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(54) **REJUVENATION OF CAR T CELL**

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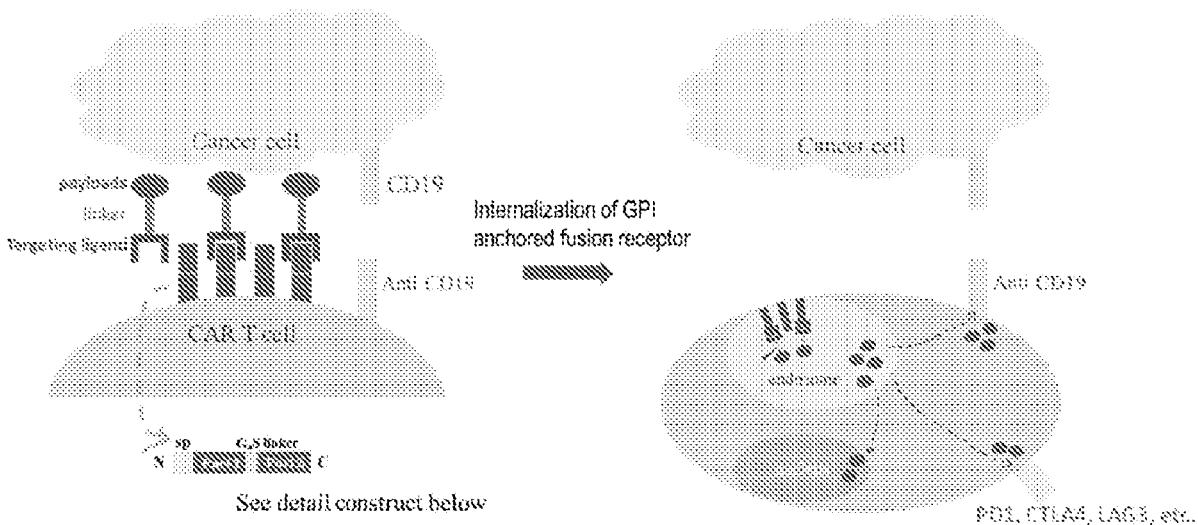
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(57) **ABSTRACT**

A payload of drug conjugated to a targeting ligand specifically designed to deliver to exhausted CAR T cells to rejuvenate these CAR T cells is provided herein. The targeted CAR T cells are modified with a fusion receptor which can bind to the targeting ligand and internalize the conjugated payload of drug to execute its regulatory function to exhausted CAR T cell.



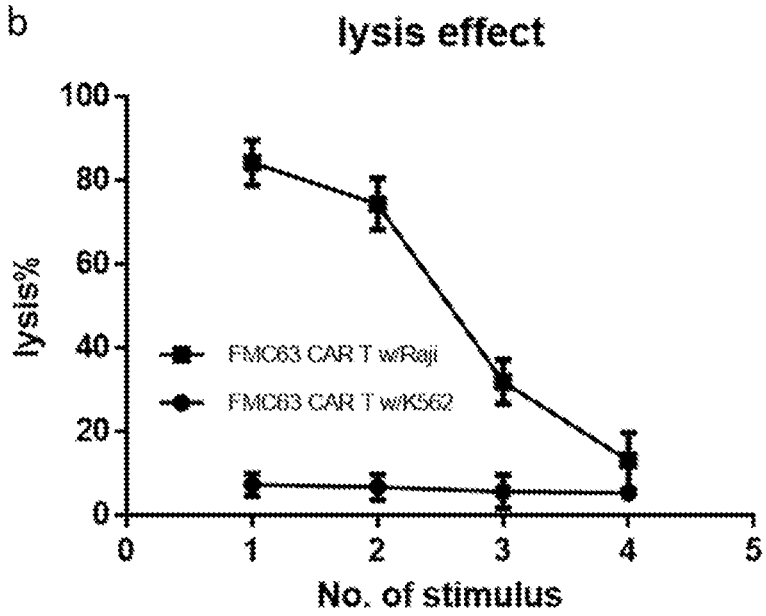


FIGURE 1

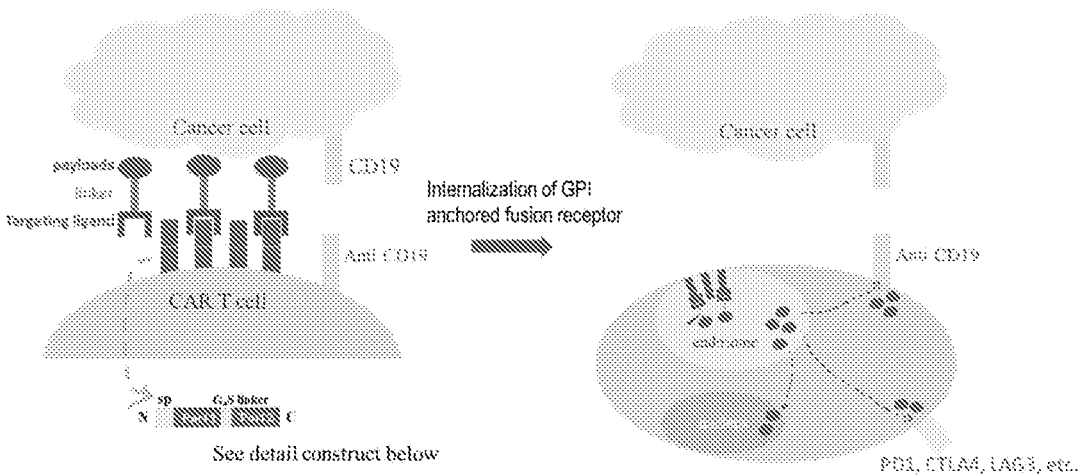


FIGURE 2

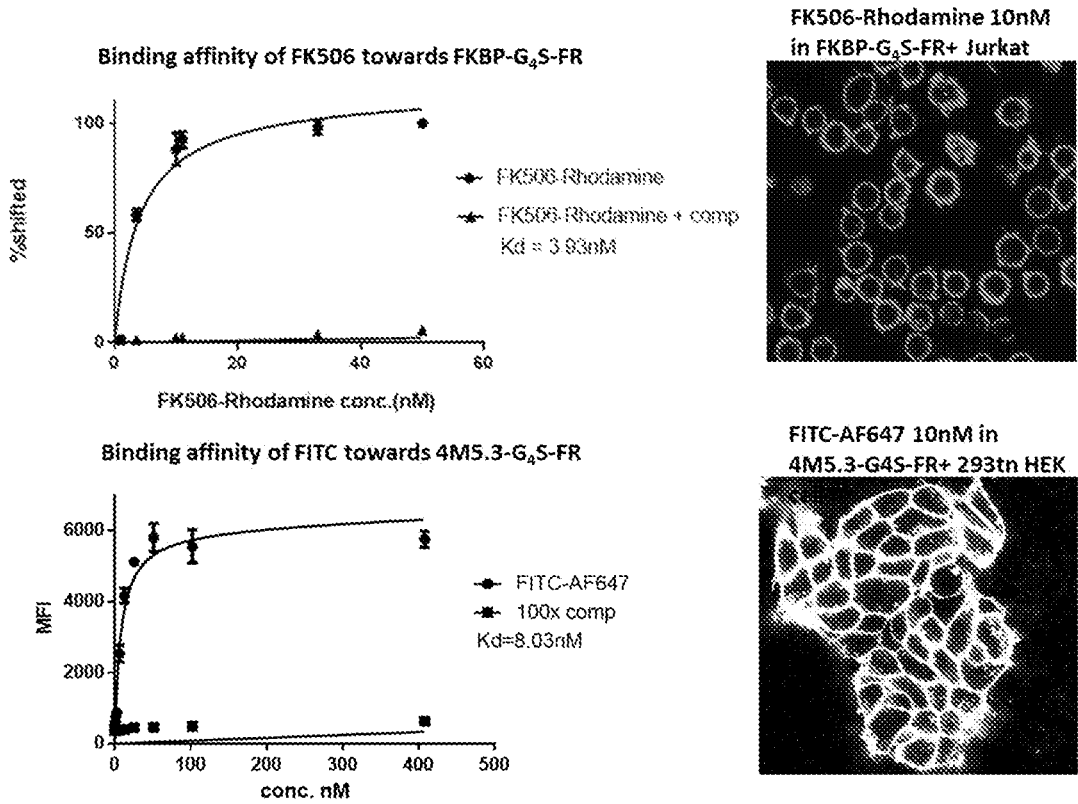
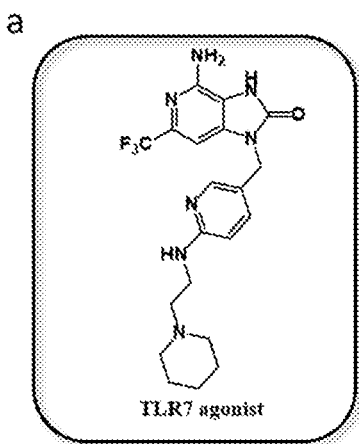


