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(54) Title: TARGETED LIGAND-PAYLOAD BASED DRUG DELIVERY FOR CELL THERAPY

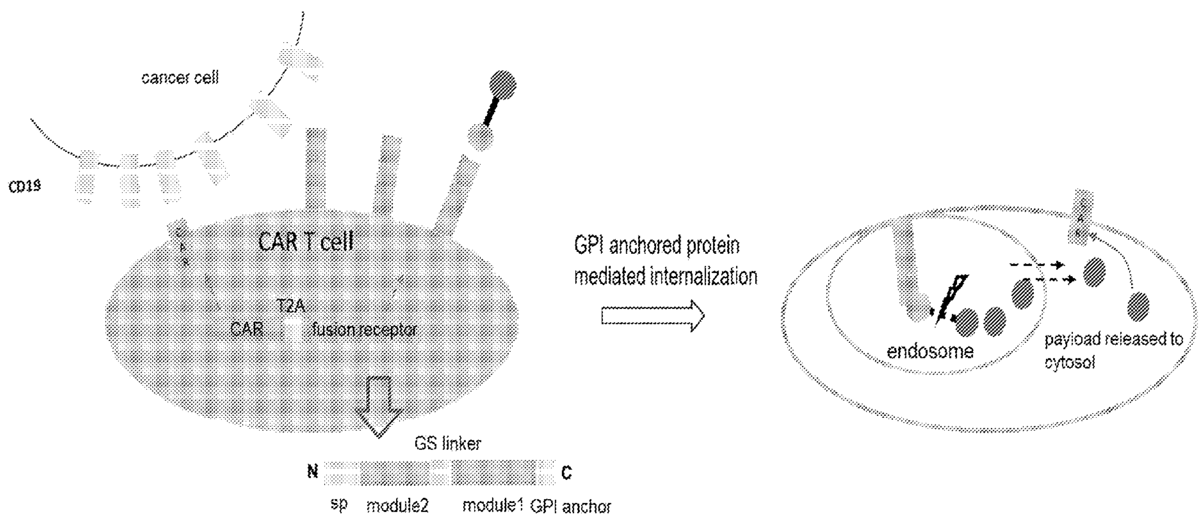

FIG. 1B

(57) Abstract: A drug delivery platform providing flexible fine tune of cell therapy is disclosed herein. Particularly, an engineered fusion protein is coupled with a high affinity ligand carrying at least one payload of drug to be internalized by the transplanted cell to observe or regulate transplanted cell therapy effects.

WO 2018/152451 A1

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TARGETED LIGAND-PAYLOAD BASED DRUG DELIVERY FOR CELL THERAPY

CROSS REFERENCE

This application claims the benefit of US provisional application 62/460,118 under 35 U.S.C. §119 (e), filed on February 17, 2017. The content of which is expressly incorporated herein entirely.

FIELD OF INVENTION

This disclosure provides a drug delivery platform for cell therapy. Particularly, an engineered protein is coupled with a high affinity ligand carrying at least one payload of drug to be internalized by the transplanted cell through the engineered protein to regulate transplanted cell therapy effects.

BACKGROUND

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.

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.

Regenerative medicine is a game-changing area of medicine with the potential to fully heal damaged tissues and organs, offering solutions and hope for people who have conditions that today are beyond repair. Advances in developmental and cell biology, immunology, and other fields have unlocked new opportunities to refine existing regenerative therapies and develop novel ones.

Stem cells have the ability to develop — through a process called differentiation — into many different types of cells, such as skin cells, brain cells, lung cells and so on. Stem cells are a key component of regenerative medicine, as they open the door to new clinical applications.

A variety of stem cells, including adult and embryonic stem cells may be used in regenerative medicine. In addition, various types of progenitor cells, such as those found in umbilical cord blood, and bioengineered cells called induced pluripotent stem cells are used in regenerative medicine. Each type has unique qualities, with some being more versatile than others.

Many of the regenerative therapies under development begin with the particular patient's own cells. For example, a patient's own skin cells may be collected, reprogrammed in a laboratory to give them certain characteristics, and delivered back to the patient to treat his or her disease.

Although the anti CD19 CAR T has received great success in clinical applications for leukemia treatment, lethal side effects such as the cytokine storm generated from the fast lysis of tumor cells, as well as the killing of normal CD19+ B cells by the fast proliferating anti-CD19 CAR T cell requires finer control of the CAR T cell. In stem cell based regenerative therapy, efforts have been put to better understand the differentiation process and trophic roles of the transplanted cells in the target tissue. Meanwhile, these processes can be potentially altered by some small molecule drugs that specifically delivered to the stem cell to further contribute to the regeneration of the target tissue.

Another long lasting concern about the CAR T cells as well as other stem cell based regenerative therapy is the tumorigenic potential of these transplanted cells. In summary, it will be ideal to have a private doorway to control the activity of the transplanted cell, either CAR T cell or stem cell, after they are being transplanted.

SUMMARY OF THE INVENTION

This disclosure provides a drug delivery platform for fine tuning cell therapy. The drug delivery system comprises:

a. an engineered protein on a target cell for transplant, wherein the fusion protein comprises a first component and a second component, the first component and the second component are connected by a peptide linker, the first component is a non-membrane protein, the second component is a membrane anchored peptide or protein;

b. at least one small ligand conjugated to a linker, wherein the at least one small ligand has intrinsic high affinity to at least one component of the engineered protein; and

c. at least one payload of drug conjugated to the linker, wherein the payload of drug is associated with the target cell when the small ligand binds to at least one component of the engineered protein.

In some embodiment, the aforementioned drug delivery platform has a payload of drug of imaging agent. Such imaging agent may be slected from from the group consisting of fluorescent dye rhodamine, fluorescein, and S0456. Alternatively, such imaging agent is selected from the group consisting of radioisotope chelating imaging moieties, EC 20 chelating head, NOTA and DOTA.

In some embodiment, the aforementioned drug delivery platform has a payload of drug of cytotoxic drug. Such cytoxic drug may be selected from the group consisting of tubulysin, DM1, DM4, and an auristatin.

In some embodiment, the aforementioned drug delivery platform has a payload of drug of a modulator of gene expression.

In some embodiment, the aforementioned drug delivery platform has a payload of drug of modulator of the cell's activity.

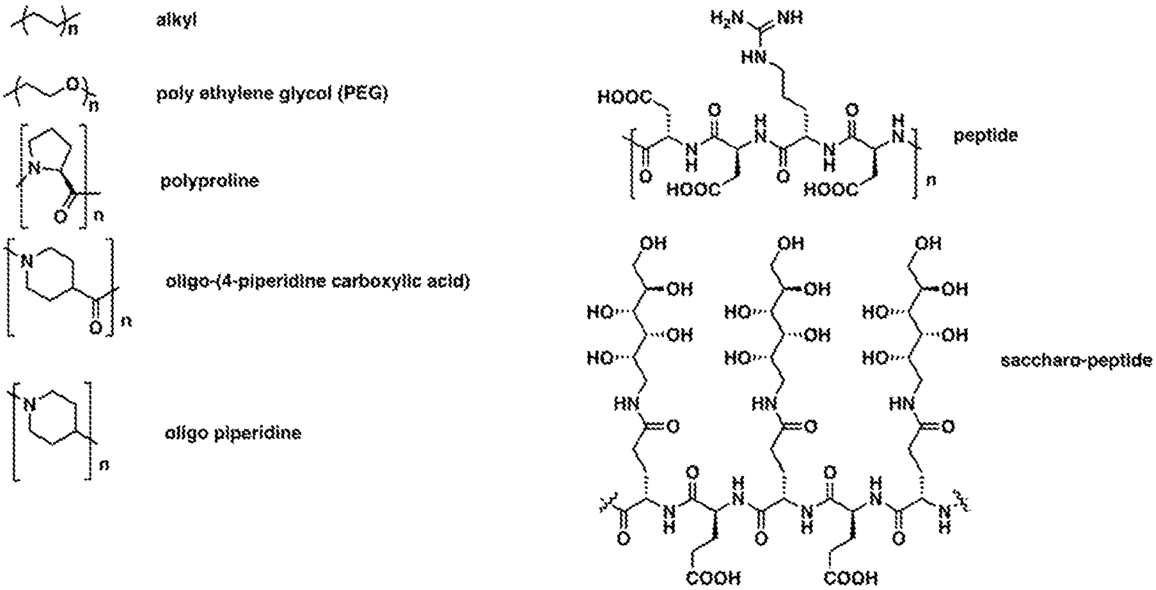
In some embodiments, the aforementioned modulator may be selected from the group of Dasatinib, MEK1/2 inhibitor, and PI3K inhibitor; group of HDAC inhibitor, kinase inhibitor and metabolic inhibitor; group of GSK3 beta inhibitor, MAO-B inhibitor and Cdk5 inhibitor.

In some embodiments, the aforementioned modulator is a phosphatase inhibitor, an RORγt agonist or an siRNA mi181a1.

In some embodiments the aforementioned payload of drug is a phosphatase inhibitor, including but not limited to inhibitors against SHP1/2, TC-PTP.

In some embodiment, the aforementioned payload of drug in the drug delivery platform is further internalized by the target cell when the small ligand binds to at least one component of the engineered protein.

