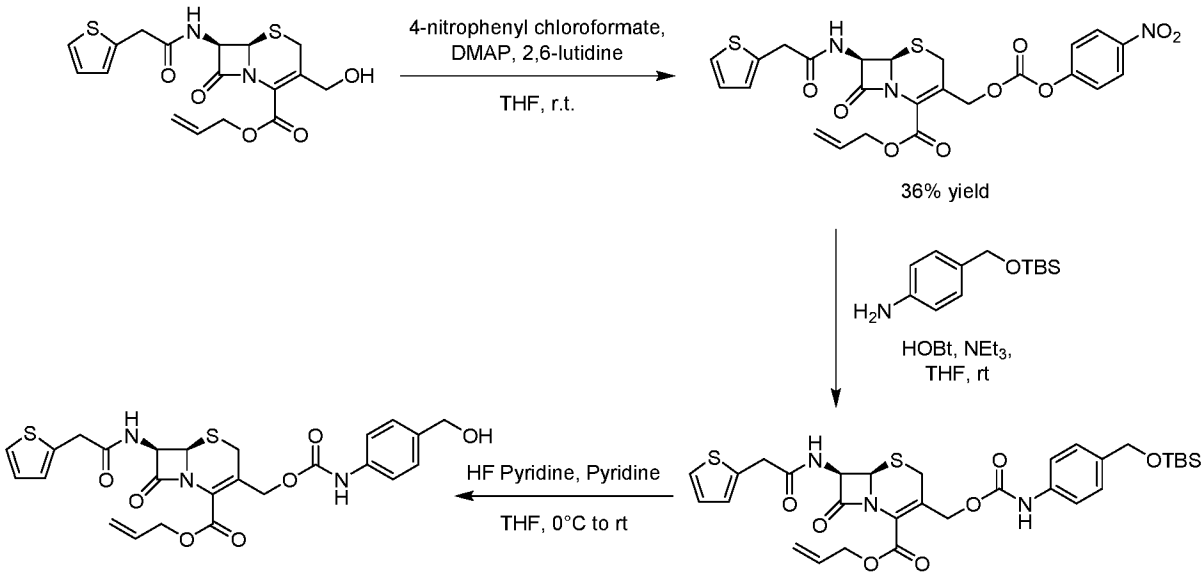


The exemplary β-lactamase prodrug precursor of Scheme 7 and the sulfoxide variant above may then be deprotected (such as by HF·pyridine and appropriate allyl deprotection conditions such as Pd(0)-catalyzed deprotection) to provide the exemplary β-lactamase prodrugs illustrated below in Scheme 9.

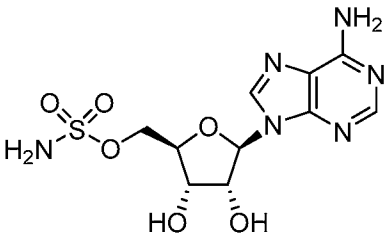
Scheme 9.



Scheme 10. Unoptimized Generation of an Exemplary PABA-Containing β-Lactamase Prodrug Precursor of the Present Technology

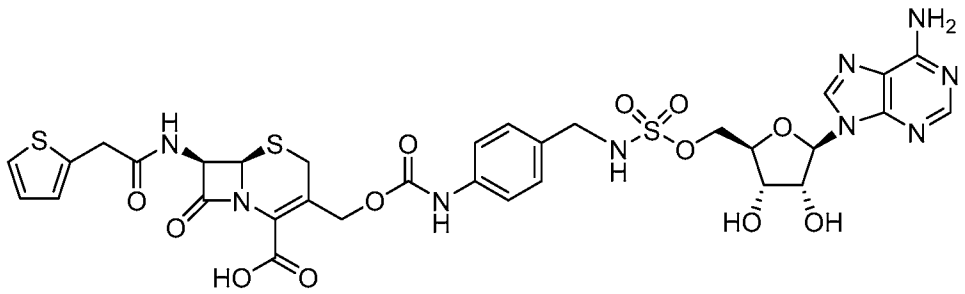


The precursor illustrated in Scheme 10 may be coupled via a Mitsunobu-type reaction with,



e.g., , and subsequently undergo deprotection (such as by HF·pyridine and appropriate allyl deprotection conditions such as Pd(0)-catalyzed deprotection) to afford the PABA-containing β-lactamase prodrug represented in Scheme 11.

Scheme 11.



WHAT IS CLAIMED IS:

1. An engineered immune cell comprising:

(a) a first prodrug converting enzyme and/or a nucleic acid encoding the first prodrug converting enzyme, wherein the first prodrug converting enzyme is expressed in the cytoplasm of the immune cell and is selected from among a carboxypeptidase, a β-lactamase, a glucosidase, a nitroreductase, and carboxypeptidase A; and

(b) a receptor that binds to a target antigen and/or nucleic acid encoding the receptor.

2. The engineered immune cell of claim 1, further comprising a second prodrug converting enzyme and/or a nucleic acid encoding the second prodrug converting enzyme, wherein the second prodrug converting enzyme is expressed on the surface of the immune cell or is secreted.

3. An engineered immune cell comprising:

(a) a first prodrug converting enzyme and/or a nucleic acid encoding the first prodrug converting enzyme, wherein the first prodrug converting enzyme is expressed in the cytoplasm of the immune cell;

(b) a second prodrug converting enzyme and/or a nucleic acid encoding the second prodrug converting enzyme, wherein the second prodrug converting enzyme is expressed on the surface of the immune cell or is secreted; and

(c) a receptor that binds to a target antigen and/or nucleic acid encoding the receptor.

4. The engineered immune cell of any of claims 1-3, wherein the receptor is a T cell receptor.

5. The engineered immune cell of any of claims 1-3, wherein the receptor is a native receptor.

6. The engineered immune cell of any of claims 1-3, wherein the receptor is a non-native receptor.

7. The engineered immune cell of any of claims 1-3, wherein the receptor is a chimeric antigen receptor.

8. The engineered immune cell of any of claims 2-7, wherein the second prodrug converting enzyme is fused to a transmembrane domain.

9. The engineered immune cell of claim 8, wherein the transmembrane domain of the second prodrug converting enzyme comprises a CD8 transmembrane domain.

10. The engineered immune cell of any of claims 2-7, wherein the second prodrug converting enzyme is attached to the surface of the cell by a GPI anchor.

11. The engineered immune cell of any of claims 2-10, wherein the nucleic acid encoding the second prodrug converting enzyme comprises a leader sequence for secretion of the prodrug converting enzyme.

12. The engineered immune cell of any of claims 2-11, wherein the first prodrug converting enzyme and the second prodrug converting enzyme have the same enzymatic activity and/or are the same enzyme.

13. The engineered immune cell of any of claims 2-11, wherein the first prodrug converting enzyme and the second prodrug converting enzyme have different enzymatic activities and/or are different enzymes.

14. The engineered immune cell of any of claims 1-13, wherein the first prodrug converting enzyme is *Pseudomonas sp.* Carboxypeptidase G2 (CPG2) or *Enterobacter cloacae* β-lactamase.

15. The engineered immune cell of any of claims 2-14, wherein the second prodrug converting enzyme is *Pseudomonas sp.* Carboxypeptidase G2 (CPG2) or *Enterobacter cloacae* β-lactamase.

16. The engineered immune cell of any of claims 1-15, wherein the nucleic acid encoding the first prodrug converting enzyme and/or the second prodrug converting enzyme is operably linked to a promoter.

17. The engineered immune cell of claim 16, wherein the promoter is a constitutive promoter.

18. The engineered immune cell of claim 16, wherein the promoter is a conditional promoter.

19. The engineered immune cell of claim 18, wherein the conditional promoter is inducible by binding of the receptor to the target antigen.

20. The engineered immune cell of any of claims 1-19, wherein the target antigen is a tumor antigen.

21. The engineered immune cell of any of claims 7-20, wherein the chimeric antigen receptor comprises (i) an extracellular antigen binding domain; (ii) a transmembrane domain; and (iii) an intracellular domain.

22. The engineered immune cell of claim 21, wherein the extracellular antigen binding domain binds to the target antigen.

23. The engineered immune cell of claim 21, wherein the extracellular antigen binding domain binds to a tumor antigen.

24. The engineered immune cell of claim 23, wherein the tumor antigen is selected from among CD19, WT1, and PRAME.

25. The engineered immune cell of any of claims 21-24, wherein the extracellular antigen binding domain comprises a single chain variable fragment (scFv).

26. The engineered immune cell of any of claims 21-25, wherein the extracellular antigen binding domain comprises a human scFv.

27. The engineered immune cell of any of claims 21-26, wherein the extracellular antigen binding domain comprises a CD19 scFv of SEQ ID NO: 19.

28. The engineered immune cell of any of claims 21-27, wherein extracellular antigen binding domain comprises a CD19 scFv having at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% sequence identity to SEQ ID NO: 19.

29. The engineered immune cell of any of claims 21-28, wherein the extracellular antigen binding domain comprises a signal peptide that is covalently joined to the N-terminus of the extracellular antigen binding domain.

30. The engineered immune cell of any of claims 21-29, wherein the transmembrane domain comprises a CD8 transmembrane domain.

31. The engineered immune cell of any of claims 21-30, wherein the intracellular domain comprises one or more costimulatory domains.

32. The engineered immune cell of claim 31, wherein the one or more costimulatory domains are selected from a CD28 costimulatory domain, a CD3ζ-chain, a 4-1BBL costimulatory domain, or any combination thereof.

33. The engineered immune cell of any of claims 1-32, wherein the engineered immune cell is a lymphocyte.

34. The engineered immune cell of claim 33, wherein the lymphocyte is a T cell, a B cell, or a natural killer (NK) cell.

35. The engineered immune cell of claim 34, wherein the T cell is a CD4+ T cell or a CD8+ T cell.

36. The engineered immune cell of any of claims 1-35. wherein the engineered immune cell is a tumor infiltrating lymphocyte.

37. The engineered immune cell of any of claims 1-36, wherein the engineered immune cell is derived from an autologous donor or an allogenic donor.

38. A method for treating cancer in a subject in need thereof comprising administering an effective amount of the engineered immune cells of any of claims 1-37.

39. The method of claim 38, further comprising administering to the subject a first prodrug that is converted to an active drug by the first prodrug converting enzyme.

40. The method of claim 39, wherein the first prodrug is administered subsequent to administration of the engineered immune cells.

41. The method of any of claims 38-40, further comprising administering to the subject a second prodrug that is converted to an active drug by the second prodrug converting enzyme.

42. The method of claim 41, wherein the second prodrug is administered prior to administering the first prodrug.

43. A method for treating or inhibiting tumor growth or metastasis in a subject comprising contacting a tumor cell with an effective amount of the engineered immune cells of any of claims 1-37.

44. The method of claim 43, further comprising administering to the subject a first prodrug that is converted to an active drug by the first prodrug converting enzyme.

45. The method of claim 44, wherein the first prodrug is administered subsequent to administration of the engineered immune cells.

46. The method of any of claims 43-45, further comprising administering to the subject a second prodrug that is converted to an active drug by the second prodrug converting enzyme.

47. The method of claim 46, wherein the second prodrug is administered prior to the administering the first prodrug.

48. The method of any of claims 38-45, wherein the engineered immune cells are administered intravenously, intraperitoneally, subcutaneously, intramuscularly, or intratumorally.

49. The method of any of claims 38-48, wherein the cancer or tumor is a carcinoma, sarcoma, a melanoma, or a hematopoietic cancer.

50. The method of any of claims 38-49, wherein the cancer or tumor is selected from among adrenal cancers, bladder cancers, blood cancers, bone cancers, brain cancers, breast cancers, carcinoma, cervical cancers, colon cancers, colorectal cancers, corpus uterine cancers, ear, nose and throat (ENT) cancers, endometrial cancers, esophageal cancers, gastrointestinal cancers, head and neck cancers, Hodgkin's disease, intestinal cancers, kidney cancers, larynx cancers, leukemias, liver cancers, lymph node cancers, lymphomas, lung cancers, melanomas, mesothelioma, myelomas, nasopharynx cancers, neuroblastomas, non-Hodgkin's lymphoma, oral cancers, ovarian cancers, pancreatic cancers, penile cancers, pharynx cancers, prostate cancers, rectal cancers, sarcoma, seminomas, skin cancers, stomach cancers, teratomas, testicular cancers, thyroid cancers, uterine cancers, vaginal cancers, vascular tumors, and metastases thereof.

51. The method of any of claims 38-50, further comprising administering an additional cancer therapy.

52. The method of claim 51, wherein the additional cancer therapy is selected from among chemotherapy, radiation therapy, immunotherapy, monoclonal antibodies, anti-cancer nucleic acids or proteins, anti-cancer viruses or microorganisms, and any combinations thereof.

53. The method of any one of claims 38-52, further comprising administering a cytokine to the subject.

54. The method of claim 53, wherein the cytokine is administered prior to, during, or subsequent to administration of the one or more engineered immune cells.

55. The method of claim 53 or 54, wherein the cytokine is selected from a group consisting of interferon α, interferon β, interferon γ, complement C5a, IL-2, TNFalpha, CD40L, IL12, IL-23, IL15, IL17, CCL1, CCL11, CCL12, CCL13, CCL14-1, CCL14-2, CCL14-3, CCL15-1, CCL15-2, CCL16, CCL17, CCL18, CCL19, CCL19, CCL2, CCL20, CCL21, CCL22, CCL23-1, CCL23-2, CCL24, CCL25-1, CCL25-2, CCL26, CCL27, CCL28,

CCL3, CCL3L1, CCL4, CCL4L1, CCL5, CCL6, CCL7, CCL8, CCL9, CCR10, CCR2, CCR5, CCR6, CCR7, CCR8, CCRL1, CCRL2, CX3CL1, CX3CR, CXCL1, CXCL10, CXCL11, CXCL12, CXCL13, CXCL14, CXCL15, CXCL16, CXCL2, CXCL3, CXCL4, CXCL5, CXCL6, CXCL7, CXCL8, CXCL9, CXCL9, CXCR1, CXCR2, CXCR4, CXCR5, CXCR6, CXCR7 and XCL2.

56. A method for preparing immune cells for cancer therapy, comprising isolating immune cells from a donor subject, transducing the immune cells with

(a) a nucleic acid that encodes a first prodrug converting enzyme, wherein the first prodrug converting enzyme is expressed in the cytoplasm of the immune cell; and
optionally,

(b) a nucleic acid that encodes (i) a second prodrug converting enzyme, wherein the second prodrug converting enzyme is expressed on the surface of the immune cell or is secreted and/or (ii) a nucleic acid that encodes a receptor that binds to a target antigen and/or a nucleic acid encoding the receptor.

57. A method of treating a cancer comprising administering the transduced immune cells of claim 56 to a recipient subject.

58. The method of claim 57, wherein the donor subject and the recipient subject are the same.

59. The method of claim 57, wherein the donor subject and the recipient subject are different.

60. The method of any of claims 56-59, wherein the immune cells isolated from the donor subject comprise one or more lymphocytes.

61. The method of claim 60, wherein the one or more lymphocytes is a T cell, a B cell, or a natural killer (NK) cell.

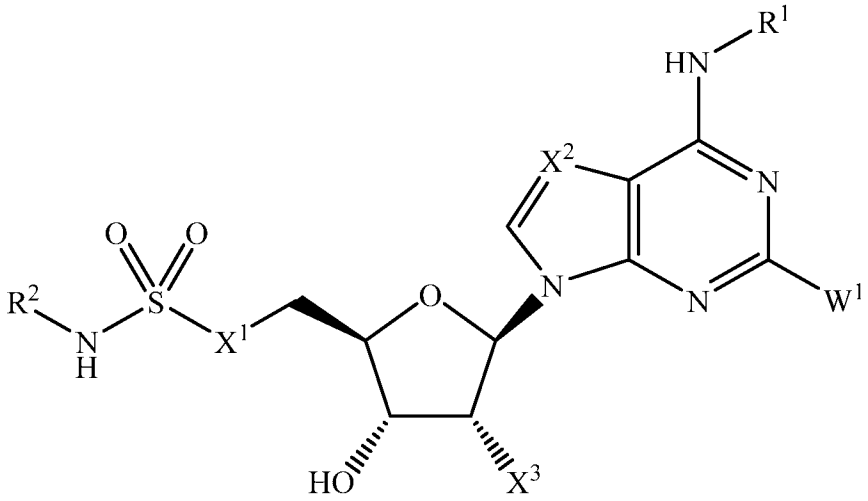
62. The method of claim 61, wherein the T cell is a CD4+ T cell or a CD8+ T cell.

63. The method of any of claims 56-62, wherein the immune cells isolated from the donor subject comprise tumor infiltrating lymphocytes.

64. Use of the engineered immune cells of any of claims 1-37 for treating a cancer.

65. Use of the engineered immune cells of any of claims 1-37 in the preparation of a medicament for the treatment of a cancer.

66. A compound of Formula I



(I)

or a zwitterion, pharmaceutically acceptable salt, and/or solvate thereof, wherein

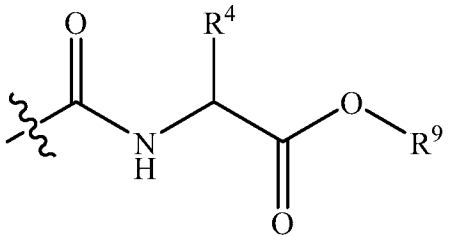
X^1 is O or NH;

X^2 is N or CH;

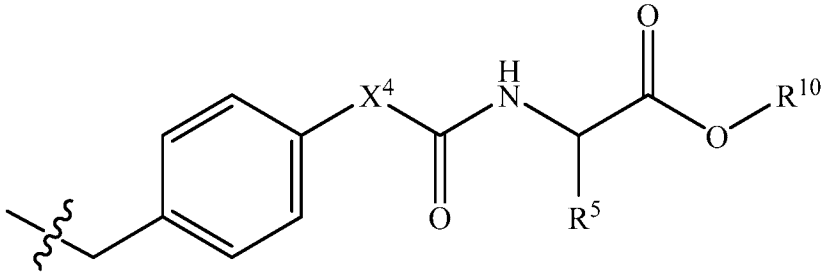
X^3 is OH or H;

W^1 is H or NH-R^3;

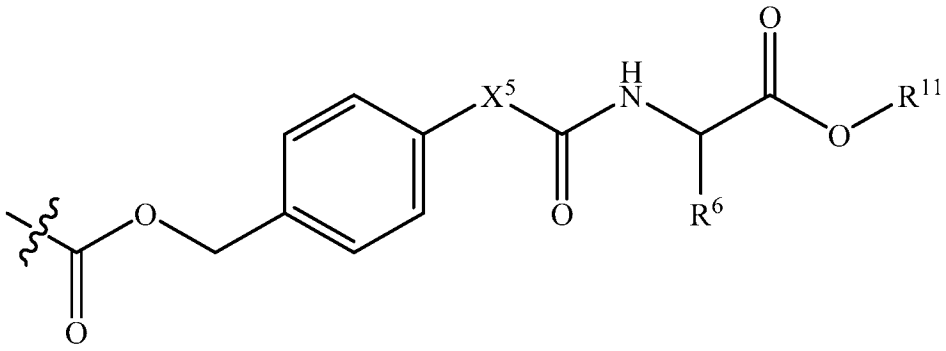
two of R^1 ,R^2, and R^3 are H and the remaining R^1 ,R^2, and R^3 is



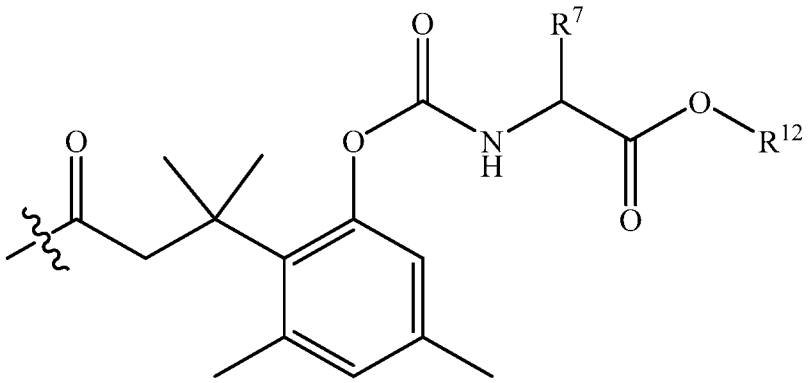
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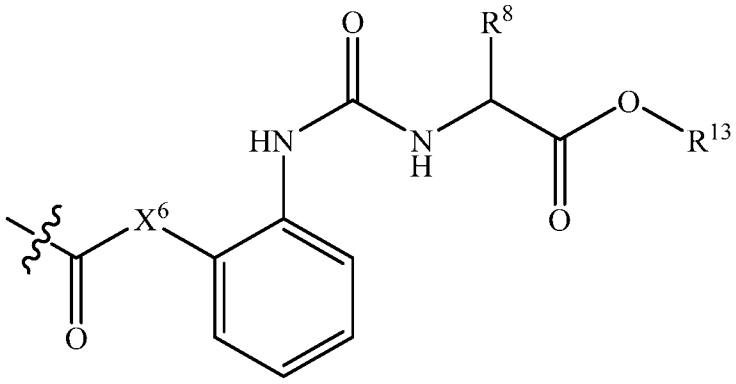
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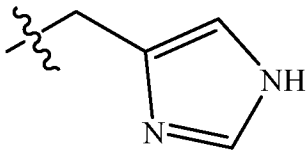
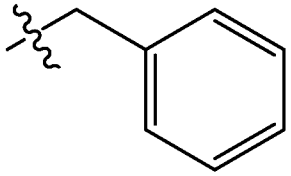


;

X^4 and X^5 are each independently O or NH;

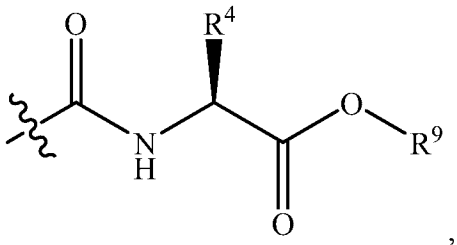
X^6 is O, NH, CH_2, or $C(Me)_2$;

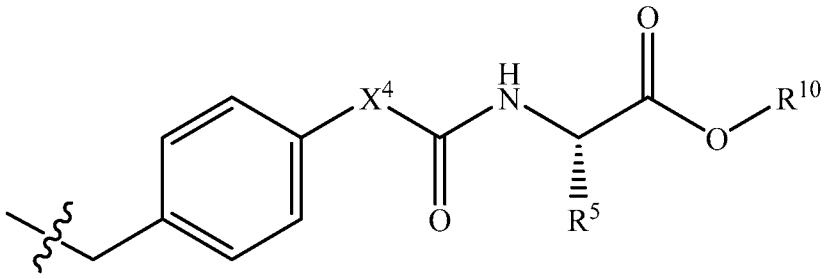
R^4, R^5, R^6, R^7, and R^8 are each independently H, $-(CH_2)_3-NH(NH)-NH_2$, $-(CH_2)_3-NH_2$, -

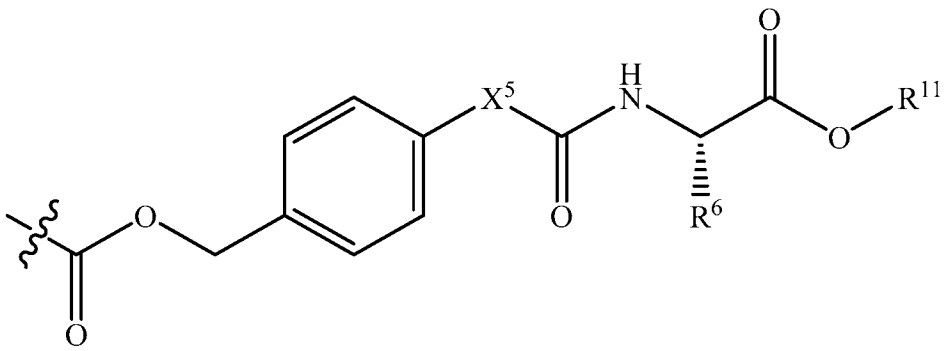
$(CH_2)_4$-NH_2, -CH_2-C(O)OR^{13a}, -$(CH_2)_2$-C(O)OR^{13b}, , or ; and

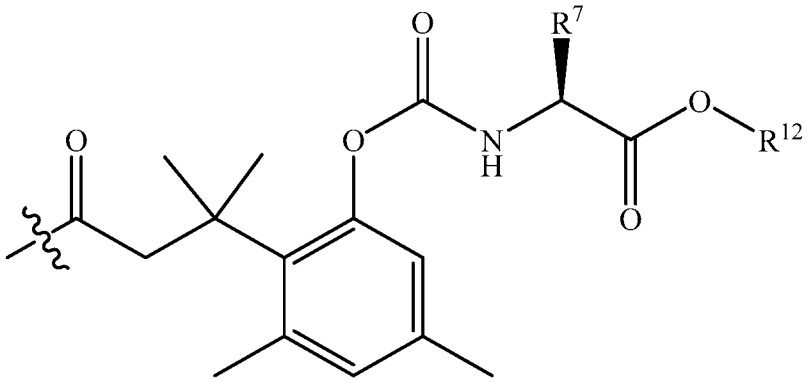
R^9, R^{10}, R^{11}, R^{12}, R^{13}, R^{13a}, and R^{13b} are each independently alkyl, aryl, heteroaryl, aralkyl, heteroaralkyl, -CH_2-OC(O)-alkyl, -CH_2-OC(O)-aryl, -CH_2-OC(O)-heteroaryl, -CH_2-OC(O)-aralkyl, or -CH_2-OC(O)-heteroaralkyl.

67. The compound of claim 66, wherein two of R^1 ,R^2, and R^3 are H and the remaining R^1 ,R^2, and R^3 is

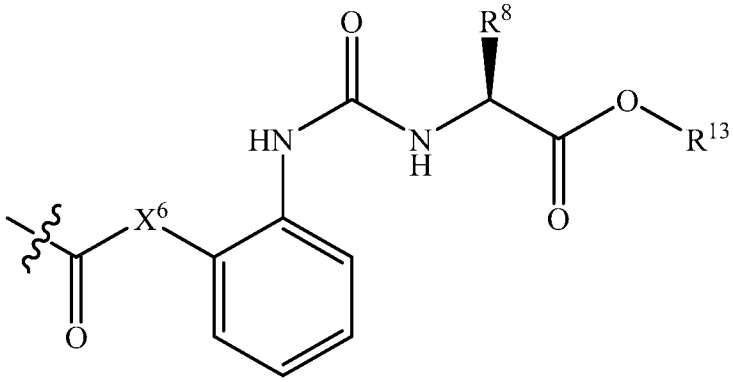
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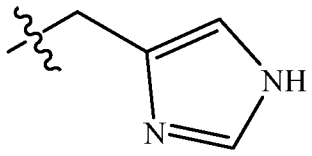


, or

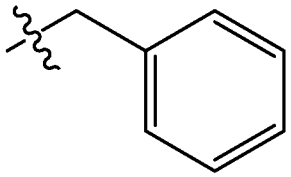


; and

R^4, R^5, R^6, R^7, and R^8 are each independently $-(CH_2)_3-NH(NH)-NH_2$, $-(CH_2)_3-NH_2$, $-(CH_2)_4-NH_2$, $-CH_2-C(O)OR^{13a}$, $-(CH_2)_2-C(O)OR^{13b}$,



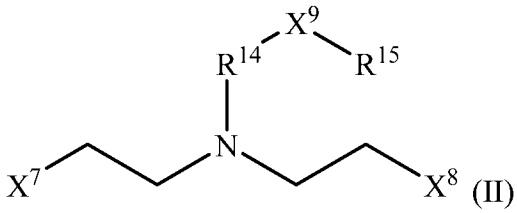
, or



.

68. The compound of claim 66 or claim 67, wherein R^9, R^{10}, R^{11}, R^{12}, R^{13}, R^{13a}, and R^{13b} are each independently neopentyl or $-CH_2-OC(O)$-*tert*-butyl.

69. A compound of Formula II



(II)

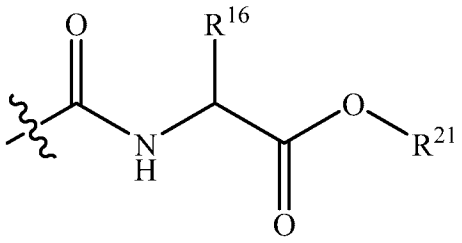
or a zwitterion, pharmaceutically acceptable salt, and/or solvate thereof, wherein

X^7 and X^8 are each independently Cl, Br, I, O-S(O)$_2$CH$_3$, or O-S(O)$_2$-tolyl;

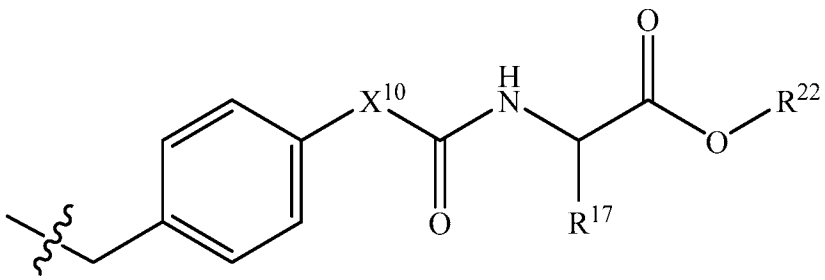
X^9 is –C(O)-O, -C(O)-NH, O, or NH;

R^{14} is a bond, alkylene, arylene, aralkylene, heteroarylene, or heteroaralkylene;

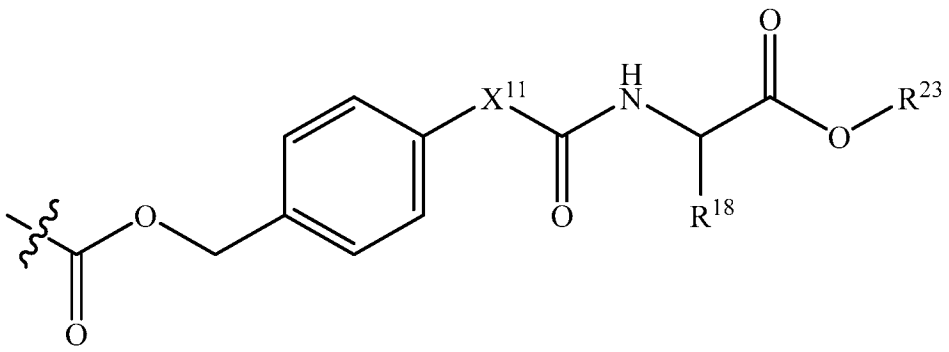
R^{15} is



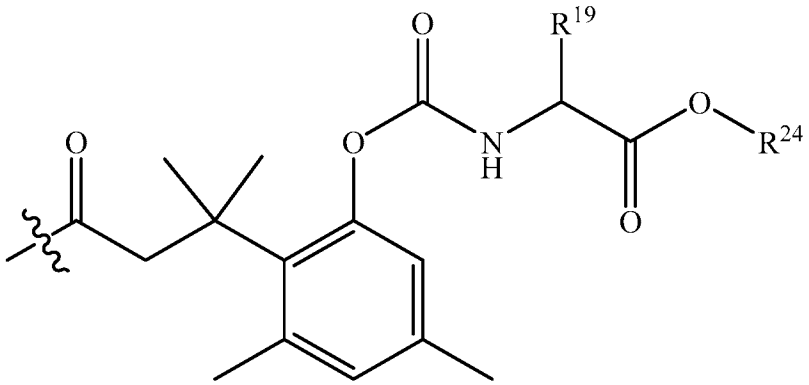
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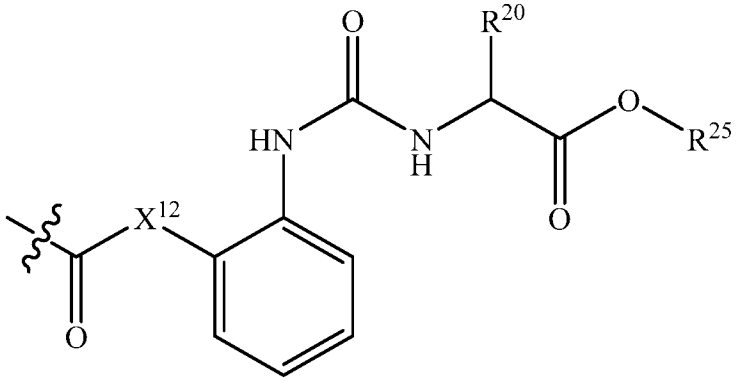
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, or

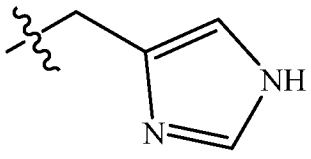


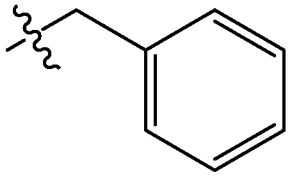
;

X^{10} and X^{11} are each independently O or NH;

X^{6} is O, NH, CH_2, or $C(Me)_2$;

X^{12} is O, NH, CH_2, or $C(Me)_2$;

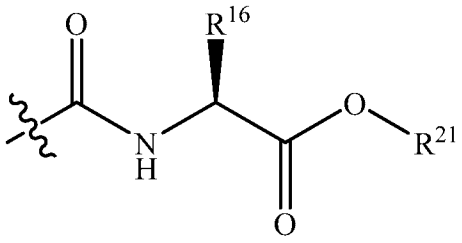
R^{16}, R^{17}, R^{18}, R^{19}, and R^{20} are each independently H, $-(CH_2)_3-NH(NH)-NH_2$, $-(CH_2)_3-NH_2$, $-(CH_2)_4-NH_2$, $-CH_2-C(O)OR^{25a}$, $-(CH_2)_2-C(O)OR^{25b}$, , or



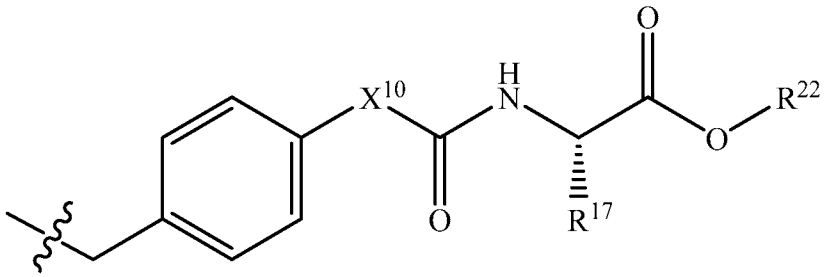
; and

R^{21}, R^{22}, R^{23}, R^{24}, R^{25}, R^{25a}, and R^{25b} are each independently alkyl, aryl, heteroaryl, aralkyl, heteroaralkyl, $-CH_2-OC(O)$-alkyl, $-CH_2-OC(O)$-aryl, $-CH_2-OC(O)$-heteroaryl, $-CH_2-OC(O)$-aralkyl, or $-CH_2-OC(O)$-heteroaralkyl, provided that R^{21}, R^{22}, R^{23}, R^{24}, R^{25}, R^{25a}, and R^{25b} are each independently not *tert*-butyl.