FIGURE 3



Chemical Formula: $C_{20}H_{24}F_3N_7O$
Molecular Weight: 435.46

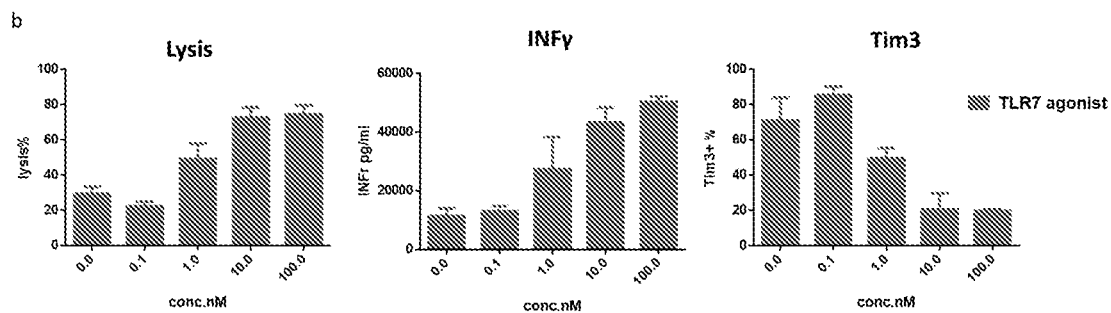


FIGURE 4

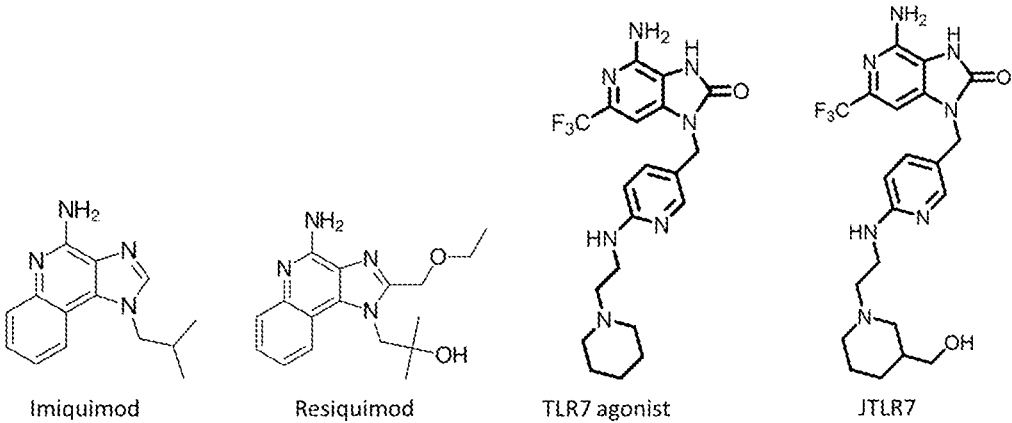


FIGURE 5

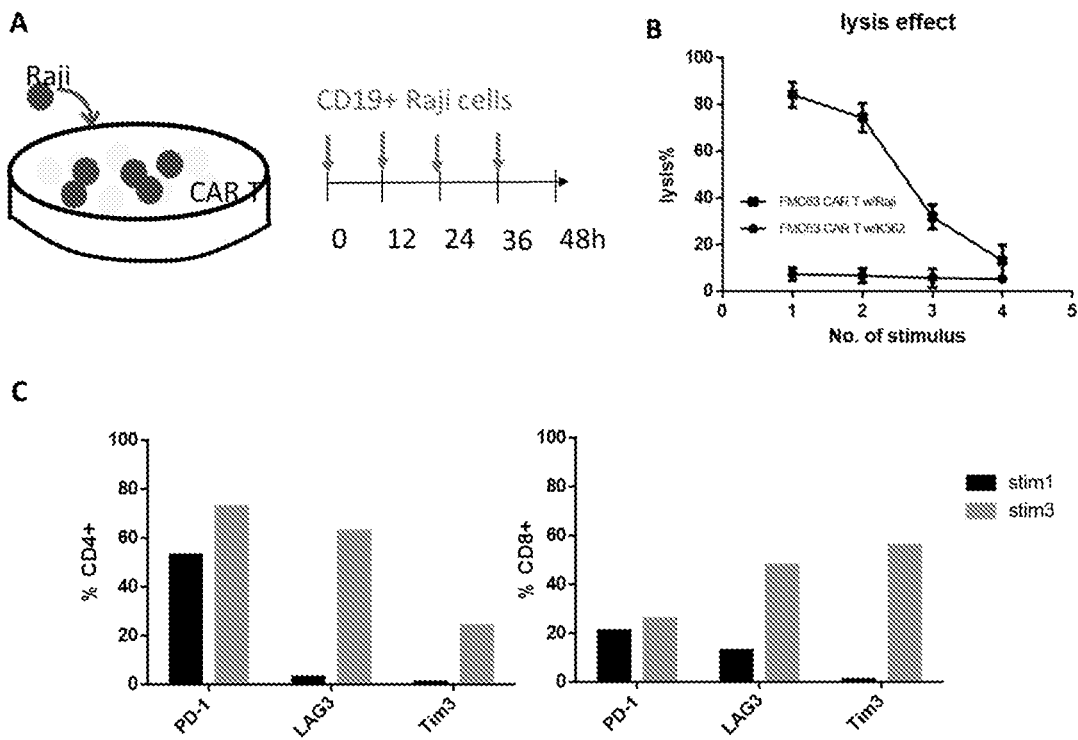


FIGURE 6

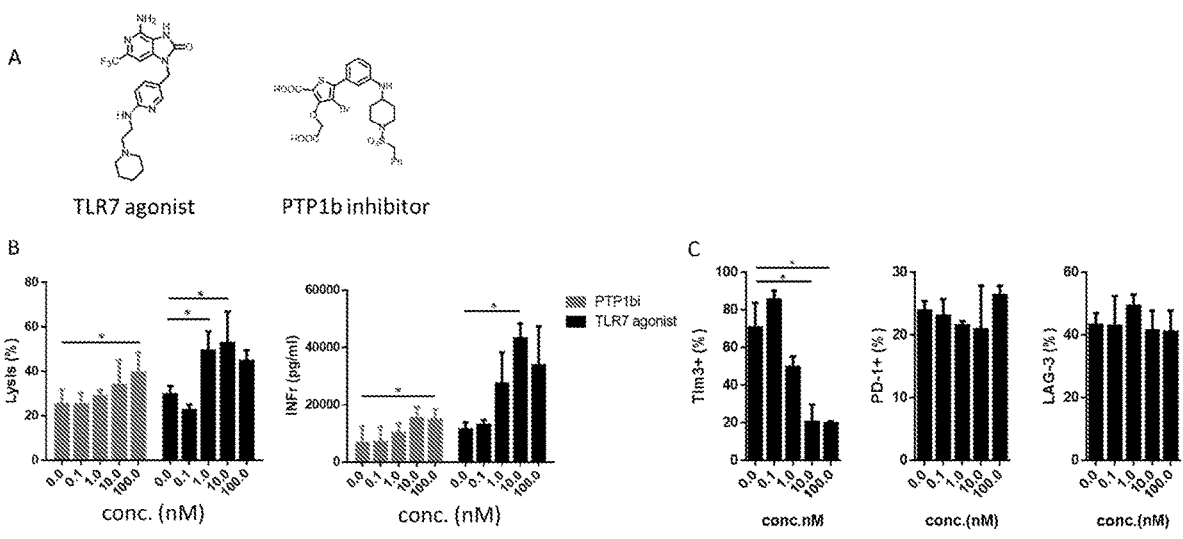


FIGURE 7

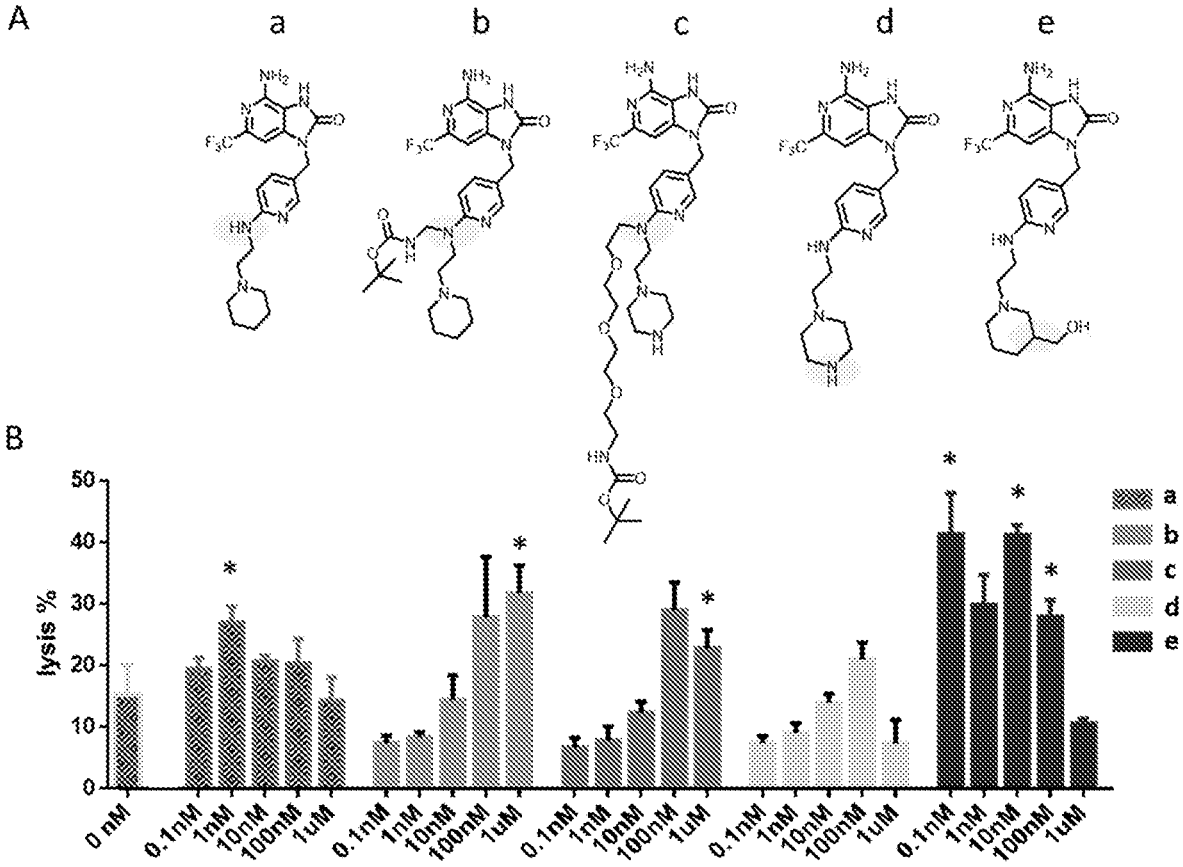


FIGURE 8

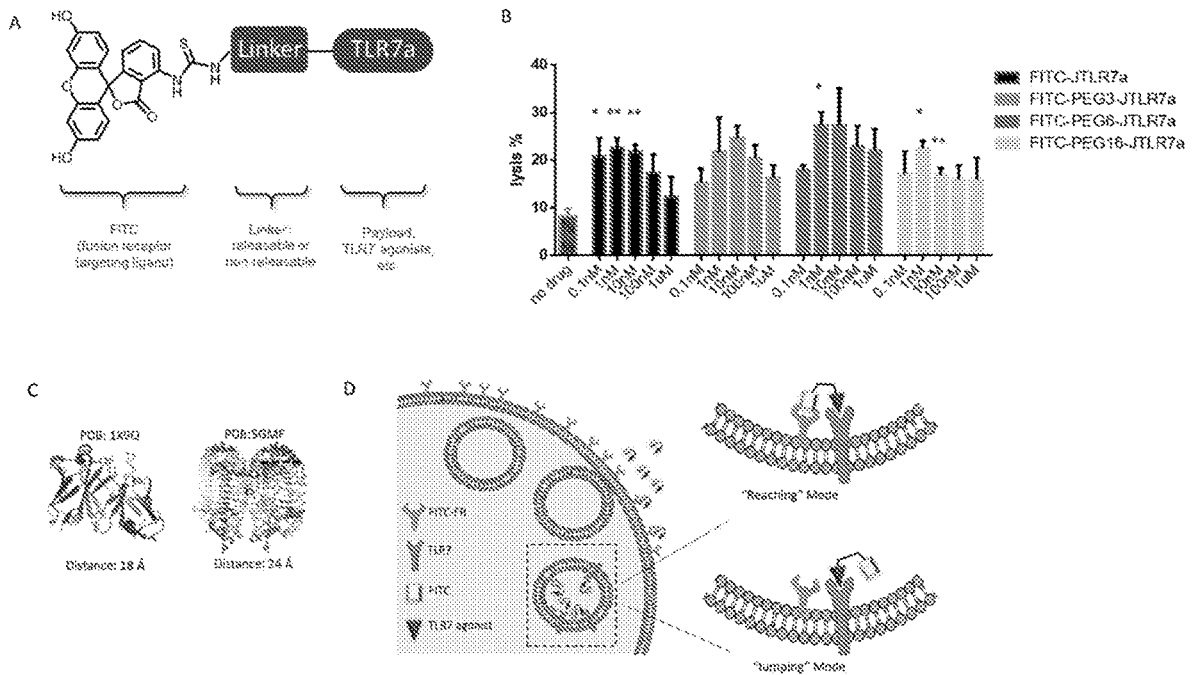


FIGURE 9

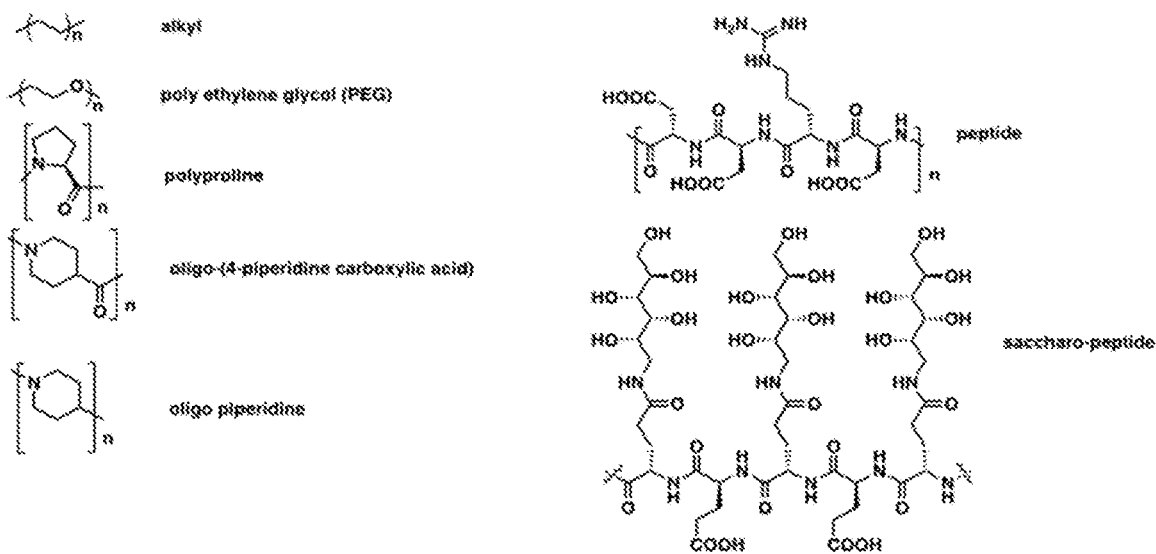


FIGURE 10

REJUVENATION OF CAR T CELL

FIELD OF INVENTION

[0001] This disclosure provides a system to rejuvenate cancer antigen exhausted chimeric antigen receptor T (CAR T) cells. Specifically, the system comprising a fusion receptor in a classical CAR construct, wherein the fusion receptor provides a ligand binding module that recognizes a high affinity ligand-payload drug conjugate to deliver payload of drugs that are designed to either block the inhibitory signaling in the exhausted CAR T, or to re-activate CAR T through an antigen independent pathway.

BACKGROUND

[0002] The field of chimeric antigen receptor (CAR) T cell therapy has made tremendous progress over the last two decades. The CAR construct consists of four parts: (1) an extracellular binding moiety against tumor specific antigen, (2) a hinge domain, (3) a transmembrane domain and (4) a combination of various activation domains, for example, CD28, 4-1BB and CD3 ζ chain. The most impressive successes have been seen in CAR T therapy against CD19 positive B cell leukemias, where more than 80% complete remission rate has been achieved in several clinical trials

[0003] In contrast, the treatment of solid tumor by CAR T has so far proven to be more challenging. Great successes have been achieved in preclinical study using syngeneic mice model or xenograft tumor model in immunodeficient mice. However, none of the clinical trials involving solid tumor, for example, breast cancer, ovarian cancer or lung cancer has shown improvement compare to control. In general, it suffers from the same limitations that are faced by other adoptive cell therapies, including (1) poor tumor penetration; (2) hypoxic pressure; (3) an immunosuppressive tumor microenvironment which includes tumor associated macrophages, fibroblasts and suppressive cytokines¹. CAR T cells, as same as tumor infiltrated lymphocytes (TIL), can be re-educated by this suppressive microenvironment and turn to a "hypofunctional" status, which is characterized by overexpression of co-inhibitory molecules (i.e., PD-1, Tim-3, LAG-3 etc.), decreased INF γ secretion and killing capability. Data from the patient samples of ovarian cancer shows that the majority of PD-1⁺CD8⁺ T cell lacked expression of CD127, which is known to be important for the effector-to-memory transition in T cell. Overexpression of LAG-3 also negatively correlated with the effector function of TCR specific CD8⁺ TIL. In addition, PD-1⁺LAG-3⁺ double positive T cell exhibited lower INF γ production². Similarly, NY-ESO-1 TCR specific human T

cell became hypofunctional in mice solid tumor model, showing high expression of the co-inhibitory molecules and less efficient anti-tumor effect, due to both the microenvironment and the constant activation of T cell by the continuous exposure to antigen³. Therefore, a reversion of the suppressive microenvironment, more importantly, a rejuvenation of the exhausted CAR T cell is highly desired for a better solid tumor treatment.

SUMMARY OF THE INVENTION

[0004] This disclosure provides system to rejuvenate an exhausted classical CAR T cell. The system comprises at least two components: a first component is a conjugate comprising a targeting ligand covalently linked to a payload of drug; and a second component is a targeting ligand binding module linked to membrane-anchoring module. The targeting ligand binding module of the second component recognizes the targeting ligand in the first component with high affinity to form a complex, and the payload drug either blocks the inhibitory signaling of the exhausted CAR T, or re-activates said CAR T through an antigen independent pathway. The membrane-anchoring module mediates internalization of the two component complex into the exhausted CAR T cell.

[0005] In some preferred embodiment, the aforementioned targeting ligand of the first component is folate, FITC or FK506.

[0006] In some preferred embodiment, the aforementioned targeting ligand binding module of the second component comprises a folate receptor, an anti-FITC antibody fragment or FKBP.

[0007] In some preferred embodiment, the aforementioned membrane-anchoring module is a folate receptor.

[0008] In some preferred embodiment, the aforementioned first component comprises a releasable linker between the targeting ligand and the payload drug.

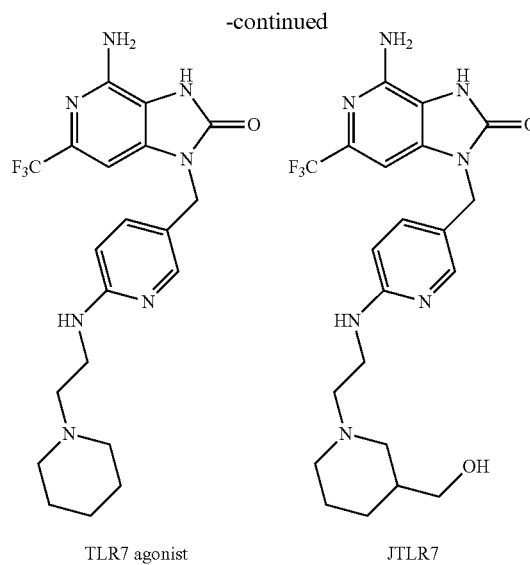
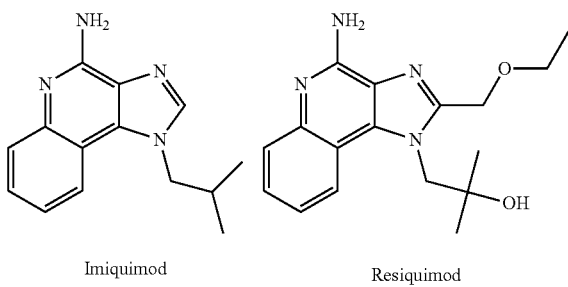
[0009] In some preferred embodiment, the aforementioned first component comprises a non-releasable linker between the targeting ligand and the payload drug.

[0010] In some preferred embodiment, the binding affinity between aforementioned targeting ligand and the targeting ligand-binding module is in sub-nanomolar range.

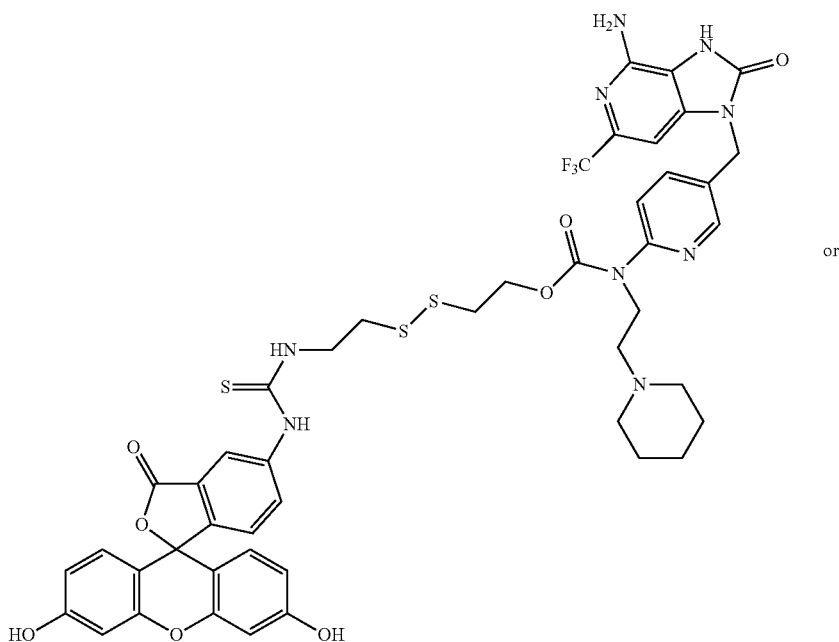
[0011] In some preferred embodiment, the aforementioned payload of drug is a Toll Like Receptor 7 (TLR7) agonist or Simulator of interferon genes (STING) agonist.

[0012] In some preferred embodiment, the aforementioned payload of drug is an inhibitor to following proteins: SHP1/2, TC-PTP or DGK α , TGF β .

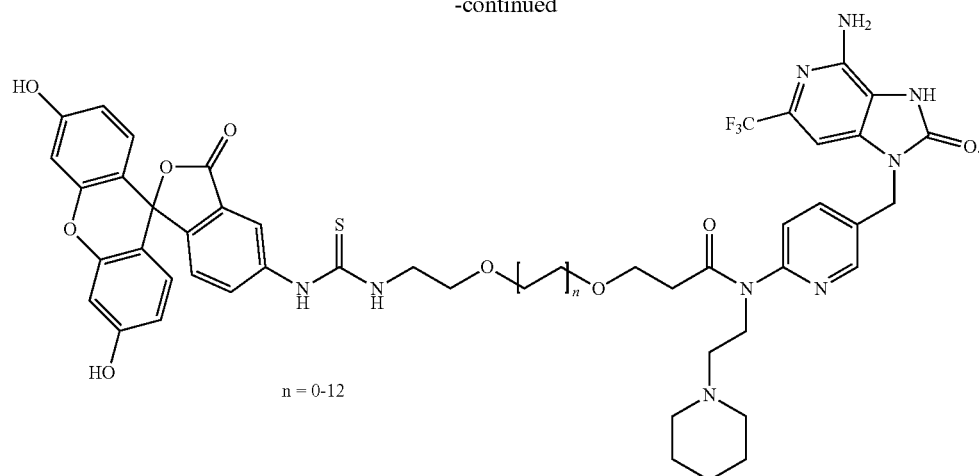
[0013] In some preferred embodiment, the aforementioned TLR7 agonist has the structure of or



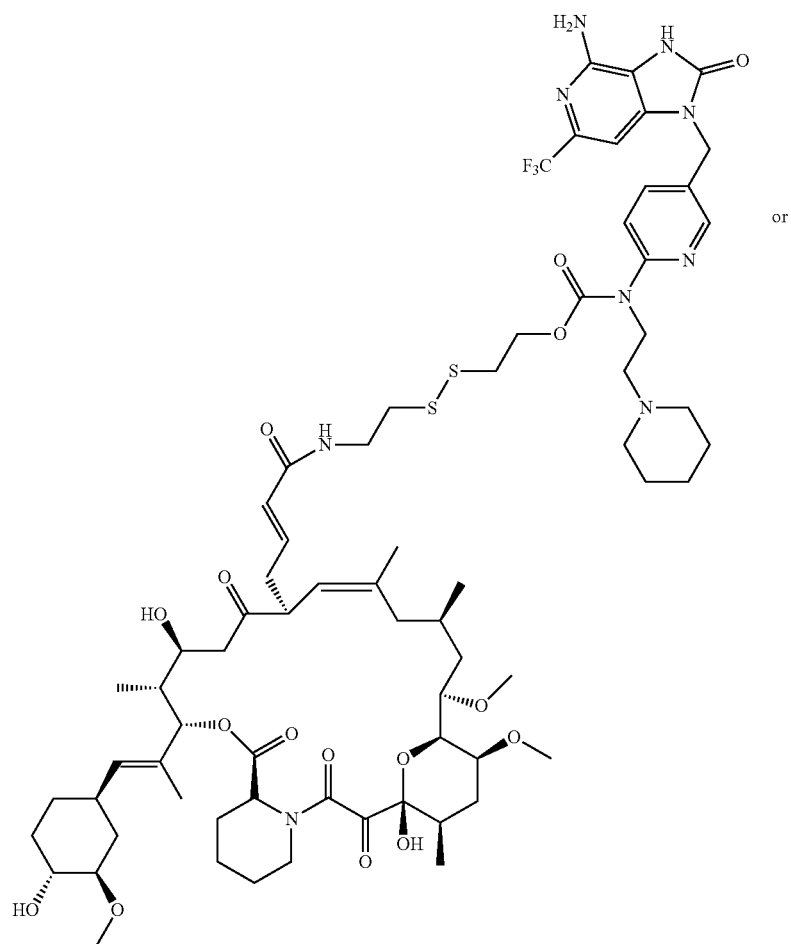
[0014] In some preferred embodiment, the aforementioned first component is a Fluorescein-TLR7 agonist having the structure of



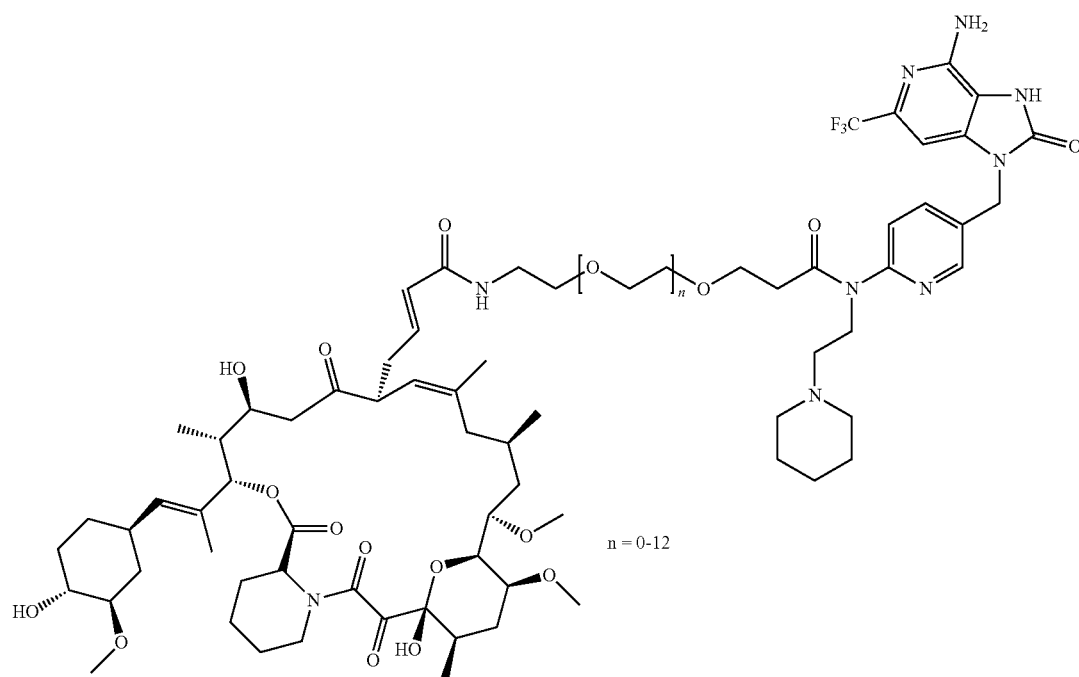
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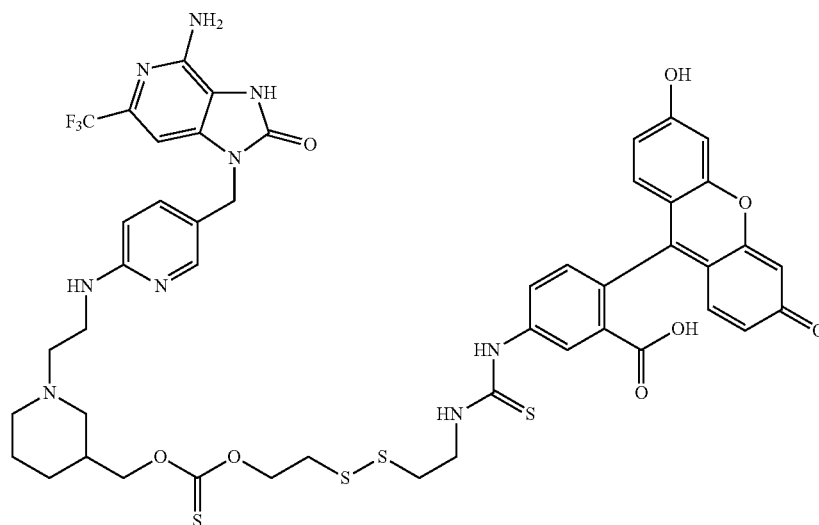
[0015] In some preferred embodiment, the aforementioned first component is a FK506-TLR7 agonist having the structure of