In some embodiment, the aforementioned drug delivery platform has a releasable linker to connect the small ligand and the payload drug. The linker can be selected from the group consisting of



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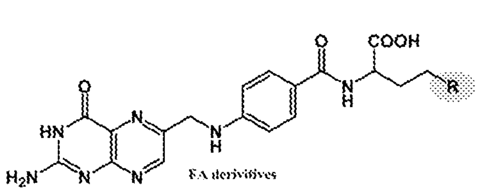
In some embodiment, the aforementioned engineered protein components are are selected from the group consisting of Folate Receptor alpha (FRa), Folate Receptor beta (FRb), Urokinase receptor (uPAR), FK506 binding protein (FKBP), dihydrofolate reductase (DHFR), Single Chain Fragment Variable against Fluorescein isothiocyanate (scFv against FITC), and Single Chain Fragment Variable against dinitrophenol (scFv against DNP).

In some embodiment, the aforementioned small ligand is selected from the group consisting of



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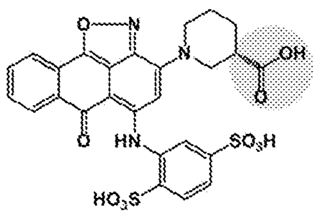

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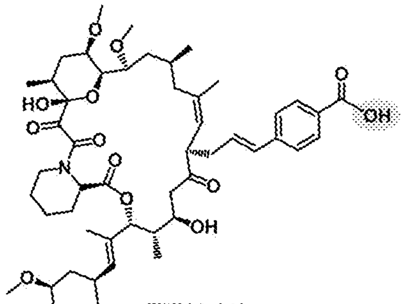
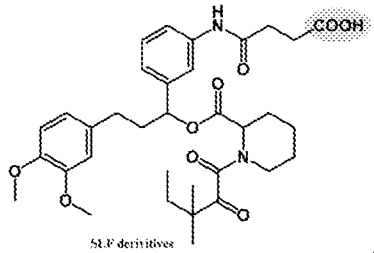
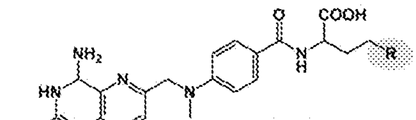
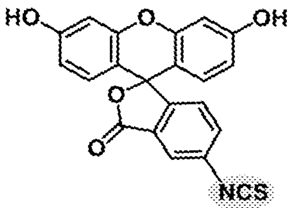
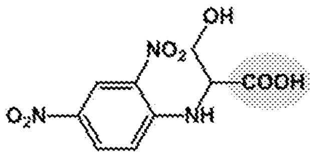

FK506 derivitives ,


SLF derivitives ,


MTX derivitives ,


FITC derivitives and


DNP-Ser .

In some embodiment, the aforementioned drug delivery platform has the first component as FKBP, the second component is a peptide that confers a glycosylphosphatidyl inositol (GPI) anchor on the first component, and the small ligand is FK506 or its derivative. In some embodiment, the FK506 derivative abolishes Calcineurin binding site.

In some embodiment, the aforementioned second component is a full length or truncated Folate Receptor (FR).

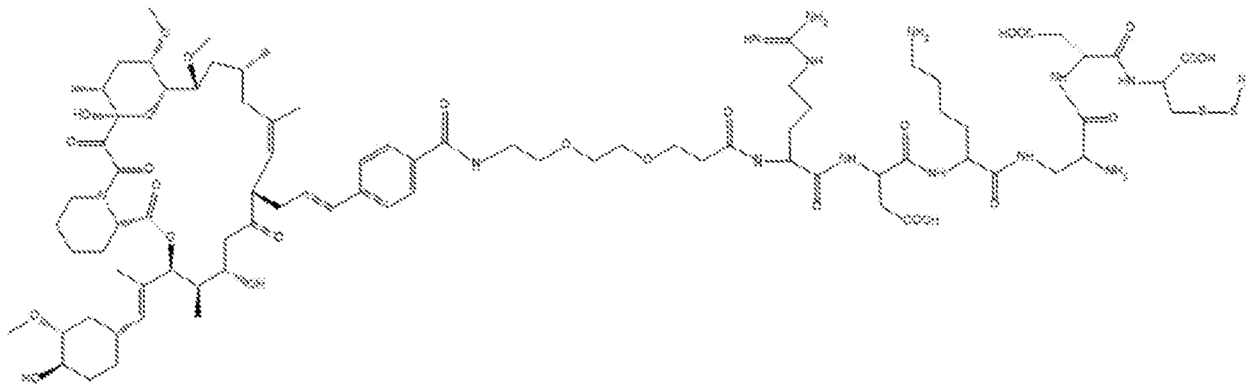
In some embodiment, the aforementioned drug delivery platform has at least one segment of flexible peptide linker SGGGS to connect the first component and the second component of the engineered protein.

In some embodiment the aforementioned drug delivery platform comprises an engineered protein selected from the group consisting of SEQ ID NOS:1-2 (amino acid sequence for mouse FKBP-FRa and amino acid sequence for human FKBP-FRa respectively).

In some embodiment the aforementioned drug delivery platform comprises an engineered protein selected from the group consisting of SEQ ID NOS:12-15.

In some embodiment the aforementioned target cell for transplant is an immune cell. For example, the immune cell can be a NK cell or a Chemeric Antigen Receptor T (CAR T) cell. Such CAR T cell may be expressing amino acid sequence selected from SEQ ID NOS: 3-4.

In some embodiment the aforementioned drug delivery platform has the small ligand conjugate with formula I.



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In some embodiment the aforementioned drug delivery platform has a target cell for transplant as CAR T cell expressing SEQ ID NO:3 (amino acid sequence for mouse antiCD19 CAR T construct) or SEQ ID NO:4 (amino acid sequence for human antiCD19 CAR T construct).

In some embodiment the aforementioned small ligand is further conjugated to a flurorescent dye or radioactive probe for tracking the drug internalization.

In some embodiment the aforementioned drug delivery platform comprises a small ligand that is further conjugated to a regulator of endogenous gene expression of a regulator of a transduced transgene expression.

In some embodiment the aforementioned drug delivery platform, the target cell for transplant is a stem cell, a progenitor cell, or a transplanted cell designed to synthesize a biochemical that is deficient in a patient.

This disclosure further provides a CAR T cell comprising a construct expressing amino acid sequences selected from SEQ ID NOS:12-15.

This disclosure further provides a DNA construct encoding an amino acid sequence selected from the group consisting of SEQ ID NOS:12-15.

This disclosure further provides a DNA construct encoding a FKBP-FRa fusion receptor comprising any of SEQ ID NOS:1-2 operably linked to an EF1a promoter in a expression vector. In some embodiment, such exprssion vector is pWPI having SEQ ID NO:5.

This disclosure further provides a DNA construct comprising any of SEQ ID NOS:6-8.

This disclosure further provides a transplanted cell comprising insert genes hFKBP-FR (SEQ ID NO:7) and human antiCD19 CAR (SEQ ID NO:9).

This disclosure further provides a transpant cell comprising insert genes mFKBP-FR(SEQ ID NO:8) and mouse antiCD19 CAR (SEQ ID NO:10).

This disclosure further provides a method to modulate cell therapy effect. The method comprises:

a. Identifying a target cell for transplant, wherein the transplanted target cell has a cell therapy function;
b. Providing an engineered fusion protein on the surface of the target cell for transplant, the fusion protein comprises a first component and a second component, the first component and the second component are connected by a flexible peptide linker, the first component is a non-membrane protein, the second component is a Glyclosylphosphatidyl inositol (GPI) anchored peptide or protein;
c. Providing a payload of drug conjugate to the target cell, wherein the payload of drug is conjugated to a small ligand through a linker, and optionally conjugated to a fluorescent dye, wherein the small ligand binds to at least one component of the engineered fusion protein with high affinity and is internalized by the target cell together with the payload of drug;
d. releasing the drug within the target cell to modulate the target cell therapy function.

In some embodiment, the aforementioned cell therapy function is to provide optically guided surgery to a subject.

In some embodiment, the aforementioned cell therapy function is to control the target cell proliferation.

In some embodiment, the aforementioned cell therapy function is to execute cytotoxicty to the target cell engaged cancer cell.

In some embodiment, the aforementioned transplanted target cell is an immune cell. For example, the target cell is a CAR T cell.

In some embodiment, the aforementioned transplanted target cell is a stem cell, a progenitor cell or a transplanted cell designed to synthesize a biochemical that is deficient in a patient.

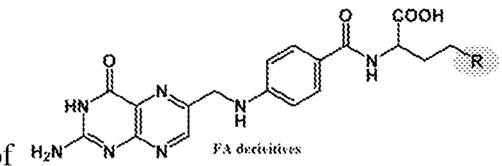
In some embodiment, the aforementioned payload of drug is an imaging agent selected from flurescent dye of Rhodamine and FITC, or a radioisotope imaging agent selected from EC20 chelating head, NOTA and DOTA.

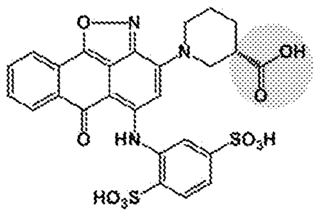
In some embodiment, the aforementioned payload of drug is a cytotoxic drug selected from the group consisting of Tubulysin, DM1, DM4 and auristatin.

In some embodiment, the aforementioned payload of drug is a modulator of gene expression selected from kinase inhibitors consisting of Dasatinib, MEK1/2 inhibitor and PI3 Kinase inhibitor, or an siRNA of mi181a1.

In some embodiment, the aforementioned transplanted target cell comprises a fusion protein selected from the group consisting of SEQ ID NOS: 12-15.