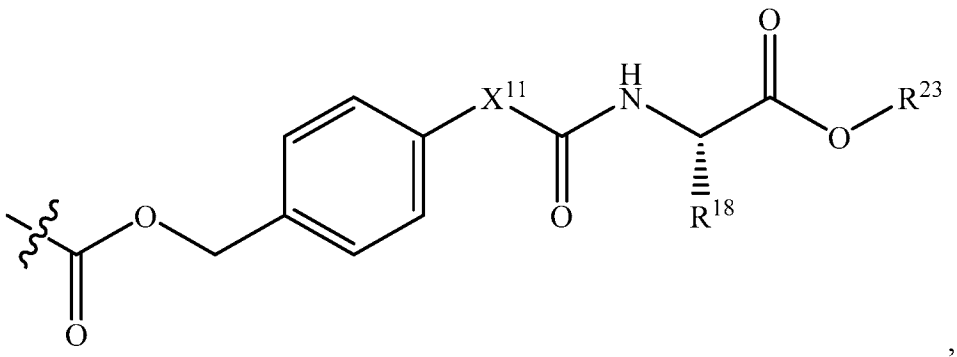
70. The compound of claim 69, wherein R^{15} is



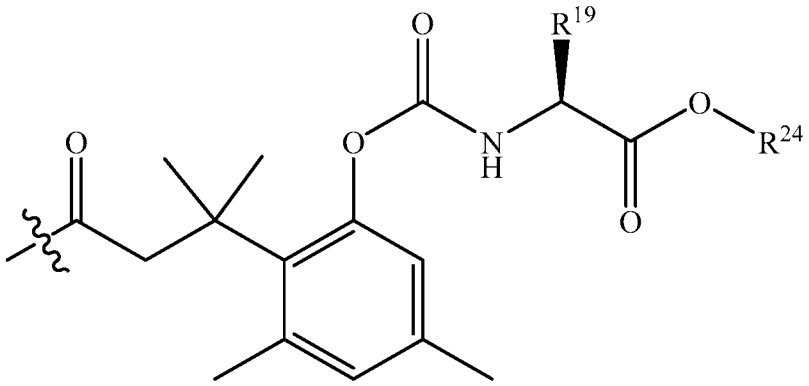
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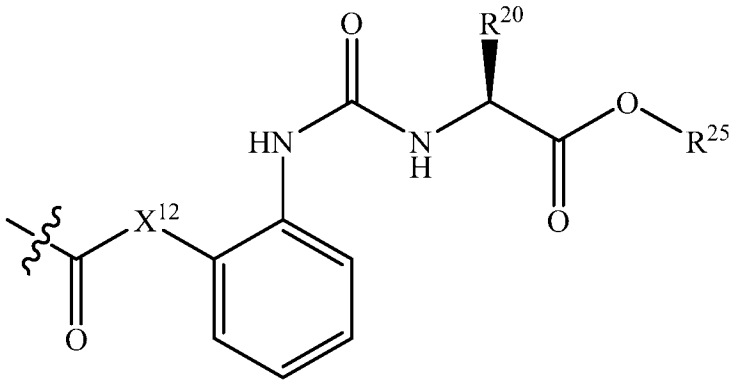
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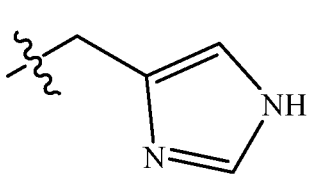


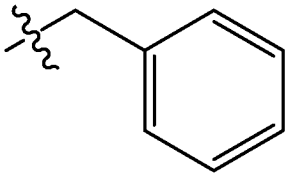
, or



, and

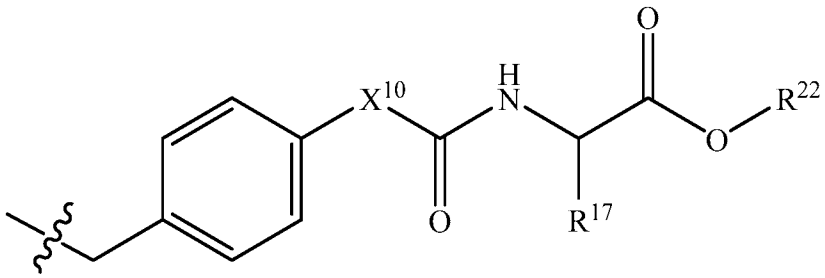
R^{16}, R^{17}, R^{18}, R^{19}, and R^{20} are each independently $-(CH_2)_3-NH(NH)-NH_2$, $-(CH_2)_3-NH_2$, $-(CH_2)_4-NH_2$, $-CH_2-C(O)OR^{25a}$,



$-(CH_2)_2-C(O)OR^{25b}$, , or .

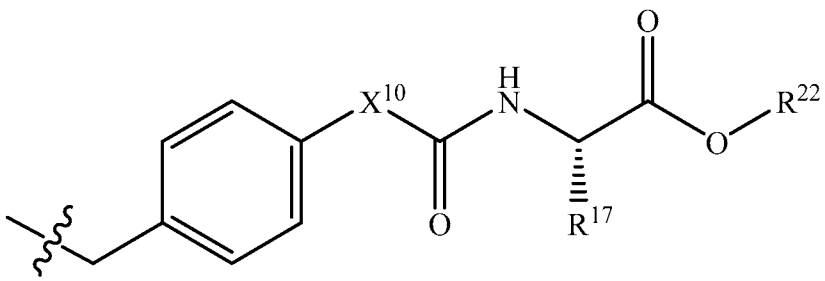
71. The compound of claim 69 or claim 70, wherein R^{21}, R^{22}, R^{23}, R^{24}, R^{25}, R^{25a}, and R^{25b} are each independently neopentyl or $-CH_2-OC(O)-$*tert*-butyl.

72. The compound of any one of claims 69-71, wherein when X^9 is –C(O)-O, R^{15} is



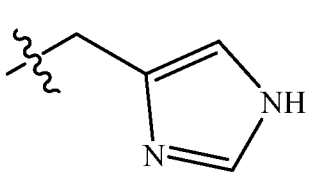
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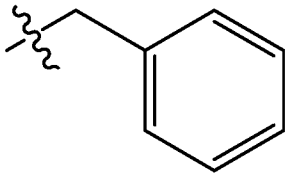
73. The compound of any one of claims 69-71, wherein X^9 is –C(O)-O, R^{15} is



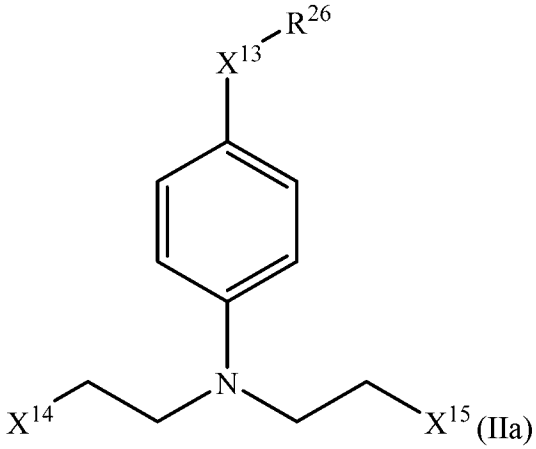
, and R^{17} is $-(CH_2)_3-$

$NH(NH)\text{-}NH_2$, $\text{-}(CH_2)_3\text{-}NH_2$, $\text{-}(CH_2)_4\text{-}NH_2$, $\text{-}CH_2\text{-}C(O)OR^{25a}$, $\text{-}(CH_2)_2\text{-}C(O)O\ R^{25b}$,

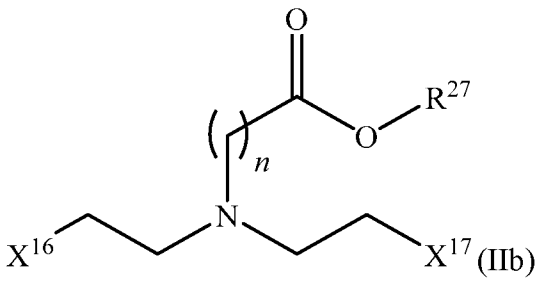


, or .

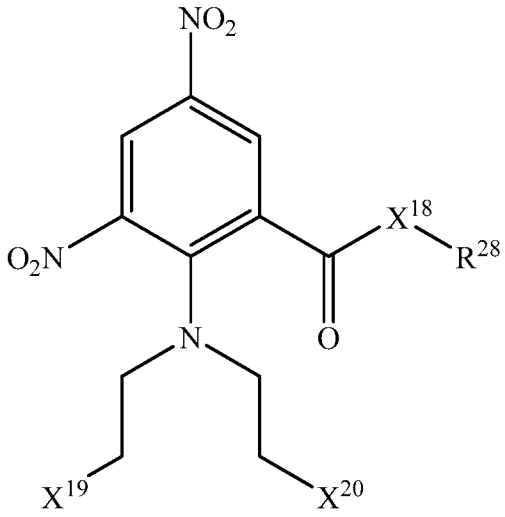
74. The compound of any one of claims 69-71, wherein the compound of Formula II is a compound of any one of Formulas IIa-IIe



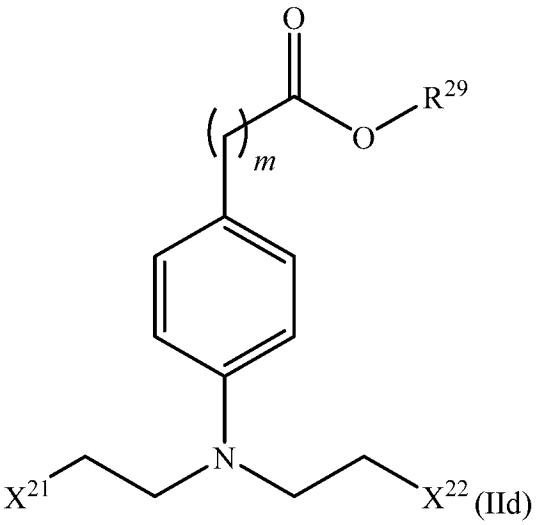
(IIa)



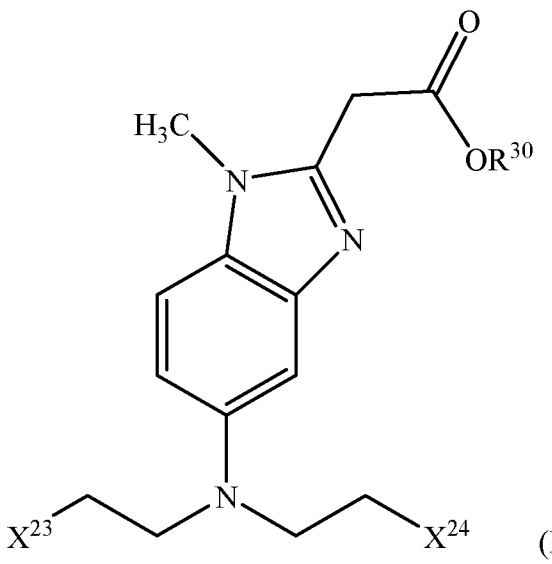
(IIb)



(IIc)



(IId)



(IIe)

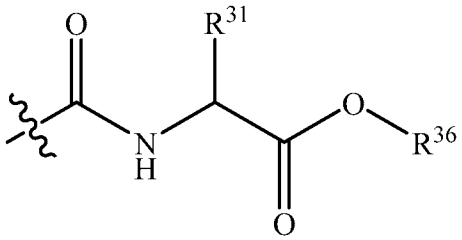
or a zwitterion, pharmaceutically acceptable salt, and/or solvate thereof, wherein

X^{13} and X^{18} are each independently O or NH;

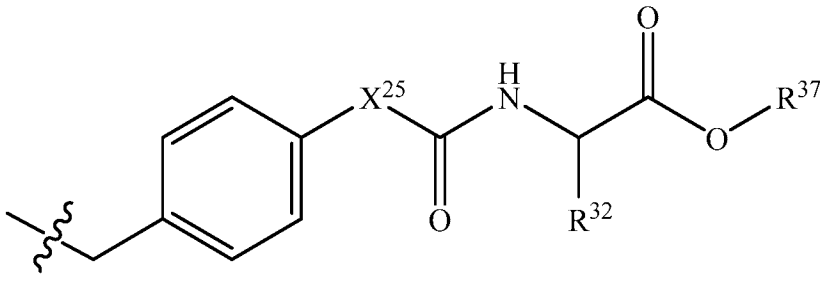
X^{14}, X^{15}, X^{16}, X^{17}, X^{19}, X^{20}, X^{21}, X^{22}, X^{23} and X^{24} are each independently Cl, Br, I, O-$S(O)_2CH_3$, or O-$S(O)_2$-tolyl;

n and *m* are each independently 0, 1, 2, 3, 4, or 5;

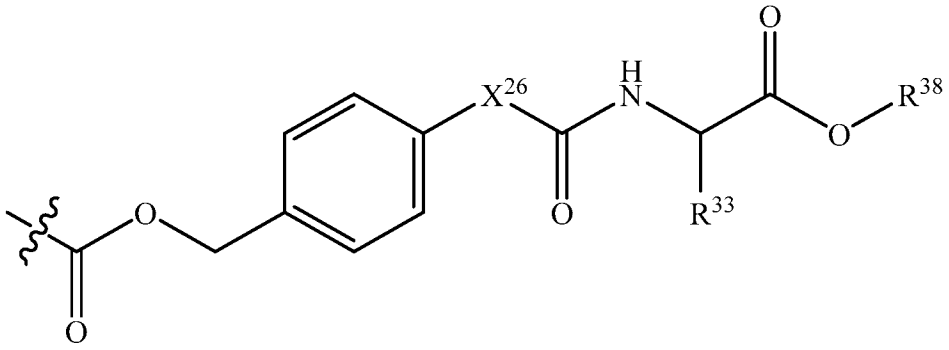
R^{26}, R^{27}, R^{28}, R^{29}, and R^{30} are each independently



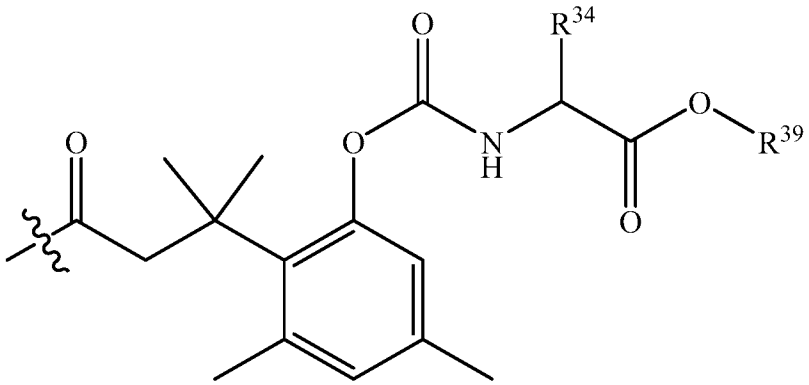
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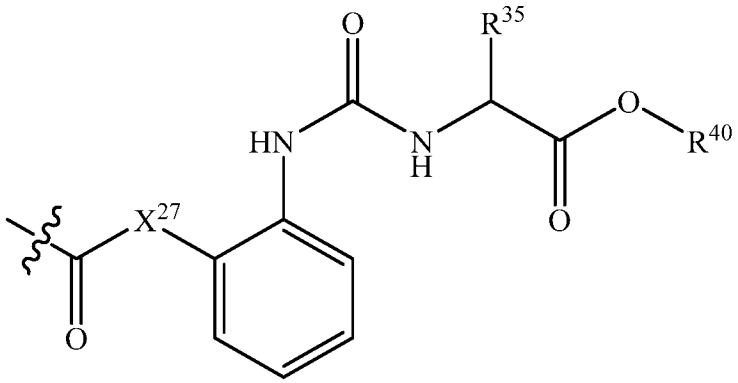
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,or

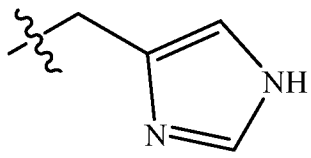


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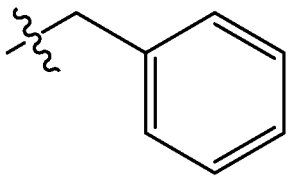
X^{25} and X^{26} are each independently O or NH;

X^{27} is O, NH, CH_2, or $C(Me)_2$;

R^{31}, R^{32}, R^{33}, R^{34}, and R^{35} are each independently H, $-(CH_2)_3-NH(NH)-NH_2$, $-(CH_2)_3-NH_2$, $-(CH_2)_4-NH_2$, $-CH_2-C(O)OR^{40a}$, $-(CH_2)_2-C(O)OR^{40b}$,



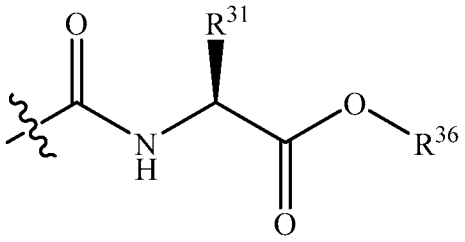
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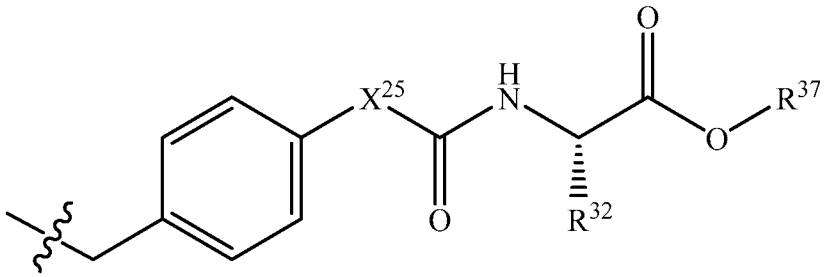


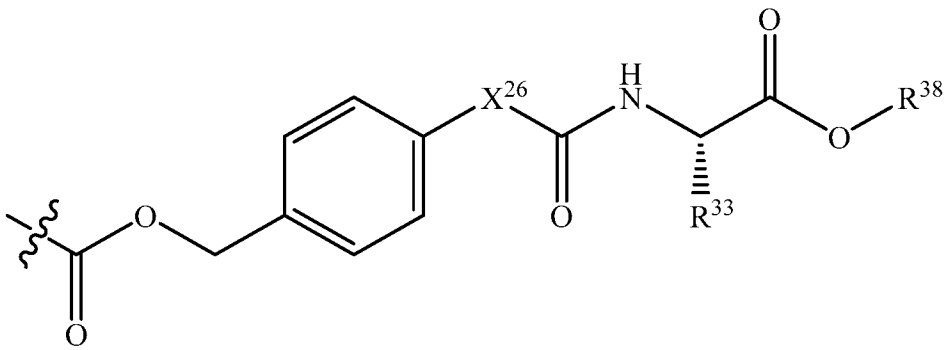
; and

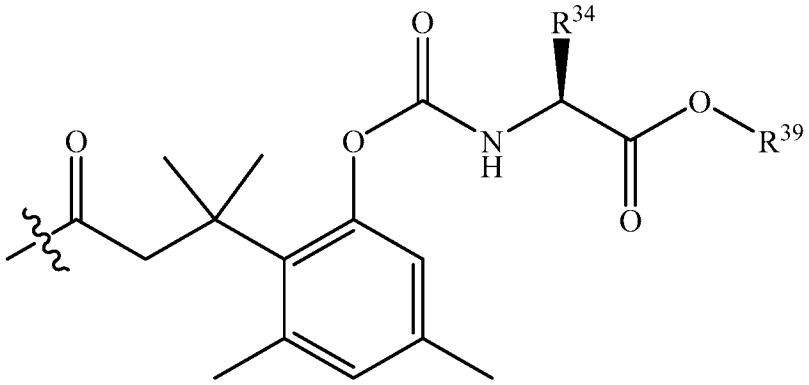
R^{36}, R^{37}, R^{38}, R^{39}, R^{40}, R^{40a}, and R^{40b} are each independently alkyl, aryl, heteroaryl, aralkyl, heteroaralkyl, $-CH_2$-OC(O)-alkyl, $-CH_2$-OC(O)-aryl, $-CH_2$-OC(O)-heteroaryl, $-CH_2$-OC(O)-aralkyl, or $-CH_2$-OC(O)-heteroaralkyl, provided that R^{36}, R^{37}, R^{38}, R^{39}, R^{40}, R^{40a}, and R^{40b} are each independently not *tert*-butyl.

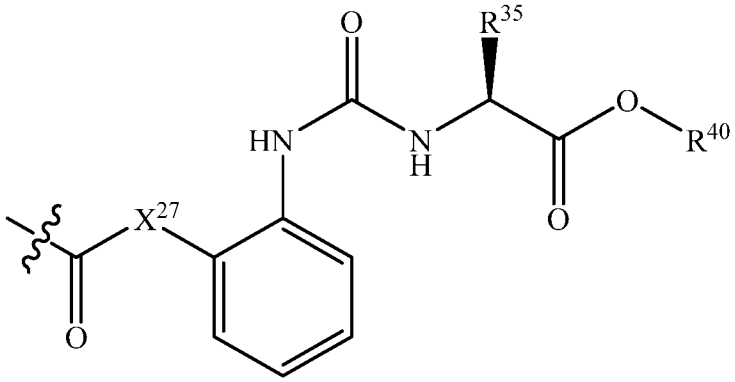
75. The compound of claim 74, wherein R^{26}, R^{27}, R^{28}, R^{29}, and R^{30} are each independently

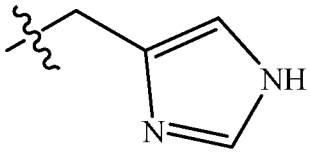
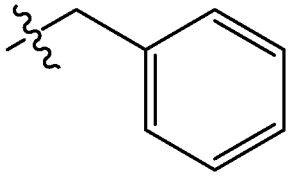

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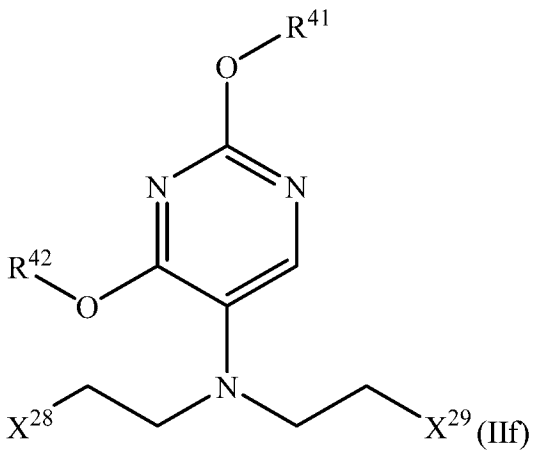

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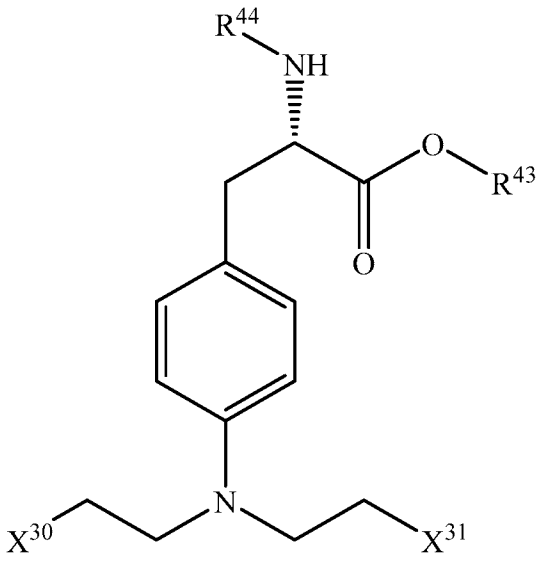


R^{31}, R^{32}, R^{33}, R^{34}, and R^{35} are each independently -$(CH_2)_3$-NH(NH)-NH_2, -$(CH_2)_3$-NH_2, -$(CH_2)_4$-NH_2, -CH_2-C(O)OR^{40a}, -$(CH_2)_2$-C(O)O R^{40b},  , or .

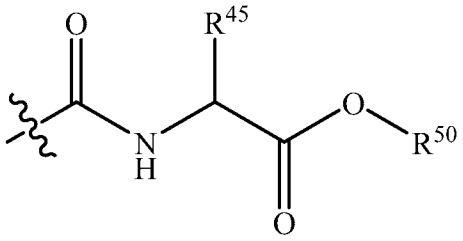
76. The compound of claim 74 or claim 75, wherein R^{36}, R^{37}, R^{38}, R^{39}, R^{40}, R^{40a}, and R^{40b} are each independently neopentyl and -CH_2-OC(O)-*tert*-butyl.

77. The compound of any one of claims 74 -76, wherein the compound of Formula II is a compound of Formula IIf or Formula IIg

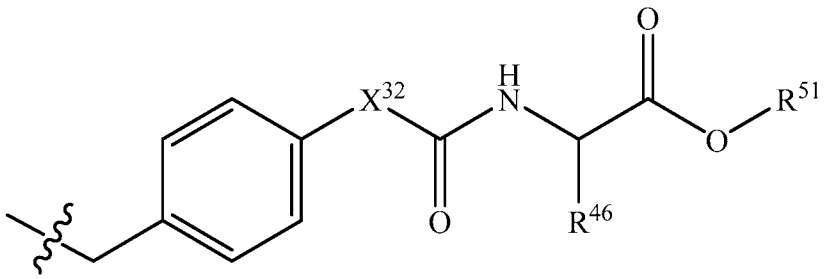
 (IIf)

 (IIg)

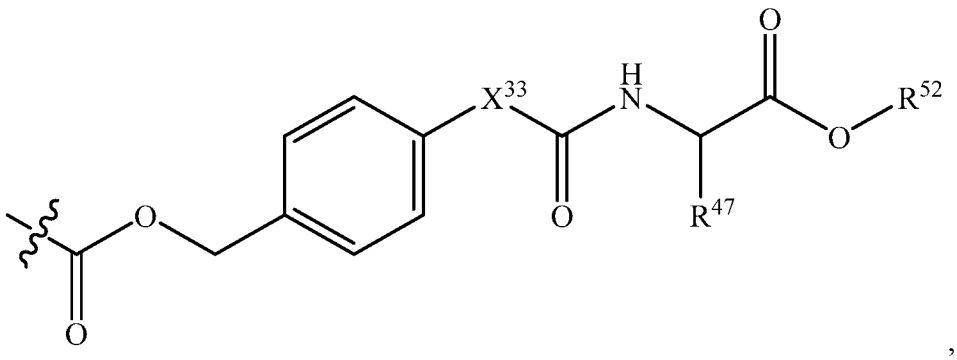
or a zwitterion, pharmaceutically acceptable salt, and/or solvate thereof, wherein X^{28}, X^{29}, X^{30}, and X^{31} are each independently Cl, Br, I, O-S(O)$_2$CH$_3$, or O-S(O)$_2$-tolyl; one of R^{41} and R^{42} is H and one of R^{43} and R^{44} is H and the remaining R^{41} and R^{42} (for Formula IIf) and R^{43} and R^{44} (for Formula IIg) are each independently



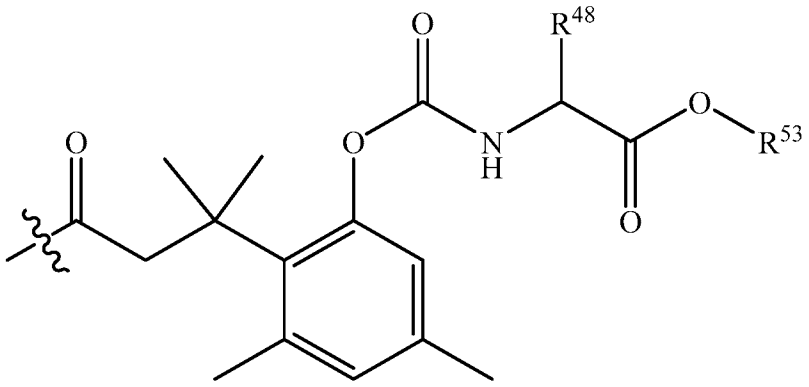
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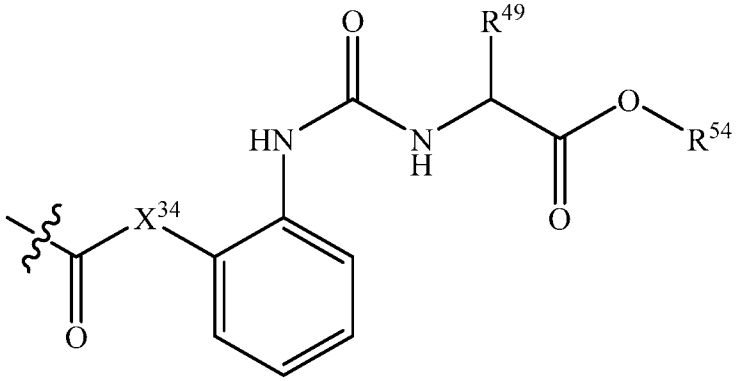
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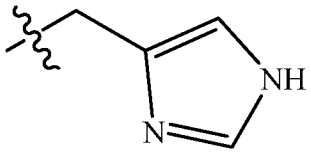
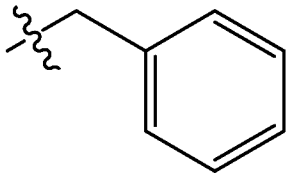
, or



;

X^{32} and X^{33} are each independently O or NH;

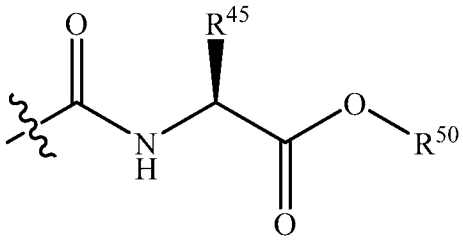
X^{34} is O, NH, CH_2, or $C(Me)_2$;

R^{45}, R^{46}, R^{47}, R^{48}, and R^{49} are each independently H, $-(CH_2)_3-NH(NH)-NH_2$, $-(CH_2)_3-NH_2$, $-(CH_2)_4-NH_2$, $-CH_2-C(O)OR^{54a}$, $-(CH_2)_2-C(O)OR^{54b}$, , or ; and

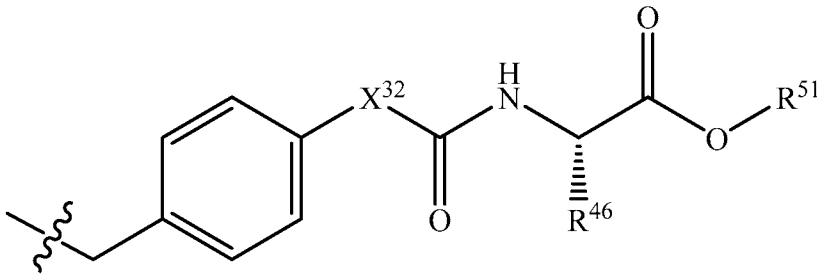
R^{50}, R^{51}, R^{52}, R^{53}, R^{54}, R^{54a}, and R^{54b} are each independently alkyl, aryl, heteroaryl, aralkyl, heteroaralkyl, $-CH_2-OC(O)$-alkyl, $-CH_2-OC(O)$-aryl, $-CH_2-OC(O)$-heteroaryl, $-CH_2-OC(O)$-aralkyl, or $-CH_2-OC(O)$-heteroaralkyl, provided that R^{50}, R^{51}, R^{52}, R^{53}, R^{54}, R^{54a}, and R^{54b} are each independently not *tert*-butyl.