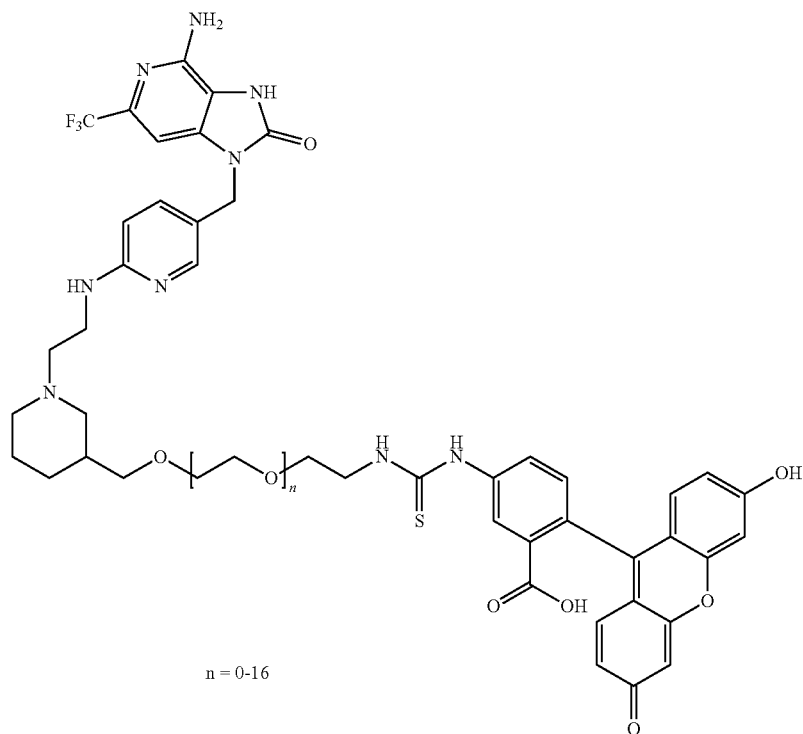
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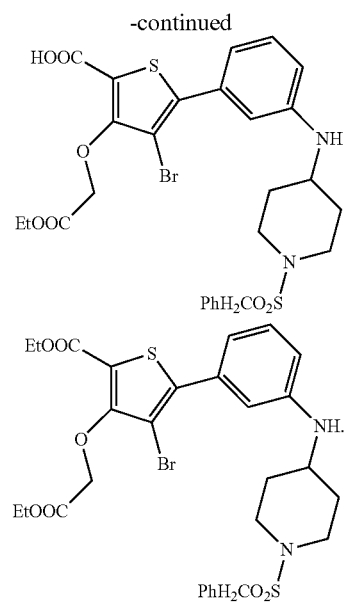
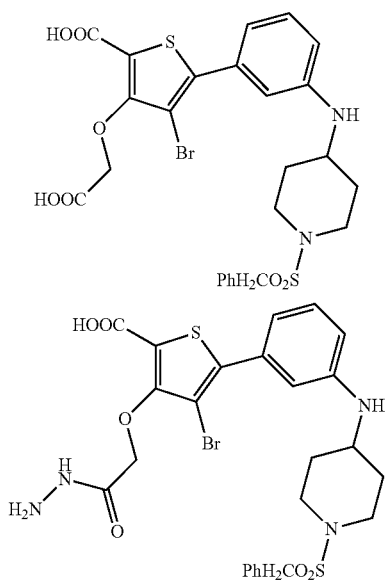
[0016] In some preferred embodiment, the aforementioned first component is one of the following:



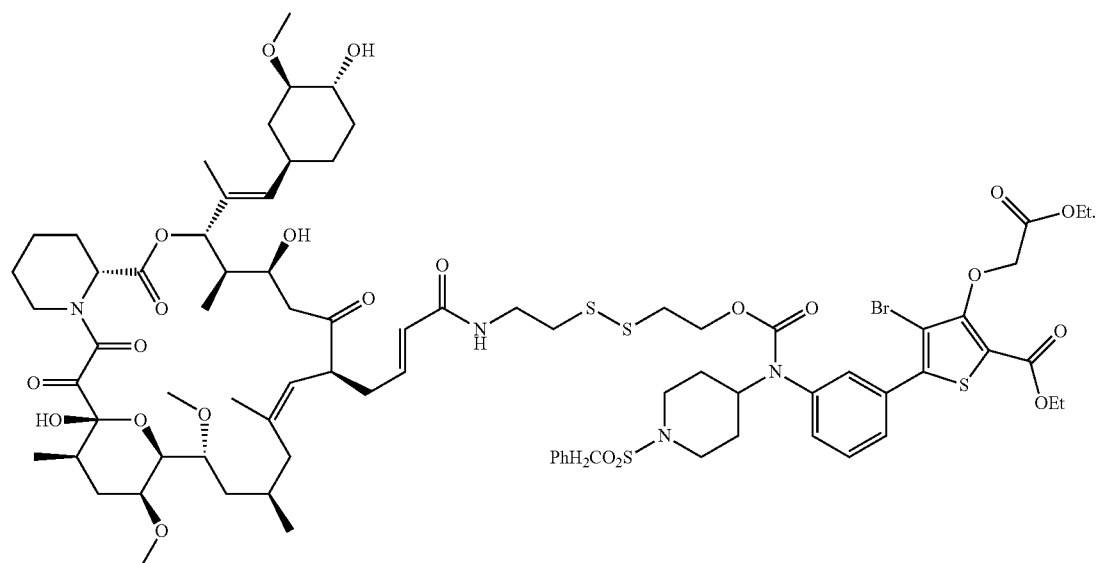
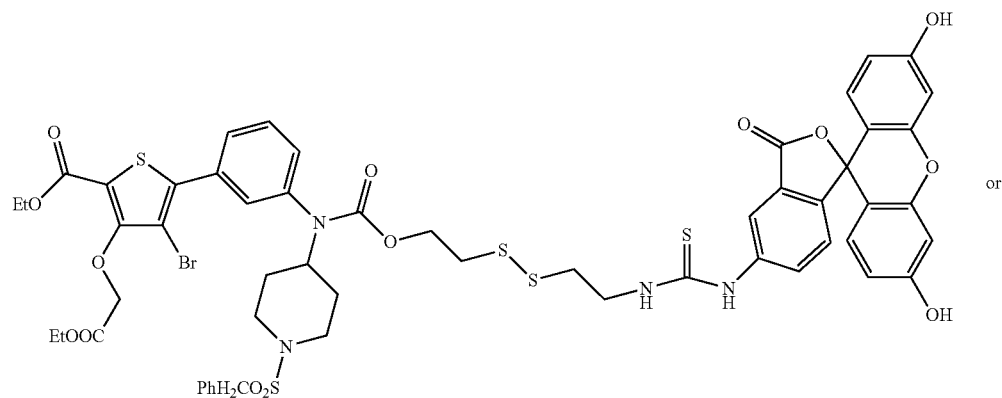
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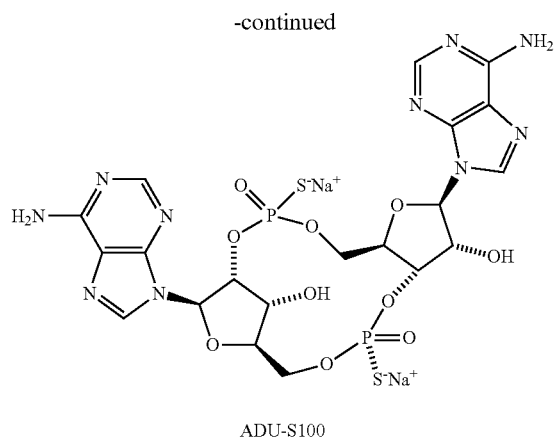
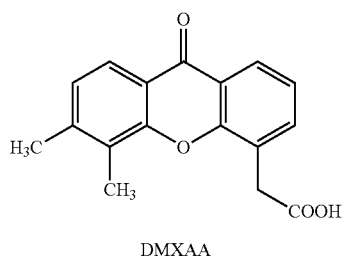
[0017] In some preferred embodiment, the aforementioned first component comprising the payload drug selected from the group consisting of following TC-PTP phosphatase inhibitors:



[0018] In some preferred embodiment, the aforementioned phosphatase inhibitor is connected to the fluorescein or FK506 (tacrolimus) to form the following structures:

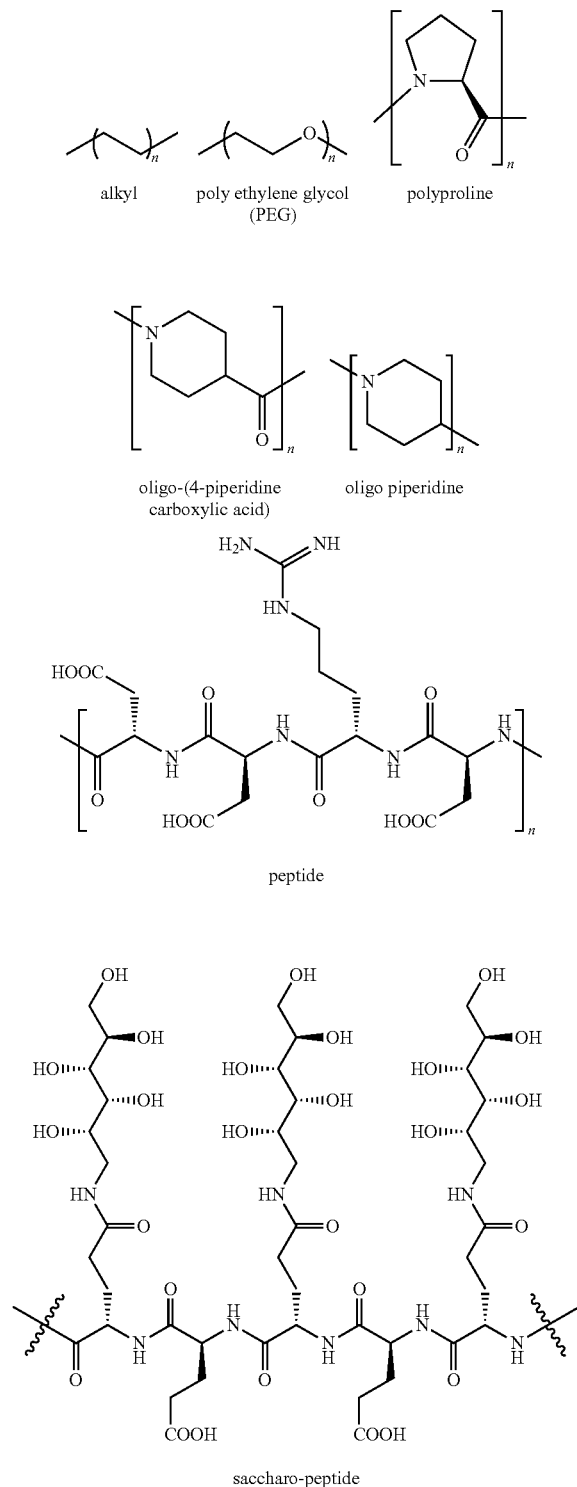


[0019] In some preferred embodiment, the aforementioned payload drug in the first component comprises a STING agonist of one of the following structures.



[0020] In some preferred embodiment, the aforementioned first component comprises a spacer between the

targeting ligand and the payload drug selected from the group consisting of the following structures:



[0021] This disclosure further provides a method to rejuvenate an exhausted CAR T cell. The method comprises the steps of:

[0022] a. providing the exhausted CAR T cell a first component comprising a conjugate, wherein the conjugate comprises a targeting ligand covalently linked to a payload of drug through a releasable or non-releasable linker;

[0023] b. providing said exhausted CAR T cell a second component comprising a fusion receptor linked to the exhausted CAR construct, wherein the fusion receptor comprises a targeting ligand binding module and a membrane-anchoring module;

[0024] c. letting the targeting ligand binding module of the second component bind to the targeting ligand in the first component to form a complex,

[0025] d. letting the membrane-anchoring module mediate internalization of the complex into the exhausted CAR T cell;

[0026] e. letting the payload drug either block the inhibitory signaling of the exhausted CAR T, or reactivate said CAR T through an antigen independent pathway.

[0027] In some preferred embodiment, the aforementioned method carries a payload drug executing its function within the endosome of the exhausted CAR T, and the targeting ligand and the payload drug are linked by a nonreleasable linker.

[0028] In some preferred embodiment, the aforementioned method carries a payload drug executing its function as a free drug in the cytosol of the exhausted CAR T, and the targeting ligand and the payload drug are linked by a releasable linker.

[0029] In some preferred embodiment, the targeting ligand of the first component is folate, FITC or FK506 in aforementioned method.

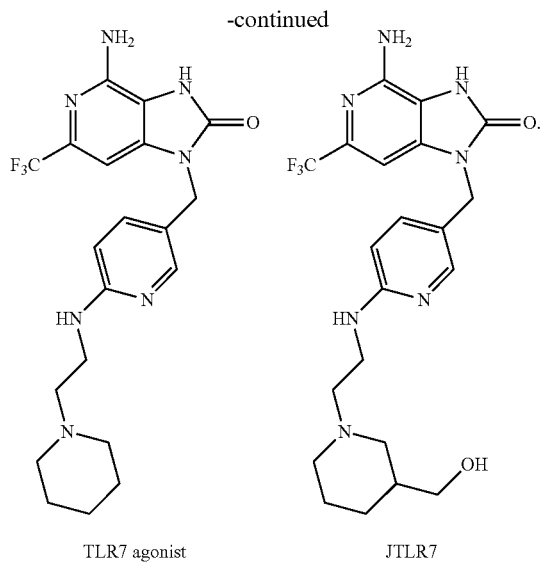
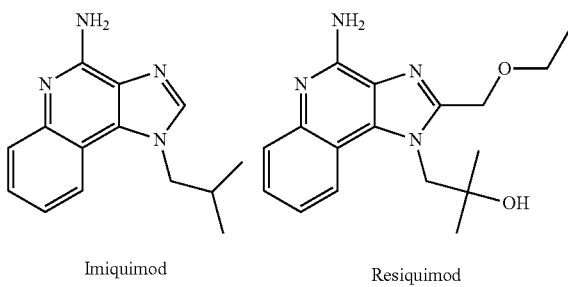
[0030] In some preferred embodiment, the targeting ligand-binding module of the second component in aforementioned method is anti-FITC, folate receptor, or FKBP.

[0031] In some preferred embodiment, the targeting ligand-binding module of the second component in aforementioned method is Folate Receptor alpha (FR α).

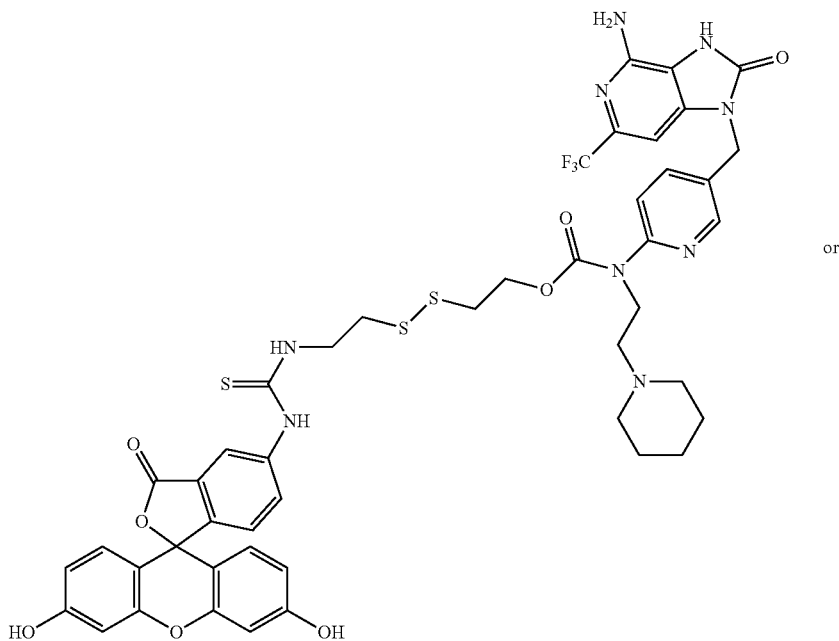
[0032] In some preferred embodiment, the payload of drug of the first component in aforementioned method is a Toll Like Receptor 7 (TLR7) agonist or Simulator of interferon genes (STING) agonist.

[0033] In some preferred embodiment the payload of drug the first component in aforementioned method is an inhibitor to following proteins: SHP1/2, TC-PTP or DGK α , TGF β .