In some embodiment, the aforementioned engineered protein components are selected from the group consisting of FRa, FRb, uPAR, FKBP, DHFR, scFv against FITC, and scFv against DNP.

In some some embodiment, the aforementioned small ligand is selected from the group consisting of 

, 

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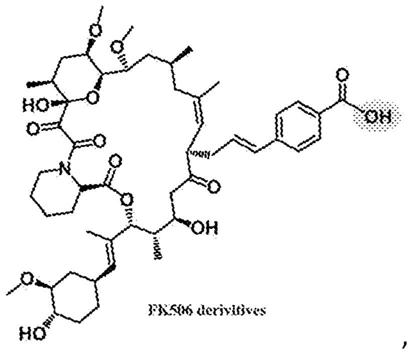

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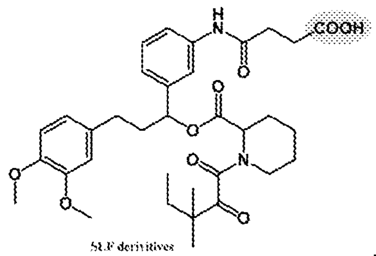

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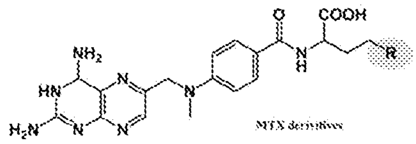

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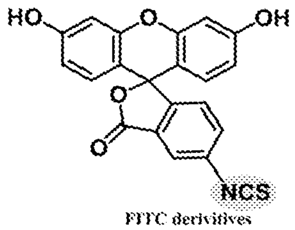

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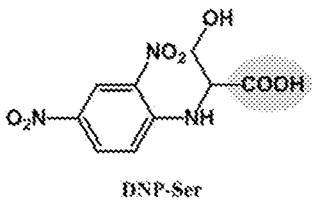

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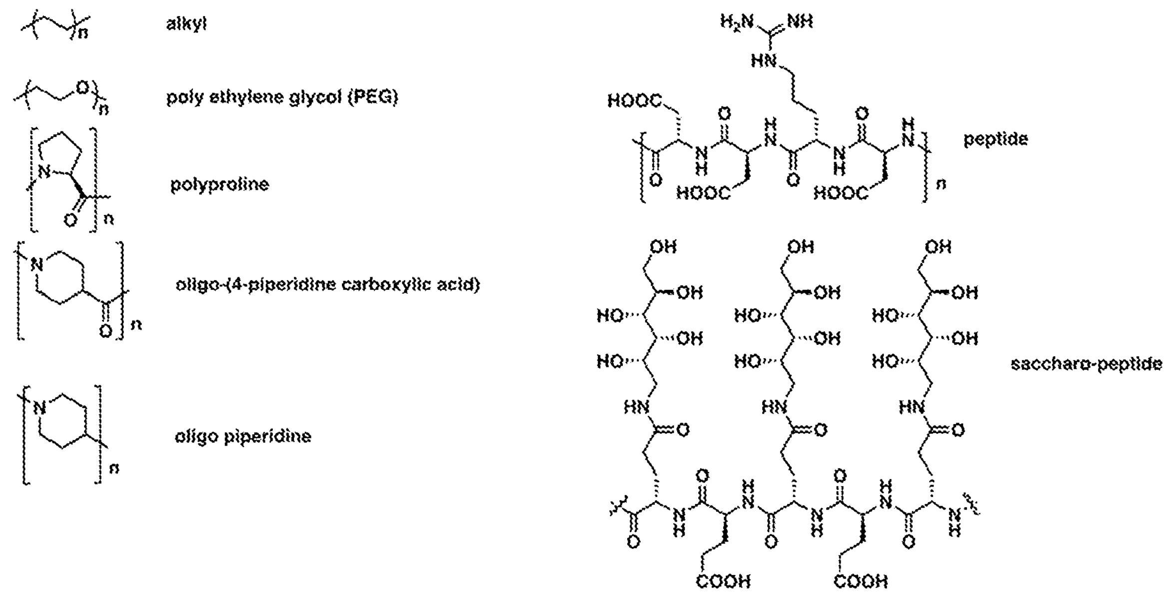
In some embodiment, the aforementioned linker to connect the small ligand and the payload drug is selected from the group consisting of



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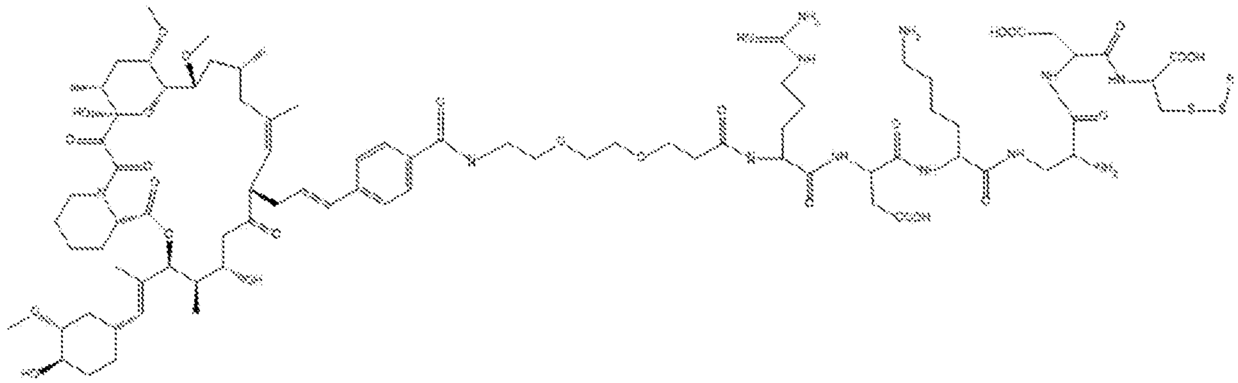
In some embodiment, the aforementioned transplanted target cell comprises an engineered FKBP-LINKER-FRa fusion protein selected from group consisting of SEQ ID NO:1 and SEQ ID NO:2.

In some embodiment, the aforementioned transplanted target cell is CAR T cell comprising an engineered antiCD19 CAR T construct selected from group consisting of SEQ ID NO:3 and SEQ ID NO:4.

In some embodiment, the aforementioned drug conjugate is FK506-releasable linker comprising formula I, wherein the binding domain of FK506 has an affinity to FKBP of about 4pM to about 100pM.



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In some embodiment the aforementioned transplanted target cell is a CAR T cell and the drug conjugate is selected from the group consisting of GSK3b inhibitor, MAPK inhibitor to control excessive cytokine storm of transplanted CAR T cell.

In some embodiment the aforementioned transplanted target cell is a CAR T cell and the drug conjugate is a modulator designed to control unwanted T cell proliferation.

In some embodiment the aforementioned transplanted target cell is a stem cell or progenitor cell and the drug conjugate is GSK3b inhibitor to boost bone fracture repair.

In some embodiment the aforementioned transplanted target cell is a stem cell, a progenitor cell or a transplanted cell designed to synthesize a biochemical that is deficient in a patient; and the drug conjugate is selected from the group consisting of MAO-B inhibitor and cdk5 inhibtior to treat Parkinson disease or other neurodegerative disease.

In some embodiment the aforementioned transplanted target cell is a NK cell and the drug conjugate is a RORγt agonist to control Th17 cell mediated immune responses.

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

Figure1: A. overview of the FKBP-FRa and FK506-payload based drug delivery platform for cell therapy; B. Graphic illustration of the secret pathway platform for CAR T cells delivery of payload.

Figure 2A.left: chemical structure of FK506 with FKBP binding sites (yellow) and derivatized site (red) highlighted. Right: co-crystal structure of ternary complex of a calcineurin A fragment (green), calcineurin B (cyan), FKBP12 (purple) and the FK506 (yellow), PDB: 1TCO

Figure 2B. Various combination options of two modules in an engineered fusion protein and their respective ligand choices, with potential derivatization sites highlighted.

Figure3: left: negative and positive regulation of the CAR T cell activity, adapted from *The quest for spatio-temporal control of CAR T cells, Sun J. etc. 2015.* Right: mechanism of AP1903 (FK506 dimer) induced FKBP-caspase9 mediated apoptosis and the structure of AP1903, adapted *from Inducible Apoptosis as a Safety Switch for Adoptive Cell Therapy, Malcolm K.B. etc. 2011*

Figure 4: **a**. pWPI expression vector map with FKBP-FRa insert (hFRa 1-24: red, FKBP: yellow, hFRa 25-258: red) **b**. FKBP-FRa transduced K562 cells show a higher band around 50kDa (37kDa for FRa plus 12kDa for FKBP) compare to FRa positive KB cells and non-transduced K562 cells. **c.** payload carrier construct and CAR T construct design **d.** The construct design of FKBPFR3GS (noted as FF3). From N terminal to C terminal, it has 1-24 aa of human FRa as the signal peptide, human FKBP protein, three Gly-Ser linker and then 25-258 aa of human FRa. In FKBPFR1GS (noted as FF1), the three Gly-Ser liner of FF3 is substituted with one Gly-Ser linker with other parts unchanged. **e**. The construct design of 4m5.3FR. From N terminal to C terminal, it has hCD8 signal peptide, scFv of 4M5.3 antibody against FITC, GS linker, 25-258aa of human FRa.