78. The compound of claim 77, wherein one of R^{41} and R^{42} is H and one of R^{43} and R^{44} is H and the remaining R^{41} and R^{42} (for Formula IIf) and R^{43} and R^{44} (for Formula

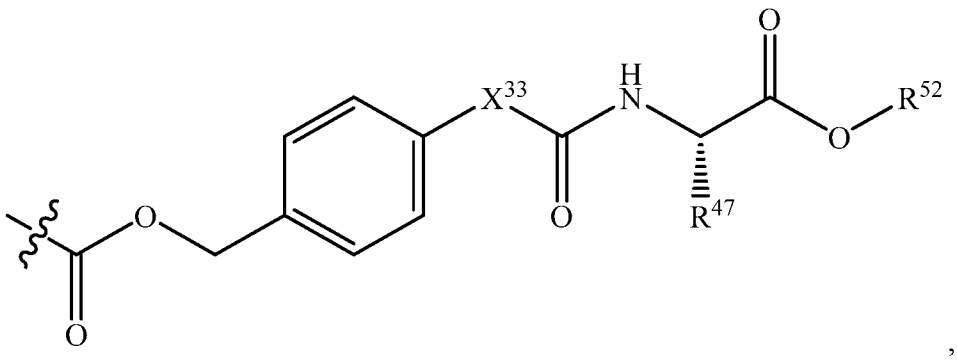
IIg) are each independently



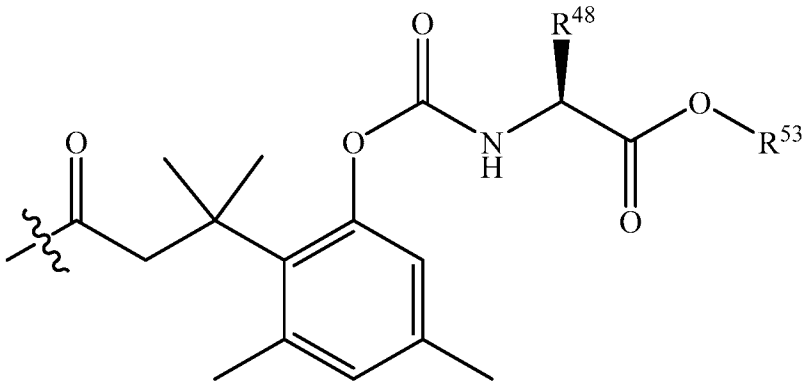
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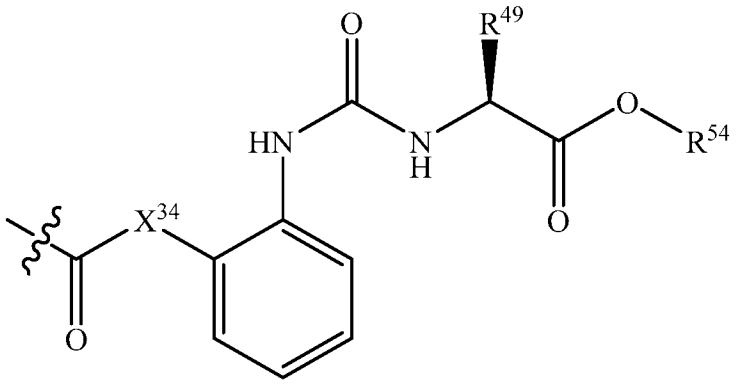
,

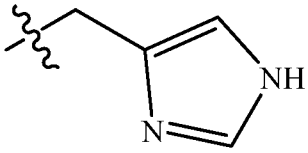
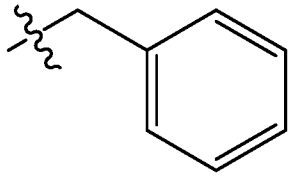


,



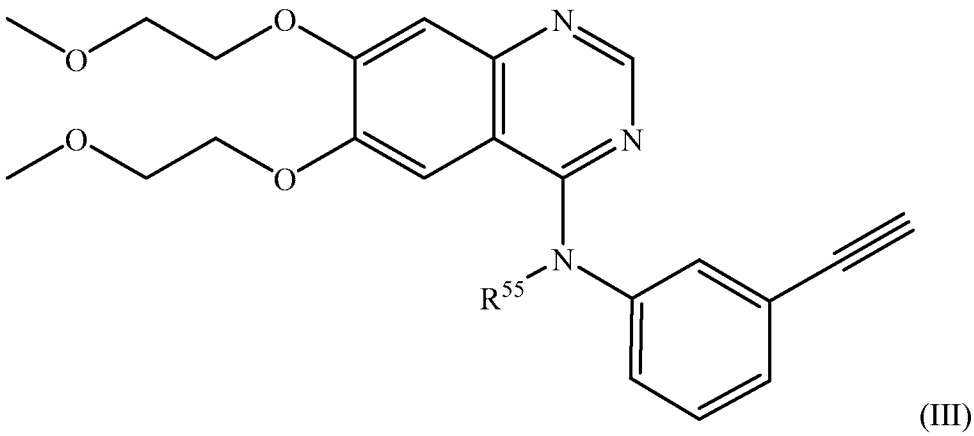
, or



; and R^{45}, R^{46}, R^{47}, R^{48}, and R^{49} are each independently $-(CH_2)_3-NH(NH)-NH_2$, $-(CH_2)_3-NH_2$, $-(CH_2)_4-NH_2$, $-CH_2-C(O)OR^{54a}$, $-(CH_2)_2-C(O)O\,R^{54b}$, , or .

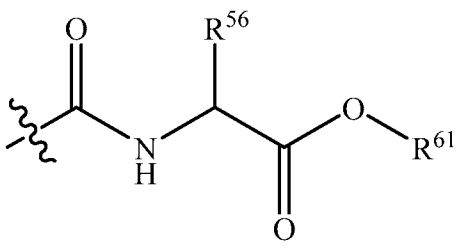
79. The compound of claim 77 or claim 78, wherein R^{50}, R^{51}, R^{52}, R^{53}, R^{54}, R^{54a}, and R^{54b} are each independently neopentyl and $-CH_2-OC(O)$-*tert*-butyl.

80. A compounds of Formula III

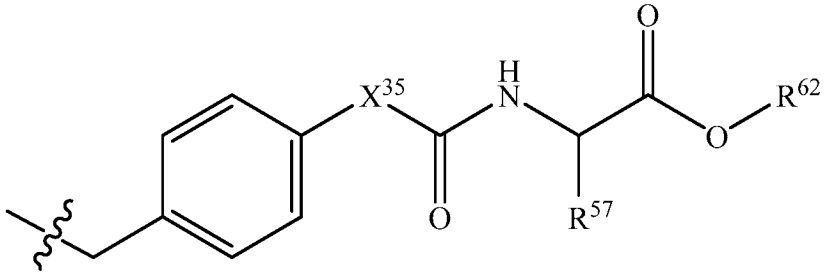


(III)

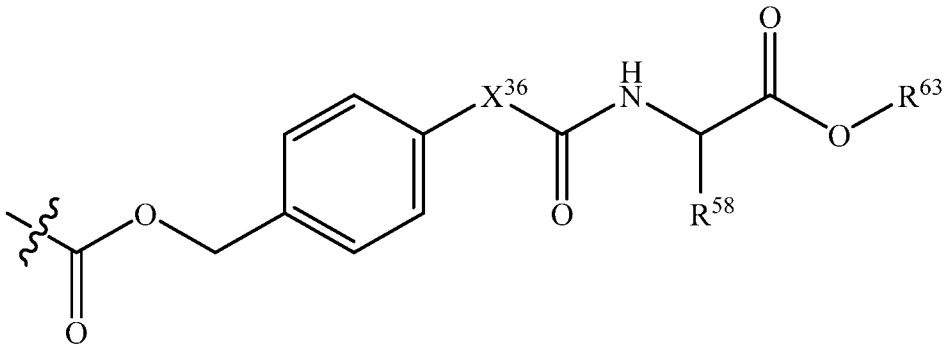
or a zwitterion, pharmaceutically acceptable salt, and/or solvate thereof, wherein R^{55} is



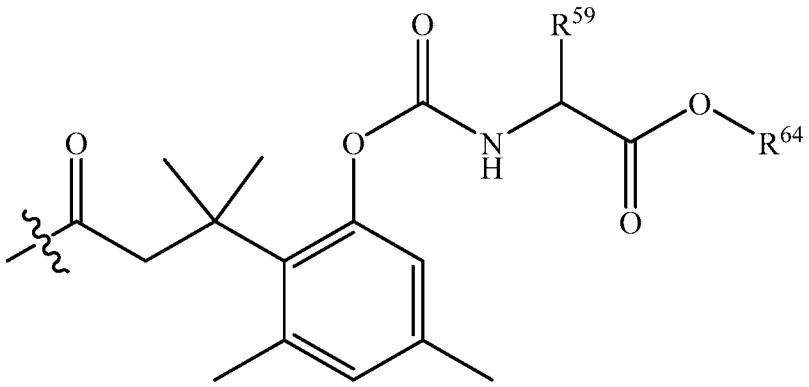
,



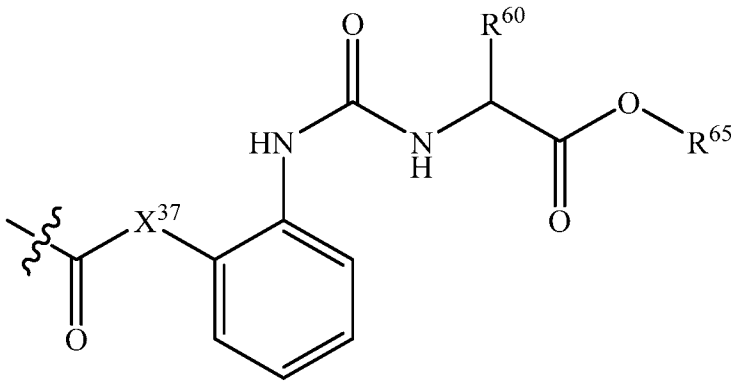
,



,



, or

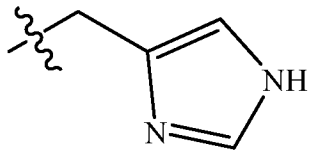


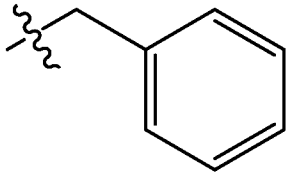
;

X^{35} and X^{36} are each independently O or NH;

X^{37} is O, NH, CH_2, or $C(Me)_2$;

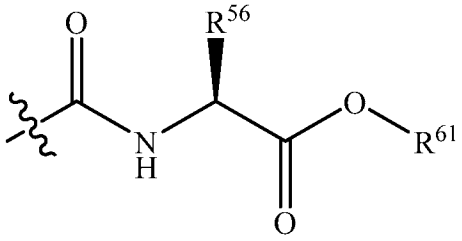
R^{56}, R^{57}, R^{58}, R^{59}, and R^{60} are each independently H, $-(CH_2)_3-NH(NH)-NH_2$, $-(CH_2)_3-NH_2$, -

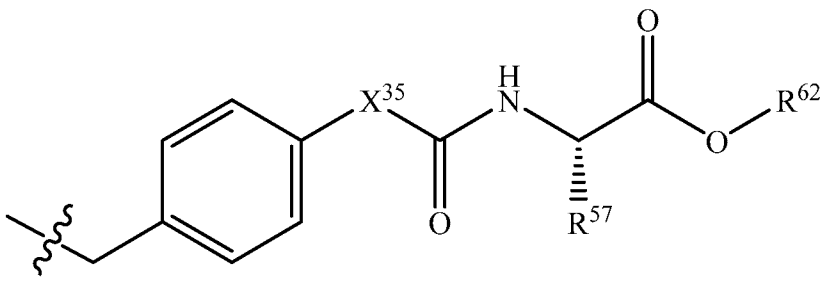
$(CH_2)_4$-NH_2, -CH_2-C(O)OR^{65a}, -$(CH_2)_2$-C(O)OR^{65b}, , or

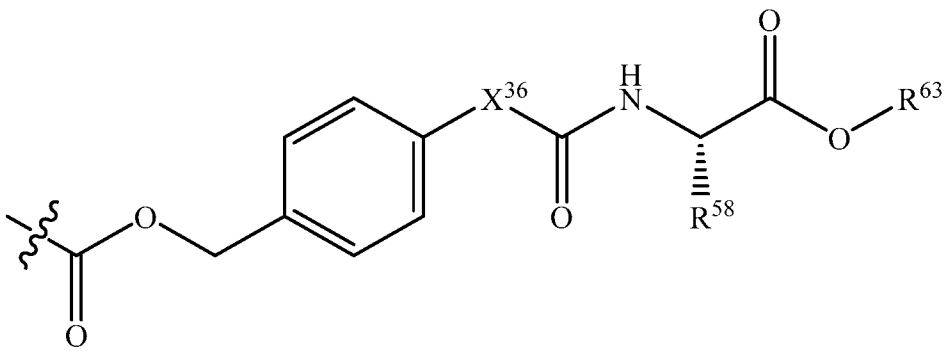
; and

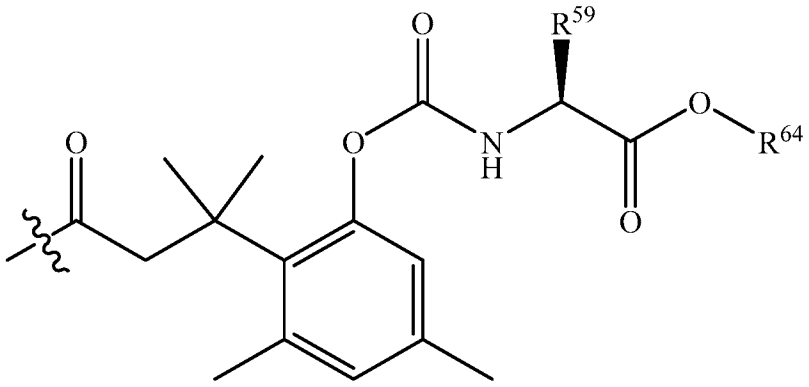
R^{61}, R^{62}, R^{63}, R^{64}, R^{65}, R^{65a}, and R^{65b} are each independently alkyl, aryl, heteroaryl, aralkyl, heteroaralkyl, -CH_2-OC(O)-alkyl, -CH_2-OC(O)-aryl, -CH_2-OC(O)-heteroaryl, -CH_2-OC(O)-aralkyl, or -CH_2-OC(O)-heteroaralkyl.

81. The compound of claim 80, wherein R^{55} is

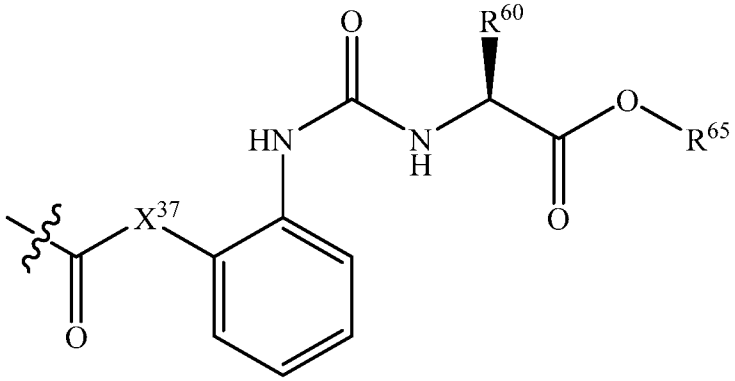
,

,

,

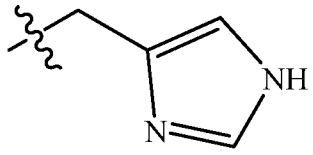


, or

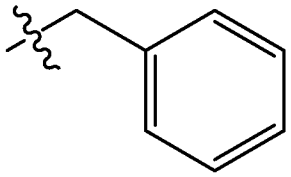


; and

R^{56}, R^{57}, R^{58}, R^{59}, and R^{60} are each independently $-(CH_2)_3-NH(NH)-NH_2$, $-(CH_2)_3-NH_2$, -



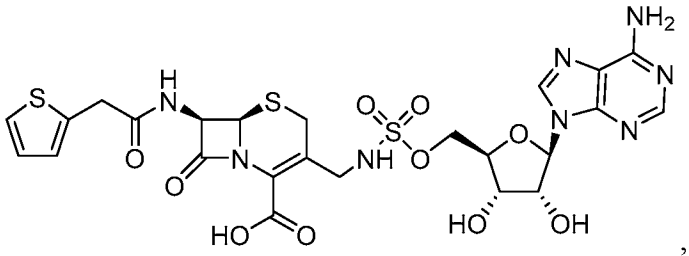
$(CH_2)_4-NH_2$, $-CH_2-C(O)OR^{65a}$, $-(CH_2)_2-C(O)OR^{65b}$, , or

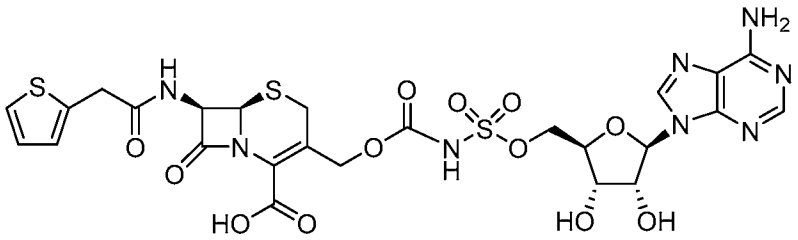


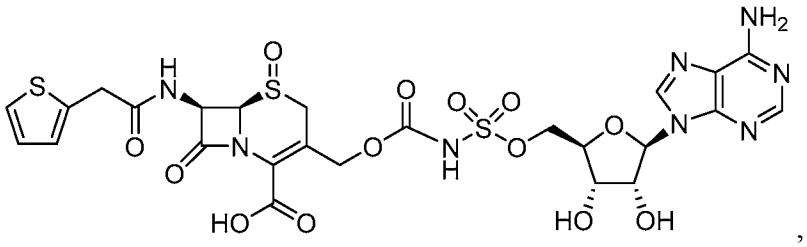
.

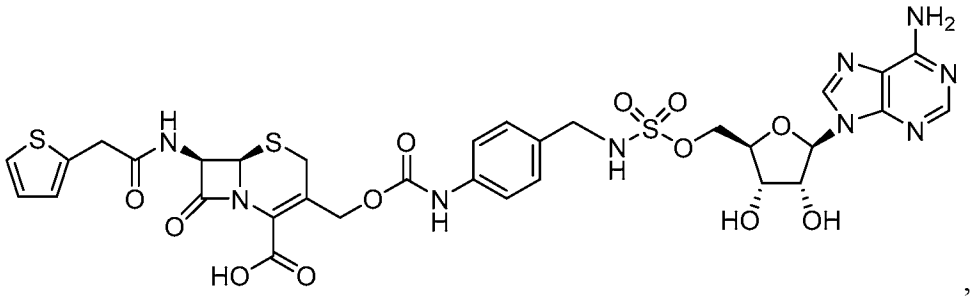
82. The compound of claim 80 or claim 81, wherein R^{61}, R^{62}, R^{63}, R^{64}, R^{65}, R^{65a}, and R^{65b} are each independently neopentyl or $-CH_2-OC(O)$-*tert*-butyl.

83. A compound that is


,


,


,


,

or a zwitterion, pharmaceutically acceptable salt, and/or solvate thereof.

FIG. 1

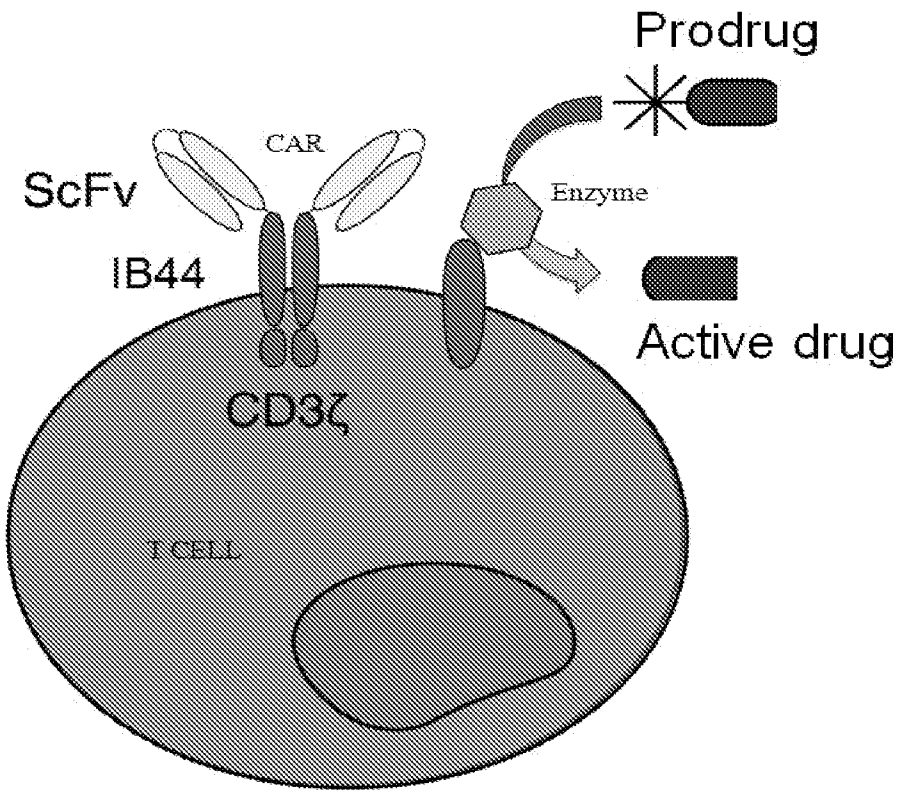


FIG. 2A

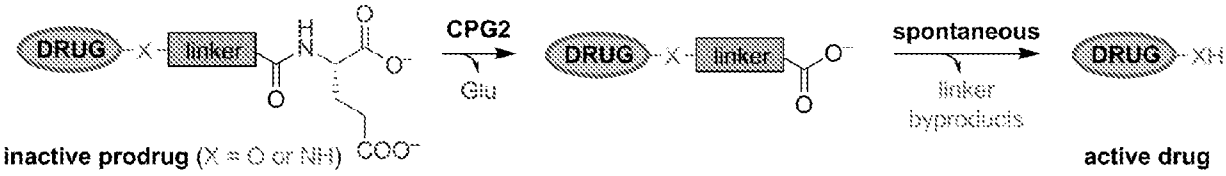

DRUG
X
linker
CPG2
Glu
spontaneous
linker byproducts
XH
inactive prodrug (X = O or NH)
active drug

FIG. 2B

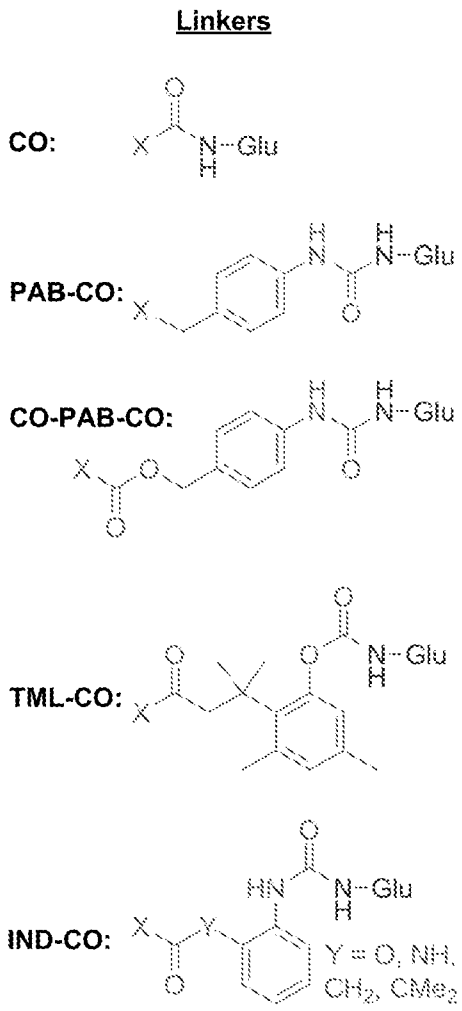

Linkers
CO:
PAB-CO:
CO-PAB-CO:
TML-CO:
IND-CO:
Glu
Y = O, NH, CH2, CMe2

FIG. 2C

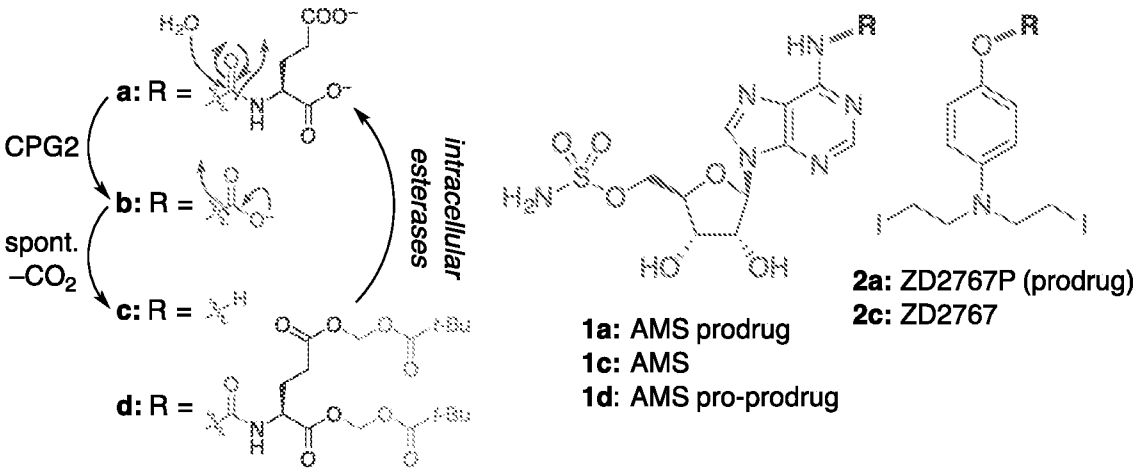

a: R =
b: R =
c: R =
d: R =
CPG2
spont.
–CO2
intracellular
esterases
1a: AMS prodrug
1c: AMS
1d: AMS pro-prodrug
2a: ZD2767P (prodrug)
2c: ZD2767

FIG. 2D

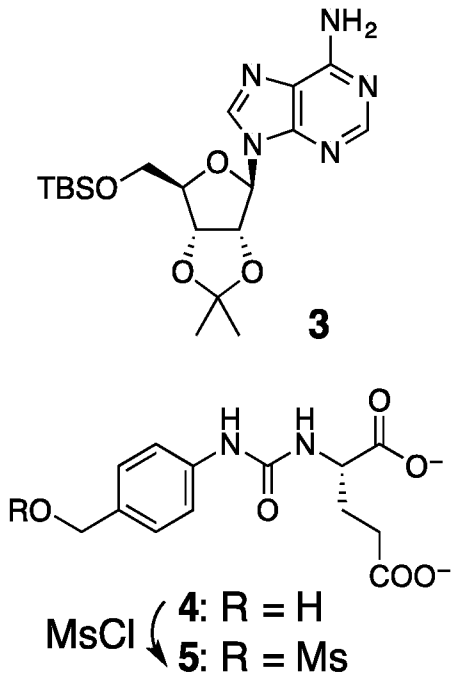

TBSO
3
RO
MsCl
4: R = H
5: R = Ms

FIG. 2E

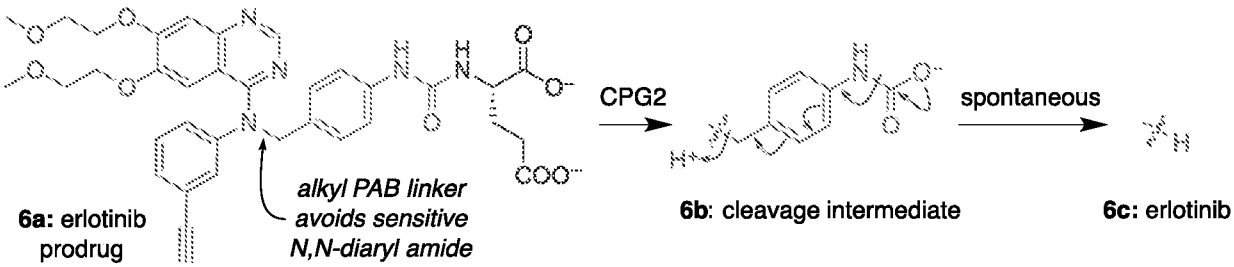

CPG2
spontaneous
alkyl PAB linker
avoids sensitive
N,N-diaryl amide
6a: erlotinib
prodrug
6b: cleavage intermediate
6c: erlotinib

FIG. 3A

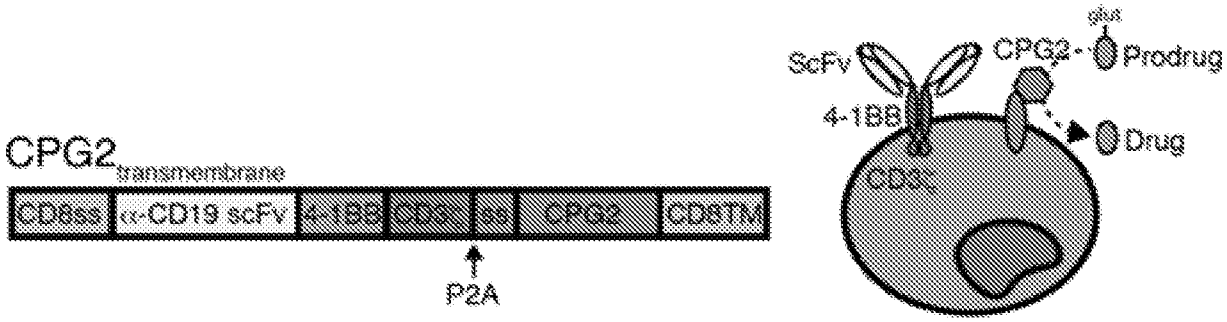

CPG2transmembrane
CD8ss
α-CD19 scFv
4-1BB
CD3ζ
ss
CPG2
CD8TM
P2A
ScFv
4-1BB
CD3ζ
CPG2
glux
Prodrug
Drug