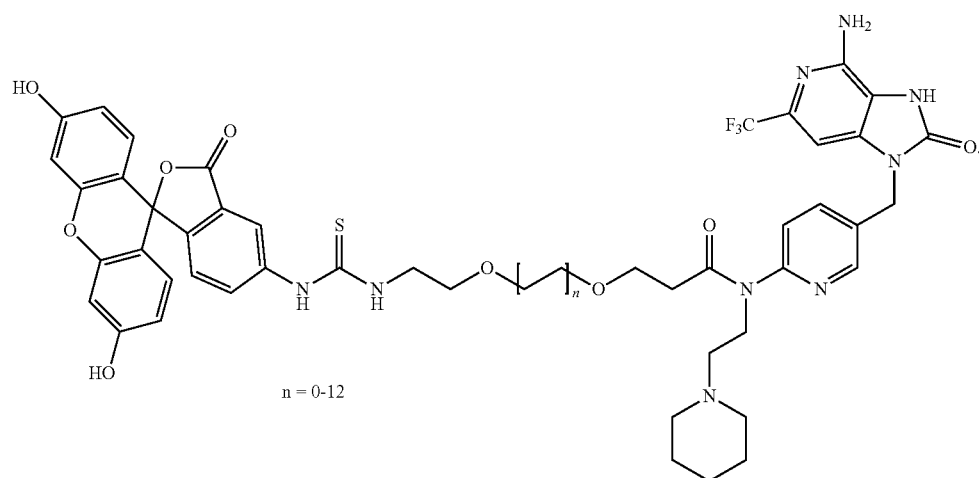
[0034] In some preferred embodiment the TLR7 agonist in aforementioned method has the structure of



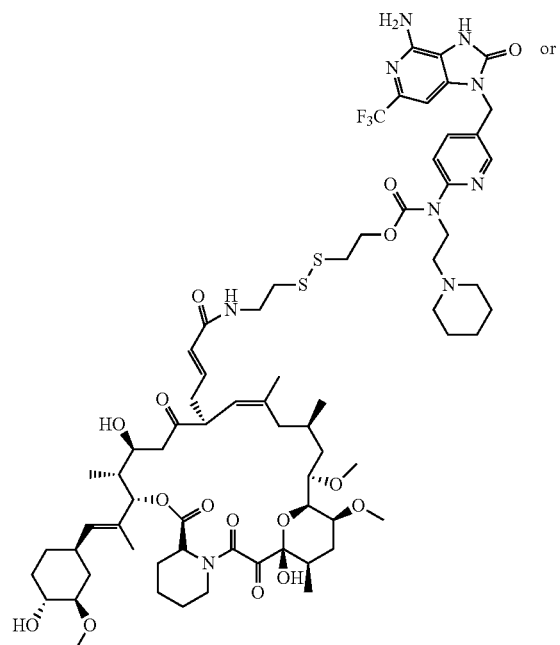
[0035] In some preferred embodiment, the first component in aforementioned method is a Fluorescein-TLR7 agonist having the structure of



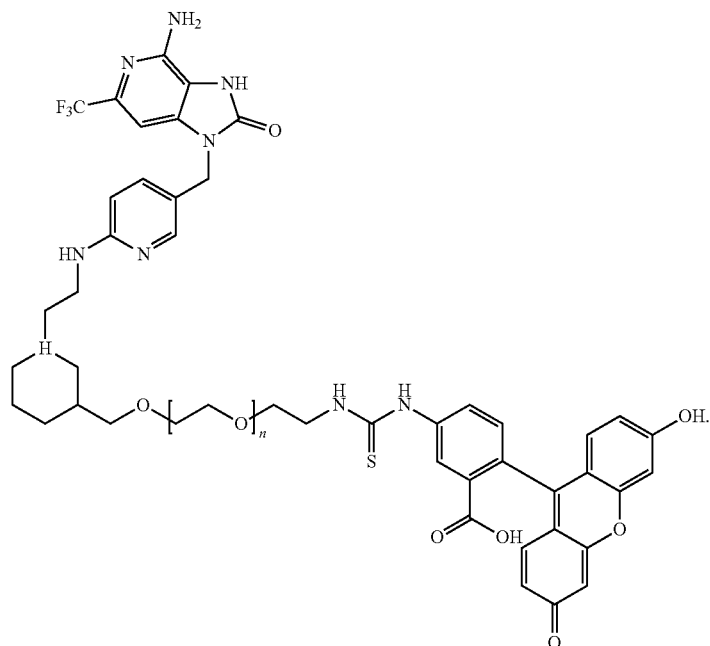
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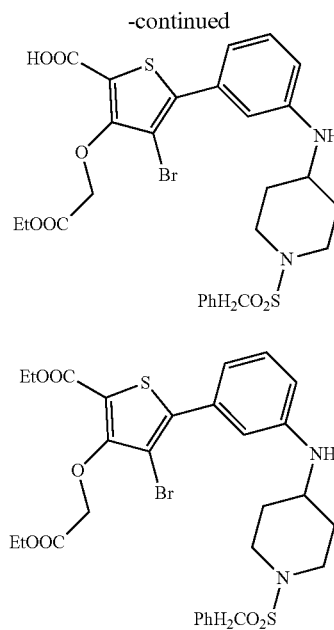
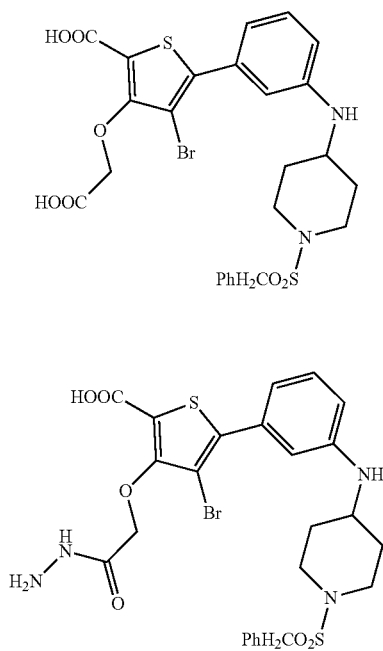
[0036] In some preferred embodiment, the first component in aforementioned method is a FK506-TLR7 agonist having the structure of



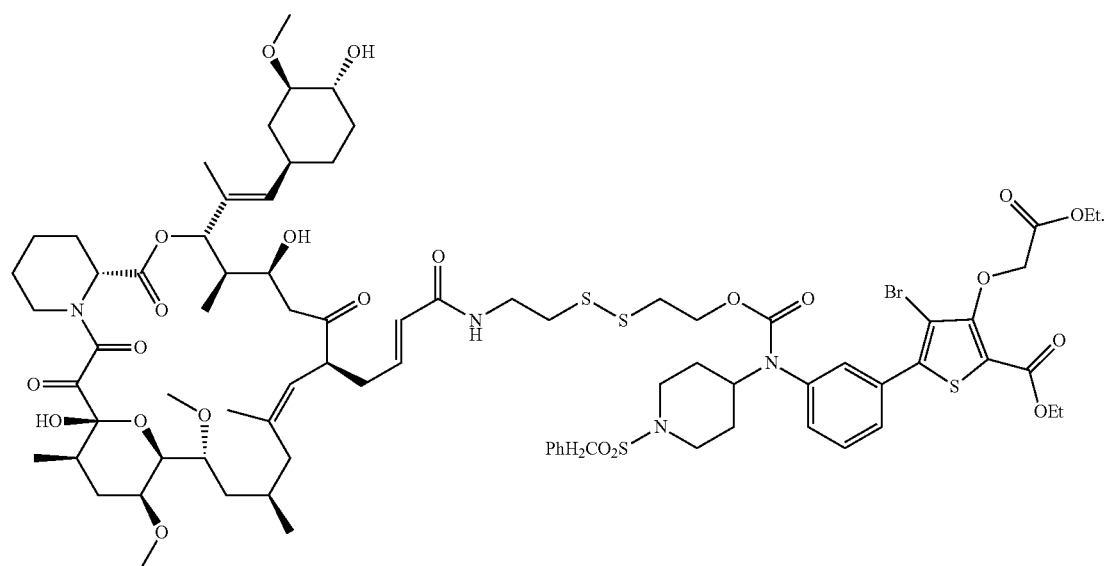
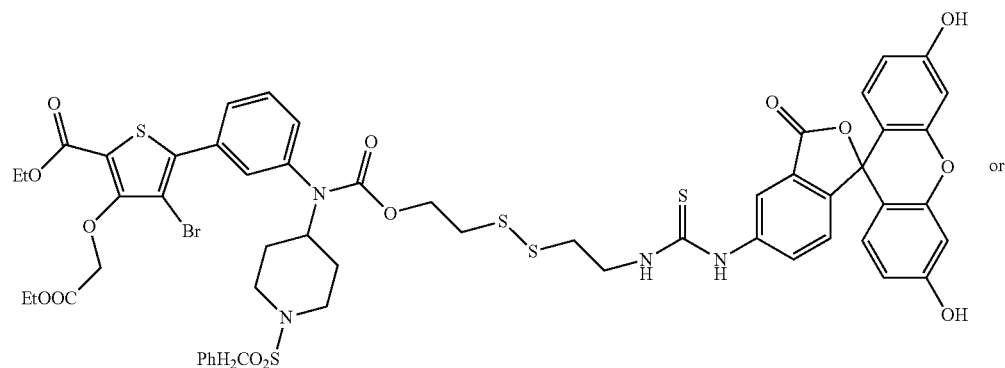
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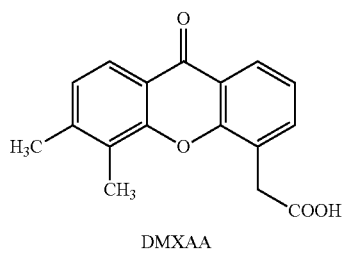
[0038] In some preferred embodiment, the first component in aforementioned method comprising payload drug selected from the group consisting of following TC-PTP phosphatase inhibitors:



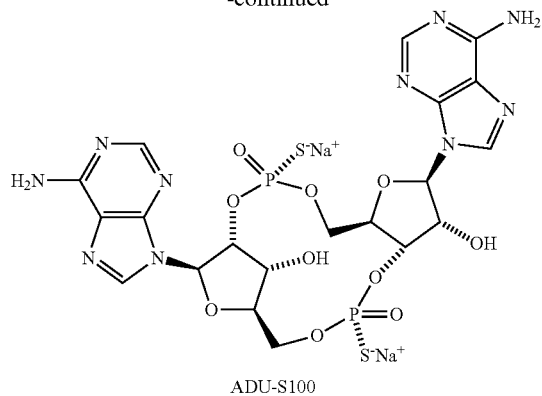
[0039] In some preferred embodiment, the first component in aforementioned method comprising the Phosphatase inhibitor connected to the fluorescein or FK506 (tacrolimus) to form the following structures:



[0040] In some preferred embodiment, the first component in aforementioned method comprising a payload drug of a STING agonist of the following structures.

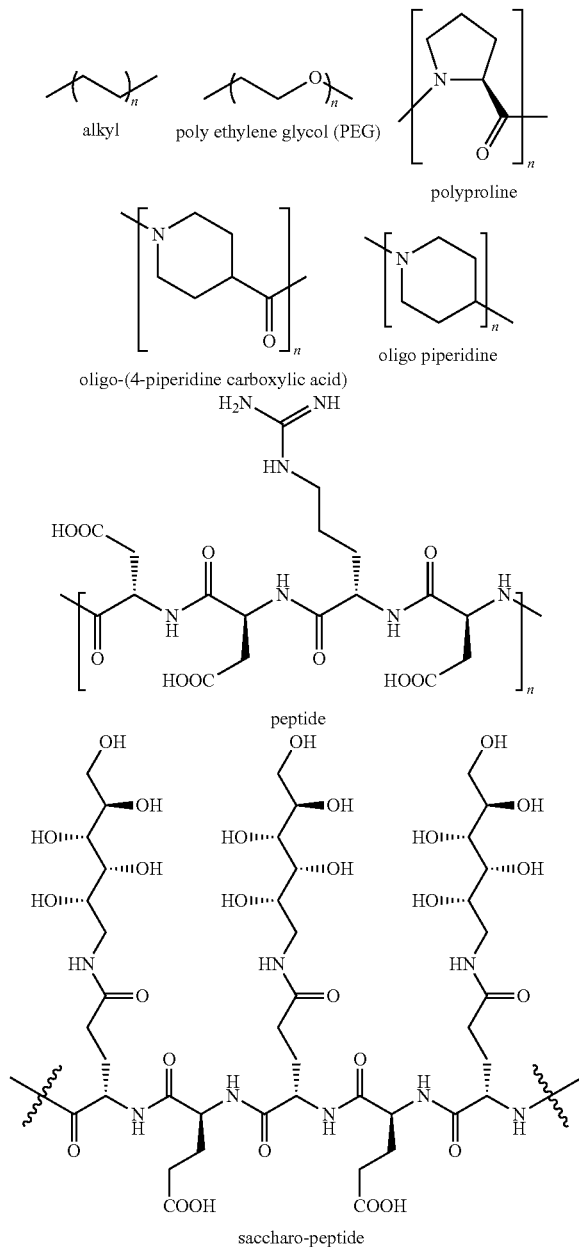


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[0041] In some preferred embodiment, the first component in aforementioned method comprising a spacer between the

targeting ligand and the payload drug that is selected from the group consisting of the following structures:



[0042] These and other features, aspects and advantages of the present invention will become better understood with reference to the following figures, associated descriptions and claims.

BRIEF DESCRIPTION OF THE DRAWINGS

[0043] FIG. 1. 1a, Graph illustration of the exhaustion model. 1b, antiCD19 CAR T cell became exhausted after 3 times of stimulation of fresh Raji cell in vitro as shown by decreased killing effect.

[0044] FIG. 2. Graph illustration of the secret passageway. FKBP or antiFITC is linked to FRa as a fusion receptor, which constantly internalizes and delivers FK506 or FITC linked payloads into the cell.

[0045] FIG. 3. Binding affinity of FK506 and FITC to the corresponding fusion receptors. FK506-Rhodamine shows $K_d=3.39$ nM towards FKBP-FR fusion receptor, while FITC-AF647 shows $K_d=8.03$ nM towards antiFITC-FR.

[0046] FIG. 4. 4a. Structure of the TLR7 agonist. 4b. rejuvenation effect of TLR7 agonist on exhausted antiCD19 CAR T cell, shown as the increased killing effect, INF γ level and decreased co-inhibitory molecule level.

[0047] FIG. 5. Chemical Structure of TLR7 agonists.

[0048] FIG. 6. in vitro model for the induction of CAR T cell exhaustion. (A) CD19 $^+$ Raji and anti-CD19 CART cells were co-cultured at 1:1 ratio with fresh Raji cells added every 12 h. (B) Lysis effect of CAR T cells gradually decreased as the number of stimulus (number of Raji cell addition) increases. CD19 $^+$ K562 cells were used as control. (C) Expression level change of co-inhibitory molecules, PD-1, LAG3 and Tim3 for stim1 and stim3 in CD4 and CD8 positive CAR T cells. FIG. 7. Evaluation of TLR7 agonist and PTP1b inhibitor effect on rejuvenation of exhausted CART cells. (A) Chemical structure of TLR7 agonist and PTP1b inhibitor. (B-C) Exhausted CAR T cells were incubated with different concentrations of TLR7 agonist and PTP1b inhibitor monitored by lysis effect and INF γ (B) and expression level of PD-1 LAG-3 and Tim3 after incubation (C). * denotes a p-value<0.05, **<0.01, ns=not significant.

[0049] FIG. 8. Evaluation of potential derivatization sites of the TLR7 agonist for non-releasable ligand targeted delivery. (A) Chemical structure of the TLR7 agonist analogs. (B) Exhausted CART cells were incubated with different concentration of TLR7 analogs and lysis effect was measured and compared to non-treated group.

[0050] FIG. 9. Design and evaluation of releasable and non-releasable targeted delivery of TLR7 agonist using FITC as a targeting ligand. (A) Illustration of chemical structure of FITC-TLR7 agonist. (B) Exhausted CAR T cells were incubated with different concentration of releasable FITC-TLR7 and non-releasable FITC-JTLR7 agonists and lysis effect was measured and compared to non-treated group. * denotes a p-value<0.05, **<0.01, ns=not significant. (C) Left, crystal structure of FITC (green) binding with FITC scFv (grey) (PDB: 1X9Q, left), the distance between FITC to the edge of FTIC scFv is measured to be around 18 Å. Right, similarly, crystal structure of R-848 (red) binding with TLR7 (grey) (PDB:5GMF) is shown with distance between R-848 and the edge of TLR7 around 24 Å. (D) Diagram illustrating the two possible working mechanisms, "Reaching" or "Jumping" Mode, for the non-releasable FITC-TLR7 agonists.

[0051] FIG. 10. Chemical linkers of variable rigidity and hydrophobicity available for usage in the design of targeting ligand-payload conjugates.

DETAILED DESCRIPTION

[0052] While the concepts of the present disclosure are illustrated and described in detail in the figures and the description herein, results in the figures and their description are to be considered as exemplary and not restrictive in character; it being understood that only the illustrative embodiments are shown and described and that all changes

and modifications that come within the spirit of the disclosure are desired to be protected.

[0053] Unless defined otherwise, the scientific and technology nomenclatures have the same meaning as commonly understood by a person in the ordinary skill in the art pertaining to this disclosure.

[0054] Chimeric antigen receptor (CAR) T cell therapies have recently experienced substantial success in the treatment of several types of hematopoietic cancers. In the meantime, one should also recognize that some of the lymphoma and most solid tumor cases still have a very low response rate or a high relapse rate with CAR T cell therapies. This mainly result from one or combinations of the following three reasons: 1. Emergence of antigen negative cancer cell colonies under the selection pressure of CAR T cells, as seen in the case of CD19 negative ALL relapse treated with anti-CD19 CART cells; 2. Hindered initial homing and proliferation of CART cells in solid tumor due to the aberrant tumor vasculature, dense stromal barrier and suppressive microenvironment; 3. Gradual exhaustion and lowered lysis effect of CART cells after continuous tumor antigen exposure. Assuming loss of antigen is not present for a given solid tumor patient (validated by biopsy sampling), the causes of a potential failure of a CAR T cell therapy are most likely to result from the latter two reasons. Therefore, to increase CAR T cells efficacy in solid tumors, practical methods for in vivo evaluation and rejuvenation of CAR T cells are highly desired.

[0055] Here we describe the novel design of a private passageway fusion receptor in CAR T cells as a universal platform to achieve both objectives. This FITC-FR fusion receptor is composed of two parts, scFv against FITC as the ligand binding domain at the N terminal and FRa as the GPI anchoring and internalizing domain at the C terminal. When independently expressed on CAR T cells, the FITC-FR fusion receptor can be specifically targeted by a FITC-immuno agonist to overcome the exhaustion status of CAR T cells in the suppressive tumor microenvironment. These immuno-agonists normally cause strong autoimmunity side effects, and can now be systemically dosed in a FITC targeted form, and safely delivered to FITC-FR positive CAR T cells. In the last few decades, great advances have been made in this field regarding the cell types, delivery methods and suitable diseases models. In terms of cell types, current cell therapies can be roughly categorized as chimeric antigen receptors (CARs), cell for tumor model and stem cell based regenerative medicine.

[0056] CAR T also known as chimeric T cell receptors, chimeric immunoreceptors or artificial T cell receptors, enable immune effector cells (usually T cells or NK cells) to recognize target cells with corresponding antigen and exercise their cytotoxic activity. The emergence and development of CAR-T technology provides promises to certain types of cancers, which turns CAR-T into a superstar in the field of both biomedical research and clinical studies. Some traditional and improved CAR T cell design are disclosed in U.S. application Ser. No. 15/296,666, the content of which is incorporated herein entirely. In '666 application, a CAR system is produced by providing a cytotoxic lymphocytes expressing CARs that target a moiety that is not produced or expressed by cells of the subject being treated. This CAR system thus allows for focused targeting of the cytotoxic lymphocytes to target cells, such as cancer cells. The targeted moiety is part of a small conjugate molecule (SCM)

that also comprises a ligand of a tumor cell receptor. Administration of a SCM along with the CAR-expressing cytotoxic lymphocytes results in the tarding of the cytotoxic lymphocyte response to only those cells expressing the tumor receptor to which the SCM is bound.

[0057] Despite the rapid progress of CAR T cell therapy in both research and clinical use field, there are concerns accompanied with CAR T therapy. One lethal side effect is cytokine storm generated from the fast lysis of tumor cells as well as it kills normal cells bearing CAR. In order to address such side effects, targeted delivery of CAR T cell with specific payload of drug to the target tumor cells to control such side effect is developed in PCT/US2018/018557, the content of which is incorporated herein entirely. Briefly, an engineered protein is coupled with a high affinity targeting ligand, wherein the targeting ligand carries at least one payload of drug to be internalized by the CAR T cell through the engineered protein to regulate transplanted cell therapy effects.