Figure 5. Interference between the FR and FKBP in FKBPFR1GS fusion receptor. Binding of Folate acid in FKBPFR1GS fusion protein blocks the binding of FK506-Rhodamine at as low as 0.01nM and totally abolish FK506-Rhodamine binding at 50nM.

Figure 6. FKBPFR1GS jurkat cell shows decreased FK506-Rhodamine intensity after binding to OTL38. FRET from FK506-Rhodamine (donor) to OTL38 (FA-S0456, acceptor, ex/em: 774/794nm) within the fusion receptor indicates the interaction between FR and FKBP.

Figure 7. Increasing the linker length between FKBP and FR significantly lowers the interference between the two parts. Compare to FF1 (1GS between FKBP and FR), FF3 (3GS between FKBP and FR) preserves the binding of FK506-Rhodamine in the presence of 10nM FA, which is comparable to the physiological concentration of FA in human body.

Figure 8. PI-PLC treatment releases the GPI anchored fusion receptor FF3. Jurkat T cell with FF3 fusion receptor shows saturated binding with 20nM FA-FITC (EC17), while after 5mU PI-PLC or 50mU PI-PLC treatment, the FA-FITC loses binding to the cell, which indicates the release of the GPI anchored FF3 fusion receptor.

Figure 9. FA-Rhodamine binding curve in FKBPFR3GS fusion receptor. FKBPFR3GS fusion receptor that stably expressed on human T cell can bind to folate acid derivative (FA-Rhodamine) with high affinity (Kd=0.95nM), which is comparable to the affinity of FA-Rhodamine in FR+ KB cell (Kd around 1nM). Therefore, the binding property of FR in the fusion receptor is preserved.

Figure 10. FK506-Rhodamine binding curve in FKBPFR3GS fusion receptor. FKBPFR3GS fusion receptor that stably expressed on human T cell is able to bind to FK506 derivative (FK506-Rhodamine) with high affinity (Kd=3.93nM), which means the binding property of FKBP in the fusion receptor is preserved.

Figure 11. SLF-FITC binds to FKBPFR3GS fusion receptor with relatively high binding affinity (Kd=62nM), while competition with free SLF (100x, preincubation) blocks this binding. SLF, a mimic of FK506, presents a 10 times lower binding affinity to FKBPFR fusion receptor, compare to the parent ligand FK506, which is consistent with previous report.

Figure 12. FA-Rhodamine binding curve in 4M5.3FR fusion receptor. FA-Rhodamine can binds to 4M5.3FR fusion receptor that stably expressed on human T cell with high affinity (Kd=2.25nM), which is comparable to the affinity of FA-Rhodamine in FR+ KB cell (Kd around 1nM). Therefore, FR binding property is preserved in 4M5.3FR fusion receptor.

Figure 13. FITC-AF647 binding curve in 4M5.3FR fusion receptor. FITC-AF647 can binds to 4M5.3FR fusion receptor that stably expression on human T cell with high affinity (Kd=8.03nM). 100x comp indicates free FITC sodium. The binding property of scFv 4M5.3 with FITC is preserved in 4M5.3FR fusion receptor.

Figure 14. FA-Tubulysin is able to mediate a receptor specific killing effect against FF3+ human T cell. Compensation with FA (100x preincubation with FA) blocks the effect. This implies the successful internalization and release of the free drug Tubulysin through the FF3 fusion receptor system.

Figure 15. FA-Tubulysin specifically kill the hFF3+ population in a mixed human T cell culture. Absolute number of hFF3+ cell decrease as the FA-Tub concentration increases, while hFF3- cells are killed also through released drugs and bystander effect at high concentration.

Figure 16. SLF-Tub specifically kill the hFF3+ Jurkat cells with a IC_{50} = 138nM. This indicates the successful internalization of SLF-Tub by the FKBPFR3GS fusion receptor and the release of the Tubulysin inside the cell.

Figure 17. Both FITC-DM4 and FITC-Tub can specifically kill the 4M5.3FR+ human T cells, while FITC-Tub has a higher IC_{50}. Compensation of free FITC sodium (100x preincubation) blocks the receptor mediated killing effect. This implies the successful internalization and release of FITC-cytotoxic drug into T cell through 4M5.3FR fusion receptor.

Figure 18 FITC-Tubulysin specifically kill the 4M5.3FR+ population in a mixed human T cell culture. Absolute number of 4M5.3FR+ cell decrease as the FITC-Tub concentration increases, while the 4M5.3FR- cells are killed also through released drugs and bystander effect at high concentration.

Figure 19 FITC-DM4 specifically kill the 4M5.3FR+ population in a mixed human T cell culture. Absolute number of 4M5.3FR+ cell decrease as the FITC-DM4 concentration increases, while the 4M5.3FR- cells are killed also through released drugs and bystander effect at high concentration.

Figure 20. Dasatinib (Lck inhibitor) and Ibrutinib (ITK inhibitor) at 10nM concentration decrease the lysis effect of antiCD19 CAR T cell (FMC63 CAR T, Effector) against CD19+ Raji tumor cell (Target). Two Effector: Target ratio (E:T) have been tested. Normal T cell and antiCD19 CAR T with CD19- K562 cell were used as negative control.

Figure 21. FITC-Dasatinib can decrease the lysis effect of FMC63+4M5.3FR+hT cell towards Raji cell. This implies the successful internalization and release of FITC-Dasatinib into T cell through 4M5.3FR fusion receptor and the release of Dasatinib into T cell.

Figure 22. TC-PTP inhibitor at 100nM concentration decrease the co-inhibitor molecule population in exhausted antiCD19 CAR T cell (generated by 7 times of stimulation with CD19+Raji cells, see detailed procedure below). Both PD-1, LAG3 and double positive population decreases upon treatment. This implies that phosphatase inhibitors, like TC-PTP inhibitor may be used as a payload for the secret gateway platform for rejuvenating the exhausted CAR T cell.

TABLE 1. Potential applications of the FKBP-FRa cell therapy platform and the corresponding payload.

SEQUENCE LISTINGS

SEQ ID NO:1 Amino acid sequence for mouse FKBP-FRa

SEQ ID NO:2 Amino acid sequence for human FKBP-FRa

SEQ ID NO:3 Amino acid sequence for mouse antiCD19 CAR T construct

SEQ ID NO:4 Amino acid sequence for human antiCD19 CAR T construct

SEQ ID NO:5 vector pWPI for human T cell transduction

SEQ ID NO:6 pMP71 gb NotIEcoRI mouse antiCD19 for mouse T cell transduction

SEQ ID NO:7 pWPI-FRa 1-24 FKBP FRa

SEQ ID NO:8 pWPI mFKBP-mFRa SGGGS

SEQ ID NO:9 pHR EcorI hAnti cd19 1D3 myc hinge cd28 cd3zeta

SEQ ID NO:10 pWPI pmei mAnti cd19 1D3 myc hinge cd28 cd3zeta

SEQ ID NO:11 FKBP-1SG-FR with GPI anchor amino acid sequence

SEQ ID NO:12 FKBP-3SG-FR with GPI anchor amino acid sequence

SEQ ID NO:13 4M5.3-FR with GPI anchor amino acid sequence

SEQ ID NO:14 FMC63-T2A-FKBP3SGFR

SEQ ID NO:15 FMC63-T2A-4M5.3SGFR

SEQ ID NO:16 FRb with signal peptide

SEQ ID NO:17 uPAR with signal peptide

SEQ ID NO:18 DHFR

SEQ ID NO:19 scFv against FITC: 4M5.3(Kd = 200pM)

SEQ ID NO:20 scFv against FITC 4D5Flu (Kd=10nM)

SEQ ID NO:21 scFv against DNP SPE7

DETAILED DESCRIPTION

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.

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.

This disclosure provides a novel platform for controlling the transplanted cell activity by genetically incorporating a fusion receptor on the transplanted cell surface. These transplanted cells will then be specifically targeted by a small molecule ligand conjugated-drug payload, using the intrinsic high affinity between the small molecule ligand and the fusion receptor on the transplanted cell surface. One part of the fusion receptor is responsible for internalizing the conjugate and the payload will be released through a releasable linker once it is inside of the transplanted cells. Depending on the transplanted cell type and the desired regulation to be imposed on the transplanted cells, the drug payload can be various functions. By changing the payload in the conjugate, for example, as cytotoxic drug or kinase inhibitors, the drug payload may be used to control the multiple aspects of the transplanted cells, such as proliferation, differentiation or cytokine release profile.

The peptidyl-proline isomerase (PPIases) family consists of FK506-binding protein (FKBP), cyclophilins and parvulins. In human, there are 18 FKBPs, 24 cyclophilins and 3 parvulins. Among these, FKBP51 and FKBP52 share high to moderate binding affinity of FK506, $KD^{FK506} \approx 104$ nM and $KD^{FK506} \approx 23$ nM, respectively, comparing to FKBP12 (KD^{FK506} ≈ 0.2 nM). Additionally, none of these two FKBPs are expressed on the cell membrane, resulting in little cross binding activity in our system. Efforts have also been made to design synthetic ligands that have higher affinity to $FKBP12^{F36V}$ than $FKBP^{WT}$, as well as to $FKBP51^{F67V}$, which preserve the overall structure of the wild type proteins. All the homolog and mutated proteins together with their ligands mentioned above, can be adapted to this disclosure.