FIG. 3B

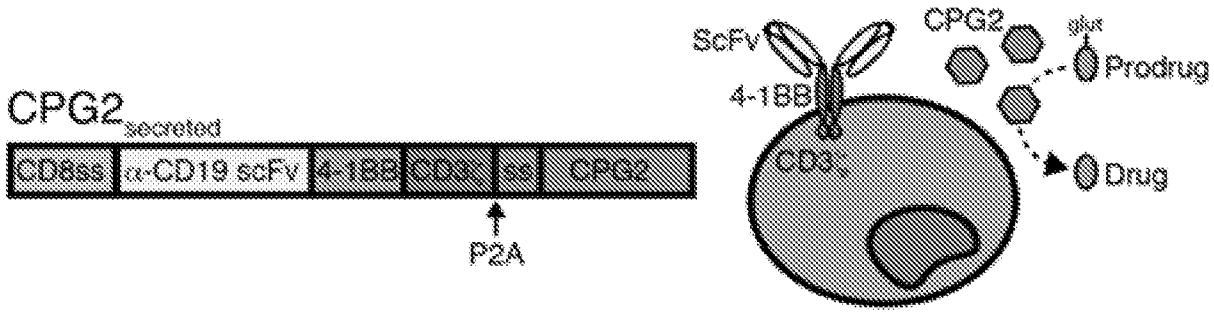

CPG2secreted
CD8ss
α-CD19 scFv
4-1BB
CD3ζ
ss
CPG2
P2A
ScFv
4-1BB
CD3ζ
CPG2
glux
Prodrug
Drug

FIG. 3C

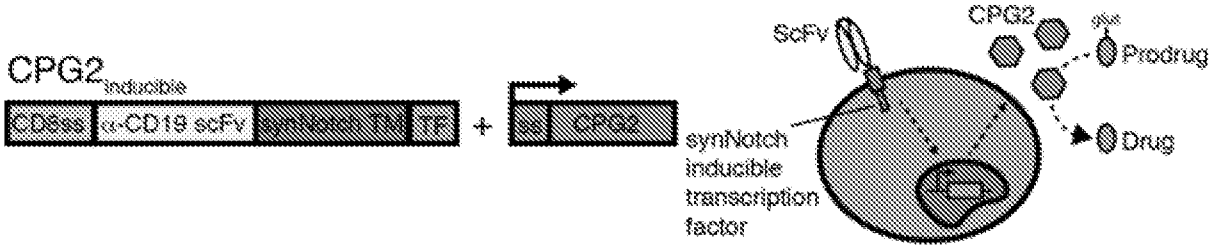

CPG2inducible
CD8ss
α-CD19 scFv
synNotch TM
TF
+
ss
CPG2
ScFv
CPG2
Prodrug
Drug
synNotch
inducible
transcription
factor

FIG. 4

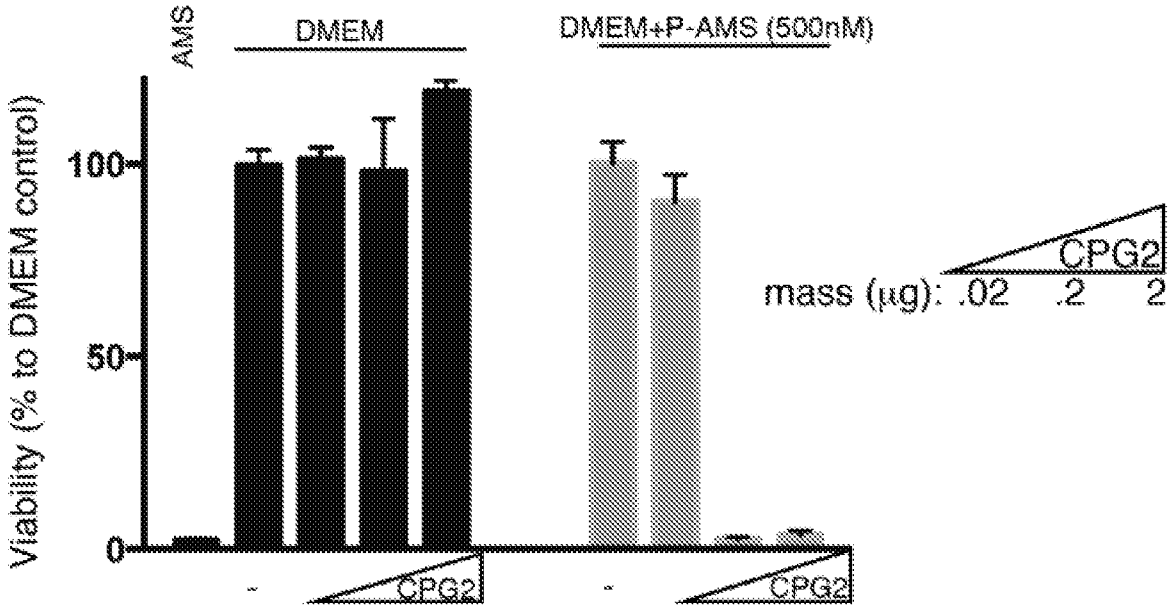

AMS
DMEM
DMEM+P-AMS (500nM)
Viability (% to DMEM control)
100
50
0
-
CPG2
-
CPG2
mass (μg): .02 .2 2
CPG2

FIG. 5

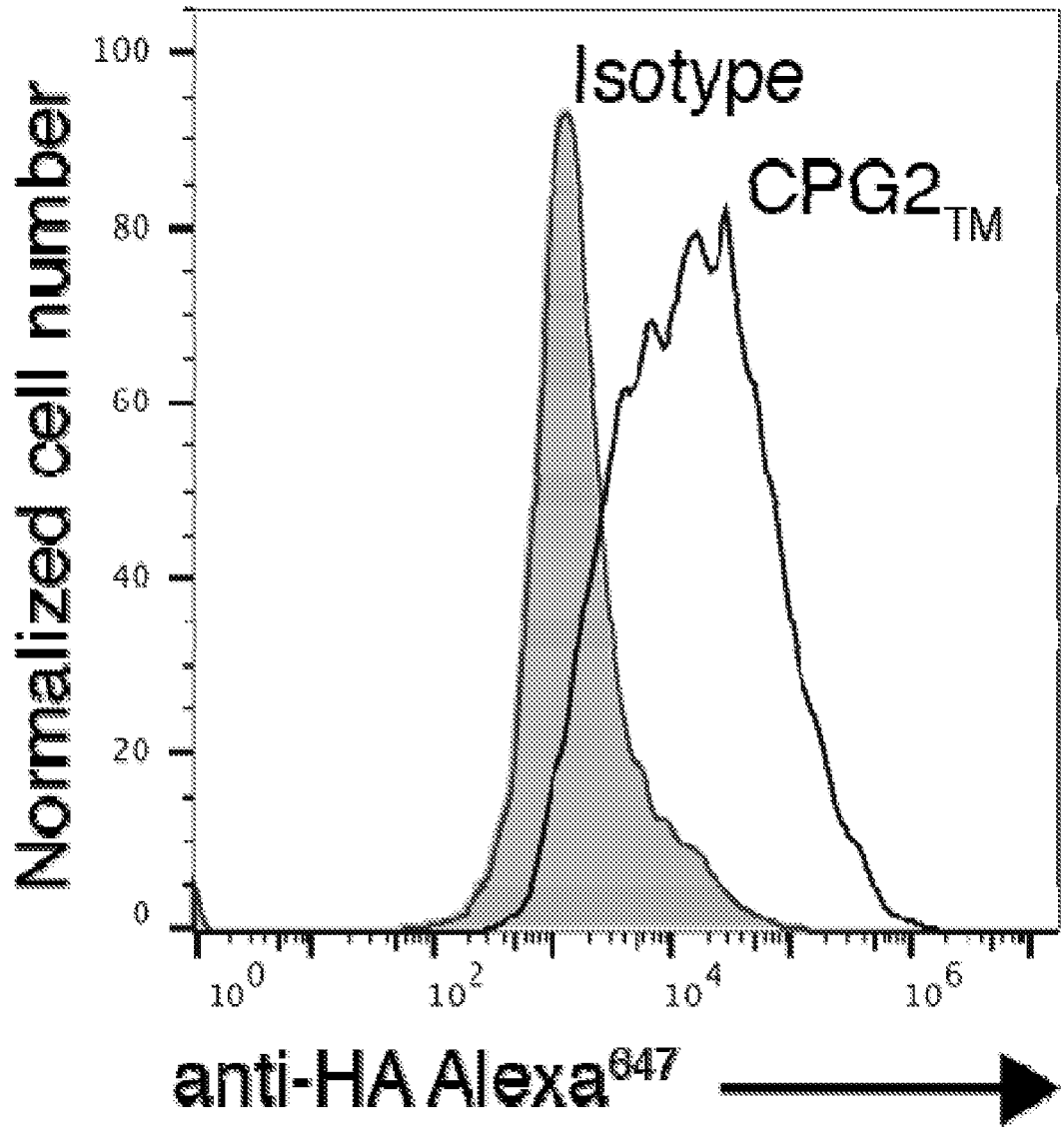

Normalized cell number
100
80
60
40
20
0
Isotype
CPG2TM
10^0
10^2
10^4
10^6
anti-HA Alexa647

FIG. 6

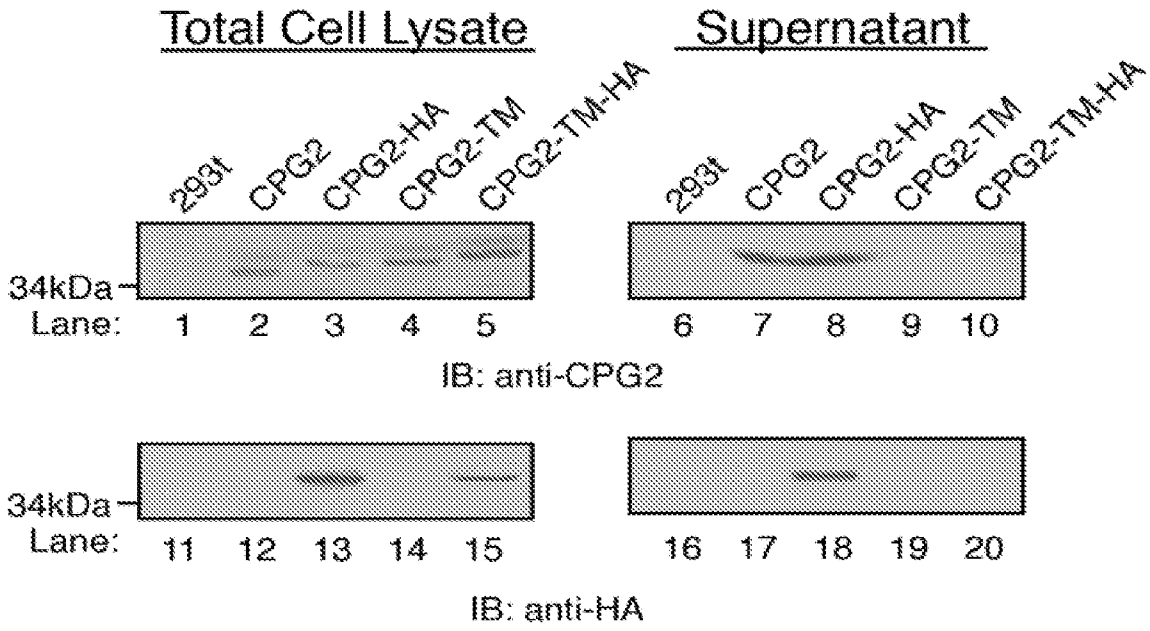

Total Cell Lysate
Supernatant
293t
CPG2
CPG2-HA
CPG2-TM
CPG2-TM-HA
293t
CPG2
CPG2-HA
CPG2-TM
CPG2-TM-HA
34kDa
Lane: 1 2 3 4 5 6 7 8 9 10
IB: anti-CPG2
34kDa
Lane: 11 12 13 14 15 16 17 18 19 20
IB: anti-HA

FIG. 7

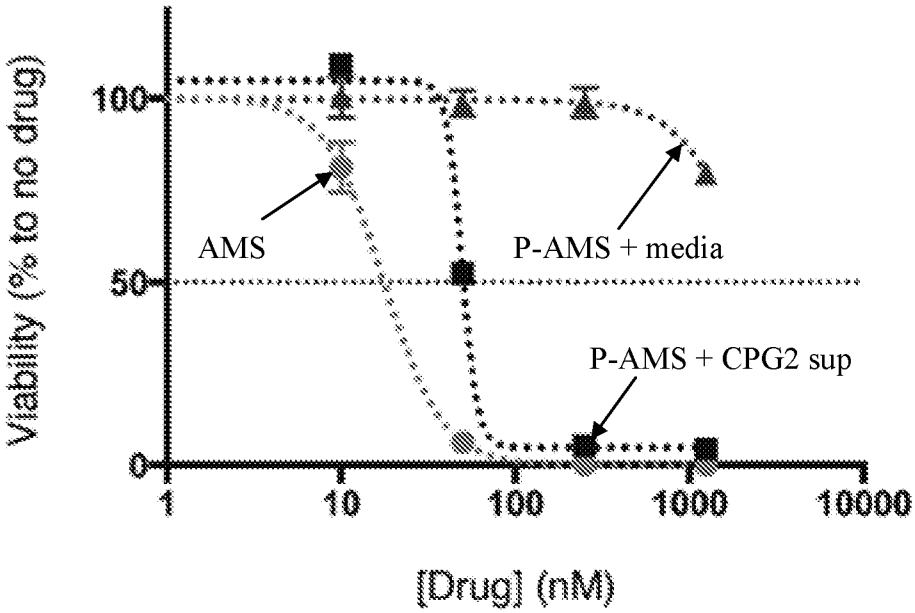

Viability (% to no drug)
100
50
0
AMS
P-AMS + media
P-AMS + CPG2 sup
1
10
100
1000
10000
[Drug] (nM)

FIG. 8

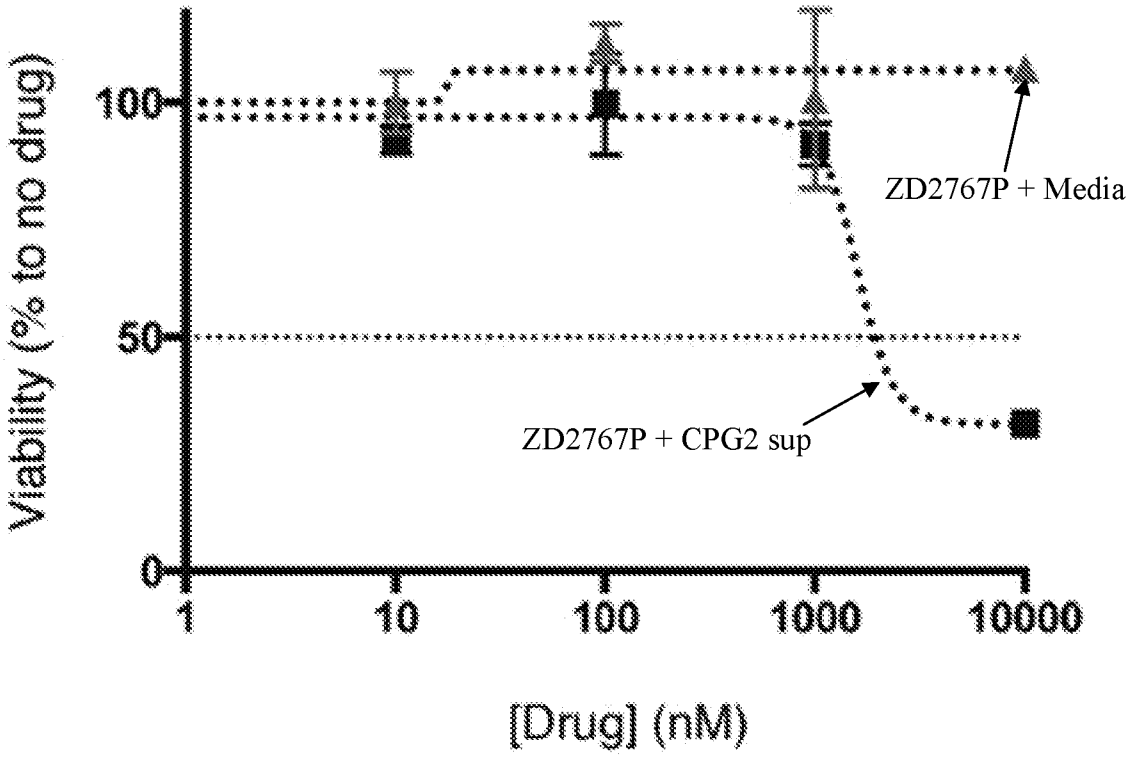

Viability (% to no drug)
100
50
0
1
10
100
1000
10000
[Drug] (nM)
ZD2767P + Media
ZD2767P + CPG2 sup

FIG. 9A

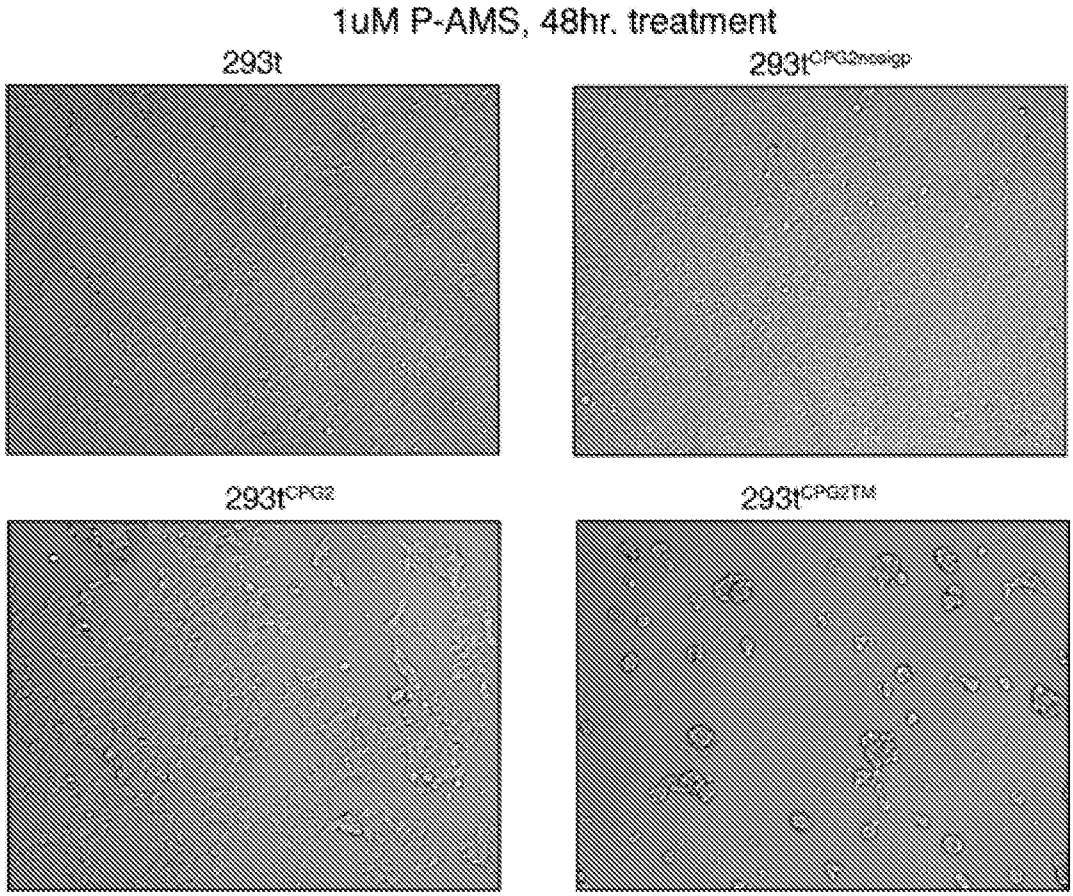

1uM P-AMS, 48hr. treatment
293t
293t^CPG2nosigp
293t^CPG2
293t^CPG2TM

FIG. 9B

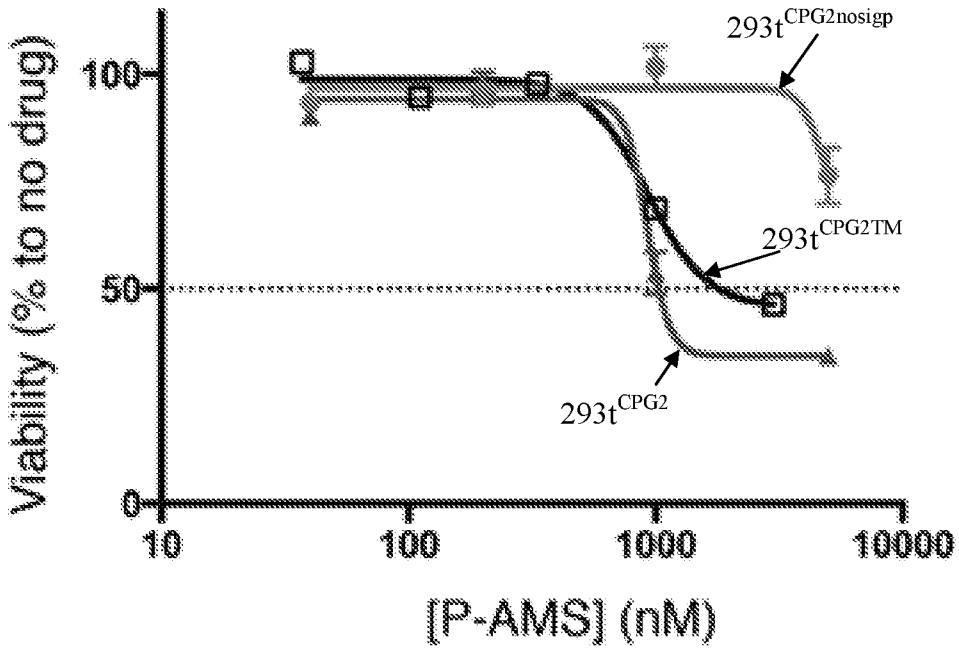

Viability (% to no drug)
100
50
0
10
100
1000
10000
[P-AMS] (nM)
293t^CPG2nosigp
293t^CPG2TM
293t^CPG2

FIG. 10

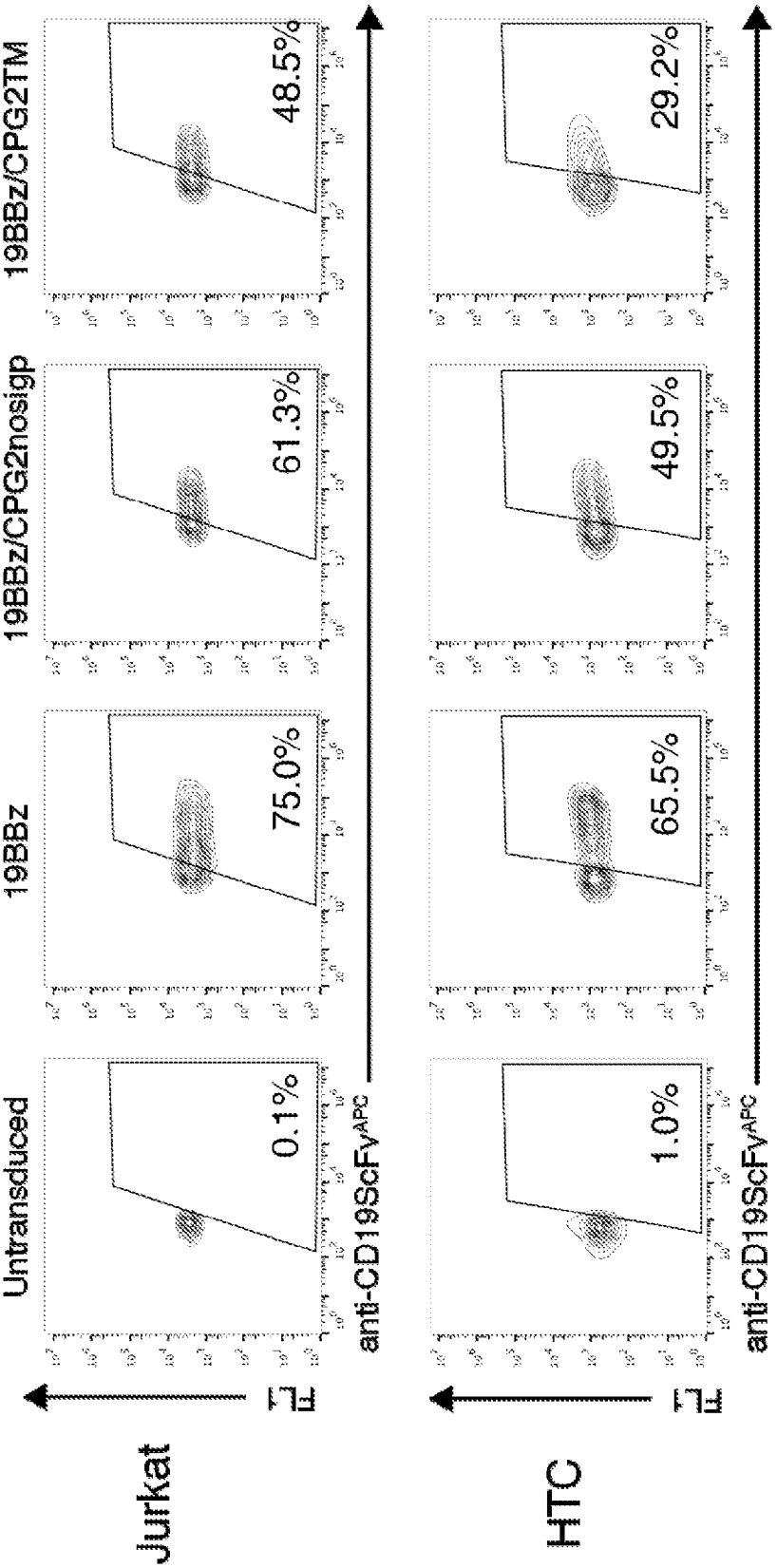

Jurkat
HTC
Untransduced
19BBz
19BBz/CPG2nosigp
19BBz/CPG2TM
0.1%
75.0%
61.3%
48.5%
1.0%
65.5%
49.5%
29.2%
anti-CD19ScFv-APC
anti-CD19ScFv-APC

FIG. 11

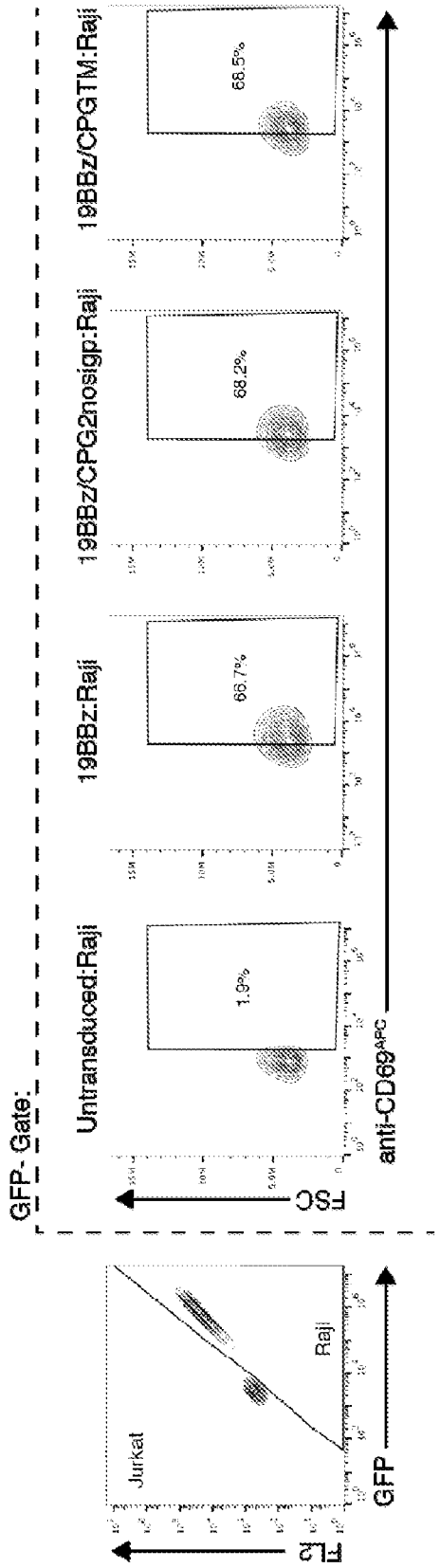

GFP- Gate:
Untransduced:Raji
19BBz:Raji
19BBz/CPG2nosigp:Raji
19BBz/CPGTM:Raji
1.9%
66.7%
68.2%
68.5%
FSC
anti-CD69APC
Jurkat
Raji
SSC
GFP