[0058] Another limitation of CAR T therapy is their tendency to get exhausted after repeated stimulation of cancer antigen. The reversibility of the exhausted phenotype of T cell is proven as T cells isolated from the solid tumor tissue show higher INF γ secretion and killing effect if kept away from antigens ("rested") overnight before re-stimulation⁴. However, it will be more appealing if rejuvenation can be achieved in a more clinical relevant way using drugs: either to block the inhibitory signaling or to activate the T cell through other pathways. Antibodies targeting checkpoint inhibitors (i.e. PD-1, CTLA-4, etc.) have shown some success in solid tumors in clinic, however, two or more targets in combination are often found to be necessary^{5,6}. Moreover, antibody therapy also suffers from poor penetration in solid tumor. Therefore, less reports have been seen for the combination therapy of CAR T and antibody for checkpoint inhibitors in solid tumor. Inhibiting the phosphatases, such as SHP1/2⁷ and TC-PTP⁸, that mediates TCR deactivating, is another way to block the inhibitory pathways. Both knockout experiments and small molecules inhibitors of these phosphatases have shown potent effect on lowering TCR threshold and increasing T cell activity, but none of them have been used in CAR T therapy. DGK α is another physiological inhibitor of TCR signaling and it's overexpressed in exhausted TIL. DGK α catabolize DAG to PA thereby reducing DAG levels, which results in attenuation of Ras and MARK ERK signaling. Inhibitor of DGK α recovers the degradation and increases the killing effect of TIL and CAR T^{3,9}. Another approach to rejuvenate the T cell is to activate it through an antigen independent pathway. It has been known that certain pathogen pattern recognition (PPR) receptors, including Toll like receptors (TLR), do express on non-myeloid cell populations, including T cells, and can be activated in a similar way. Research has also shown that TLR2¹⁰, 4¹¹ and 7/8^{12,13} agonists can activate CD8 T cells and increase INF γ secretion. However, due to the strong side effects of systemic dosing of TLR agonists¹⁴, none of these agonists have been used in CAR T therapy to re-activate the T cell or change the immunosuppressive microenvironment. It's also hindered by the controversial effect of TLR agonists on tumor cell itself¹⁵. Stimulator of interferon genes (STING) is a cytosolic DNA sensor (CDS) that widely expressed in hematopoietic cells in peripheral lymphoid tissues, including T cell, myeloid cells and monocytes. STING agonists have been used as an immune stimulator for

many immunotherapies, and may also have a profound effect in CAR T therapy. However, although the mentioned inhibitors and agonists may have a profound rejuvenation effect on CAR T cell, it's also highly possible that it may induce severe side effects if systematically dosed due to their highly potent pro-inflammatory functions. Therefore, a targeted delivery of the potential payloads to the CAR T cell is highly desired.

[0059] To solve the specific delivery problem, we designed a secret passageway platform, which can be expressed in T cell together with the CAR construct, so that certain payloads can be systemically dosed and specifically accumulated within the CAR T cell only, rendering other cells untouched. The system consists a fusion receptor and a classical CAR construct, linked through a T2A self-cleavable sequence. The fusion receptor contains two parts: (1) a ligand binding module, which can recognize a high affinity ligand-payload conjugate; (2) a membrane bound receptor module, which can mediate the internalization of the receptor/conjugate complex into the cell. Two protein/ligand pairs have been chosen for part 1, FKBP/FK506 and scFv against FITC (4M5.3)/FITC, for the following reasons: (1) the absence of FKBP or 4M5.3 on natural cell membrane guarantees the specific delivery of payload to fusion receptor positive CAR T cell, thus reduces the side effects to other cells; (2) the sub-nanomolar binding affinity between protein/ligand pairs promote sufficient payload accumulation inside the targeted cells. For the membrane bound receptor in part 2, Folate Receptor alpha (FRa) was chosen for its constitutive internalization properties regardless of Folate Acid binding¹⁶. We also designed a target-payload linkage system, where payload can be linked to targeting ligands with either non-releasable linker or a disulfide releasable linker, depending on the target. More specifically, since TLR7 locates in the endosome, secret passageway delivered TLR7 agonist can exert its function as soon as it enters the endosome through receptor mediated internalization. Therefore, releasable linker is not necessary in this case. While for other targets that are located in the cytosol instead of the endosome, such as SHP1/2, TC-PTP, DGK, TGF β and STING, release of the free drug from the target-payload conjugate is necessary for its escape from the endosome. Together, this secret passageway system provides a versatile platform for specific in vivo delivery of numerous payloads to the exhausted CAR T cell.

[0060] These and other features, aspects and advantages of the present invention will become better understood with the following experiments examples.

Methods:

[0061] Exhaustion of the antiCD19 CAR T Cell and Drug Treatment:

[0062] AntiCD19 CAR T cell were co-cultured with Raji at 1:1 ratio in 6 well plate, while fresh Raji cell were added every 12 h to the same well. Killing effect and co-inhibitory markers were quantified by flow cytometry counting. For drug treatment, after 4 rounds of stimulation with Raji cells, exhausted antiCD19 CAR T were further incubated with drugs at different concentration for 12 h, and then quantified similarly

Targeting Ligand Binding Assay

[0063] Fusion receptor positive cells were incubated with certain ligand-dye molecule at different concentrations for

30 min at 4 degree. After incubation, cells were washed twice with PBS and then submitted to flow cytometry. MFI or percentage of shift is used for binding curve and calculation of Kd.

Materials

Cell Lines and Human T Cells

[0064] DMEM (Gibco) containing 10% heat-inactivated fetal bovine serum and 1% penicillin-streptomycin was used for the culture of MDAMB-231 and MDA-MB-231 CD19⁺ cells. Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll density gradient centrifugation (GE Healthcare Lifesciences, #17-5442-02) from human whole blood obtained from healthy volunteers. Pure CD3⁺ T cells were enriched from PBMCs using an EasySepTM Human T Cell Isolation Kit (STEM CELL technologies, #17951).

Evaluation of Potential Payloads for In Vitro Rejuvenation of Exhausted CAR T Cells

[0065] anti-CD19 CAR T cells were co-incubated with CD19⁺ Raji cell at 1:1 ratio in 12-well plate at density of 2×10^6 CAR T and 2×10^6 Raji per well, new Raji cell were added every 12 h for 3 times, Raji cell population, lysis effect and co-inhibitory receptors were then tested to confirm the exhaustion of the CART cells. Both flow cytometry and luciferase-based assays were used to quantify the lysis effect. To test the rejuvenating efficacy of the potential payloads, this cell mixture was then transferred to 96-well plate, around 2×10^5 cells per well, and different concentration of drugs were added. After 12 h, Raji cell population, lysis effect and co-inhibitory receptors were tested again and compared to the PBS treatment group.

EXAMPLES

Example 1. Exhaustion of the antiCD19 CAR T Cell In Vitro

[0066] In this Example, we illustrated a model of exhausted CAR T cell. Briefly, FIG. 1a shows Raji cells, a type of B-lymphoma cells were co-cultured with FMC63 CAR T cells, a type of anti CD19 CAR T cells as described in the method section, and fresh Raji cells were added to the co-culture every 12 hours for consecutive three days. FIG. 1b shows these CAR T cells became exhausted after 3 times of stimulation by fresh Raji cells in vitro, indicated by decreased killing effect. Co cultured K562 cells served as negative control.

Example 2. Design of the Secret Passageway

[0067] In this Example, we show a graphic illustration of the secret passageway of delivery payload drug to targeted cell types. FKBP or antiFITC is linked to FRa as a fusion receptor, which is able to engage a targeting ligand FK506 or FITC linked to a specific payload of drug. Due to the nature of FRa, it constantly internalizes and delivers FK506 or FITC linked payloads into the CART cell. Therefore, when CAR T is engaged to its target cells through CAR, in this example, anti-CD 19 molecule on the CAR T surface engages CD-19 of the cancer cell, the delivered payload drug inside of CAR T may execute its function, i.e. regulate CAR T activity based on the payload function. For example, some payload drugs may be directed to act on PD-1,

CTLA4, or LAG3 T cell function regulatory molecules to rejuvenate CAR T when necessary.

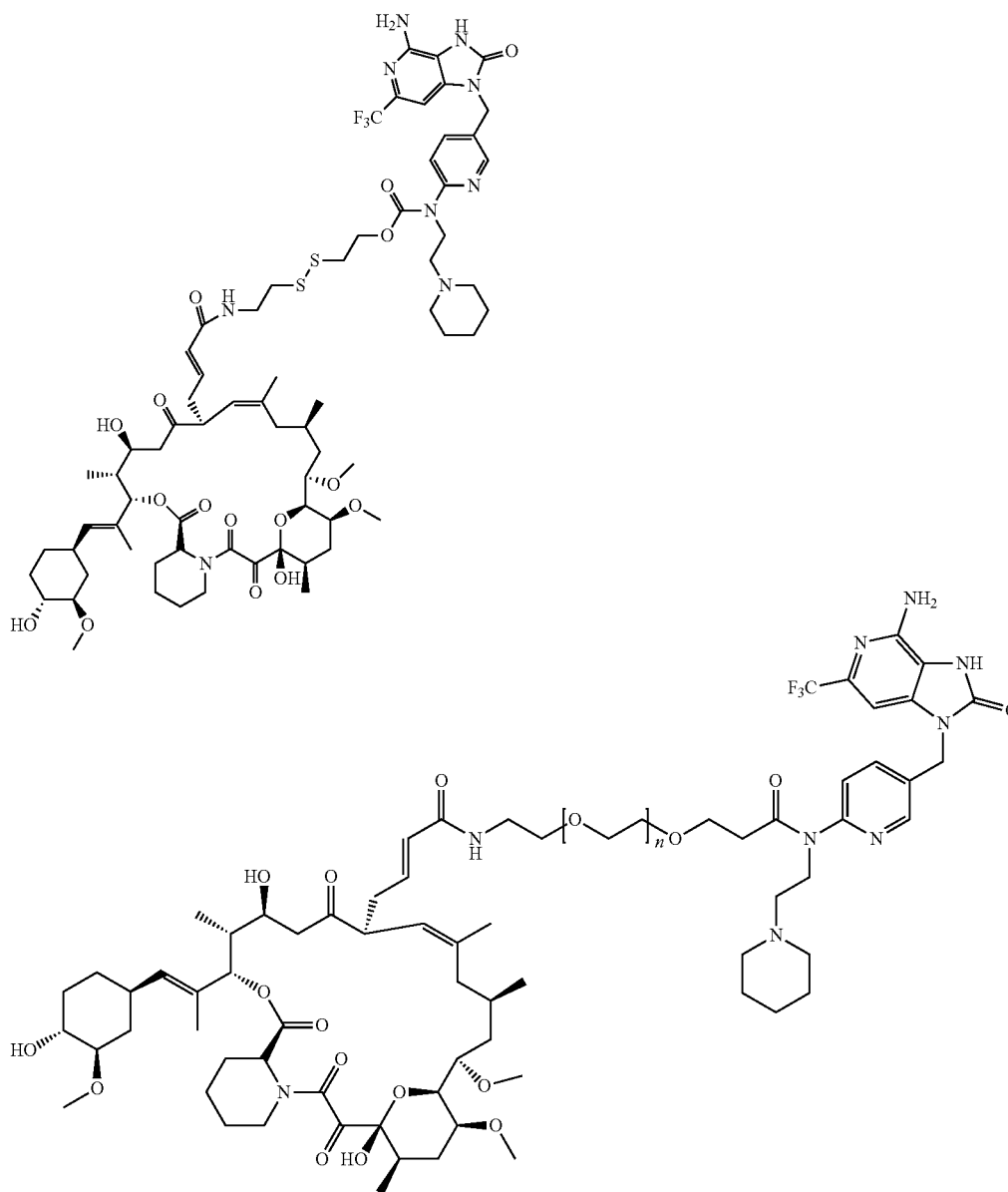
Example 3. Reversion of the Exhausted antiCD19 CAR T Cell by TLR7 Agonist

[0068] In this Example, we show that targeting ligand FK506 or FITC successfully engages its respective fusion receptor (FKBP or FITC-AF647 linked to Folate Receptor by G₄S). In this example the payload drug is an imaging agent Rhodamine to show the payload distribution in fusion receptor transfected Jurkat cells. The binding affinity of FK506 and FITC are calculated by competitive binding and FK506-Rhodamine shows K_d=3.39 nM towards FKBP-FR fusion receptor, while FITC-AF647 shows K_d=8.03 nM towards antiFITC-FR.

Example 4. Design of Potential Releasable and Non-Releasable FK506-TLR7 Agonist and FITC-TLR7 Agonist

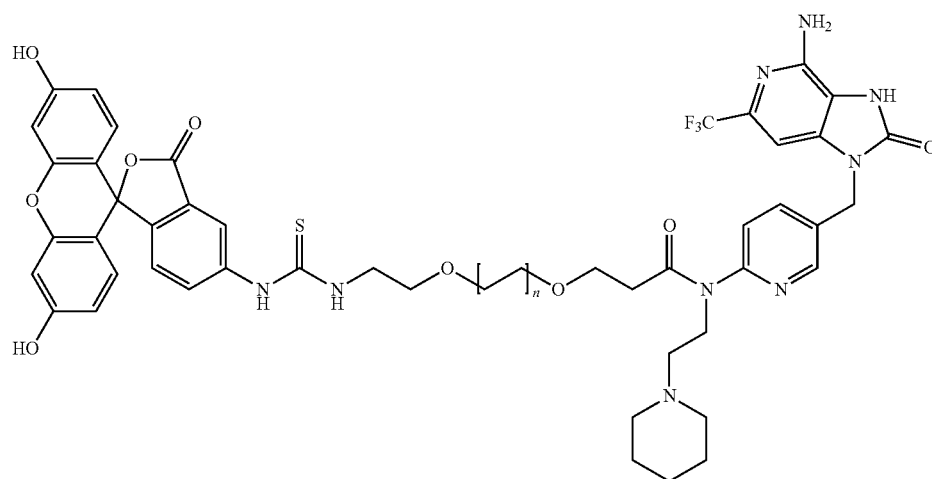
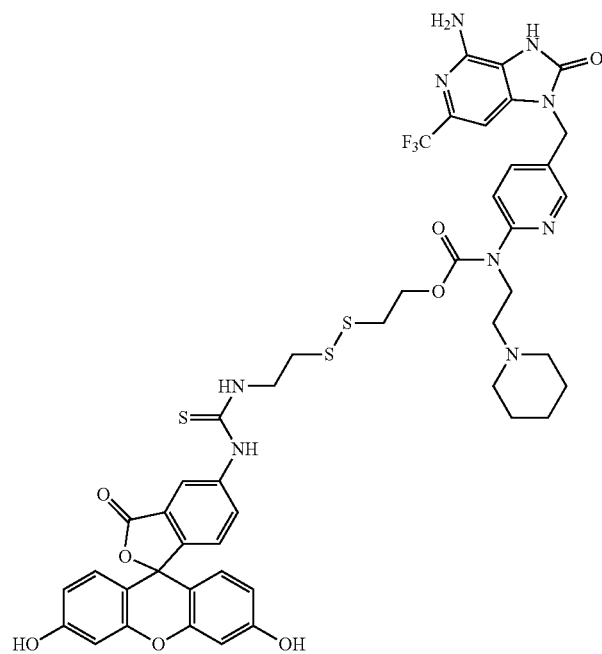
[0069] In this Example, we provided targeting ligand FK506 conjugated to a Toll Like Receptor 7 agonist with the structure below to treat exhausted CAR T cells. A dose dependent pattern of increased killing effect is observed when TLR7 agonist payload drug is targeted to exhausted CAR T cells. Accordingly, the indicator of CAR T activity, IFN γ expression level, increased in a dose dependent manner relative to payload drug concentration; and the expression level of a T cell effect inhibitory molecule, Tim 3, had showed a reverse dose dependence manner, i.e. Tim 3 expression decreased as the concentration of payload drug is increased.

FK506-TLR7 agonist conjugate should have one of the structures given below:



n = 0-12

Similarly, Fluorescein-TLR7 agonist conjugate should have one of the structures given below and rejuvenate exhausted CAR T if the functional CAR T has an anti-FITC fusion receptor.

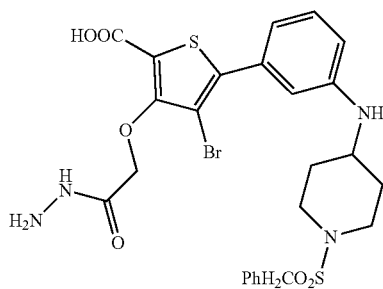
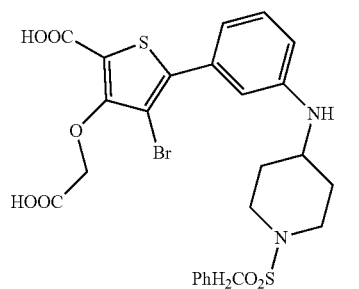


n = 0-12

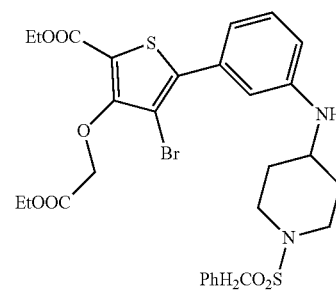
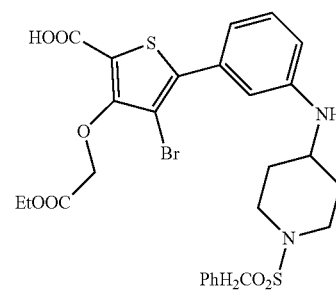
Example 5. Design of Other Potential Payloads for the Rejuvenation of the Exhausted CAR T Cell

[0070] In this Example we provide a list of structures of other potential payloads that may revert CAR T exhaustion with lower nanomolar range potency.