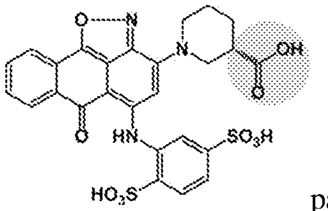
Particularly, in one embodiment, an exemplified pair of small molecule ligand and fusion receptor is chosen as FK506-FKBP. The entire process can be generalized in Figure 1A, where the FK-506-payload binds to the FKBP-FRa engineered cell first; upon this binding, the transmembrane fusion protein will internalize the payload-linker-FK506. Next, the internalized FK506-payload is cleaved at the linker, and the payload got released in the cell. Depending on the cell type and the payload type, the released payload drug can exert its desired function.

In Figure 1B, a specific Chimeric Antigen Receptor T cell mediated cell therapy is illustrated. In this model, a CAR T cell expressing a fusion protein with the structure from N terminus to C terminus comprising a suitable signal peptide, a protein module 2 linked to a protein module 1 of GPI anchor is presented to a cancer cell. In some embodiment, the cancer cell has CD19 surface protein, which will be recognized by the CAR T cell and engaged with the payload associated with the CAR T cell, when a targeting ligand binds to at least one module of the fusion receptor. Typically, with high affinity of the targeting ligand toward any one of these modules, the payload associated with the ligand may be internalized into the target cell and be released to engage the cancer cell through the chimeric antigen receptor.

There can be many different combinations of GPI anchored proteins, represented as Module 1 and its ligands, and Module 2 target cell surface protein and its respective ligands, represented in Figure 2B. It is contemplated that either module 1 protein or module 2 protein, or both can engage a high affinity targeting ligand to facilitate the ligand conjugated payload delivery. For example, it is feasible to have a fusion protein comprising FRa-Linker-FKBP structure, wherein FRa engaging with an FA derivative, at the same time, FKBP engaging a

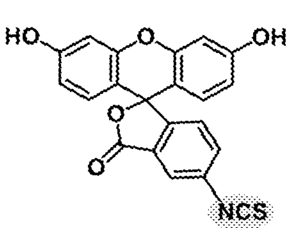
FK506 derivative, either FA derivative or FK 506 derivative or both can be linked to a payload, such as a cytotoxic drug, or an imaging agent, or a modulator. With such flexibility of carrying same or different payload, one can achieve some unexpected, synergy or regulatory effect of different or same payload, or to observe the target cell if the payload is an imaging agent. The advantages of flexibility and diversity of payload delivery of this system will be appreciated with more examples.

Similarly, another embodiment of ligand paired with a GPI anchored protein can be



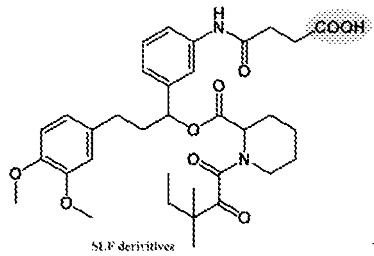
paired with uPAR.

It is also contemplated that FITC or its derivatives may bind to a single chain fragment of variant (scFv) of an antibody against FITC, for example, a ligand structure of



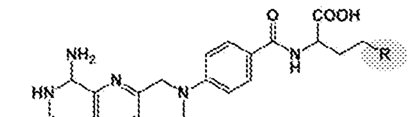
FITC derivitives

, or a structure of



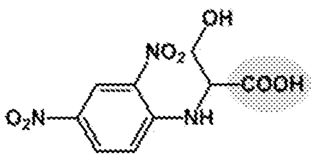
SLF derivitives

paired with FKBP, or



MTX derivitives

paired with DHFR, or



DNP-Ser

paired with scFV against DNP.

It is worth mentioning a few advantages of choosing FK506-FKBP as an exemplary ligand-protein pair in this delivery system. 1. FKBP is not a membrane protein that naturally present on the mammalian cell membrane, so that the FK506-payload conjugate will specifically bind to the target cell; 2. FKBP protein is a relatively small protein with molecular weight of 12kDa, which makes it easier to be fused with other receptors with minimum perturbation to the receptors' structure and internalization properties. 3. FK506 is not naturally present inside human being, so that the fusion receptor will not be blocked; 4. The binding affinity between FK506-FKBP is around 4pM so that the payload drug can be delivered with high affinity; 5. The co-crystal structure of FK506-FKBP is available and the well-established derivative site of FK506 preserves the binding of FK506-FKBP while abolishes the unwanted binding between FK506 and calcineurin (See Figure 2A).

It is contemplated that the sequence of FKBP can be modified, and the corresponding FK506 ligand can be modified accordingly to the extent that the modified versions of FKBP and modified version of FK506 still have the desired affinity as exemplified herein or better than the current disclosure.

For the other portion of the fusion protein, folate receptor (FR) is chosen for its well understood internalization process as a monomer. Prior research shows that 'magic carbonate' linked folic acid conjugate can be internalized through FR and cleaved by the reductive environment inside the cell. Using this mechanism, the FK506-FKBP conjugated drug payload is internalized by FR and will then be released to the cytoplasm.

Due to the great potential of CAR T therapy as well as the severe side effects, several controlled CAR T cell designs have been reported. Most of them focused on the ON/OFF switch by incorporating either a Boolean gate or a cascade pathway for the T cell activation (see Figure 3, left, which depicts negative and positive regulation of the CAR T cell activity, adapted from *The quest for spatio-temporal control of CAR T cells, Sun J. etc. 2015*). Malcolm K.B. group designed the FKBP-caspase9 fusion protein and use FK506 dimer to induce the apoptosis of the target cell (see Figure 3, right, which depicts mechanism of AP1903 (FK506 dimer) induced FKBP-caspase9 mediated apoptosis and the structure of AP1903, adapted *from Inducible Apoptosis as a Safety Switch for Adoptive Cell Therapy, Malcolm K.B. etc. 2011*).

The current disclosure has several advantages over these reported methods**:** 1. Instead of a binary ON/OFF switch, our platform can delivery multiple kinds of regulating payloads, modifying many aspects of the target cells, thus it has great flexibility compared to binary ON/OFF switch; 2. the controlling moiety, FK506-payload, is a small molecule, which makes it possible for linear control and dosage optimization compared to pre-engineered cells. 3. The platform can be utilized in not only CAR T cell, but many other stem cell based regenerative therapies.

The greatest novelty and most important part of this platform is the multifunctional payloads, which can be selected to either address the potential side effects or improve the efficiency of the cell therapy. For instance, cytotoxic drugs can be delivered to the transplanted cells if: 1. the cells over-proliferate and affect the normal organ or system, like anti CD19 CAR T cell. 2. The cells become tumorigenic, which lies in the lentivirus based gene modification and the intrinsic characteristic of stem cells.

On the other hand, some cell therapies receive less success because of the suppressive microenvironment of the target tissue. For instance, in CAR T cell therapy against solid tumor, aside from the low penetration rate, the proliferation and activation of CAR T is highly suppressed by the MDSC and the tumor cells. This can be potentially alleviated by the RORrt agonist or MAP kinase inhibitor induced T cell activation as well as TLR8 agonist induced expression of granzyme B in CAR T cells. Although intracellular targets, like RORrt, may be more suitable for our payload, membrane receptors such as TLR8 may also be accessible due to the proximity on the cell membrane.

In stem cell regenerative therapy, the payload is more diverse according to the disease models. Instead of delivering a pre-fixed gene, which has been developed by using stem cell as a gene delivery platform, the current disclosure provides a fine tune to transplanted cells and their microenvironment, and obtains the desired phenotype through diverse small molecule payloads. Since the small molecule is conjugated to FK506, which specifically target the FKBP-FRa overexpressed transplant cell, the non-specific targeting of normal tissues of the small molecule is also avoided.

One of the many examples is to induce the overexpression of BMP2 in mesenchymal stem cell (MSC) for bone fracture repair in skeletal regenerative therapy. Meanwhile, the expression level of BMP2 and/or VEGF can be increased by introducing GSK3 beta inhibitor to the transplanted cells through this drug payload delivery system. GSK3 beta inhibitor is a desired drug for bone fracture repair. Therefore, GSK3 beta inhibitors is ideal as a potential payload for further modulating the function of the transplanted MSC as well as the microenvironment within the bone fracture sites.