FIG. 12A

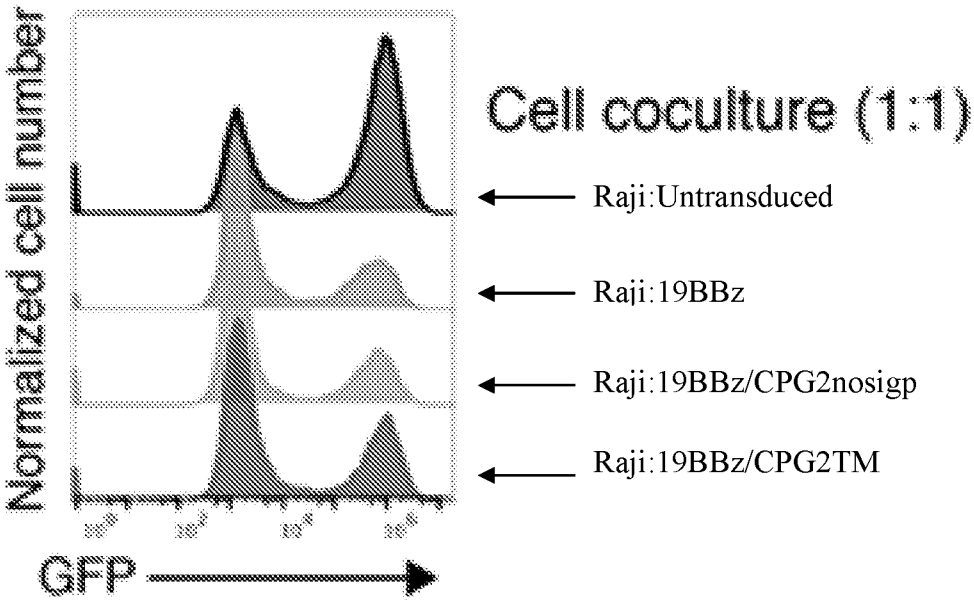


FIG. 12B

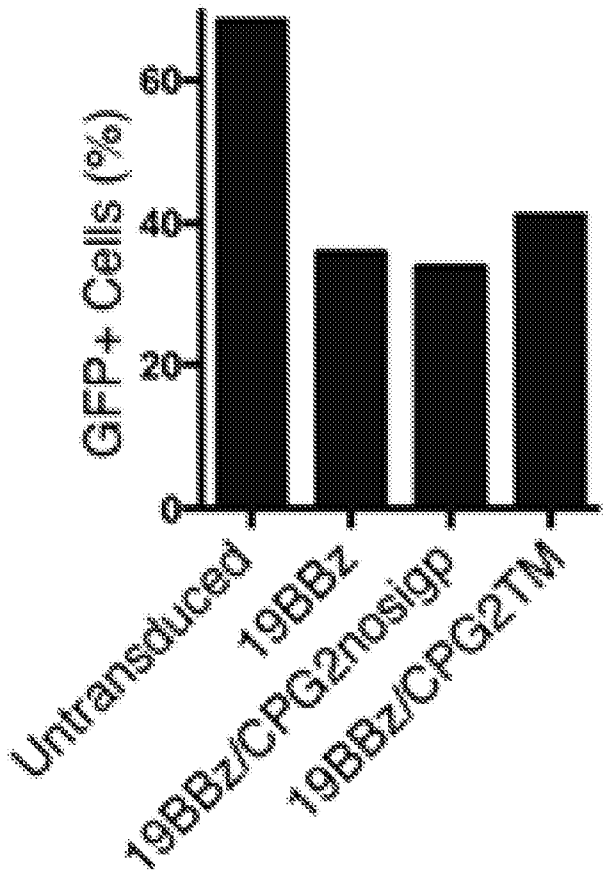


FIG. 13

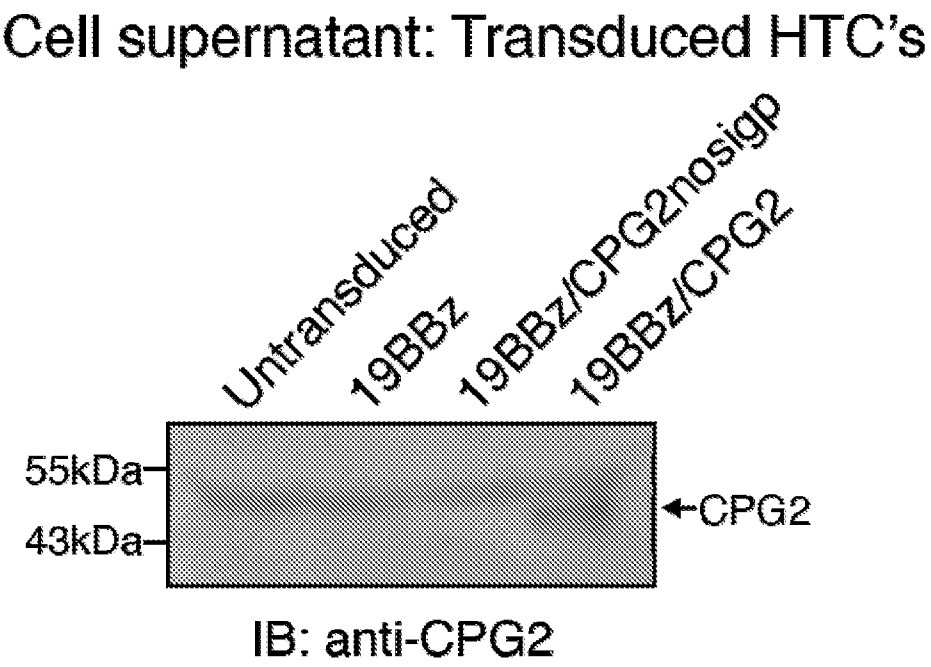

Cell supernatant: Transduced HTC's
Untransduced
19BBz
19BBz/CPG2nosigp
19BBz/CPG2
55kDa
43kDa
CPG2
IB: anti-CPG2

FIG. 14A

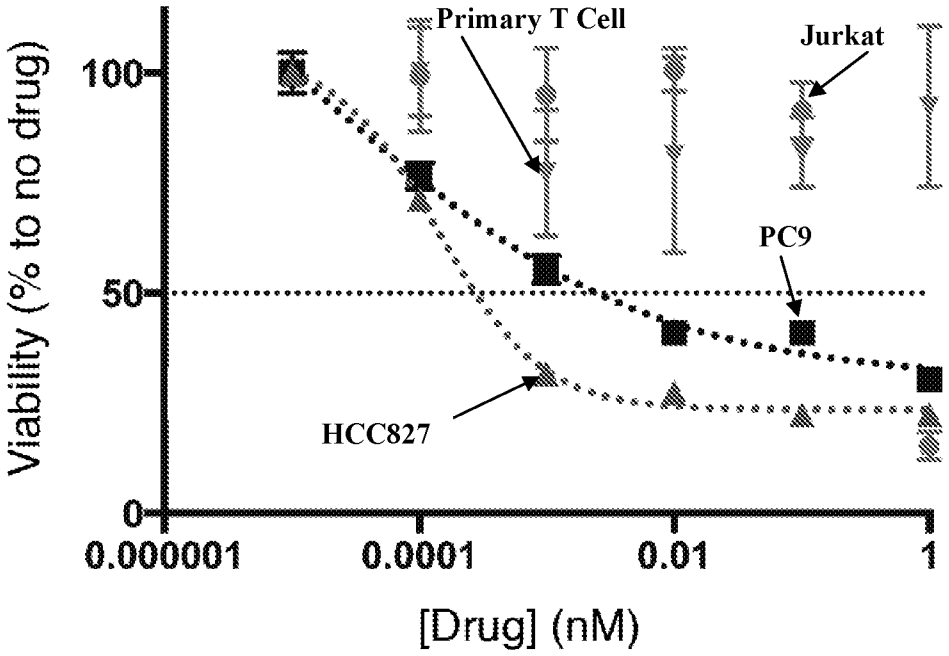


FIG. 14B

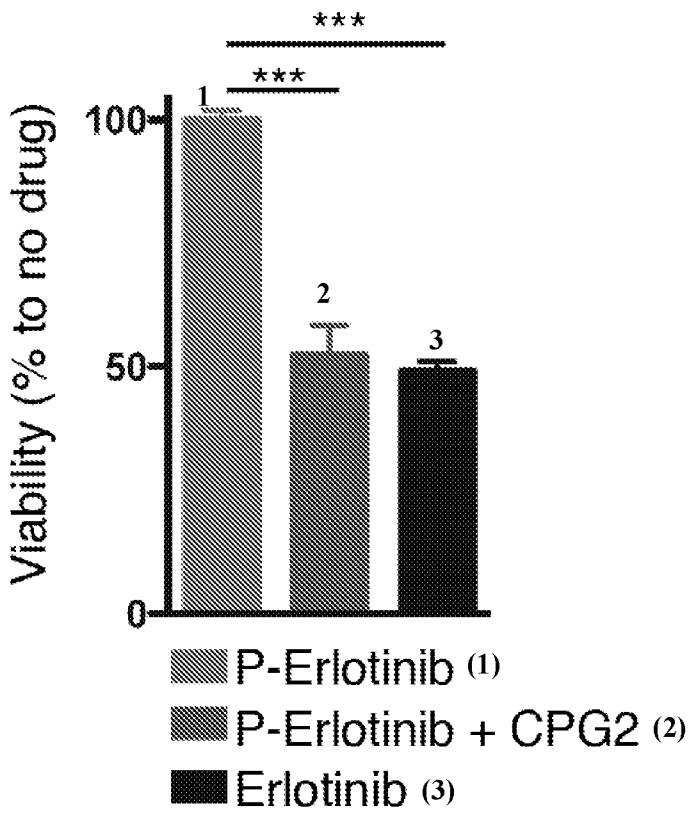


FIG. 15A

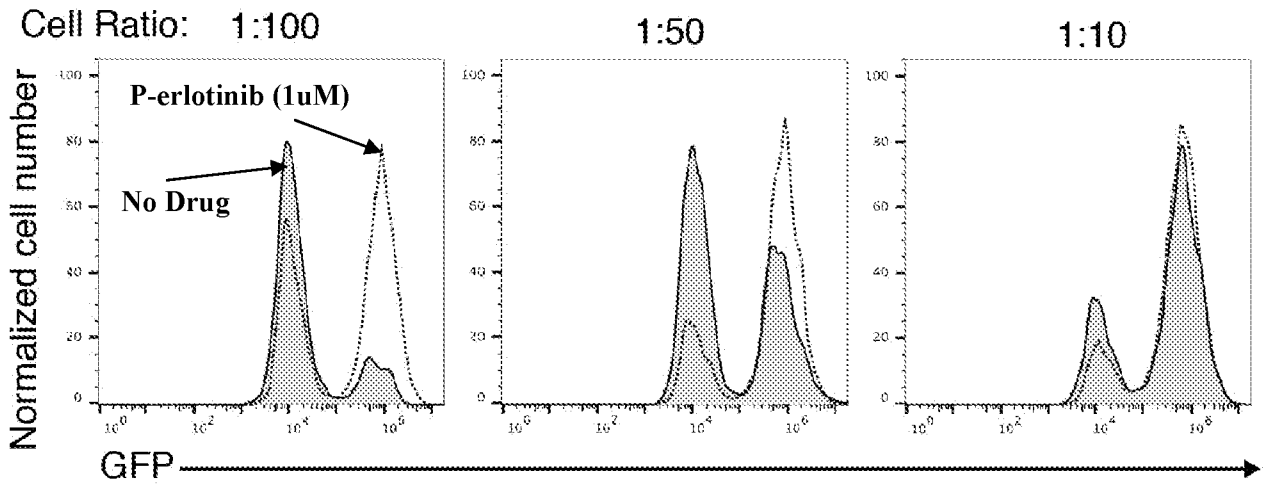


FIG. 15B

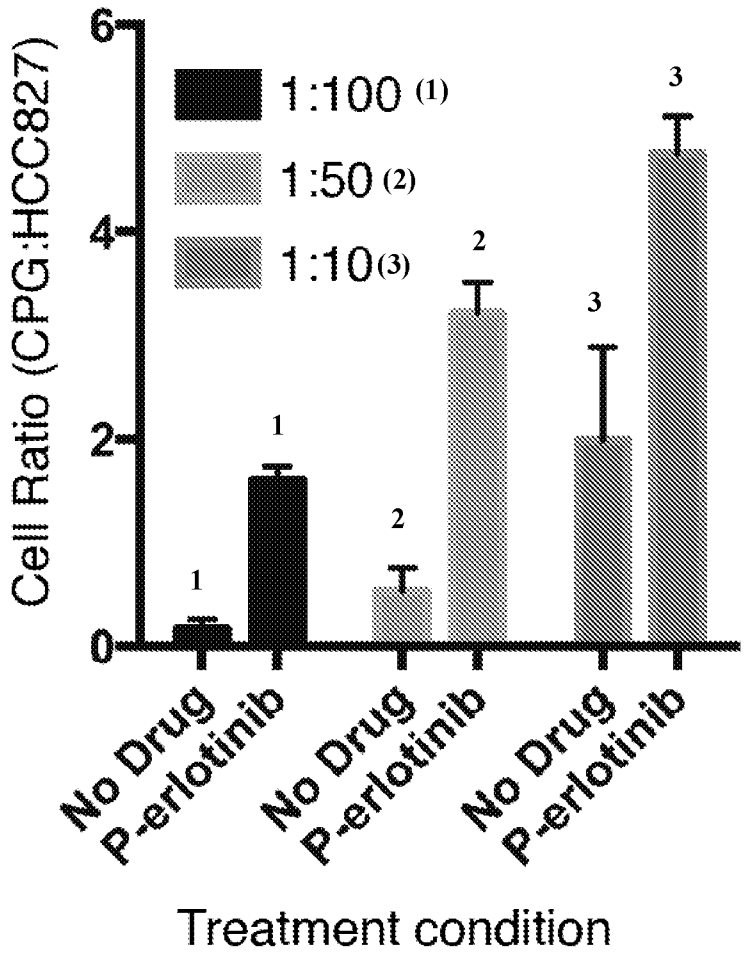


FIG. 16

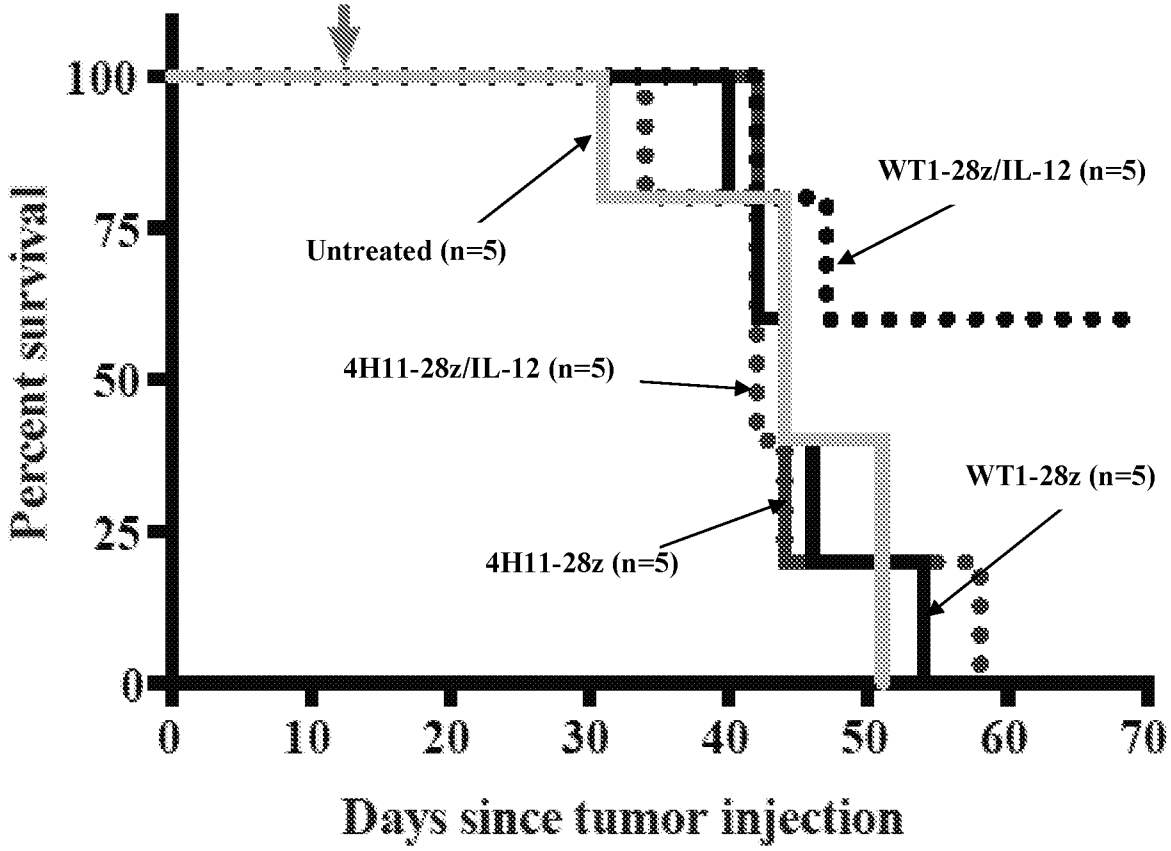

100
75
50
25
0
Percent survival
0
10
20
30
40
50
60
70
Days since tumor injection
Untreated (n=5)
WT1-28z/IL-12 (n=5)
4H11-28z/IL-12 (n=5)
WT1-28z (n=5)
4H11-28z (n=5)

FIG. 17A

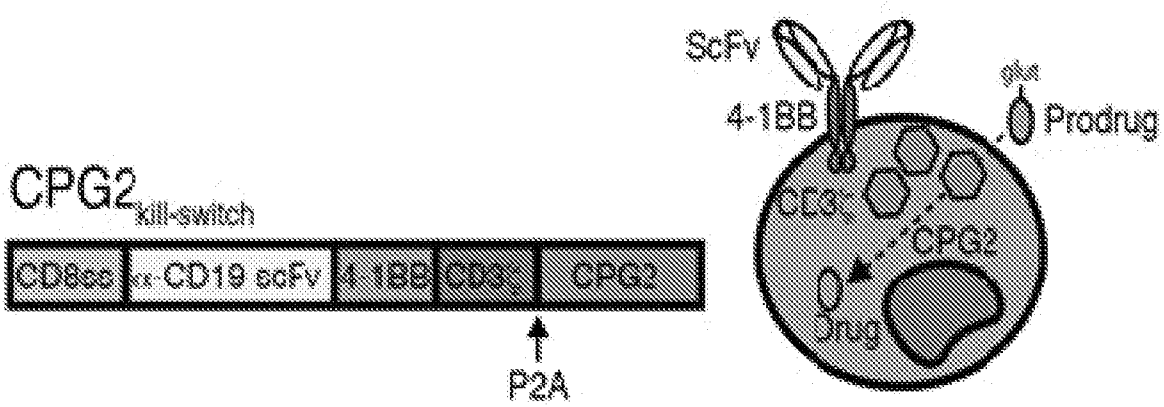


FIG. 17B

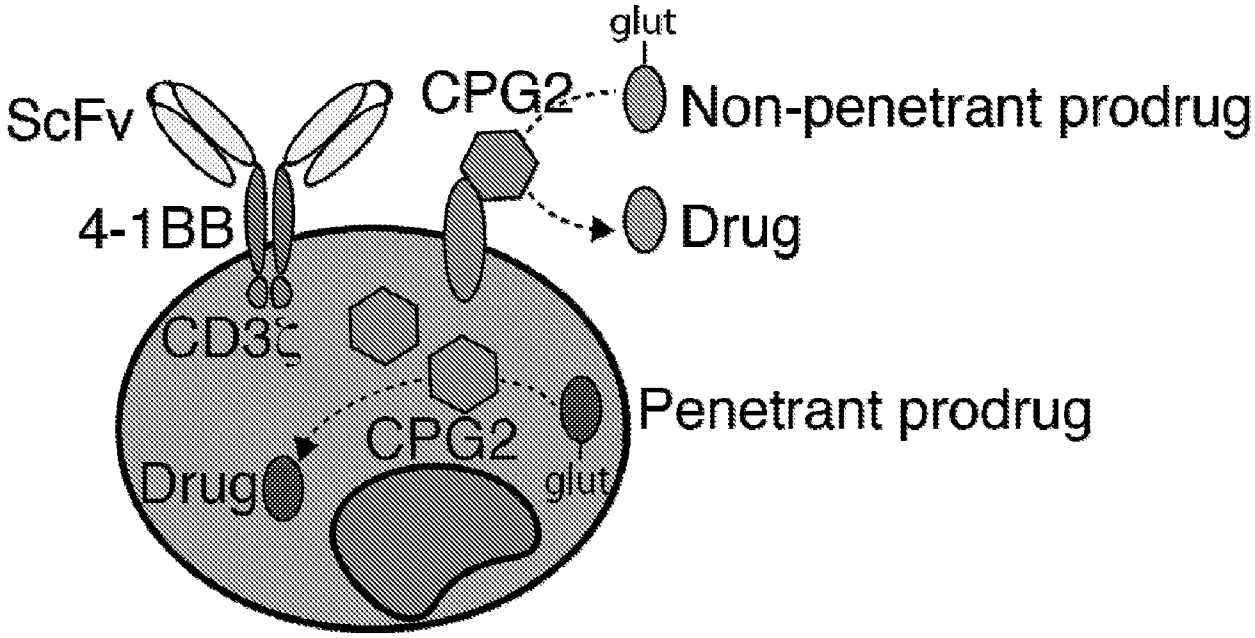


FIG. 18

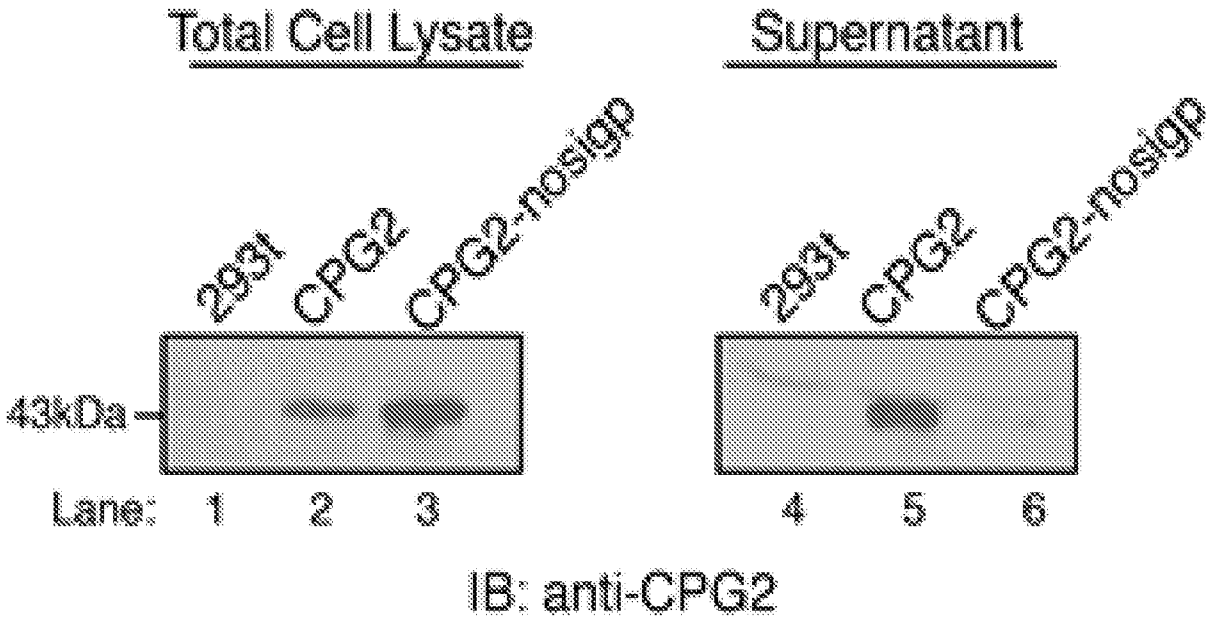
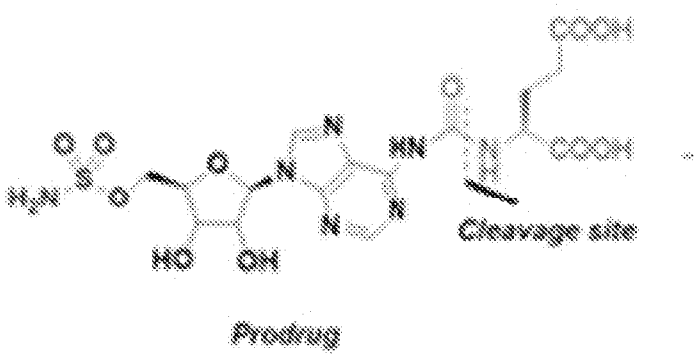

Total Cell Lysate
Supernatant
293t
CPG2
CPG2-nosigp
293t
CPG2
CPG2-nosigp
43kDa
Lane: 1 2 3 4 5 6
IB: anti-CPG2