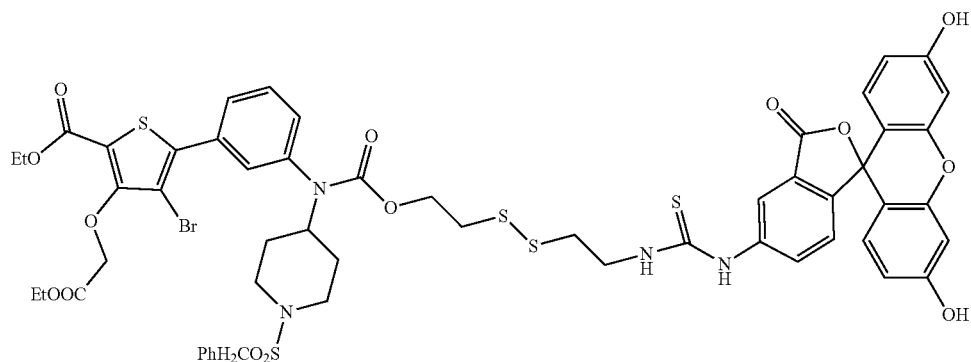
[0071] 1) TC-PTP phosphatase inhibitor should have the following structures,



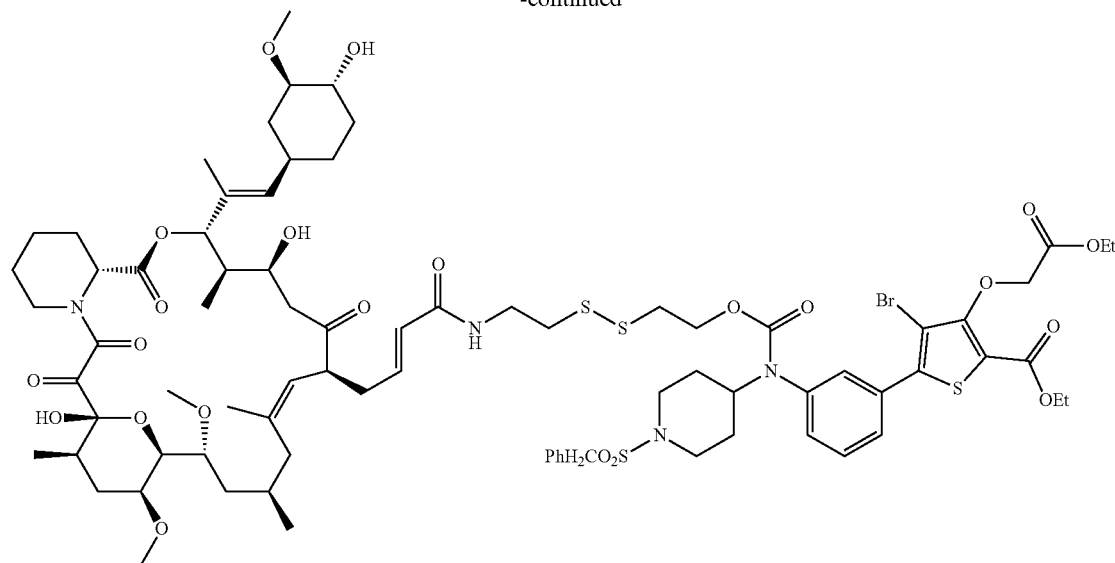
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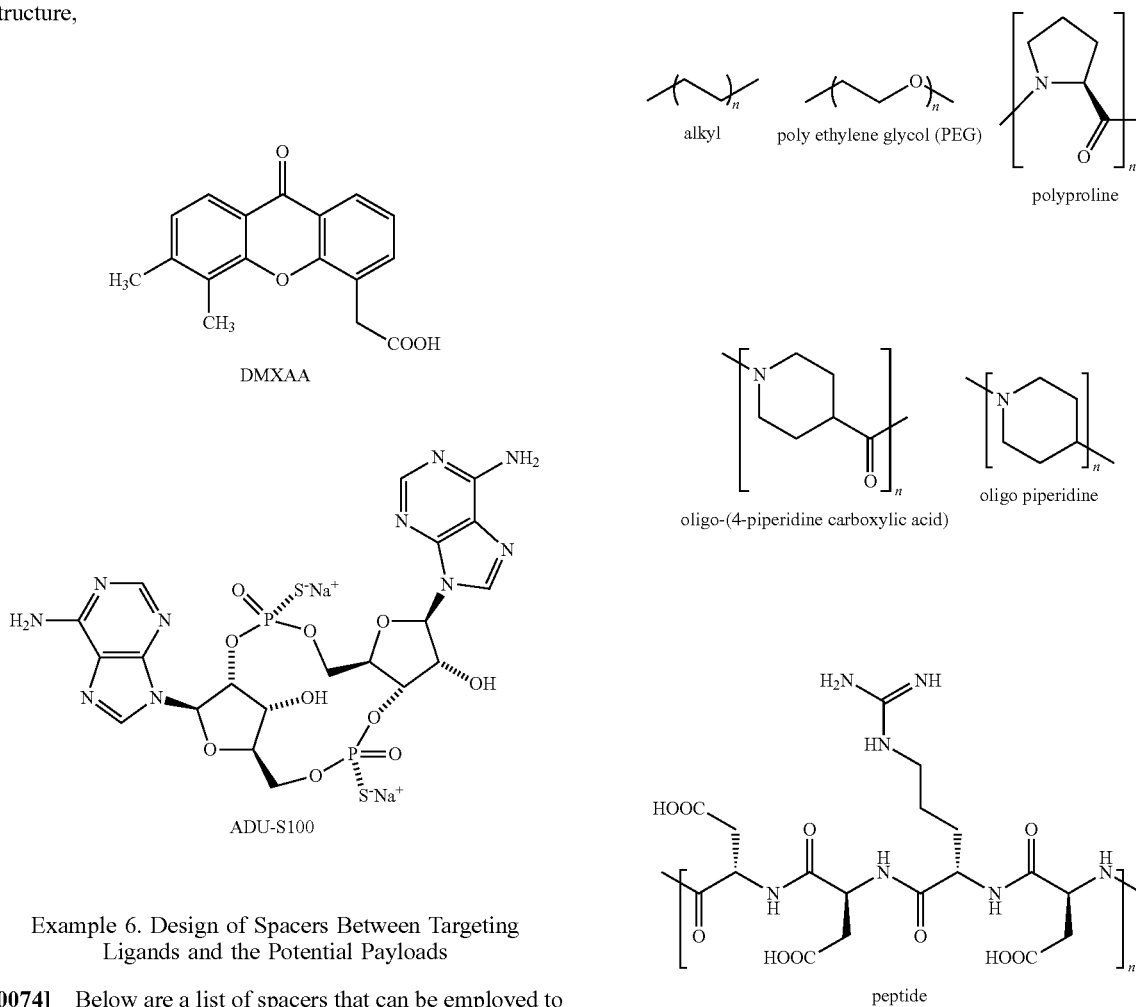
[0072] Phosphatase inhibitor mentioned above may be connected to the fluorescein or FK506 (tacrolimus) in the following way.



-continued

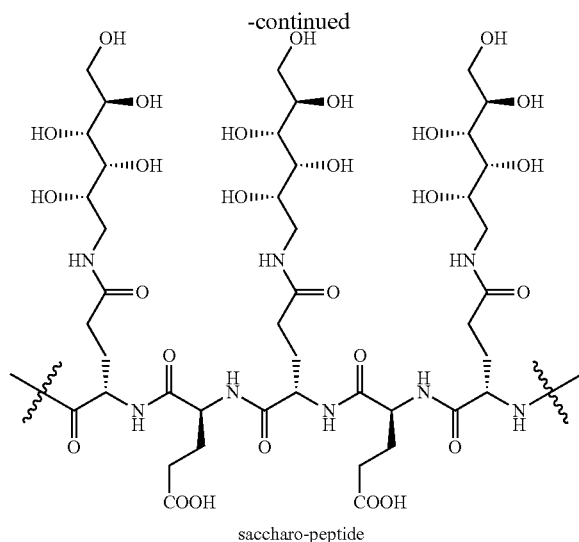


[0073] 2) The STING agonist should have the following structure,



Example 6. Design of Spacers Between Targeting Ligands and the Potential Payloads

[0074] Below are a list of spacers that can be employed to link the targeting ligands and any potential payloads.

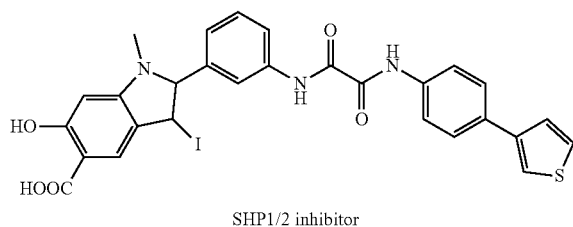


Example 7. Evaluation of the Ability of Phosphatase Inhibitors and TLR7 Agonists to Rejuvenate Exhausted CAR T Cells

[0075] One major limitation of CAR T cell therapies in solid tumors is their tendency to become exhausted after repeated stimulation with cancer antigens. This phenomenon however, is not specific to CAR T cells, but has been described in both chronic virus infections⁴ and tumor infiltration lymphocytes⁵. The reversibility of the exhausted phenotype of T cells has been proven in studies where T cells isolated from the solid tumor tissue show a higher INF γ secretion and a killing effect if kept away from antigens (“rested”) overnight before re-stimulation⁵⁷. However, it would be more appealing if rejuvenation could be achieved in a more clinically relevant way using commercially available therapeutics: either to block the inhibitory signaling or to activate the T cells through other pathways. Antibodies targeting checkpoint inhibitors (i.e. PD-1, CTLA-4, etc.) have shown some success in solid tumors in-clinic¹⁵¹, however, two or more targets in combination often have been found to be necessary. Moreover, antibody therapy also suffers from poor penetration in solid tumors and this may have led to less reports for the combination therapy of CAR T cells and checkpoint blockades (ICB) in solid tumors.

[0076] The inhibition of the phosphatases, such as SHP1/2 and TC-PTP, that mediate TCR deactivation, is a potential way to block tonic CAR T signaling. SHP1/2 phosphatase is responsible for mediating the signal from PD-1 and other exhaustion markers. Data has shown that SHP1/2 phosphatase inhibitor or silencing can increase the activity of T cells and CAR T cells⁶⁹. TC-PTP is known to be an important player in T cell activity signaling. Mice harboring a T cell specific TC-PTP deficiency have increased susceptibility to inflammation and autoimmunity due to heightened antigen-driven T cell activation. TC-PTP inactivates Src family kinase downstream of the TCR, thereby contributing to the threshold of TCR activation¹¹. Although both knock-out experiments and small molecule inhibitors of these phosphatases have shown potent effect on lowering TCR

threshold and increasing T cell activity, none of them have been used in CAR T therapy. A representative SHP1/2 inhibitor has the structure of



[0077] Another approach to rejuvenate the T cells is to augment their activity through the engagement of antigen independent innate immune receptors. It has been known that certain pathogen pattern recognition (PPR) receptors, including toll like receptors (TLR), do express on non-myeloid cell populations, including T cells, and can be activated in a similar way. Research has also shown that co-stimulation of TLR7/8 agonists and TCR signaling can activate CD8 T cells and increase INF γ secretion¹³. However, due to the strong side effects of systemic dosing of TLR agonists, none of these agonists have been used in CAR T therapy to reactivate the T cell or change the immunosuppressive microenvironment. The employment of TLR agonists for cancer immunotherapy is also hindered by the controversial effect of TLR agonists on the tumor cells. Therefore, a targeted delivery of the potential payloads to the CAR T cell is highly desired. A potent TLR7 agonist was found in the literature (FIG. 7A), which is around 40 fold stronger than the FDA approved imiquimod.

[0078] To set up an in vitro screening model, as shown in FIG. 6, anti-CD19 CART cells were exposed to 4 rounds of addition of CD19 positive Raji cells, and became exhausted as marked by gradual decreased lysis activity as well as increased co-inhibitory markers in an in vitro co-culture model. It’s worth noticing that the culture medium is important for the introduction of CART exhaustion and needs to be kept the same without new replenish or change during the whole process. It indicates that the soluble components that are released by cancer cells and/or CAR T cells into the medium, most likely immunosuppressive cytokines and modulators (adenosine etc.), play a pivotal role in this process. It also suggests that the exhaustion of CAR T cells generated by this in vitro model is at a rather pliable than irreversible status.

[0079] Treatment of the TLR7 agonist and PTP1b (highly homologous to TC-PTP²⁶) inhibitor²⁷ of choice with the already exhausted CAR T cells was shown to be able to reactivate them compared to the no treatment group (FIG. 7). No significant changes, however, were observed in the expression level of co-inhibitory markers except for Tim3. As shown by FIG. 7C, the PTP1b inhibitor in general does not show as strong of a reactivation effect as the TLR7 agonist. Without being limited by any theory, this result could be due to the current in vitro screening model where “Reversion” rather than “Prevention” of exhaustion is studied, and the phosphatases are already “silenced” at the exhausted status, therefore their inhibition will have little to no effect. A modified screening model for a future study will test the effects of phosphatase inhibitors and other drugs

with a focus on the “prevention” of exhaustion by adding the drugs at the beginning of all cultures and keeping the rest of the settings the same. In this way, it may be possible to see whether the inhibition of phosphatase can lower the tonic signaling of CAR T while still keeping a functional killing effect. We will mainly focus on TLR7 agonist for the following study.

[0080] Since TLR7 is one of the 4 TLR family members that resides inside the endosome, it is speculated that a non-releasable linker between the TLR7 agonist and our secret passageway targeting ligand would preserve its TLR7 agonist function²⁸. To achieve that, several TLR7 agonist analogs were prepared and tested to find the proper derivatization sites for linkage. As shown in FIG. 8, the TLR7 agonist with a CH₂OH extension at the piperidine ring has an even higher activity compared to the parent drug. Therefore, this derivative site will be used for a non-releasable conjugate. A disulfide bond linked self-immolative form also has been synthesized. In order to understand the distance needed for this TLR7 agonist to reach its own target, three different lengths of linker (PEG3, 6, 16) between the FITC and TLR7 agonist were made for the non-releasable FITC-TLR7. As shown in FIG. 9 C, all of the non-releasable forms had some effect, while the PEG6 compound showed the best dose-dependent response. These results indicate that TLR7 agonists may dock with TLR7 either by reaching out while binding with FITC-FR or jumping between TLR and FITC-FR, under which conditions the length of the linker in between is not a crucial factor (FIG. 9 D-E). Since the non-releasable FITC-TLR7 is trapped inside the endosome and the volume of each endosome is much smaller than the cytosol, the intra-endosome TLR7 can get to its functional concentration much faster and quicker, resulting in a smaller IC₅₀.

Example 8. Other Potential Payloads for Revert/Prevent the Exhaustion of CAR T Cells

[0081] Other than TLR7 agonist, there are several other potential payloads that may revert/prevent the exhaustion of CAR T cells as described below. Some of the targets may not have agonists or inhibitors with IC₅₀ suitable for our targeted drug delivery approach for now, but are still worth noticing and may be explored through other inhibitory mechanisms, such as CRISPR or targeted microRNA delivery approaches.

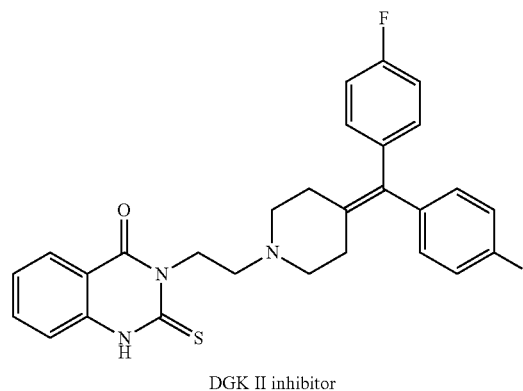
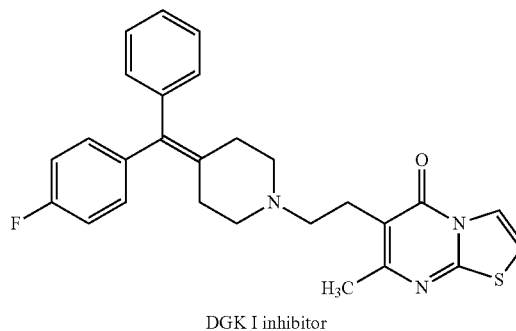
[0082] STING Agonist

The Simulator of IFN Genes (STING) is a master adaptor involved in cytosolic DNA sensing and the following IFN- β production. STING associates weakly to ssDNA, but strongly binds the endogenous cyclic dinucleotide GMP-AMP (cGAMP) synthesized by the cGMP-AMP synthase (sGAS). It is predominantly expressed in macrophages, T cells, a variety of DCs, endothelial cells, and select fibroblasts and epithelial cells. Studies of STING have mainly focused on its function in macrophages and dendritic cells, and recently some groups have noticed the direct effect of STING activation in T cells⁴⁰. It is possible that a STING agonist will have a similar pro-inflammatory effect on T cells. ADU-S100 is one of the many STING agonists that has been pursued in clinics.

[0083] DGK- α Inhibitor

Diacylglycerol Kinase- α (DGK- α) converts diacylglycerol (DAG), a second messenger in TCR signaling together with IP₃, to phosphatidic acid (PA). DGK is more highly expressed in CD8⁺TIL than in CD8⁻NIL, and its inhibition

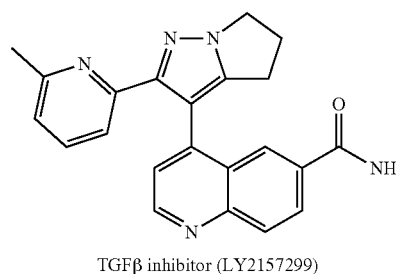
promotes ERK phosphorylation and lytic degranulation⁴¹; it also restores lytic functions of CAR TIL that are isolated from *in vivo*⁵. Some DGK inhibitor structures are as following;



[0084] TGF β RI (ALK5) Inhibitor

TGF β is known for its immunosuppressive function in many immune cells, such as the T cell, B cell, and macrophages. The blockage of TGF β type I receptor (TGF β RI, also called ALK5) in T cells reverts the immunosuppressive environment of the tumor⁴². Small molecule inhibitors have been pursued with galunisertib (LY2157299 monohydrate) and EW-7197 tested in clinics⁴³⁻⁴⁴.

[0085] A TGF β inhibitor structure is as following:



[0086] EZH2 Inhibitor

Enhancer of Zeste Homolog 2 (EZH2) is a histone H3K27 methyltransferase with a strong coregulator with the Treg function. Genetic or pharmacological disruption of EZH2 drove acquisition of proinflammatory function of tumor infiltrating Treg⁴⁵. Since exhausted CTL in chronic virus infections is also characterized by unique epigenetic changes¹⁷⁴, it is

possible that EZH2 inhibitors will be able to reverse this exhaustion status. Several small molecules of EZH2 inhibitors have been developed, including CPI1205, EPZ6438 and GSK126.

[0087] Therefore, the FITC-FR fusion receptor and the corresponding FITC targeted immune-agonists payloads provide a universal platform for the monitor and control of CAR T cells homing and persistence in solid tumor. This approach can be easily incorporated into CAR T cells for any antigen since the FITC-FR fusion receptor is independently expressed to the CAR construct. The modular design of targeting ligand-payload conjugates also makes it easier for the switching and modification. This approach combines the benefits of cell therapy and small molecule-based targeted drug delivery and may require extra characterization of both the engineered cells and the corresponding ligands.

[0088] The success of CAR T cells in solid tumor most likely requires the combination of multiple approaches targeting other players within the microenvironment as well, such as breaking down of the extracellular matrix by PI3K kinase inhibitors, reprogramming of anti-inflammatory M2 macrophages to a proinflammatory M1 phenotype, and upregulation of the decreased MHC molecules level on cancer cells. Therefore, more and more combinational therapy studies will be conducted both preclinically and clinically. However, at the same time, a careful examination and control of the CAR T cell itself cannot be neglected and should be optimized by using simple but robust systems like FITC-FR fusion receptors in preclinical research first before it reaches to humans.

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1. A system to rejuvenate an exhausted classical CAR T cell, comprising at least two components: a first component is a conjugate comprising a targeting ligand covalently linked to a payload drug; and a second component is a targeting ligand binding module linked to a membrane-anchoring module, wherein the targeting ligand binding module of the second component recognizes the targeting ligand in the first component with high affinity to form a

complex, the payload drug either blocks the inhibitory signaling of the exhausted classical CAR T, or re-activates said CAR T through an antigen independent pathway, and wherein the membrane-anchoring module mediates internalization of the two component complex into the exhausted CAR T cell.

2. The system according to claim 1, wherein the targeting ligand of the first component is folate, FITC or FK506.

3. The system according to claim 1, wherein the targeting ligand binding module of the second component comprises an anti-FITC antibody fragment or FKBP or folate receptor.

4. The system according to claim 1, wherein the membrane anchoring module is a folate receptor.

5. The system according to claim 1, wherein the first component comprises a releasable linker between the targeting ligand and the payload drug.