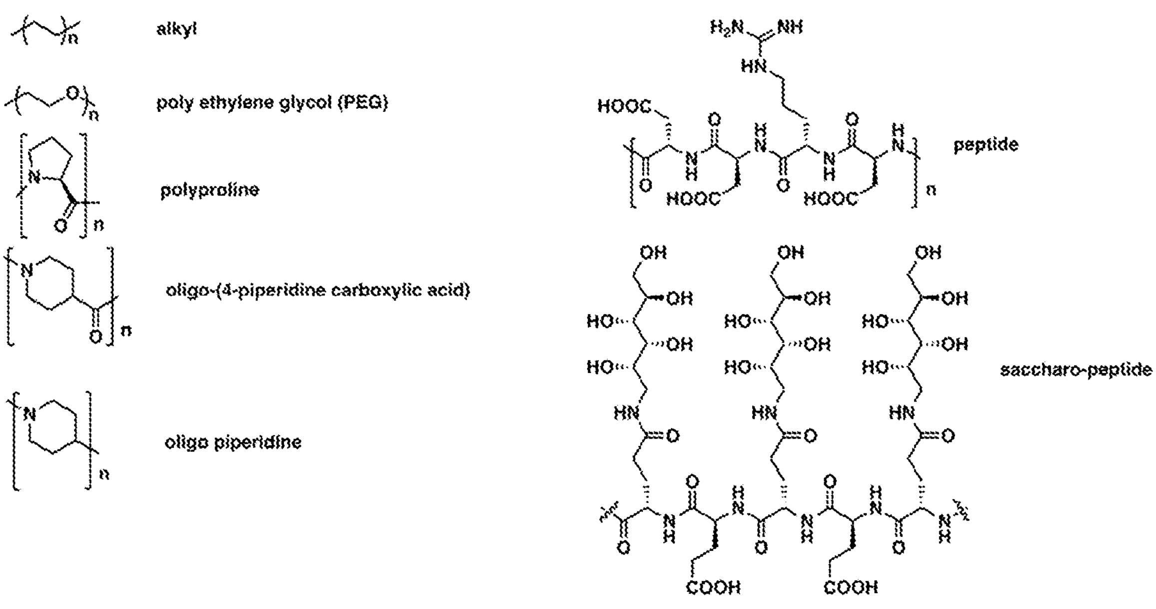
Another example of this drug payload delivery system goes to the neurodegenerative disease, including Alzheimer, Parkinson disease and etc., where MSC based therapy holds a promising future. MSC has been modified to overexpress GDNF, VEGF and many other cytokines to promote the neuronal regeneration. Meanwhile, small molecules like MAO-B inhibitors has been confirmed to increase the expression of GDNF, NGF and BDNF in astrocytes. Several kinase inhibitors have also been proposed for the treatment of Alzheimer disease, such like PI3K inhibitor (BEZ235), Cdk5 inhibitor (roscovitine) and GSK3b inhibitor (NP-12). Using FK506-FKBP pair targeted specific delivery of these small molecules to MSC in neurodegenerative models will improve the regeneration efficacy while avoiding the side effects of these potent inhibitors and agonists in other non-targeted tissues.

Material and Methods:

Compounds and Synthesis procedure:

Targeting ligand is linked with payload through linkers. The linker optimization options are listed below. payloads are characterized into 3 classes: I imaging, II cytotoxic drug, III regulatory small molecule drug.

Linker optimization:

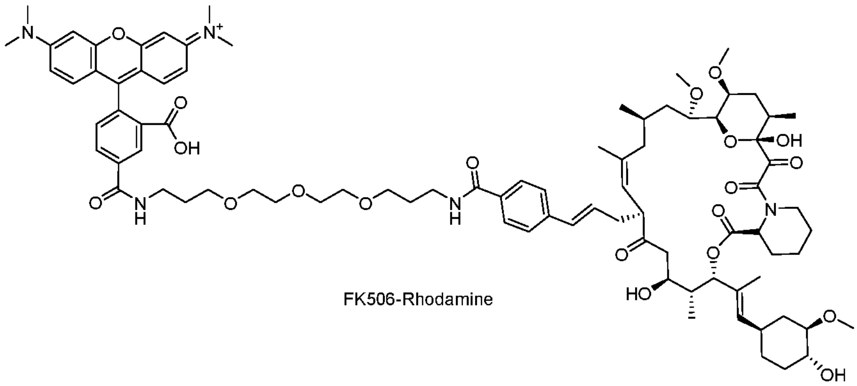


Compounds Classification:

Class	function	example
I	Imaging	Fluorescent dye: Rhodamine, FITC Radioisotope imaging: EC20 chelating head, NOTA, DOTA
II	Cytotoxic drug	Anti-microtubule drug: Tubulysin, DM1, DM4
III	Modulator	Kinase inhibitor: Dasatinib, MEK1/2i, PI3Ki siRNA: mi181a1

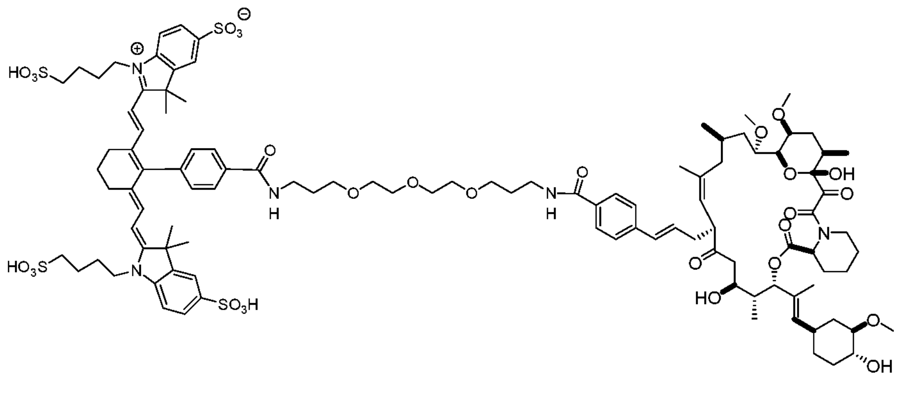
Detailed compound structure and synthesis route

FK506-Rhodamine:



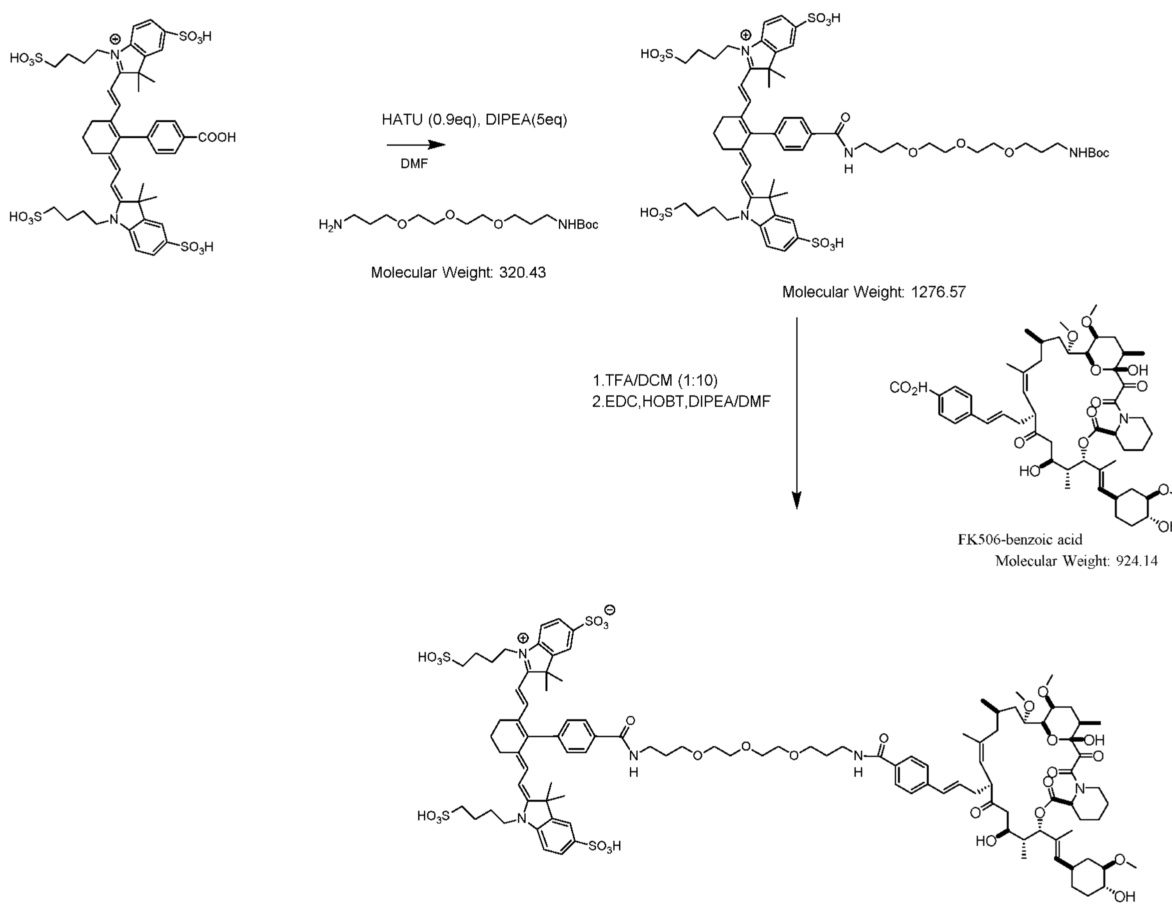
Procedure: Rhodamine-NHS ester (1.0 equiv.) in dimethylformamide was reacted with Boc-NH-PEG_3-NH_2 (1.2 equiv.) and diisopropylethylamine (3.0 equiv.) for 2h at room temperature. The product was purified by preparative reverse-phase HPLC with a UV detector. The purified Rhodamine-PEG_3-NH-Boc conjugate (1.0 equiv.) was subjected to Boc deprotection by stirring in a 1:10 TFA-dichloromethane system for 2h. The crude free amine product was then dissolved in dimethylformamide and activated with EDC (2.0 equiv.), HOBT (2.0 equiv.) in the presence of diisopropylethylamine (3.0 equiv.). After 15 minutes, FK506-CO_2H (1.2 equiv., synthesized using the procedure in the reference: Bioorg. Med. Chem., 17 (2009) 5763-5768) was added and the reaction mixture was stirred overnight. The final FK506-Rhodamine conjugate was isolated after purification on preparative reverse-phase HPLC with a UV detector (monitored at wavelength of 280 nm). The crude product was loaded onto an Xterra RP18 preparative HPLC column (Waters) and eluted with gradient conditions starting with 95% 5 mM sodium phosphate (mobile phase A, pH7.4) and 5% acetonitrile (mobile phase B) and reaching 0% A and 50% B in 35 min at a flow rate of 12mL/min. Retention time of the product peak = 2.5 min during the gradient (0-50%B) in a 7 min analytical HPLC-MS analysis. ESI m/z = 1539.6. Abbreviations: PEG = polyethylene glycol; EDC = 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide; HOBT = Hydroxybenzotriazole; HPLC = High Performance Liquid Chromatography.