FIG. 19



Prodrug

FIG. 20A

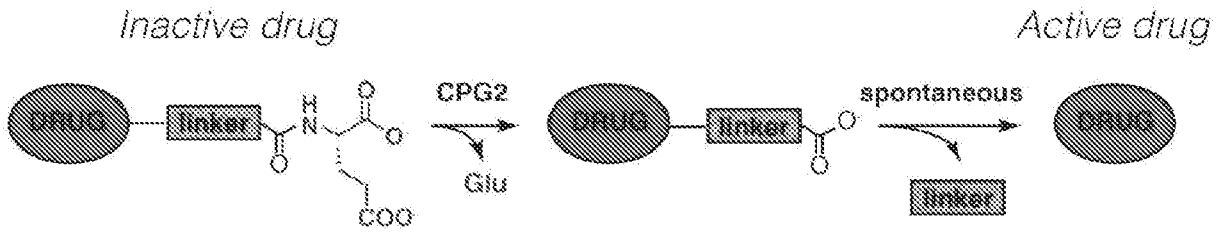


FIG. 20B

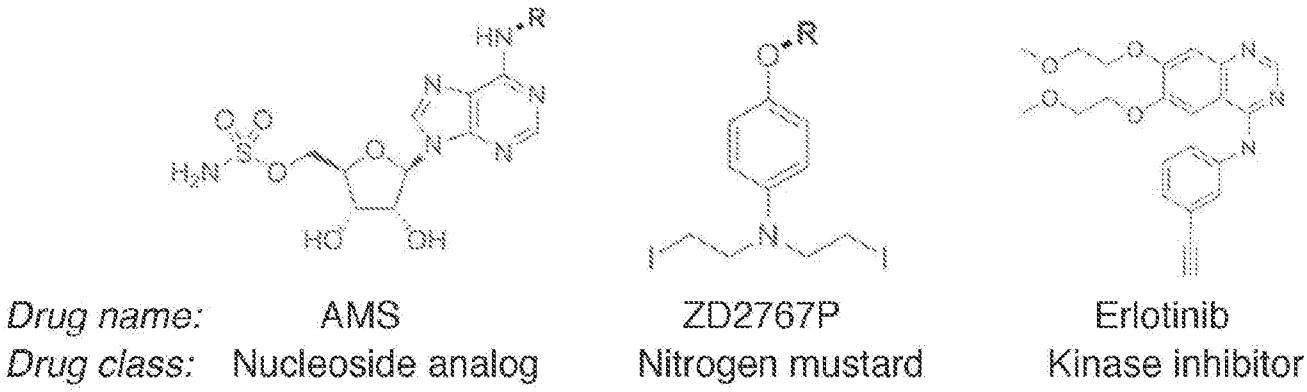


FIG. 20C

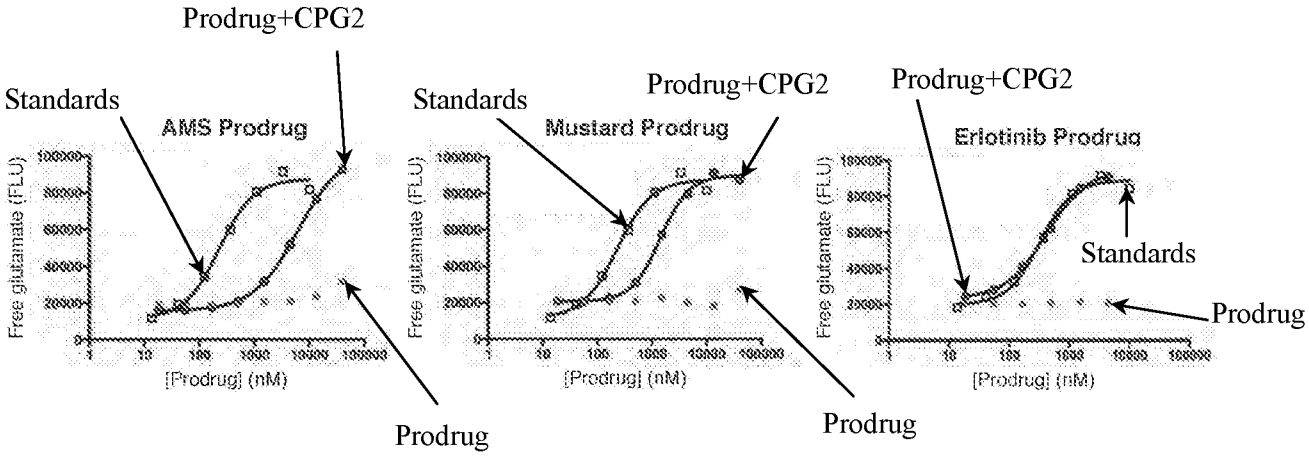


FIG. 20D

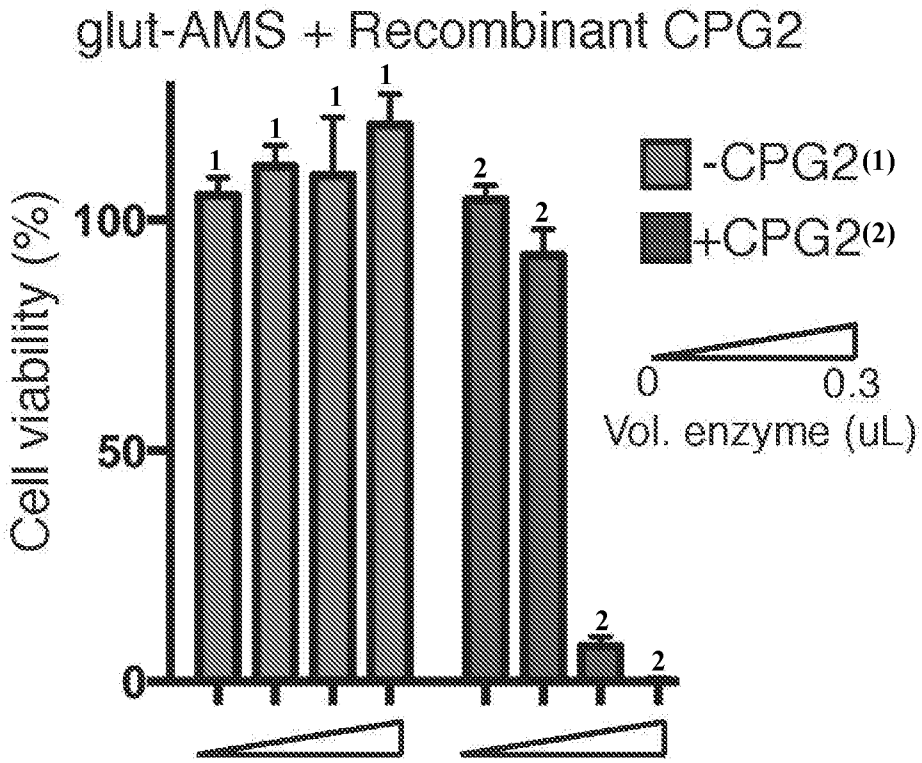


FIG. 20E

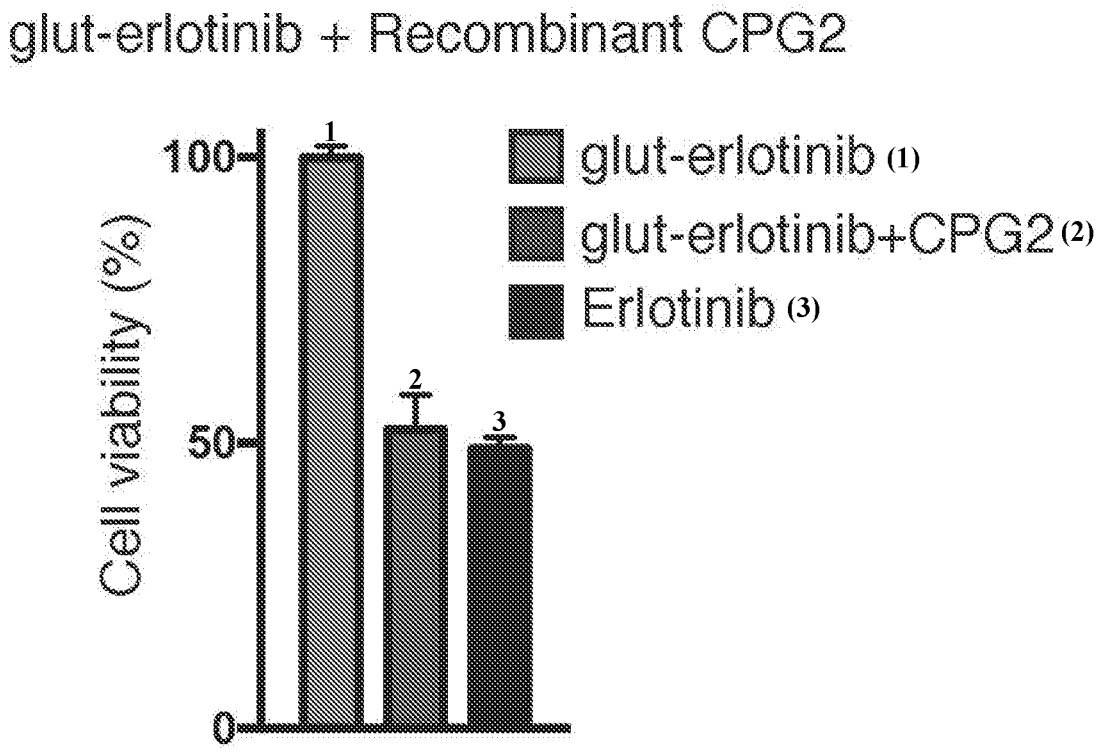


FIG. 20F

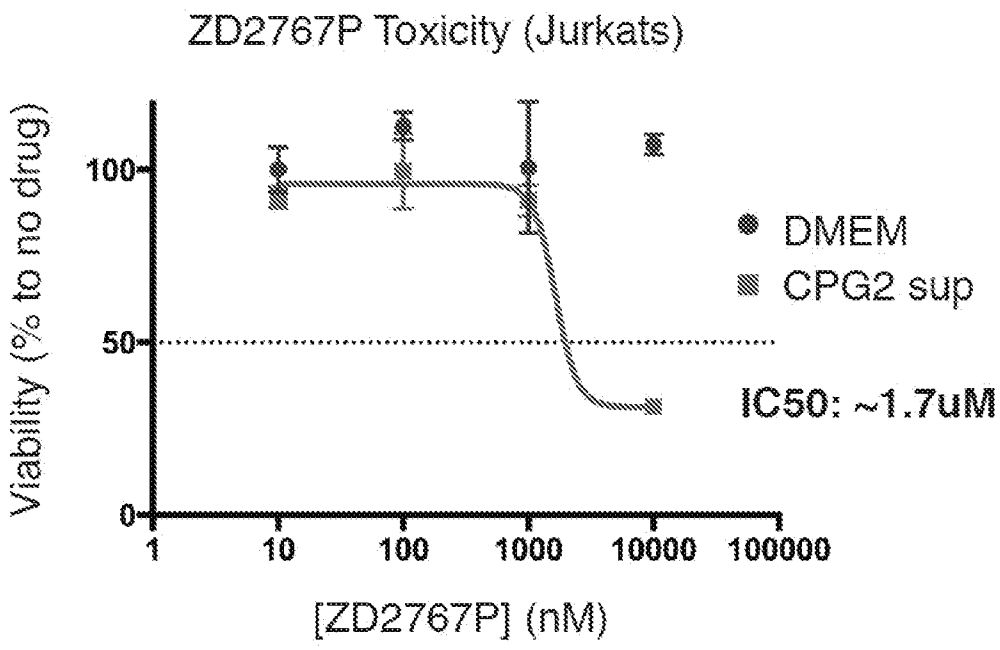


FIG. 21A

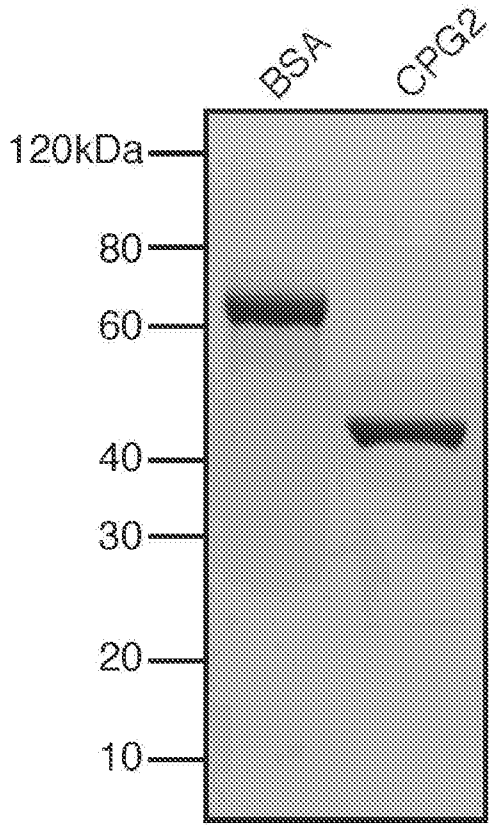


FIG. 21B

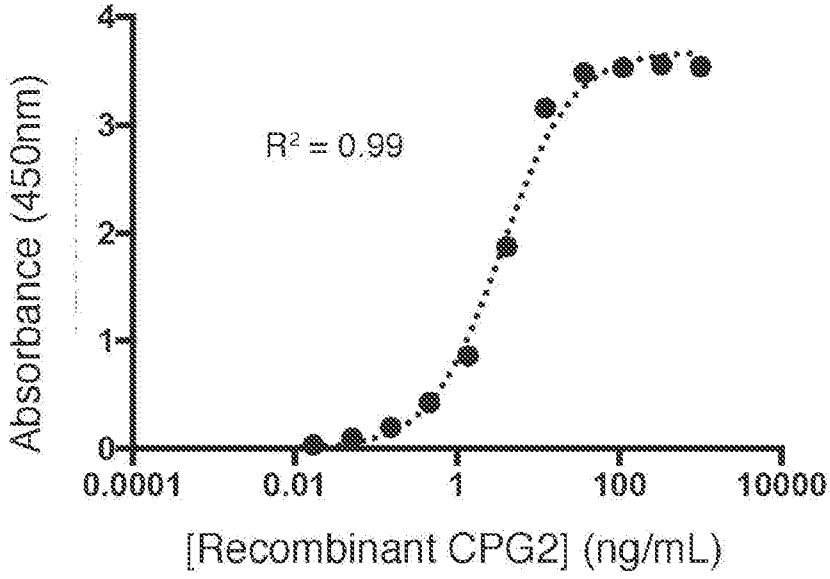


FIG. 21C

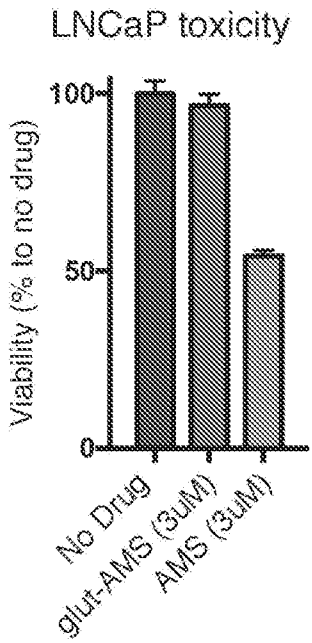


FIG. 21D

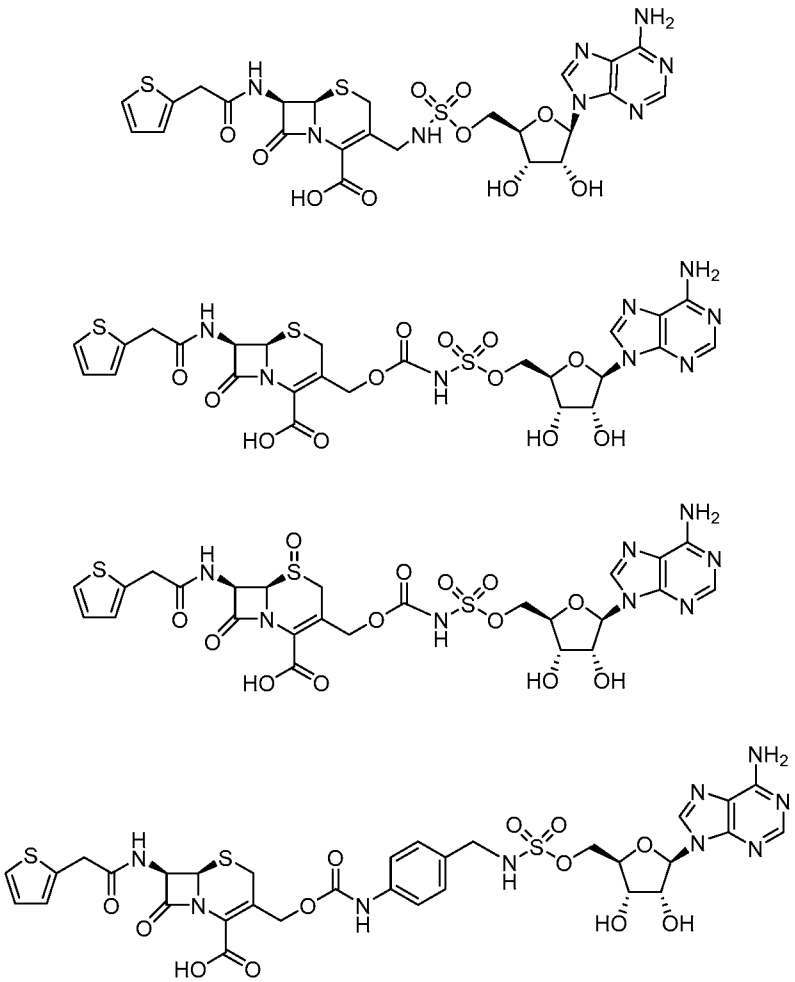


FIG. 21E

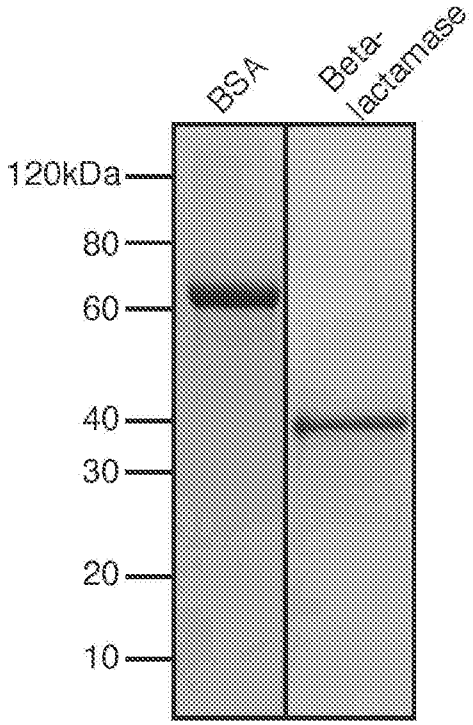


FIG. 21F

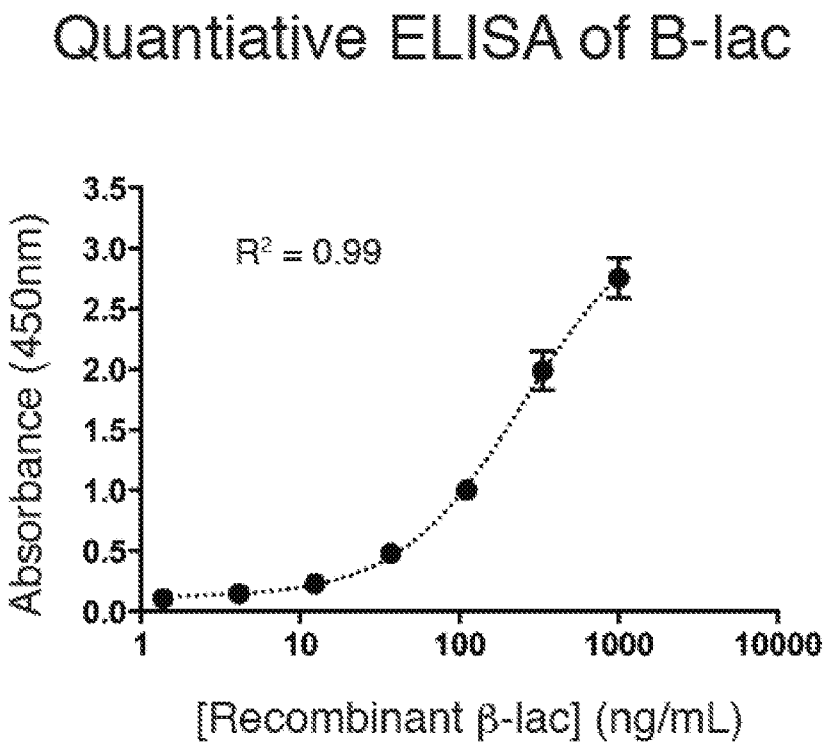


FIG. 22A

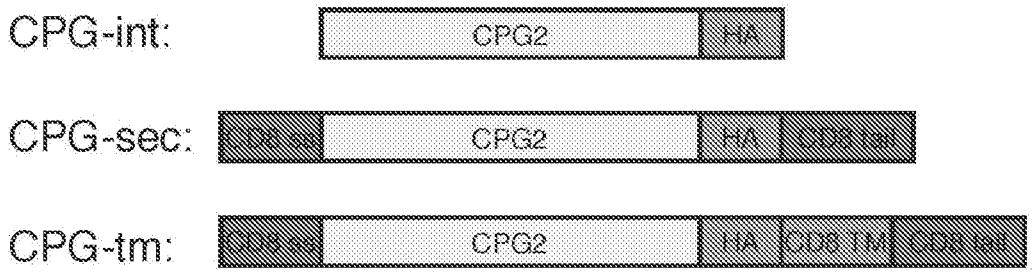


FIG. 22B

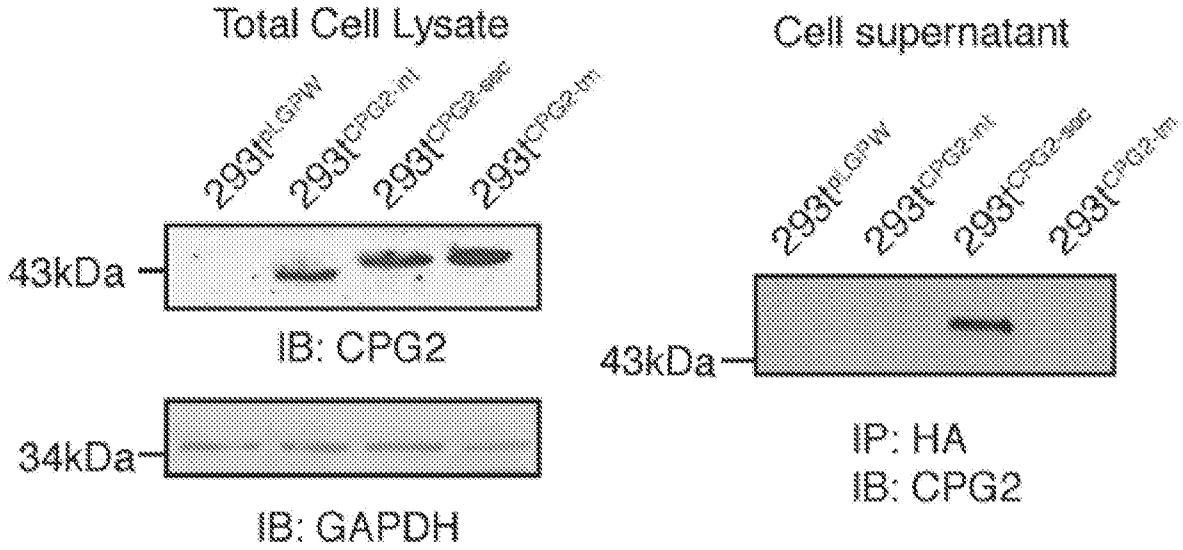


FIG. 22C

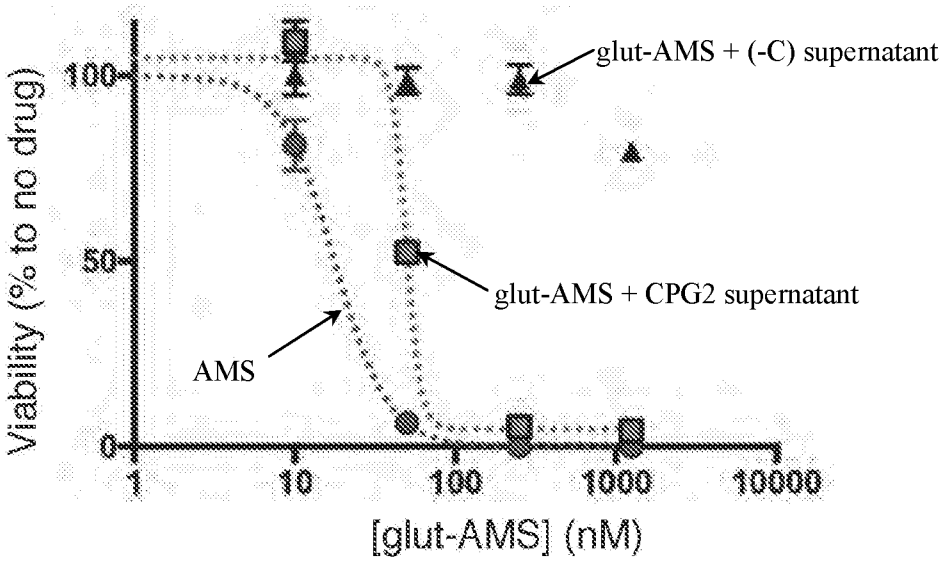


FIG. 22D

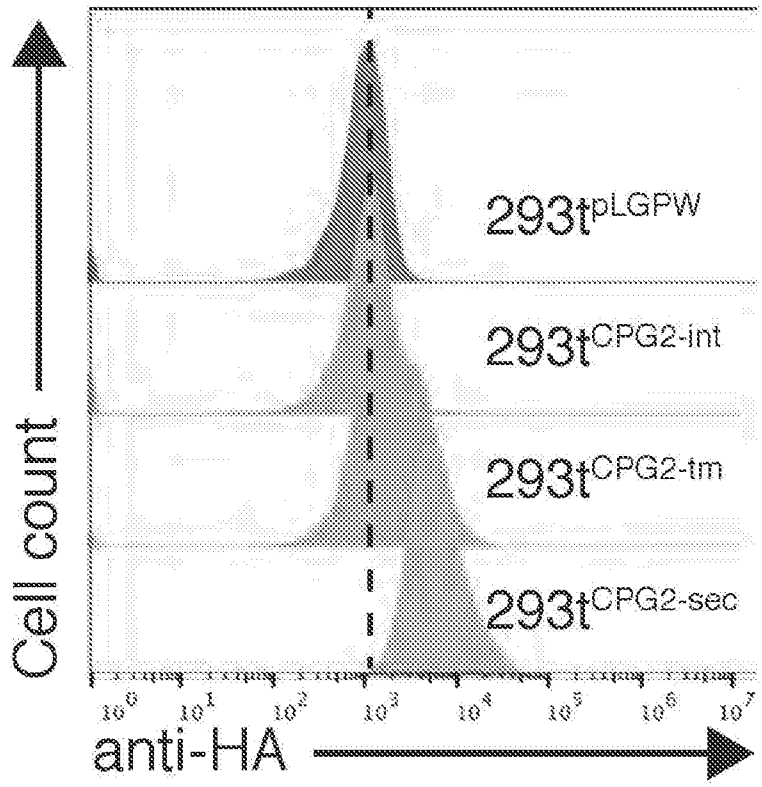


FIG. 22E

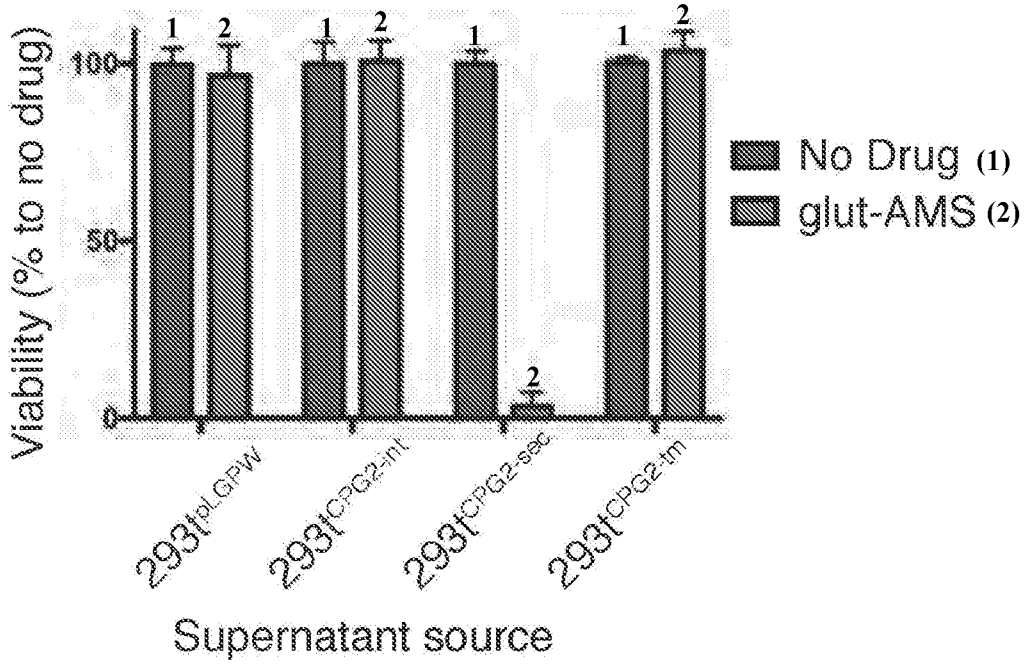


FIG. 22F

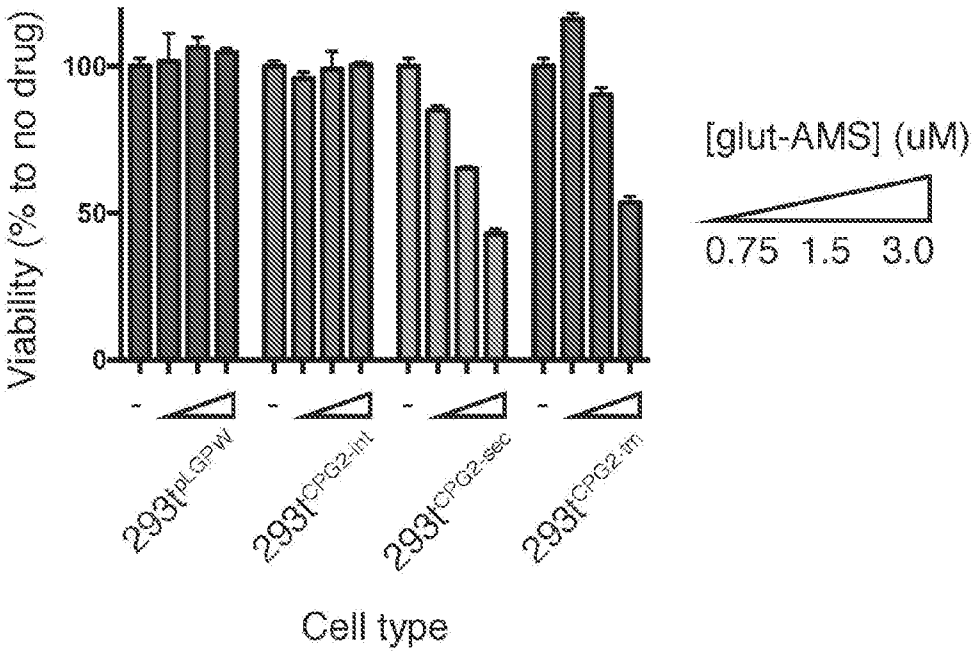


FIG. 22G

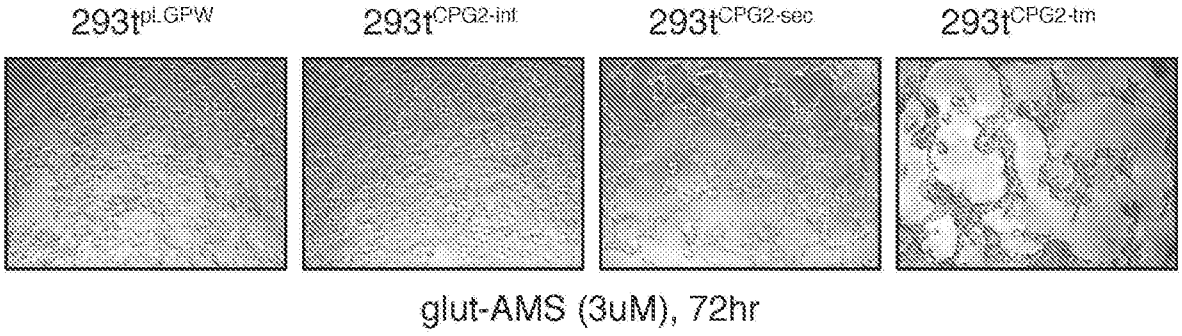


FIG. 22H

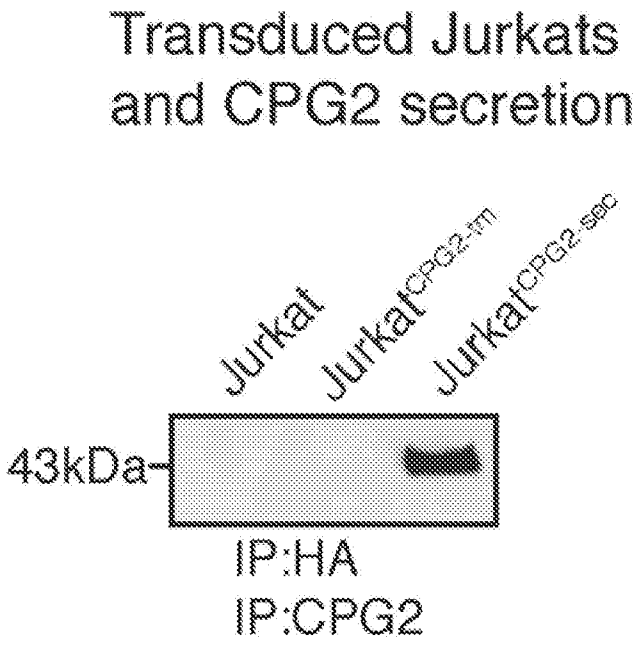


FIG. 23A

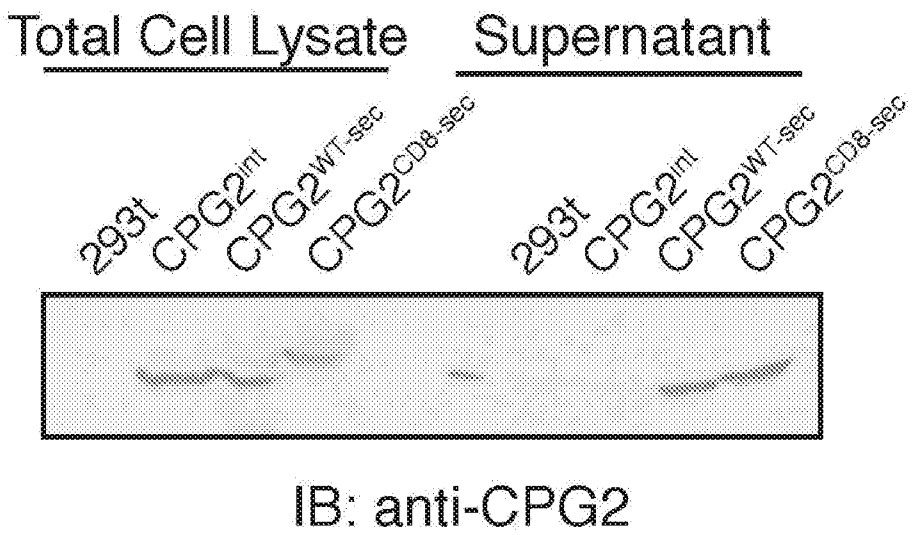


FIG. 23B

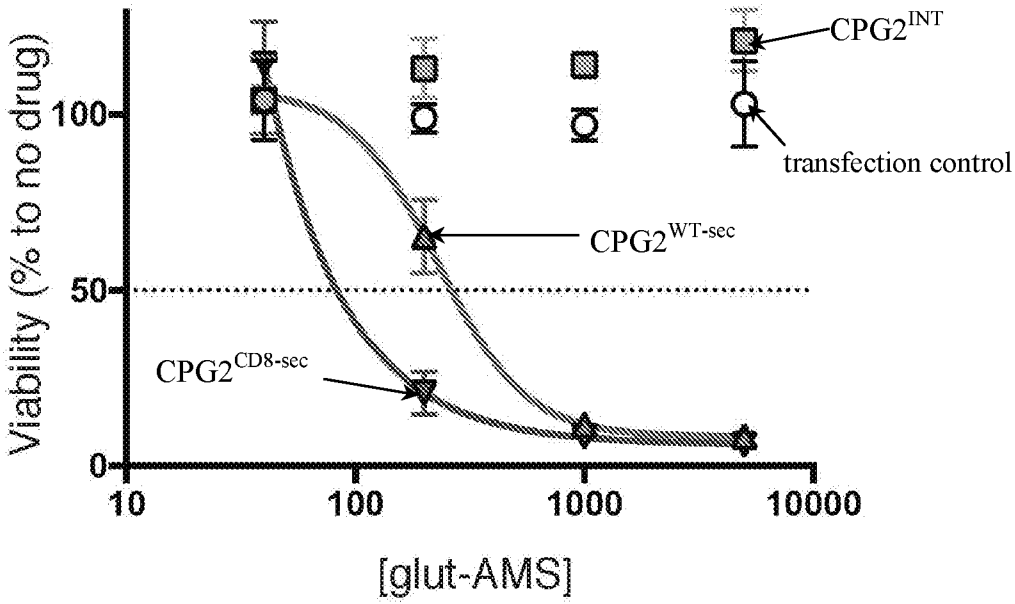


FIG. 23C

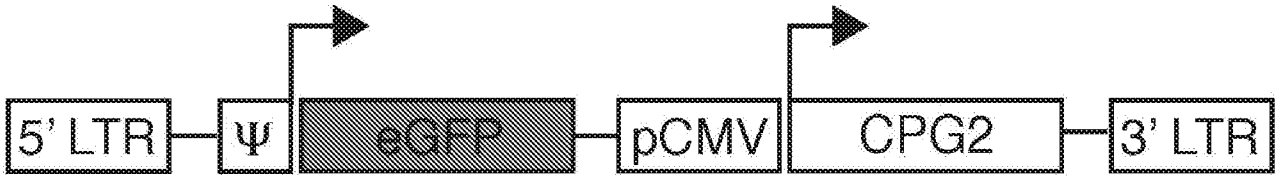