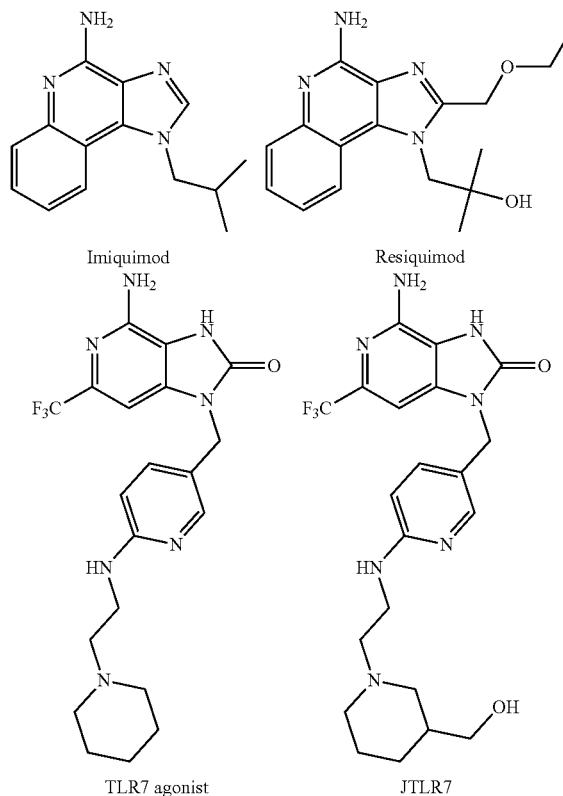
6. The system according to claim 1, wherein the first component comprises a non-releasable linker between the targeting ligand and the payload drug.

7. The system according to claim 1, wherein the binding affinity between the targeting ligand and the ligand-binding module is in sub-nanomolar range.

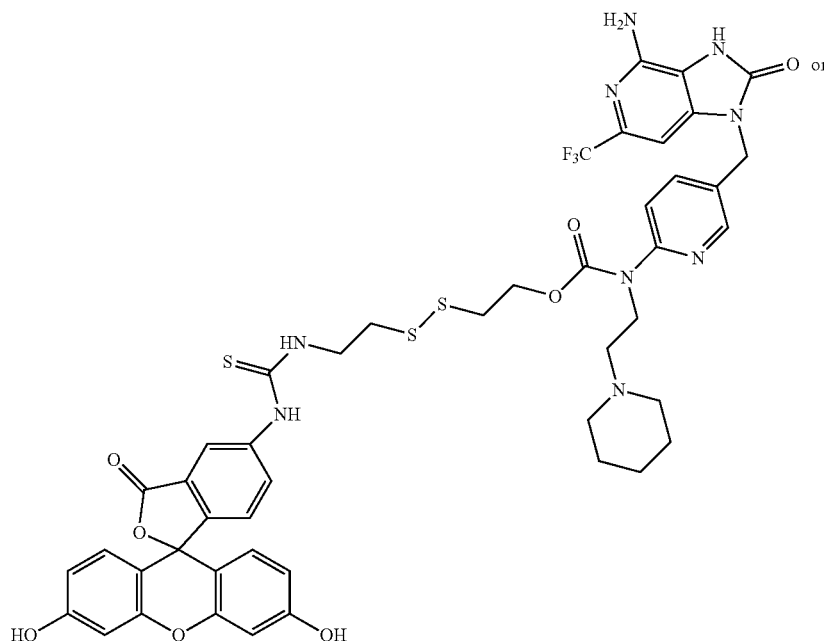
8. The system according to claim 1, wherein the payload of drug is a Toll Like Receptor 7 (TLR7) agonist or Simulator of interferon genes (STING) agonist.

9. The system according to claim 1, wherein the payload of drug is an inhibitor to following proteins: SHP1/2, TC-PTP or DGK α , TGF β .

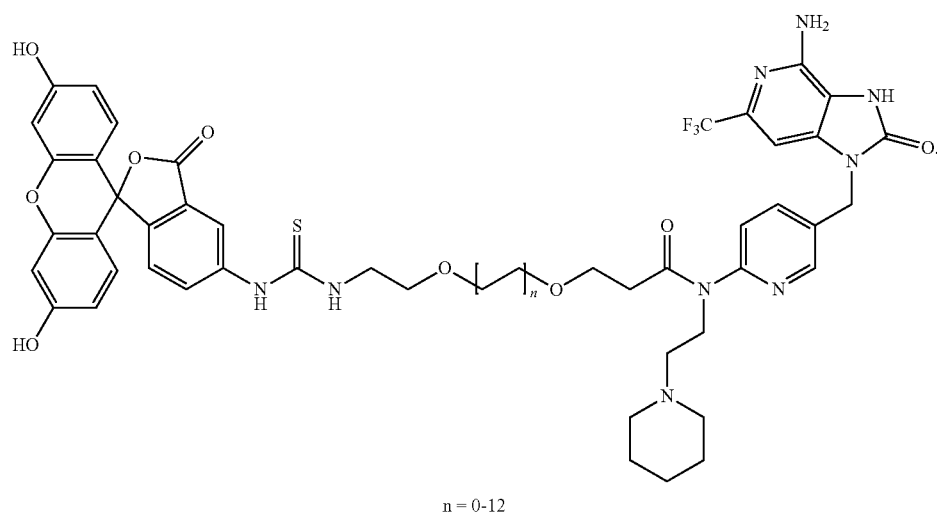
10. The system according to claim 8, wherein the TLR7 agonist has the structure of



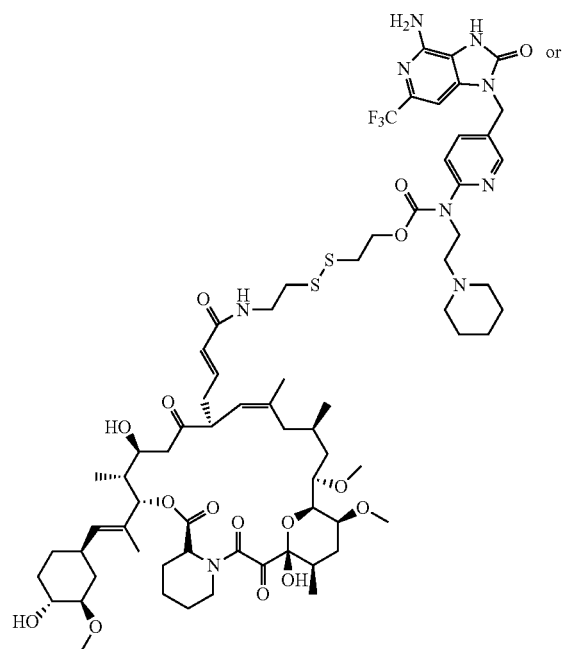
11. The system according to claim 1, wherein the first component is a Fluorescein-TLR7 agonist having the structure of



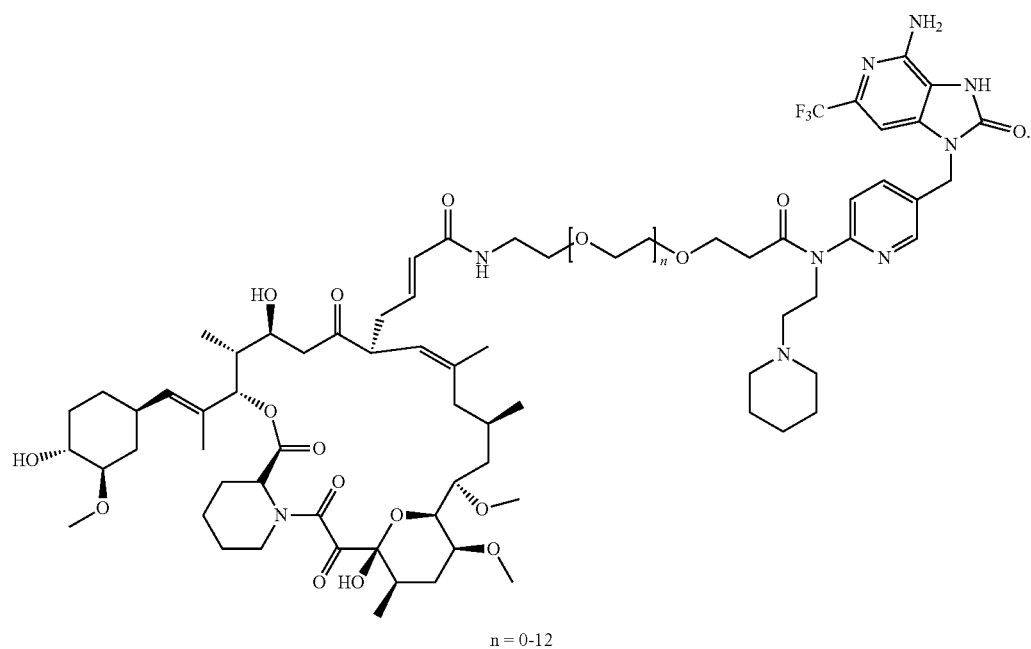
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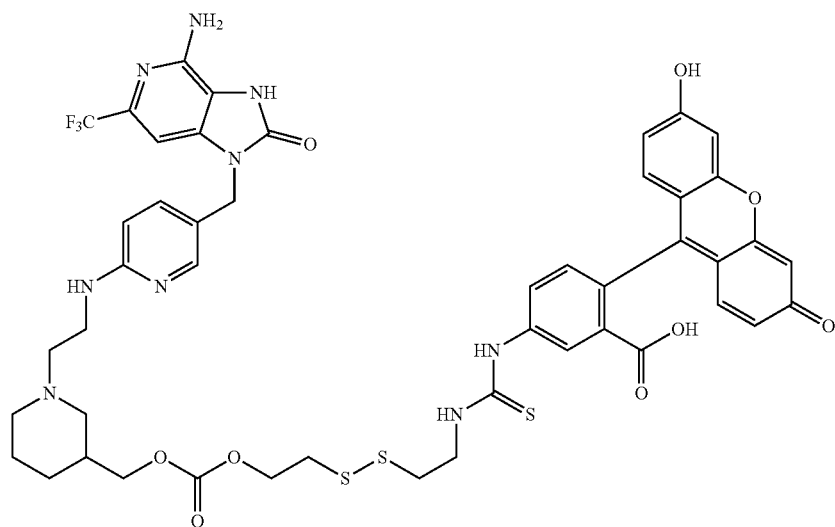
12. The system according to claim 1, wherein the first component is a FK506-TLR7 agonist having the structure of



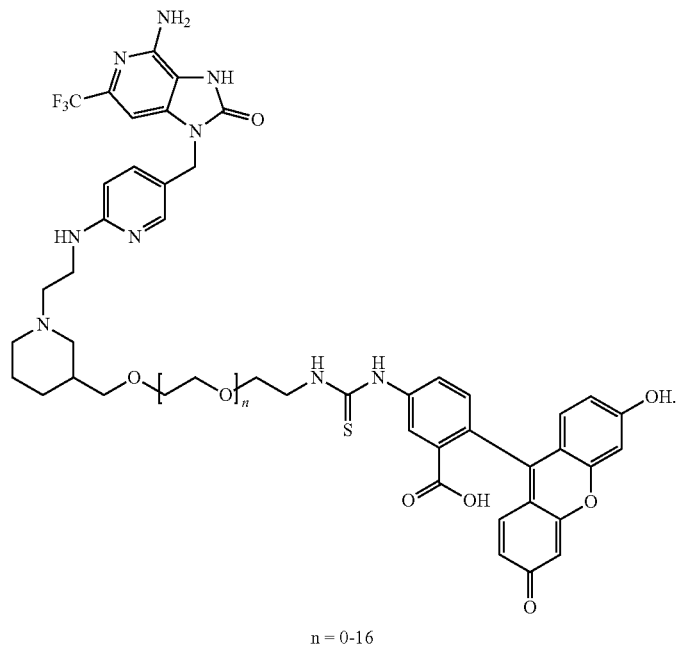
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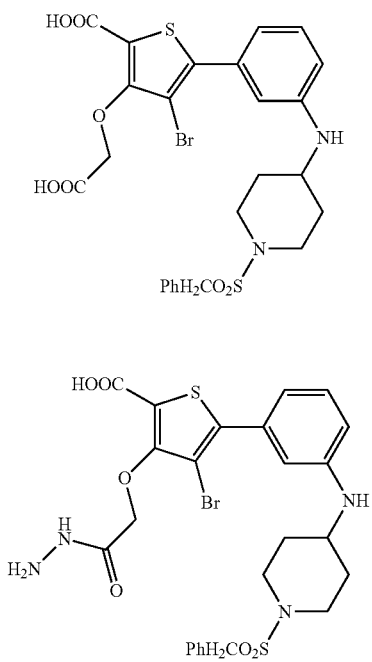
13. The system according to claim 1, wherein the first component is one of the following:



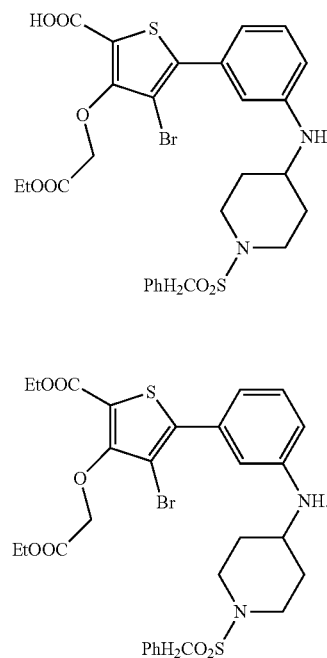
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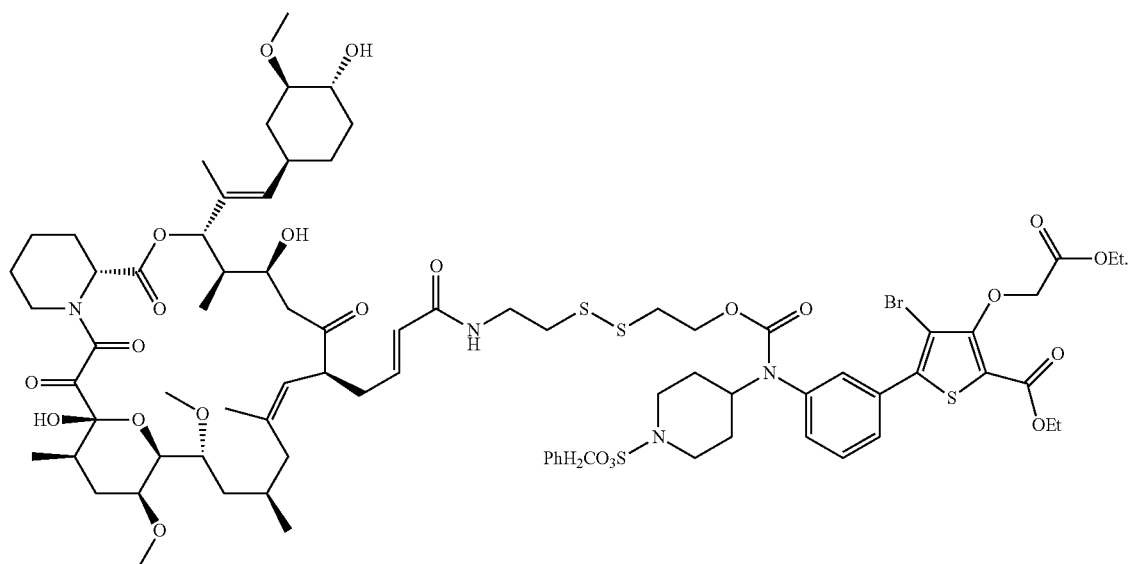
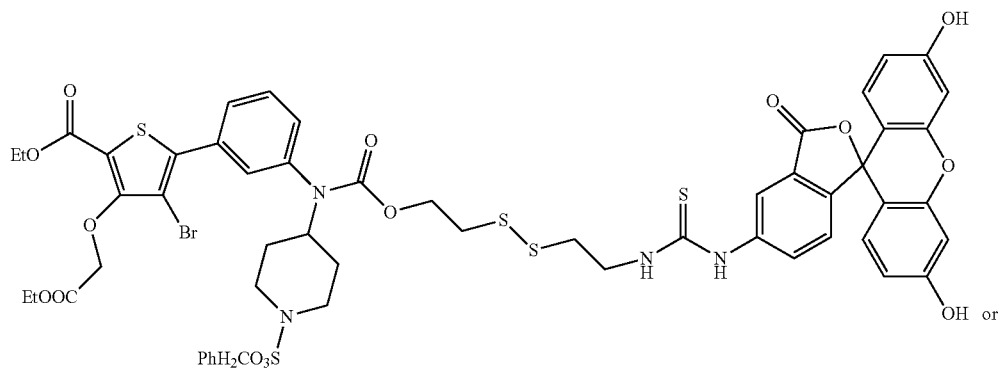
14. The system according to claim 1, wherein the first component comprising the payload drug selected from the group consisting of following TC-PTP phosphatase inhibitors:



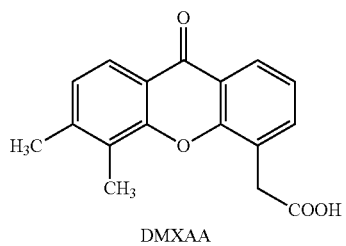
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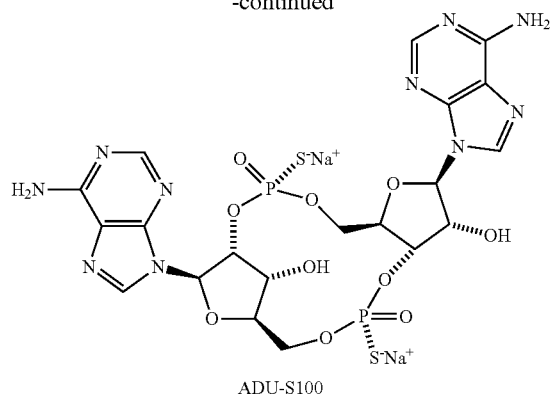
15. The system according to claim 14, wherein the phosphatase inhibitor is connected to the fluorescein or FK506 (tacrolimus) to form the following structures:



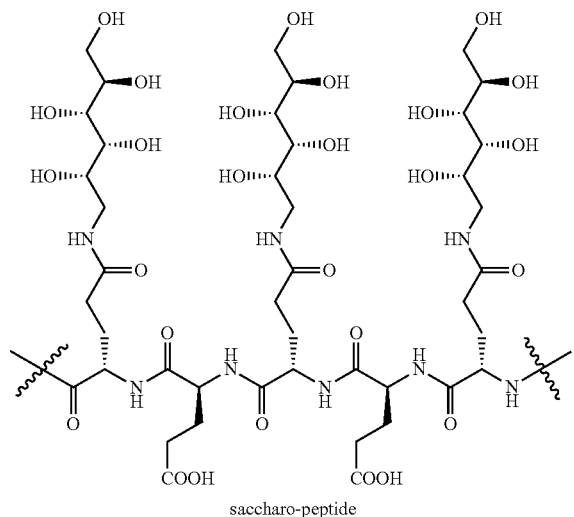
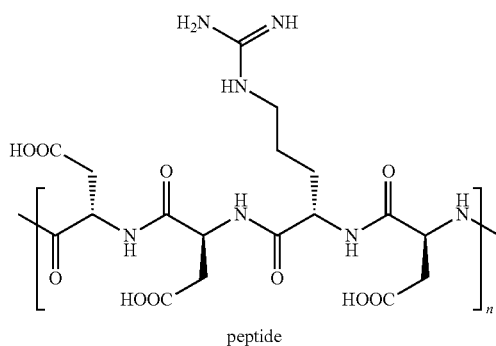
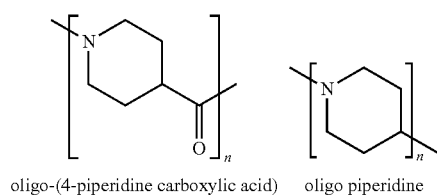
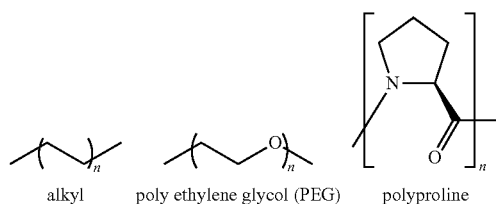
16. The system according to claim 1, wherein the payload drug in the first component comprises a STING agonist of one of the following structures.



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17. The system according to claim 1, wherein the first component comprises a spacer between the targeting ligand and the payload drug selected from the group consisting of the following structures:



18. A method to rejuvenate an exhausted CAR T cell, comprising:

- a. providing said exhausted CAR T cell a first component comprising a conjugate, wherein the conjugate comprises a targeting ligand covalently linked to a payload of drug through a releasable or non-releasable linker;
- b. providing said exhausted CAR T cell a second component comprising a fusion receptor linked to the exhausted CAR construct, wherein the fusion receptor comprises a targeting ligand binding module and a membrane bound receptor module;
- c. letting the targeting ligand binding module of the second component bind to the targeting ligand in the first component to form a complex,
- d. letting the membrane bound CAR module mediate internalization of the complex into the exhausted CAR T cell;
- e. letting the payload drug either block the inhibitory signaling of the exhausted CAR T, or re-activate said CAR T through an antigen independent pathway.

19. The method according to claim **18**, wherein the payload drug executes its function within the endosome of the exhausted CAR T, and the targeting ligand and the payload drug are linked by a nonreleasable linker.

20. The method according to claim **18**, wherein the payload drug executes its function as a free drug in the cytosol of the exhausted CAR T, and the targeting ligand and the payload drug are linked by a releasable linker.

21. The method according to claim **18**, wherein the targeting ligand of the first component is folate, FITC or FK506.

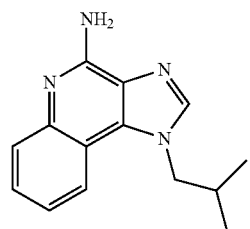
22. The method according to claim **18**, wherein the targeting ligand binding module of the second component anti-FITC, folate receptor, or FKBP.

23. The method according to claim **18**, wherein the ligand binding module is Folate Receptor alpha (FRa).

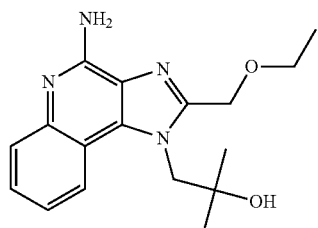
24. The method according to claim **18**, wherein the payload of drug is a Toll Like Receptor 7 (TLR7) agonist or Simulator of interferon genes (STING) agonist.

25. The method according to claim **18**, wherein the payload of drug is an inhibitor to following proteins: SHP1/2, TC-PTP or DGK α , TGF β .

26. The method according to claim 18, wherein the TLR7 agonist has the structure of

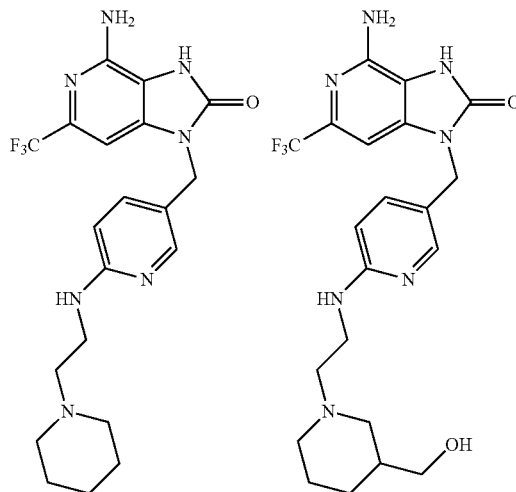


Imiquimod



Resiquimod

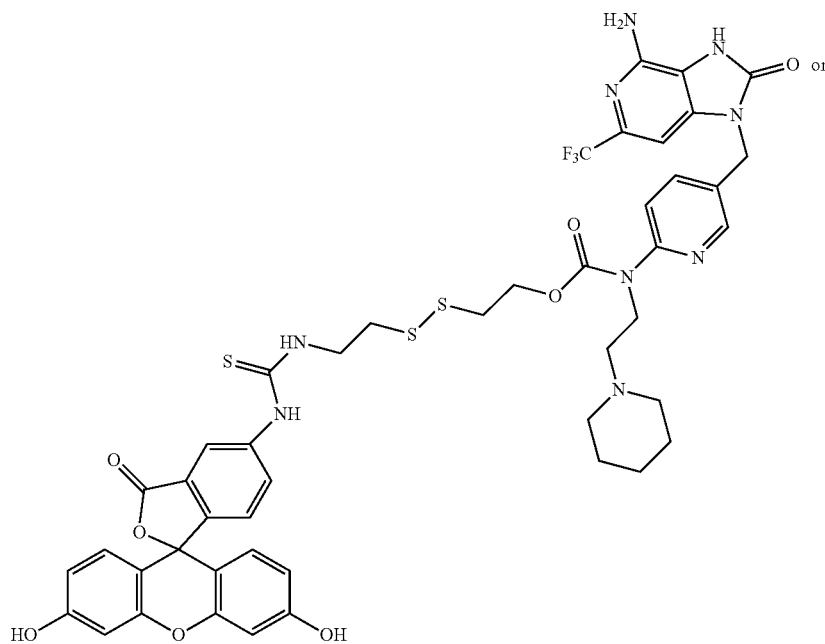
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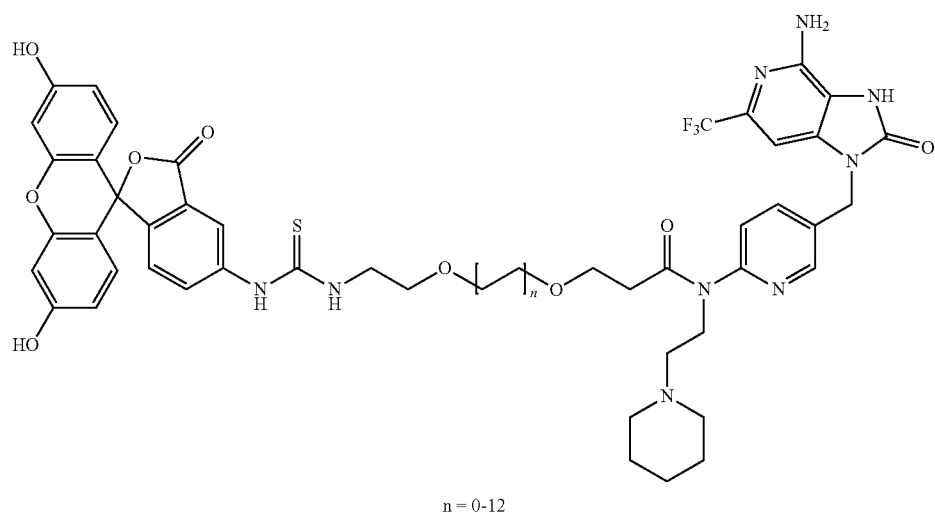
TLR7 agonist

JTLR7

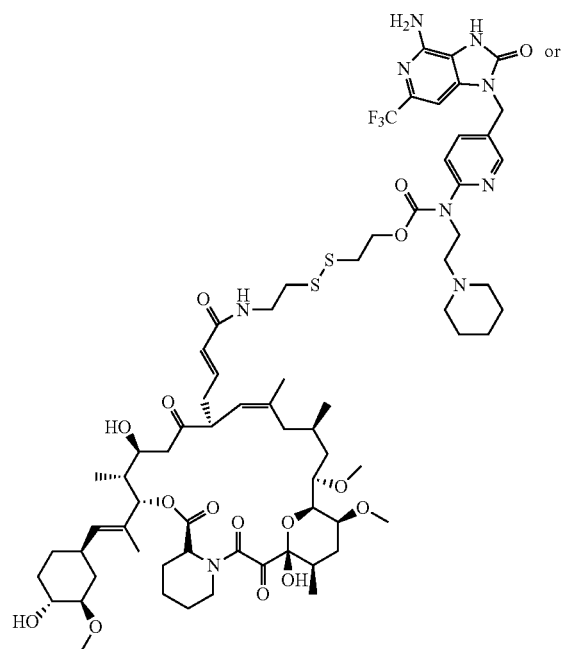
27. The method according to claim 18, wherein the first component is a Fluorescein-TLR7 agonist having the structure of



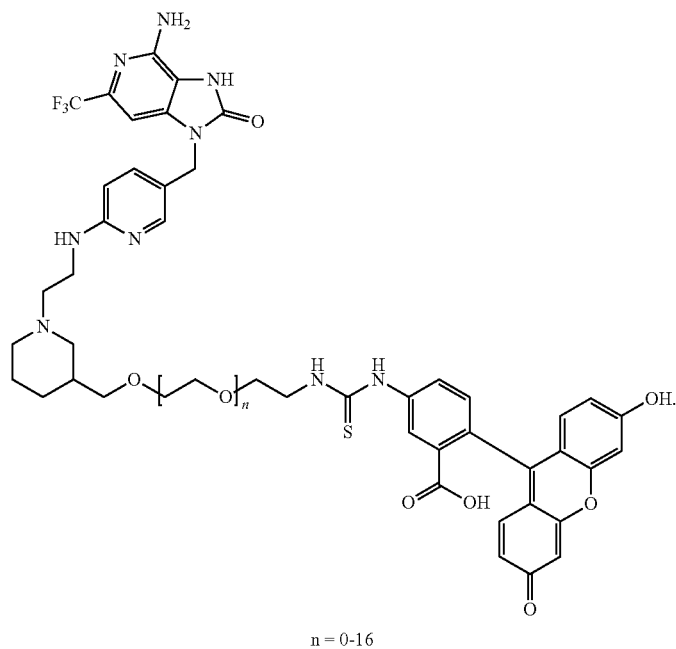
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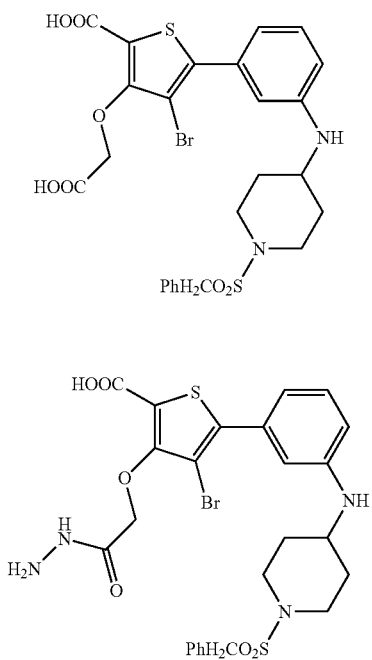
28. The method according to claim 18, wherein the first component is a FK506-TLR7 agonist having the structure of



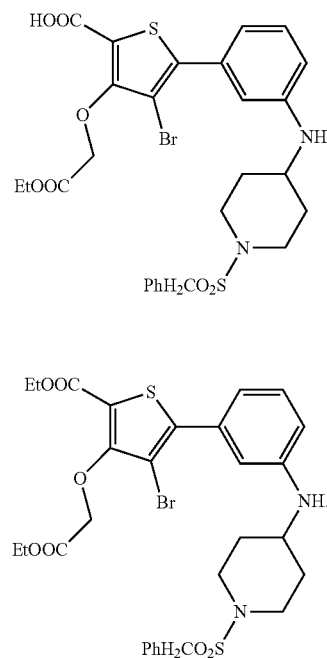
-continued



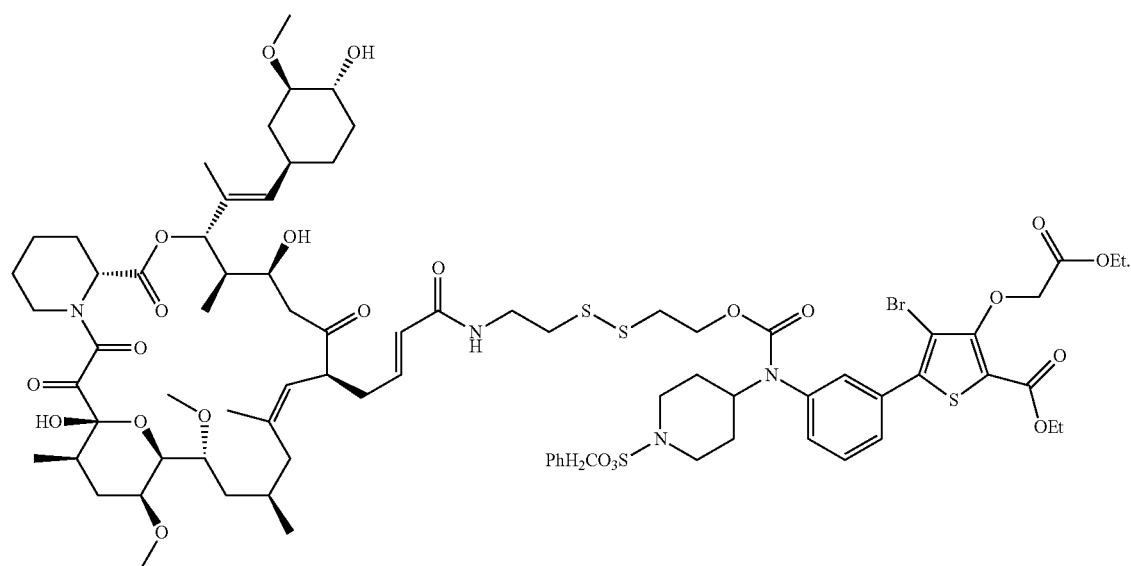
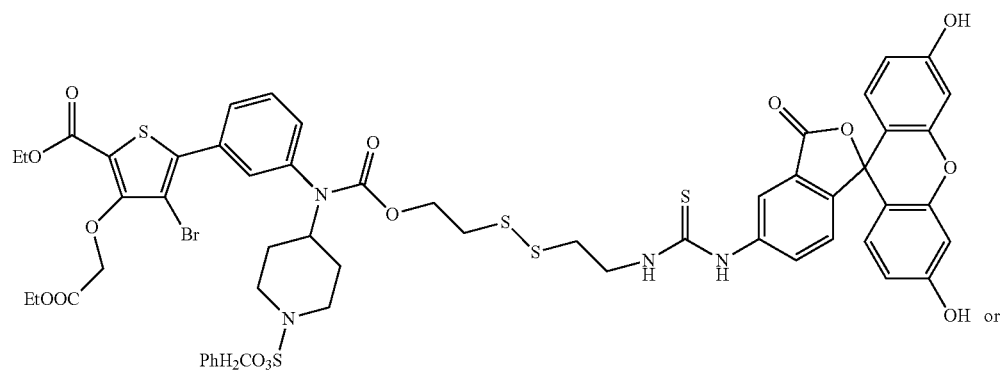
30. The method according to claim 18, wherein the first component comprising the payload drug selected from the group consisting of following TC-PTP phosphatase inhibitors:



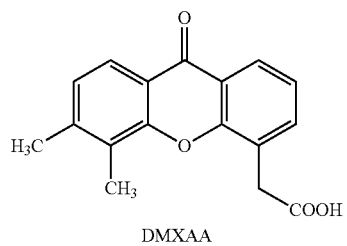
-continued



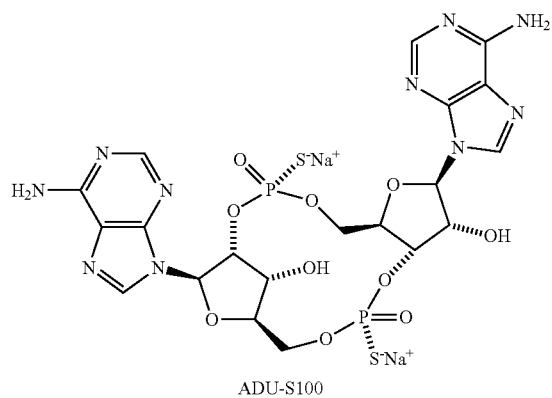
31. The method according to claim 30, wherein the Phosphatase inhibitor is connected to the fluorescein or FK506 (tacrolimus) to form the following structures:



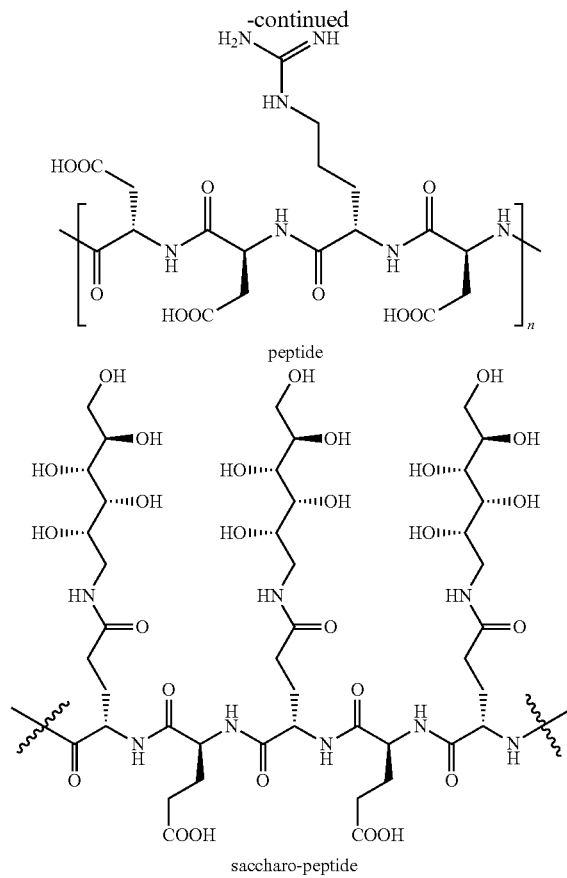
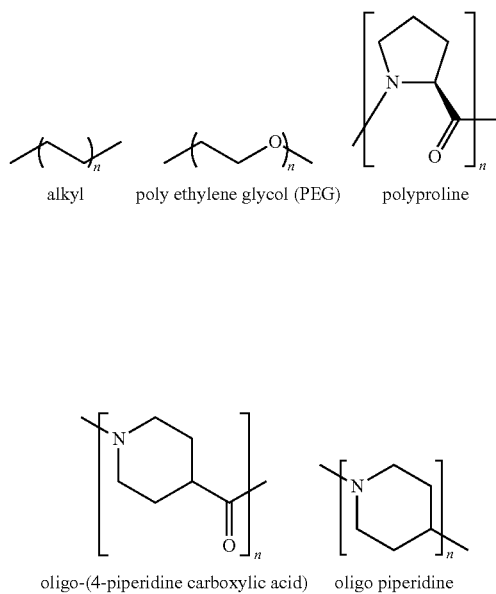
32. The method according to claim 18, wherein the payload drug in the first component comprising a STING agonist of the following structures.



-continued



33. The method according to claim 18, wherein the first component comprising a spacer between the targeting ligand and the payload drug selected from the group consisting of the following structures:



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