FIG. 24A

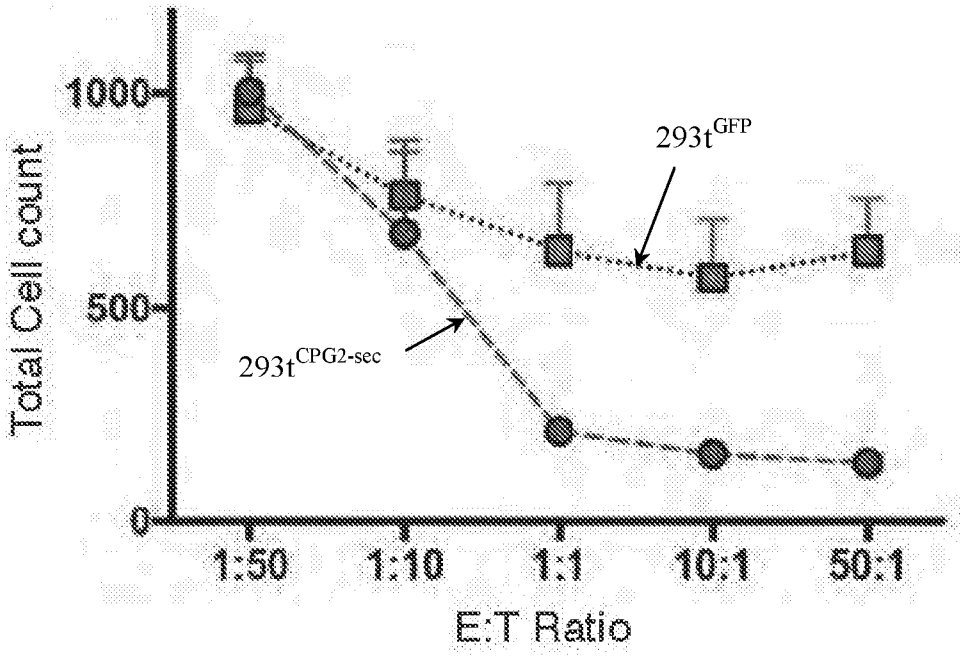


FIG. 24B

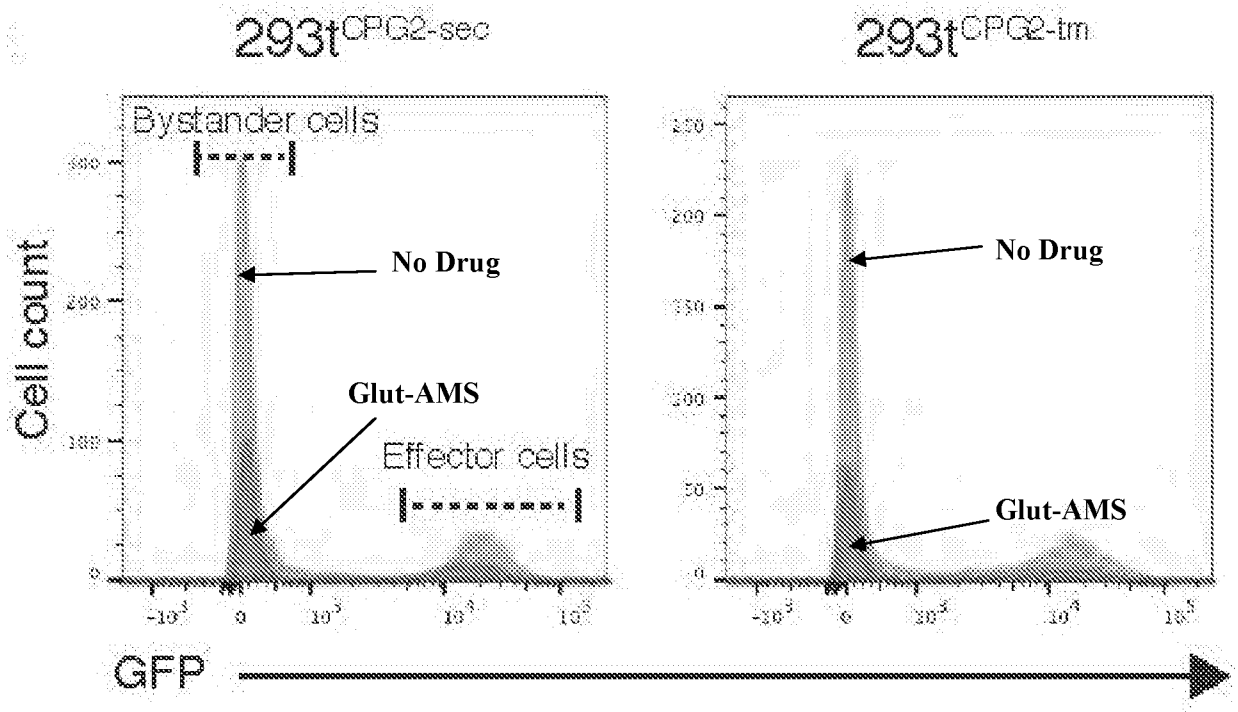


FIG. 24C

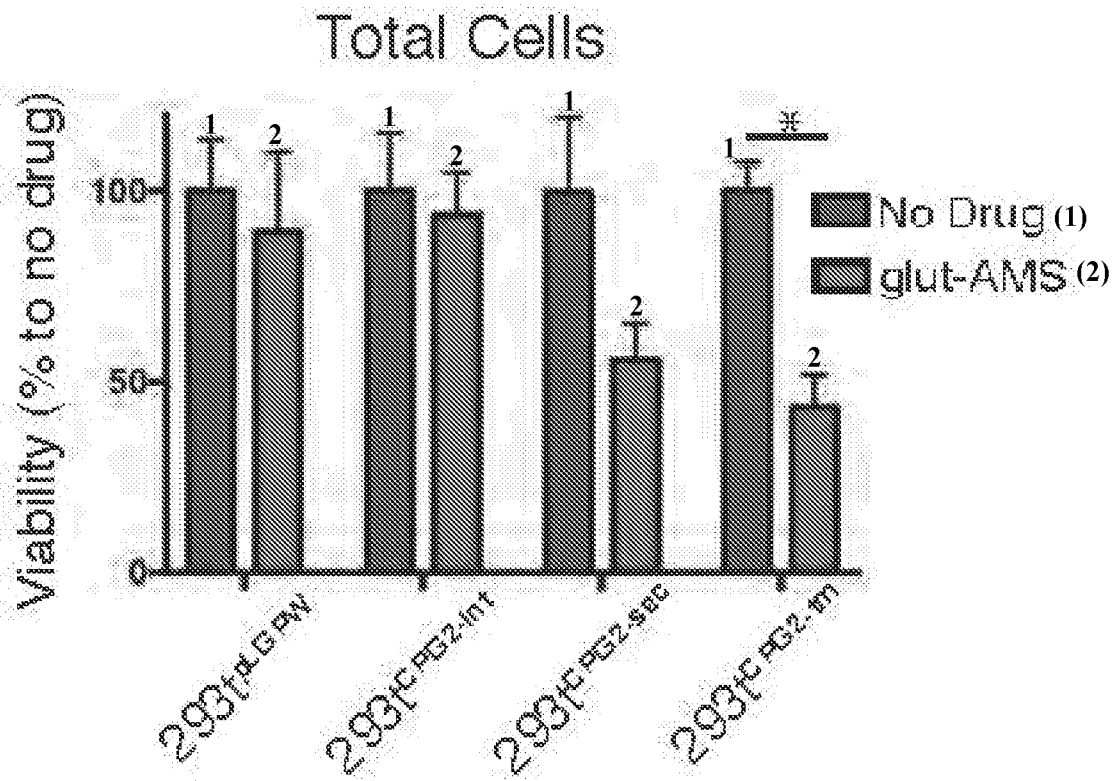


FIG. 24D

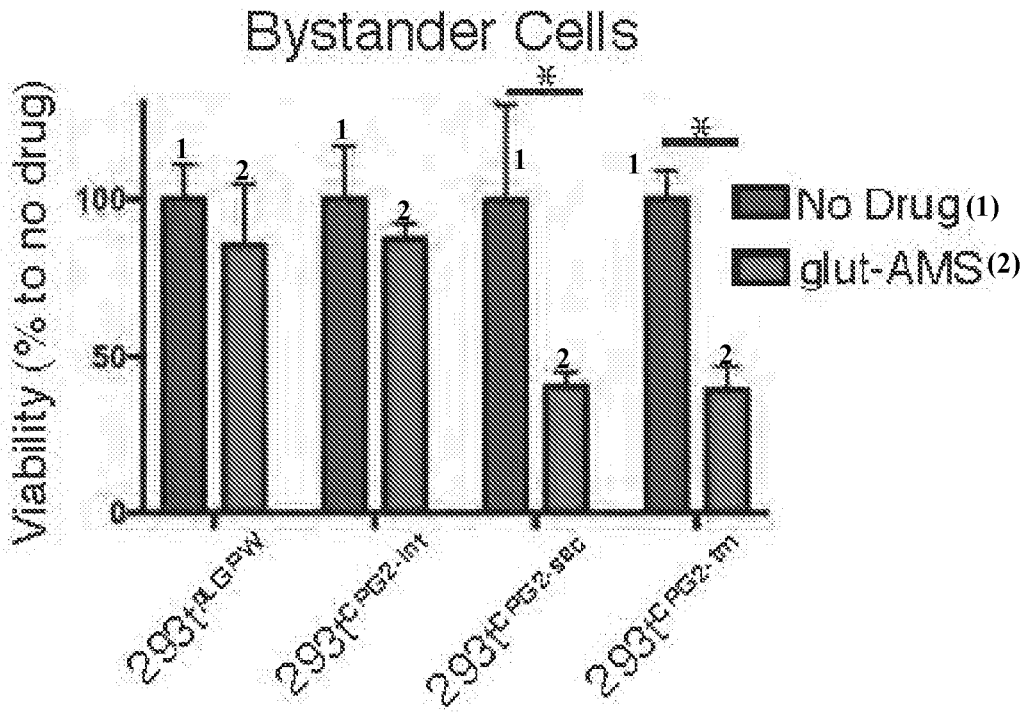


FIG. 25A

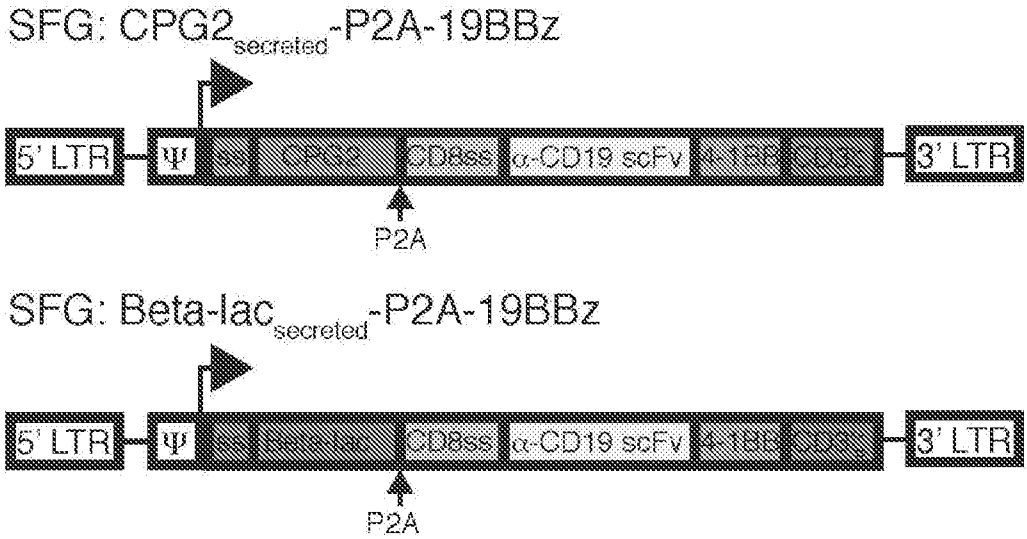


FIG. 25B

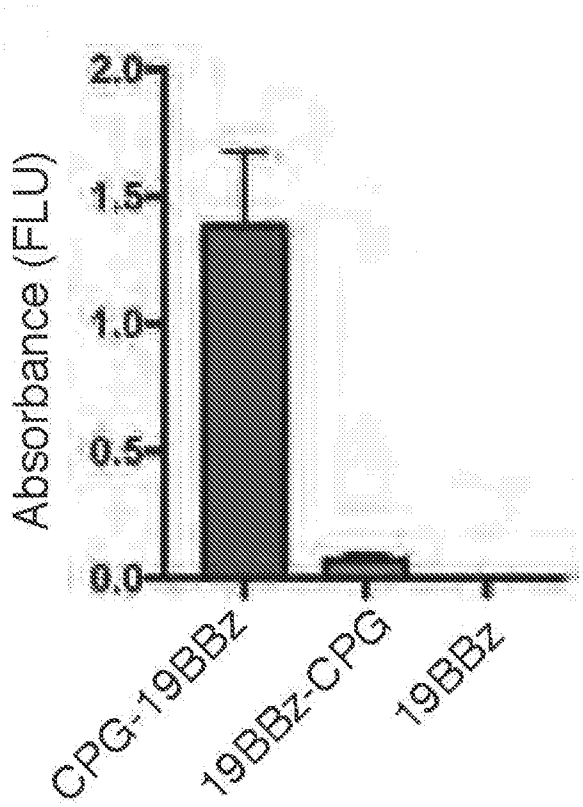


FIG. 25C

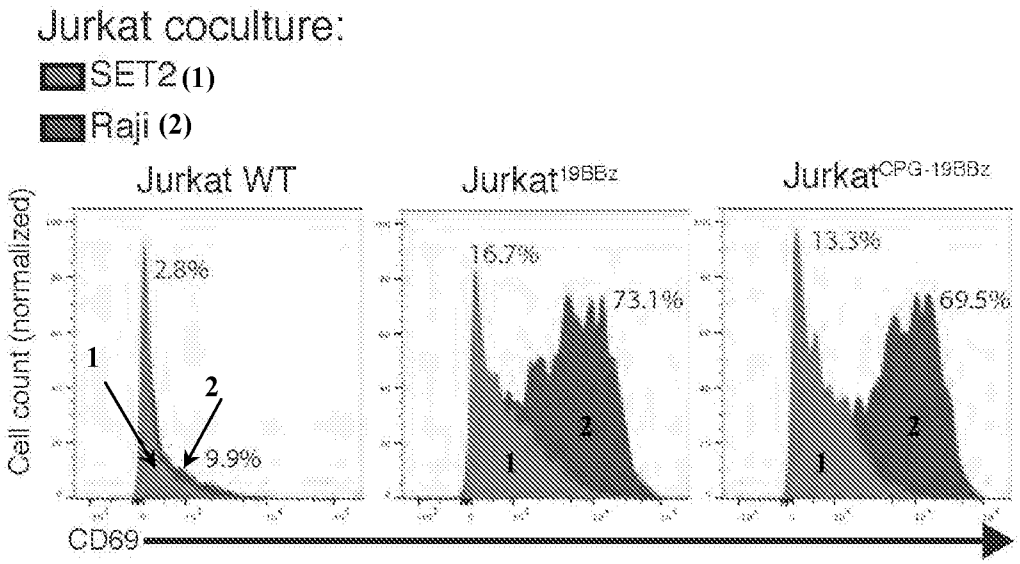


FIG. 25D

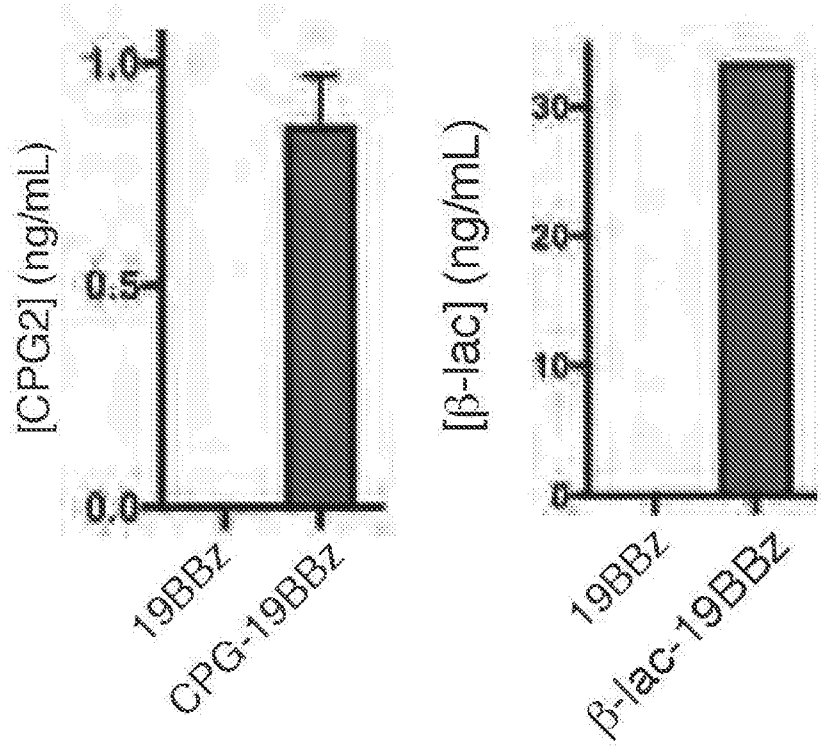


FIG. 25E

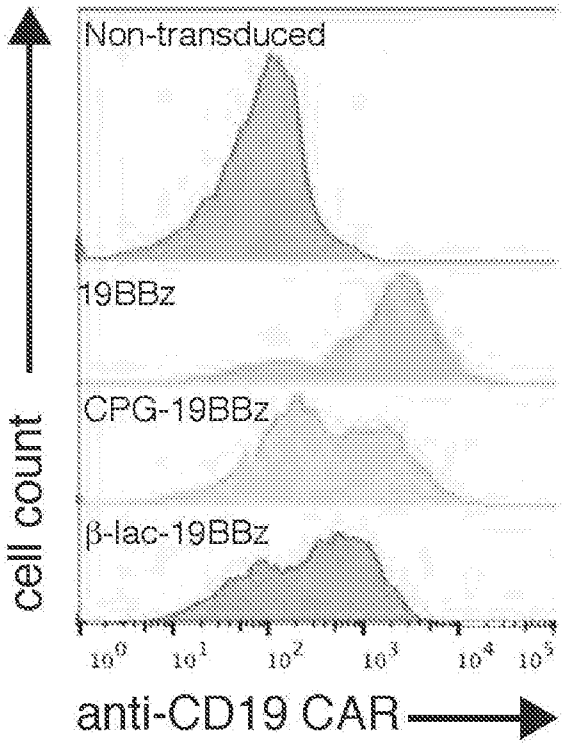


FIG. 25F

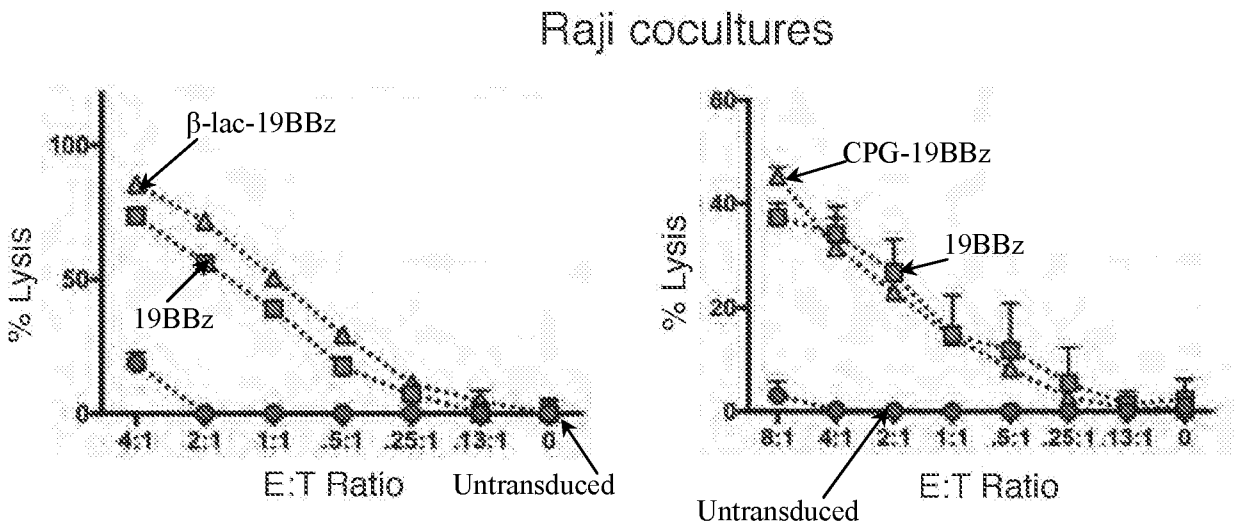


FIG. 25G

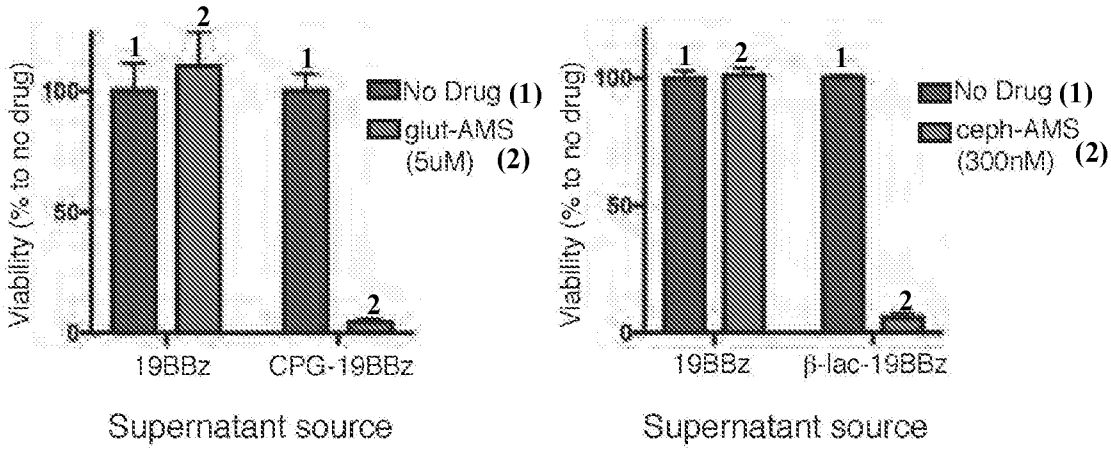


FIG. 25H

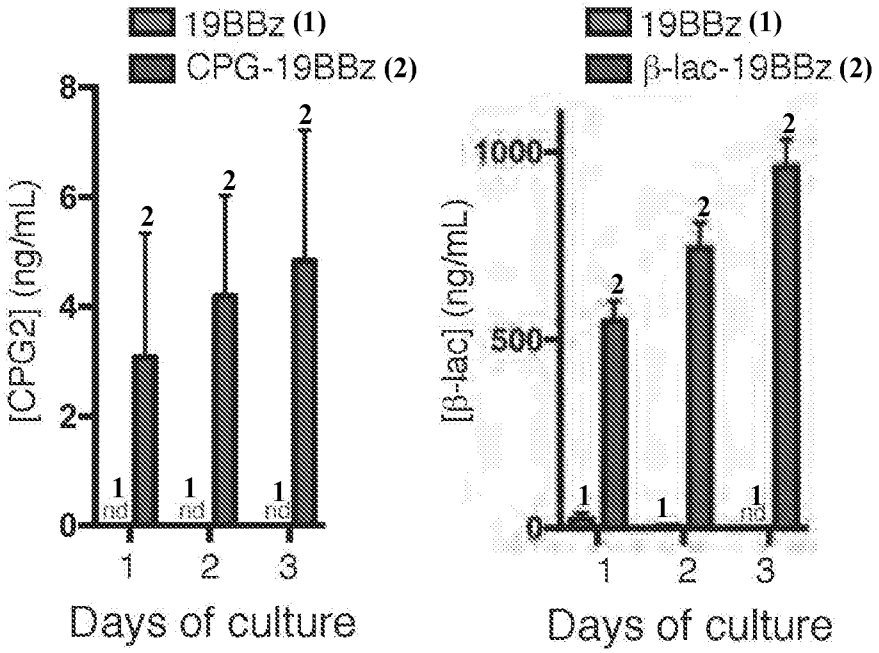


FIG. 25I

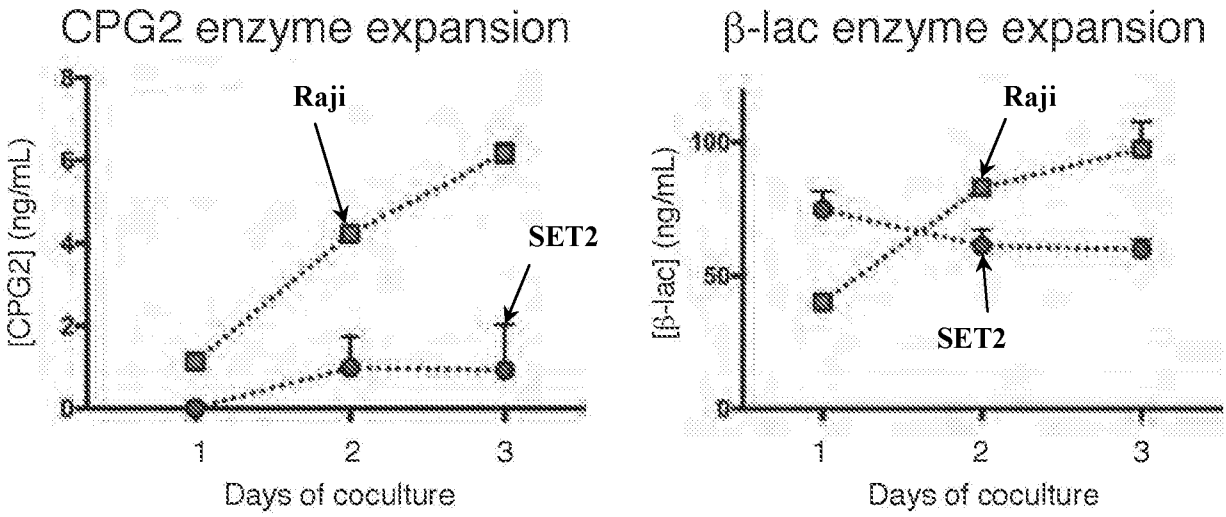

CPG2 enzyme expansion
[CPG2] (ng/mL)
Raji
SET2
0
1
2
3
Days of coculture
β-lac enzyme expansion
[β-lac] (ng/mL)
100
50
0
Raji
SET2
1
2
3
Days of coculture

FIG. 26A

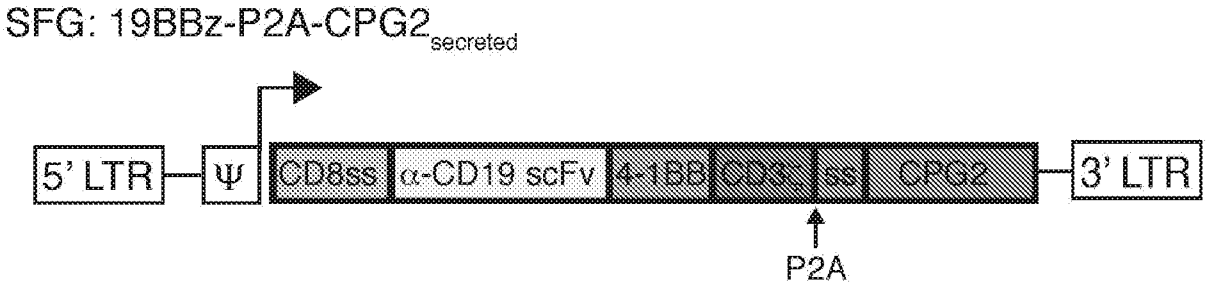


FIG. 26B

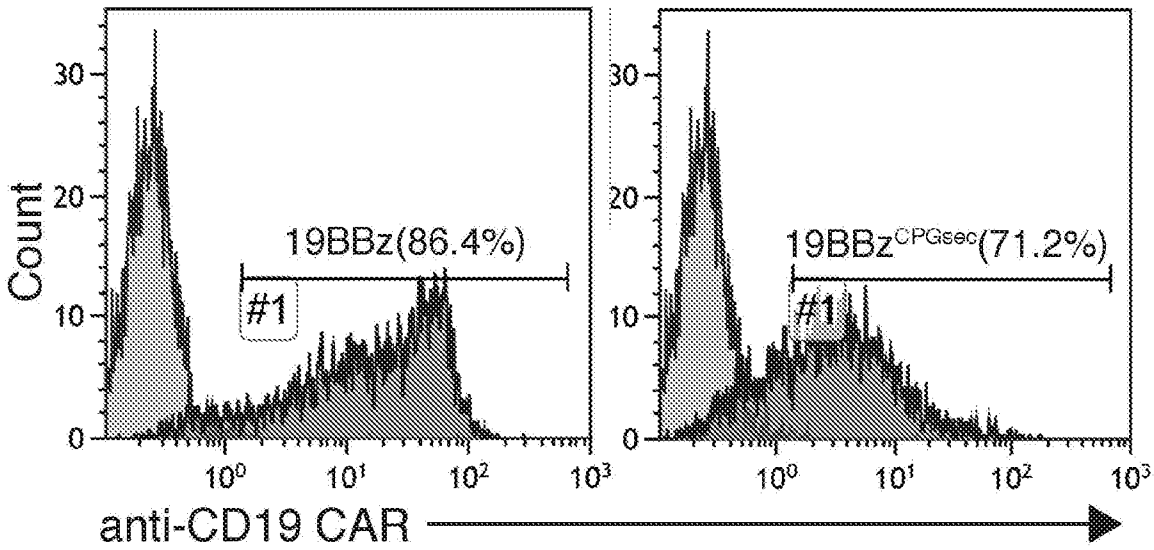


FIG. 26C

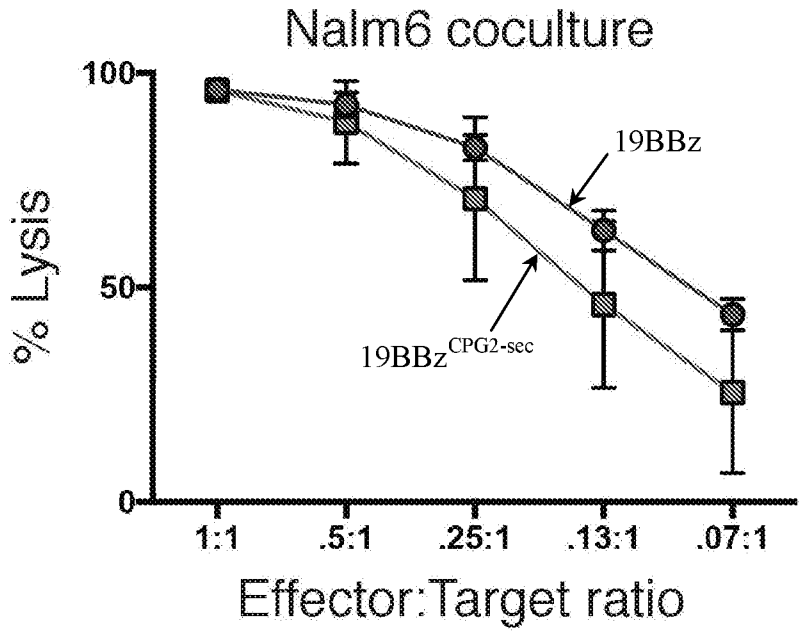


FIG. 26D

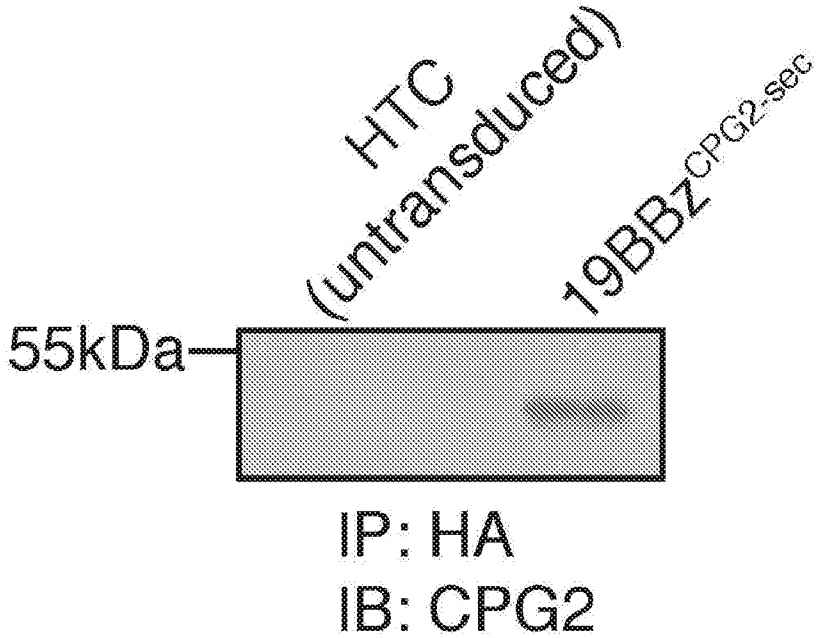


FIG. 26E

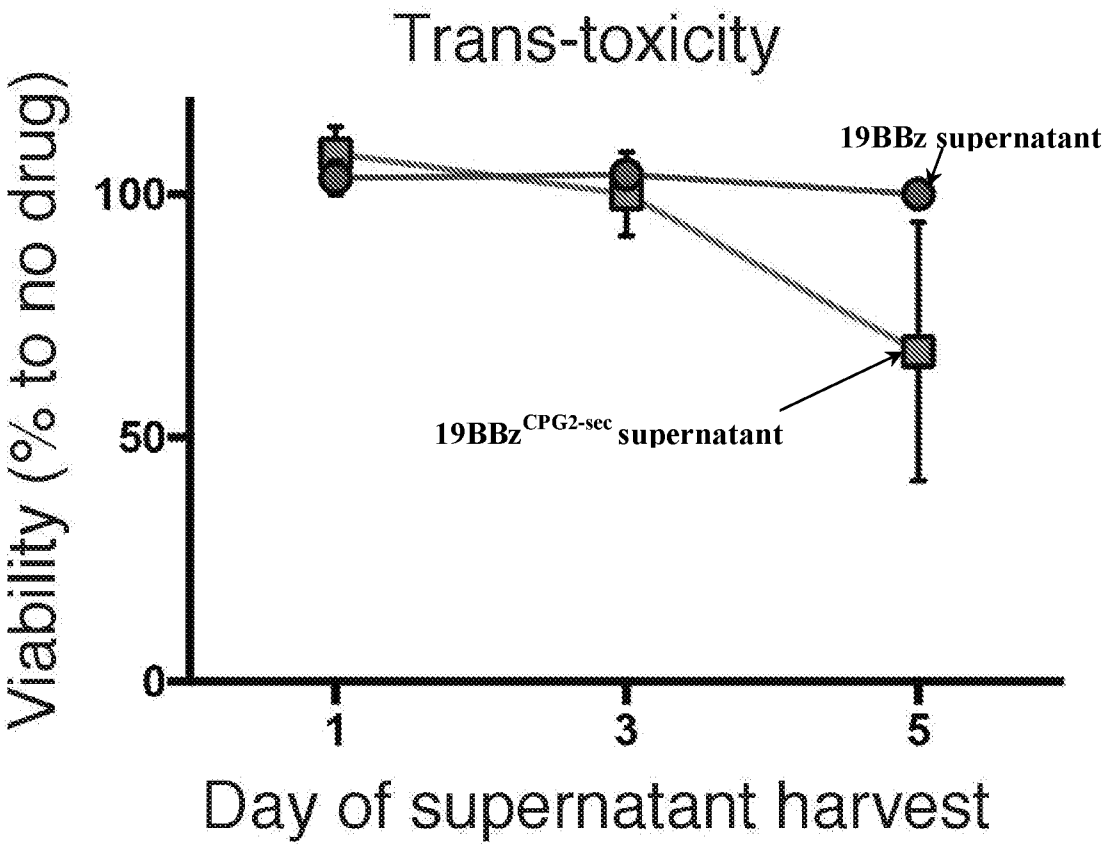

Trans-toxicity
Viability (% to no drug)
100
50
0
1
3
5
Day of supernatant harvest
19BBz supernatant
19BBz^CPG2-sec supernatant

FIG. 27A

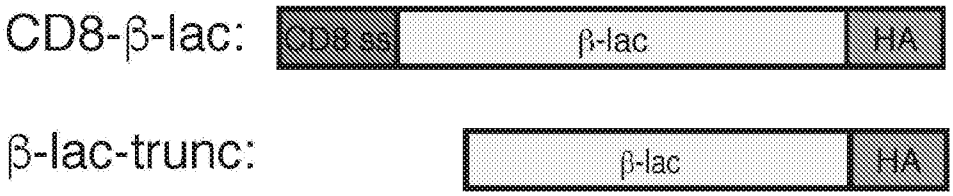


FIG. 27B

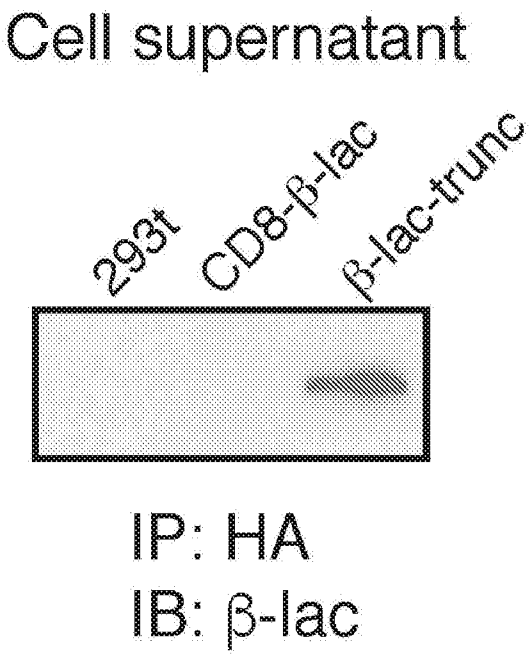


FIG. 27C

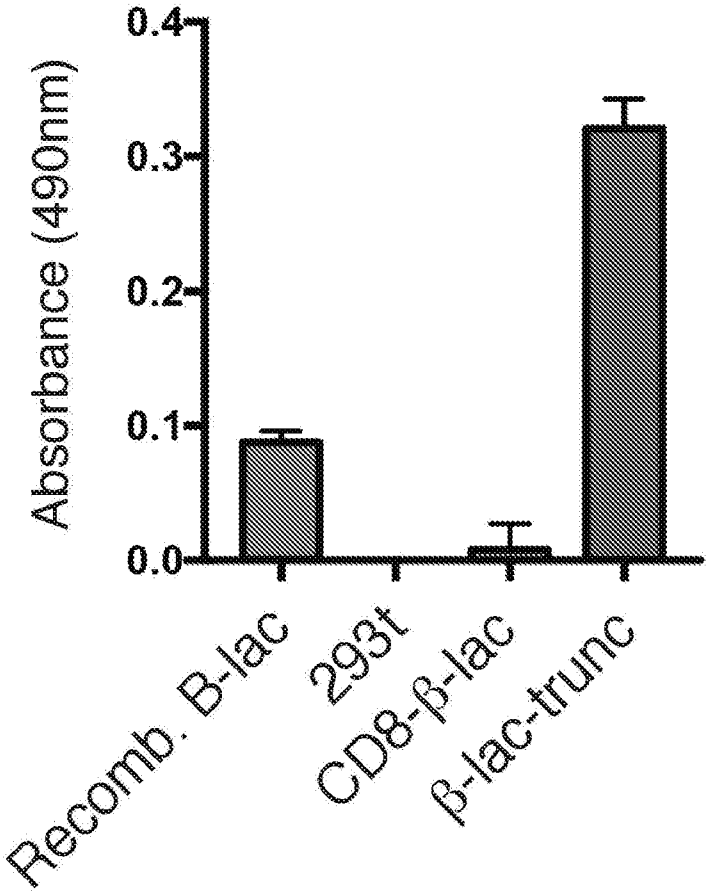

Absorbance (490nm)
0.4
0.3
0.2
0.1
0.0
Recomb. B-lac
293t
CD8-β-lac
β-lac-trunc

FIG. 28A

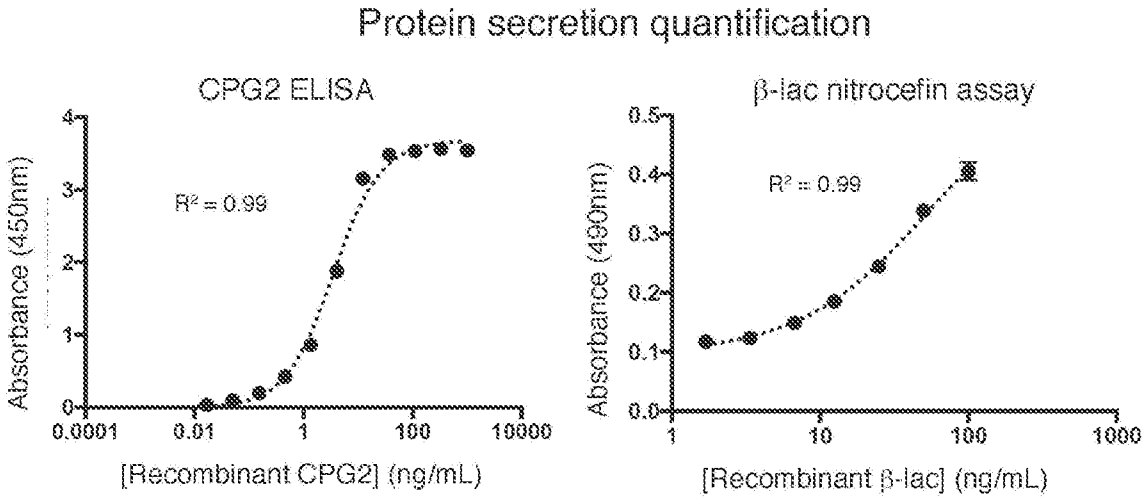


FIG. 28B

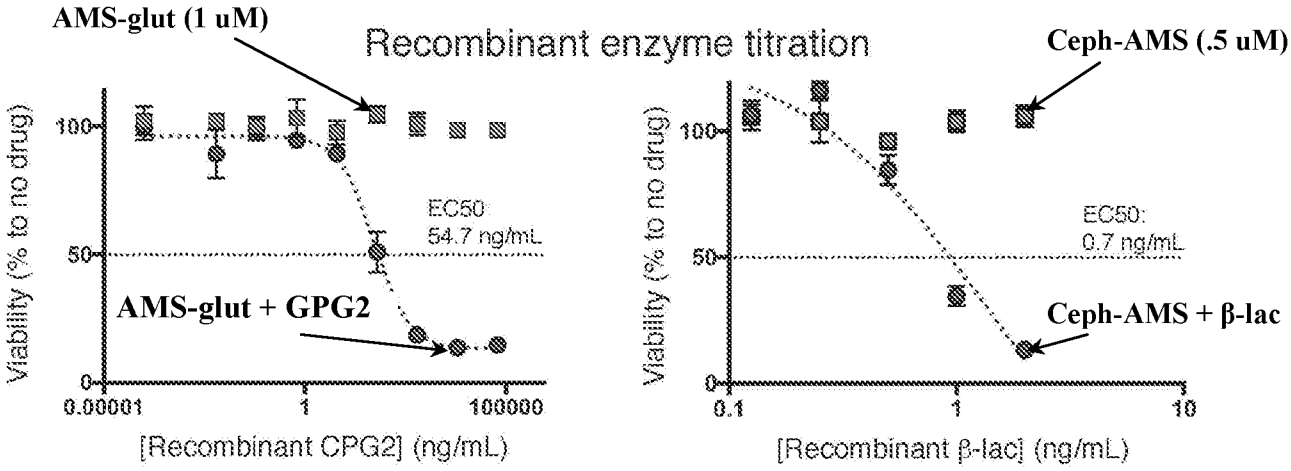


FIG. 28C

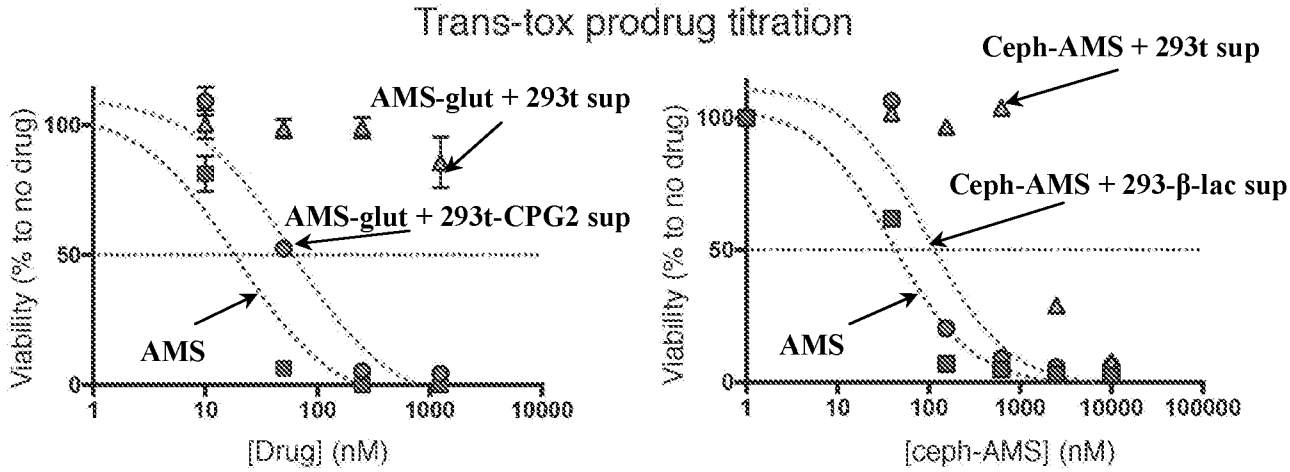


FIG. 28D

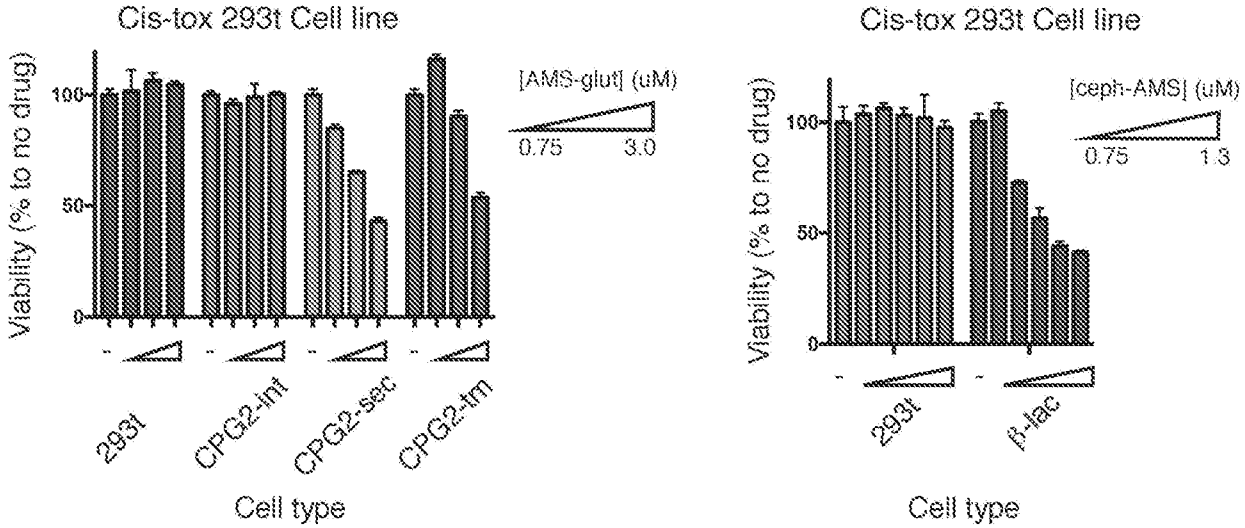


FIG. 28E

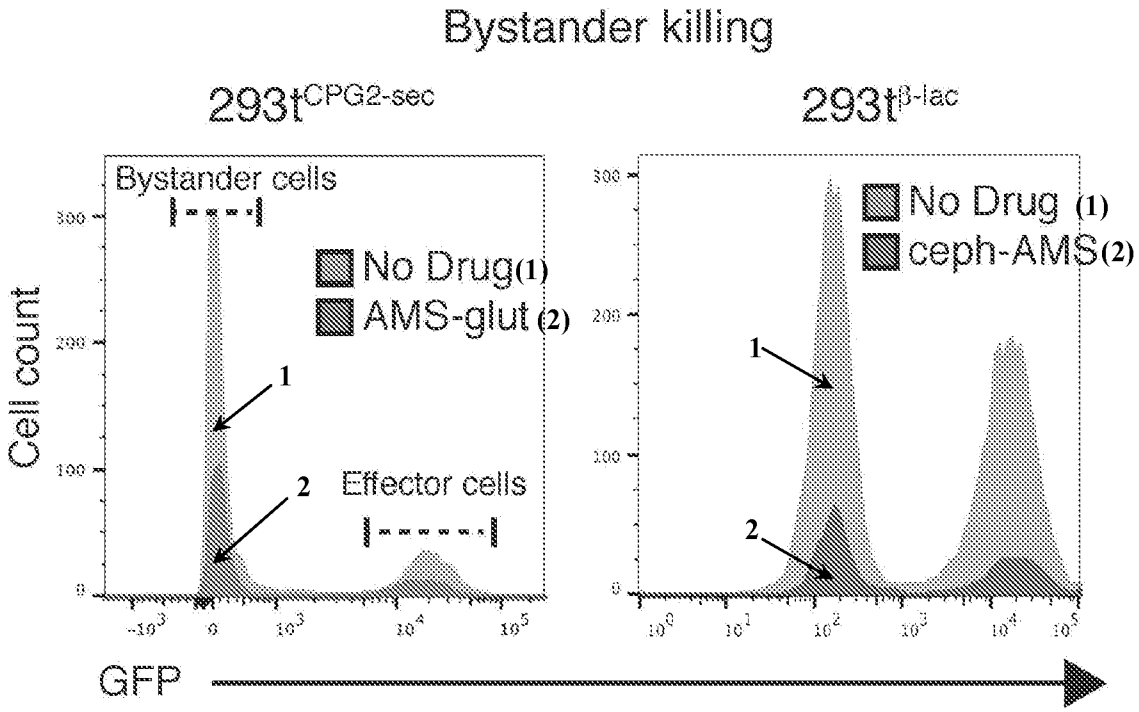

Bystander killing
293t^CPG2-sec
293t^β-lac
Bystander cells
No Drug(1)
AMS-glut (2)
1
2
Effector cells
Cell count
GFP
No Drug (1)
ceph-AMS(2)

FIG. 29A

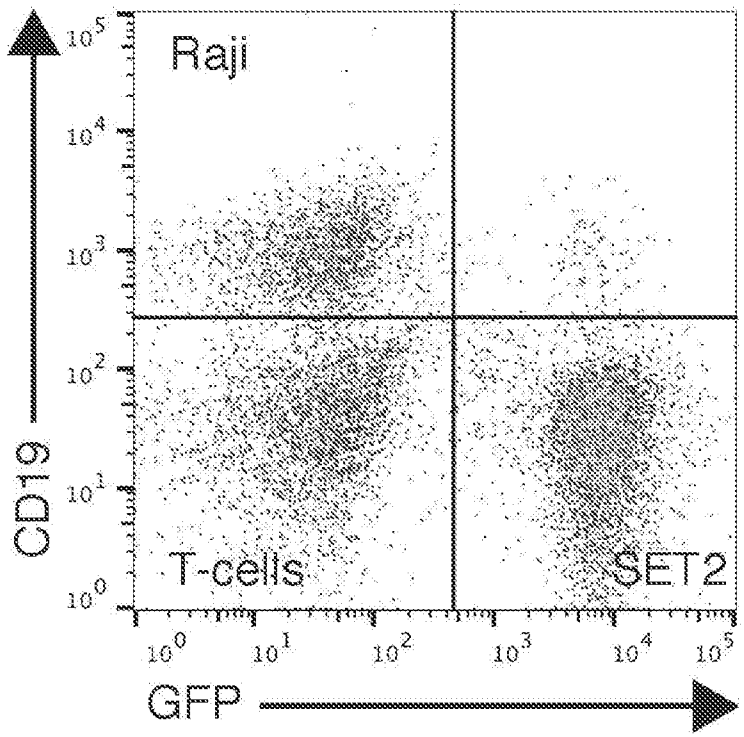


FIG. 29B

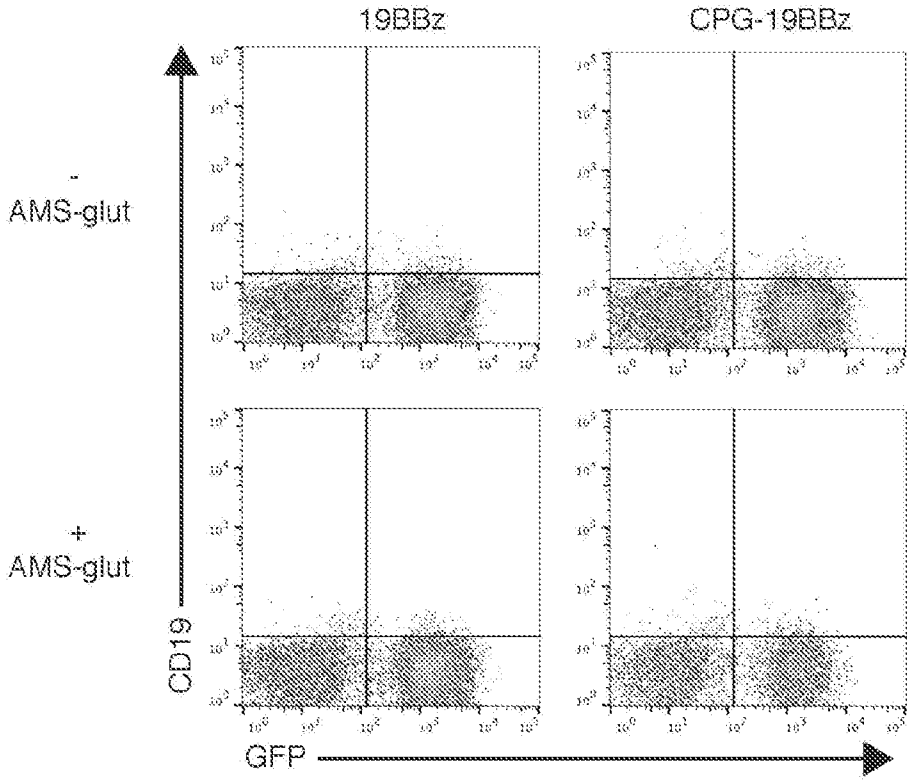


FIG. 29C

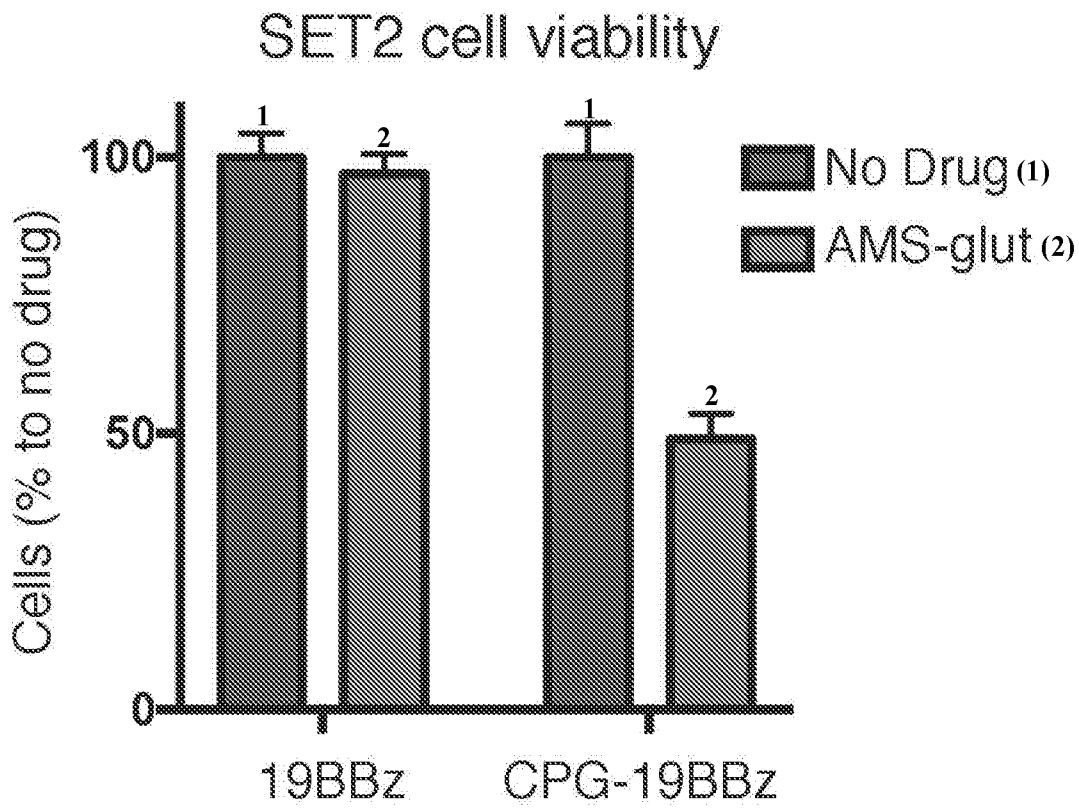

SET2 cell viability
Cells (% to no drug)
100
50
0
1
2
1
2
No Drug (1)
AMS-glut (2)
19BBz
CPG-19BBz

FIG. 30A

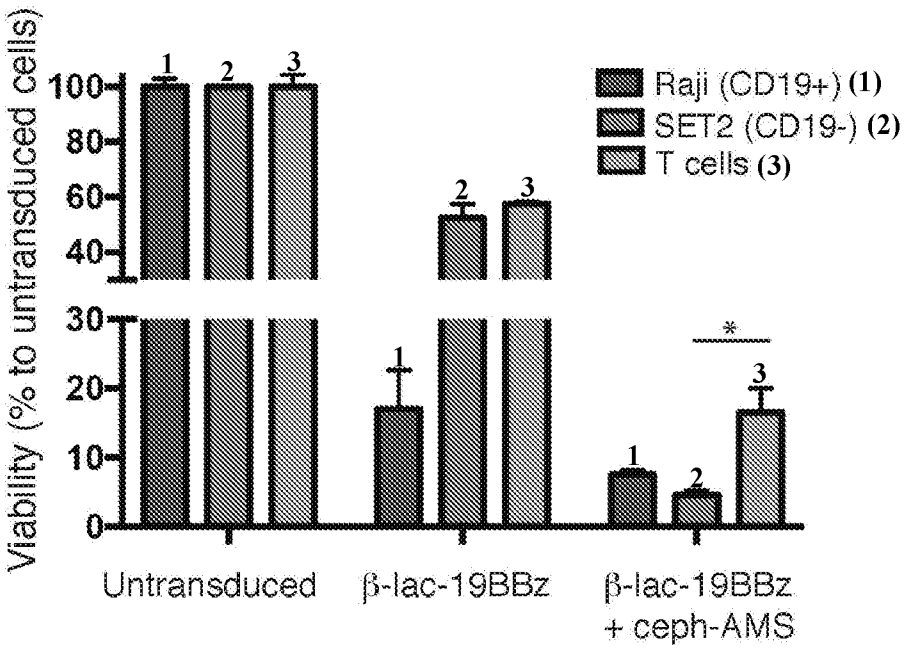


FIG. 30B

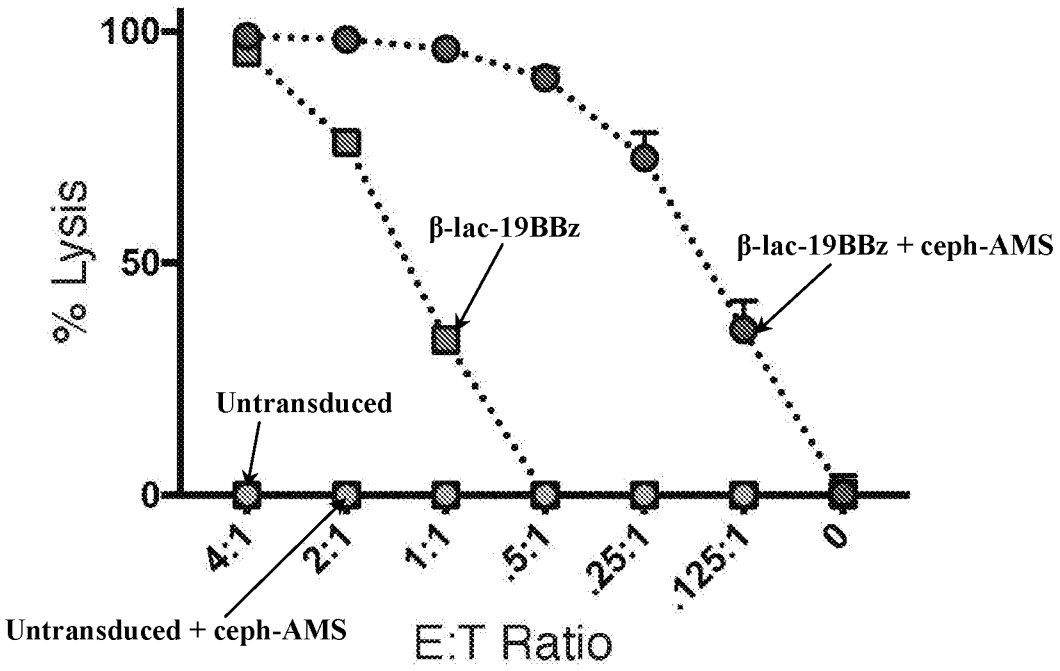


FIG. 31

Route	Group	Pretreatment	Analyte	Dose (mg/kg)	T_{max} (hr)	C_{max} (ng/mL)	AUC_{last} (hr*ng/mL)	AUC_{inf} (hr*ng/mL)	$T_{1/2}$ (hr)
IP	Group 1	Probenecid	Glutamate AMS	10	0.5	24997.27	32129.31	32221.26	0.49
			AMS	-	0.08	154.91	276.92	302.74	-
	Group 2	No pretreatment	Glutamate AMS	10	0.08	17635.83	12039.35	12103.91	0.64
			AMS	-	0.08	43.54	23.77	25.84	-
	Group	**Pretreatment**	**Analyte**	**Dose (mg/kg)**	**T_{max} (hr)**	**C_{max} (µM)**	**AUC_{last} (hr* µM)**	**AUC_{inf} (hr* µM)**	**$T_{1/2}$ (hr)**
	Group 1	Probenecid	Glutamate AMS	10	0.5	48.16	61.89	62.07	0.49
			AMS	-	0.08	0.45	0.8	0.87	-
	Group 2	No pretreatment	Glutamate AMS	10	0.08	33.97	23.19	23.32	0.64
			AMS	-	0.08	0.13	0.07	0.07	-

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US18/40633

A. CLASSIFICATION OF SUBJECT MATTER
IPC - C07K 16/00; C12N 5/10, 15/63 (2018.01)
CPC - C07K 16/00; C12N 5/10, 15/63

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
See Search History document

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

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
See Search History document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 7,094,572 B2 (RAMANATHAN, CS et al) 22 August 2006; column 18, lines 50-55; column 19, lines 57-62; column 20, lines 5-15; column 31, lines 15-25; column 49, lines 5-15; column 77, lines 40-45; column 109, lines 50-60; column 117, lines 60-65; column 118, lines 1-5; column 135, lines 1-5, 25-30; column 158, lines 25-40; column 193, lines 30-45; column 199, lines 40-45; column 210, lines 35-55	1-3, 5/1-3, 6/1-3, 7/1-3, 56-59, 60/56-59, 61/60/56-59
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Y		4/1-3, 62/61/60/56-59
Y	US 2011/0301331 A1 (GLASER, S et al) 8 December 2011; paragraphs [0103], [0115], [0123], [0125], [0375], [0687], [0709], [0769]; claims 61, 87	4/1-3
Y	US 5,426,029 A (RITTERSHAUS, CW et al) 20 June 1995; column 13, lines 1-5; column 36, lines 35-45; column 51, lines 30-40	62/61/60/56-59

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

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

Date of the actual completion of the international search	Date of mailing of the international search report
15 August 2018 (15.08.2018)	18 OCT 2018
Name and mailing address of the ISA/ Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-8300	Authorized officer Shane Thomas PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

Form PCT/ISA/210 (second sheet) (January 2015)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US18/40633

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

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

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

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

3. ☒ Claims Nos.: 8-55, 63-65, 72-79
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

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

This International Searching Authority found multiple inventions in this international application, as follows:
-***-Please See Extra Sheet Below-***-

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

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

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

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-7, 56-62

Remark on Protest

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

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

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.
PCT/US18/40633

-***-Continued from Box III: Observations where unity of invention is lacking-***-

This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Group I: Claims 1-7 and 56-62 disclose an engineered immune cell comprising a prodrug converting enzyme expressed in the cytoplasm and a method of preparing thereof, where the engineered immune cell is not a compound of Formula I, II or III.

Group II: Claims 66-68 and 83 disclose the compound of Formula I, where the compound of Formula I is not a compound of Formula II or III or the engineered immune cell.

Group III: Claims 69-71 disclose the compound of Formula II, where the compound of Formula II is not a compound of Formula I or III or the engineered immune cell.

Group IV: Claims 80-82 disclose the compound of Formula III, where the compound of Formula III is not a compound of Formula I or II or the engineered immune cell.

The inventions listed as Groups I-IV do not relate to a single general inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: the special technical features of Group I include an engineered immune cell comprising a prodrug converting enzyme expressed in the cytoplasm and a method of preparing thereof, where the engineered immune cell is not a compound of Formula I, II or III and is not present in Groups II-IV; the special technical features of Group II include the compound of Formula I, where the compound of Formula I is not a compound of Formula II or III or the engineered immune cell and is not present in Groups I and III-IV; the special technical features of Group III include the compound of Formula II, where the compound of Formula II is not a compound of Formula I or III or the engineered immune cell and is not present in Groups I-II and IV; and the special technical features of Group IV include the compound of Formula III, where the compound of Formula III is not a compound of Formula I or II or the engineered immune cell and is not present in Groups I-III.

No technical features are shared among Groups I-IV, accordingly, these groups lack unity a priori.