FK506-NIR dye:



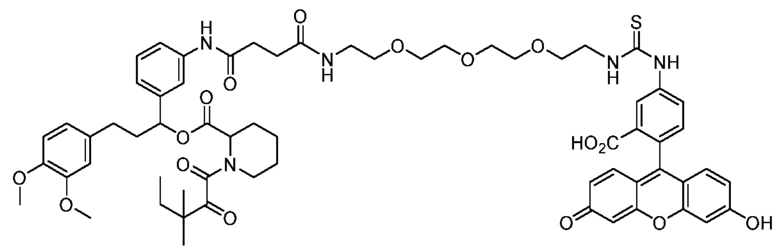
Molecular Weight: 2081.57

Synthesis procedure:



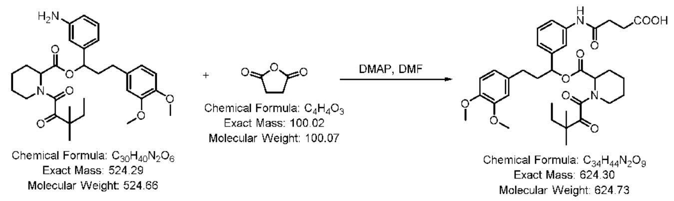
Molecular Weight: 2081.57

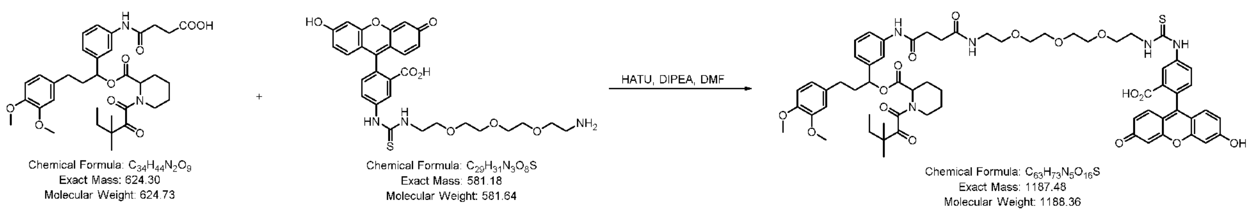
SLF-FITC:



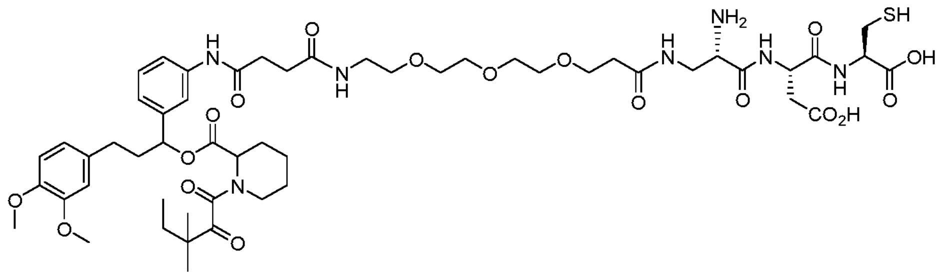
Chemical Formula: $C_{63}H_{73}N_5O_{16}S$
Exact Mass: 1187.48
Molecular Weight: 1188.36

Synthesis procedure:



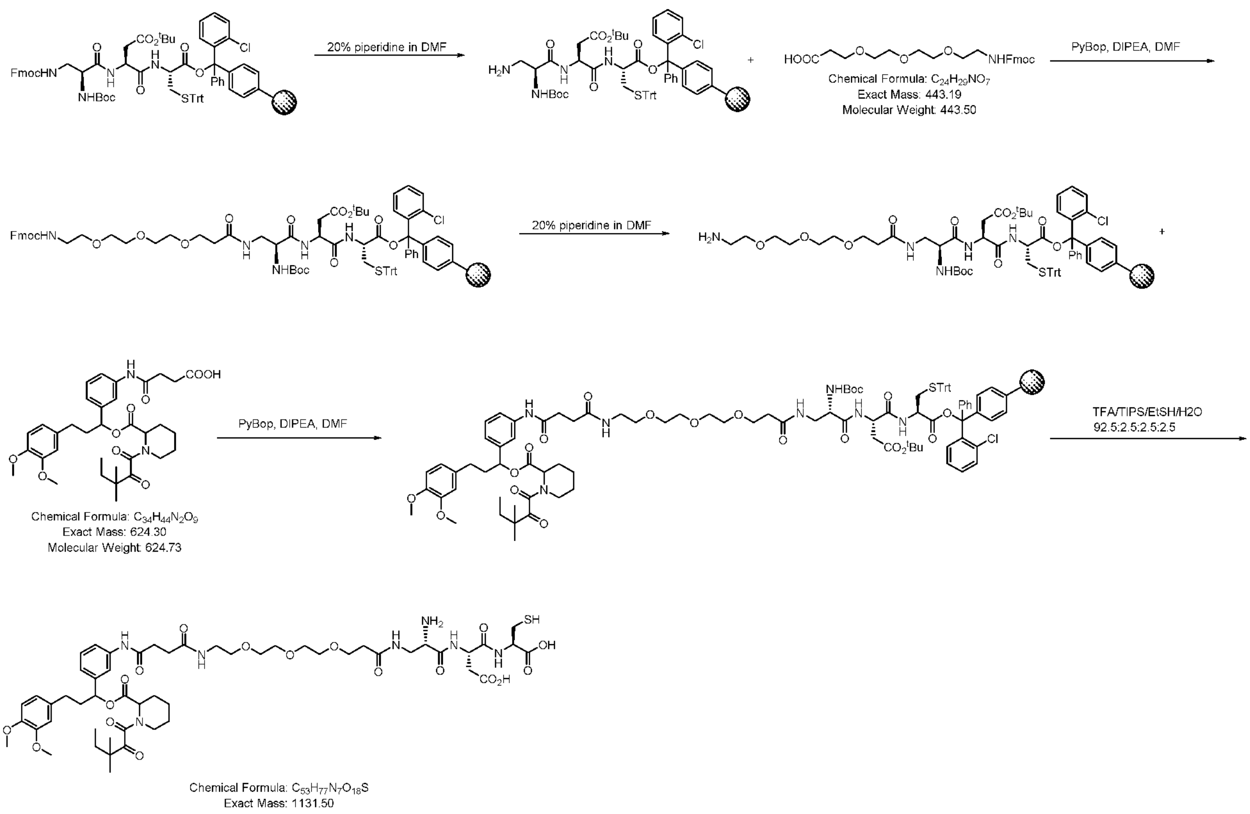


SLF-EC20:

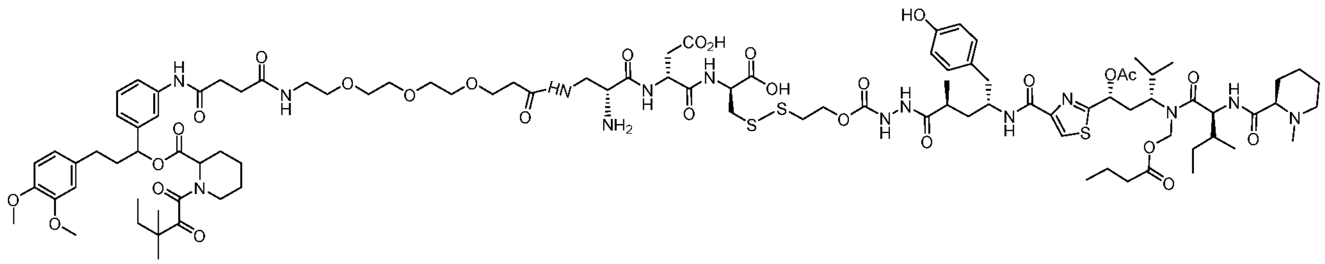


Chemical Formula: $C_{53}H_{77}N_7O_{18}S$
Exact Mass: 1131.50
Molecular Weight: 1132.29

Synthesis procedure:

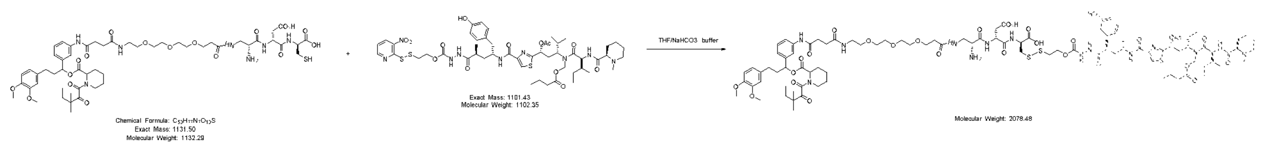


SLF-Tubulysin:

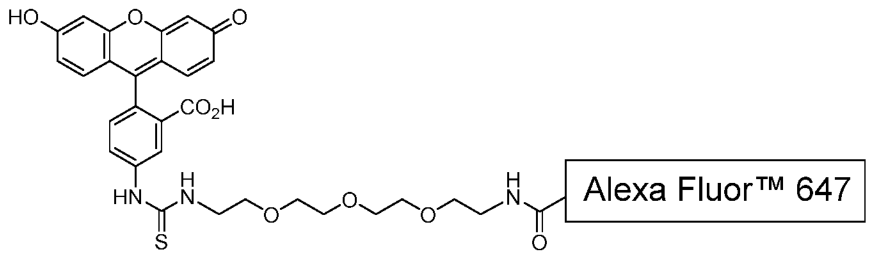


Molecular Weight: 2078.48

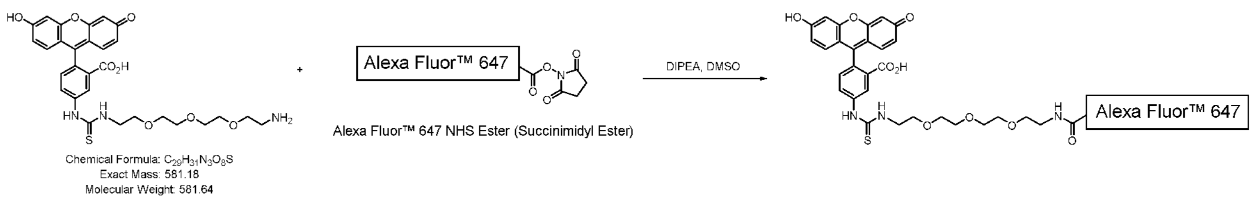
Synthesis procedure:



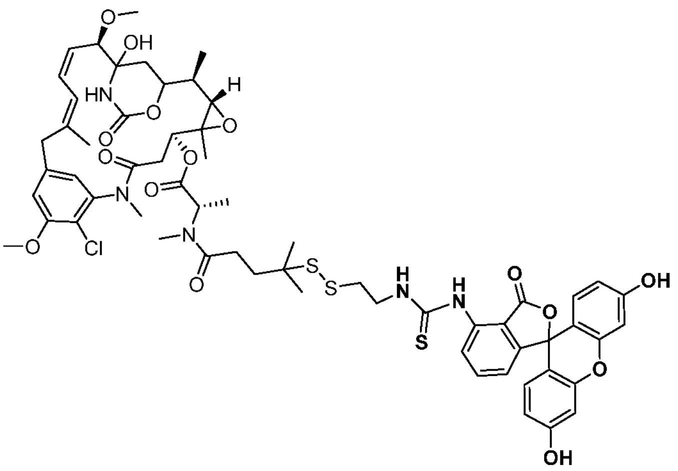
FITC-AF647:



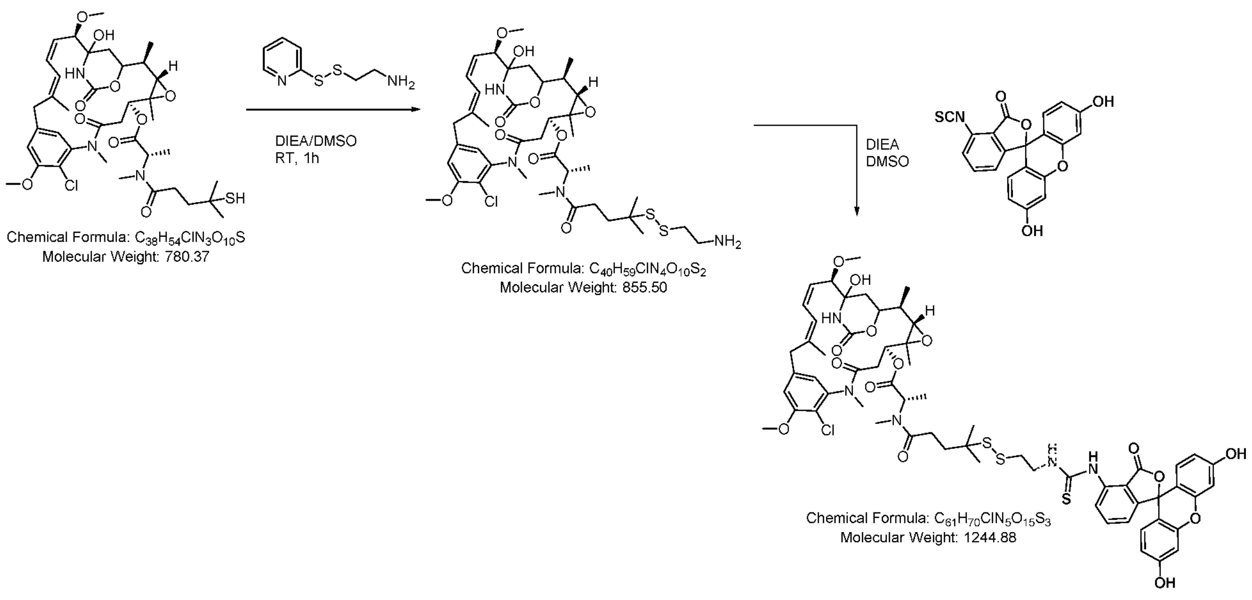
Synthesis procedure:



FITC-DM4:

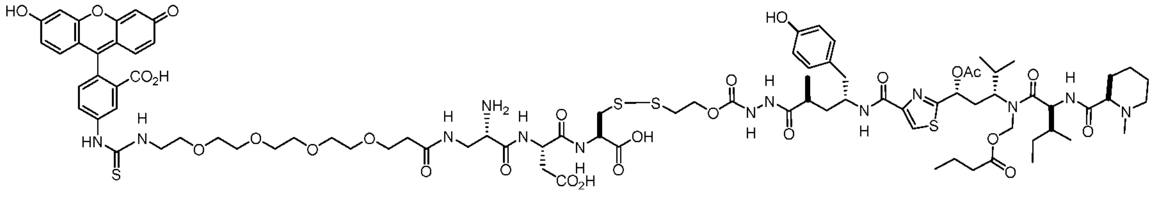


Synthesis procedure:



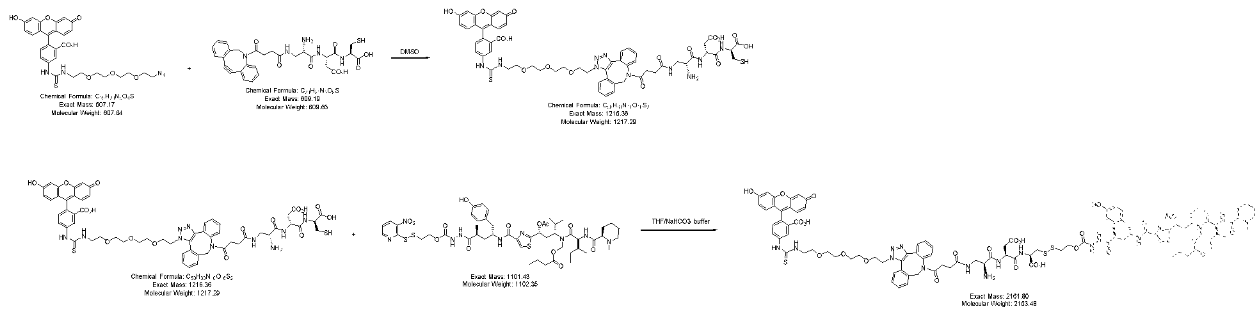
Procedure: DM4 (1.0 equiv.) in dimethylsulfoxide was reacted with 2-(pyridin-2-yldisulfaneyl)ethan-1-amine (1.0 equiv.) and diisopropylethylamine (3.0 equiv.) for 1h at room temperature. The resulting crude product was then reacted with FITC (1.0 equiv.) and the reaction mixture was stirred for 1h. The final FITC-DM4 conjugate was isolated after purification on preparative reverse-phase HPLC with a UV detector (monitored at wavelength of 280 nm). The crude product was loaded onto an Xterra RP18 preparative HPLC column (Waters) and eluted with gradient conditions starting with 95% 5 mM sodium phosphate (mobile phase A, pH7.4) and 5% acetonitrile (mobile phase B) and reaching 0% A and 100% B in 10 min at a flow rate of 12mL/min. Retention time of the product peak = 4.23 min during the gradient (0-100%B) in a 7 min analytical HPLC-MS analysis. ESI m/z = 1244.8. Abbreviations: FITC = fluorescein isothiocyanate; HPLC = High Performance Liquid Chromatography.

FITC-Tub

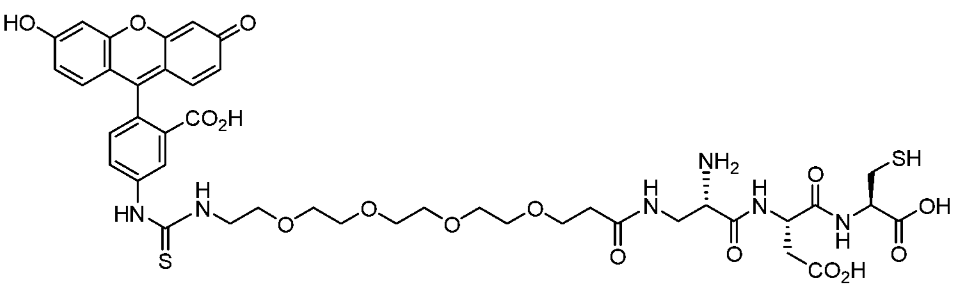


Chemical Formula: $C_{87}H_{117}N_{13}O_{27}S_4$
Exact Mass: 1903.71
Molecular Weight: 1905.20

Synthesis procedure:

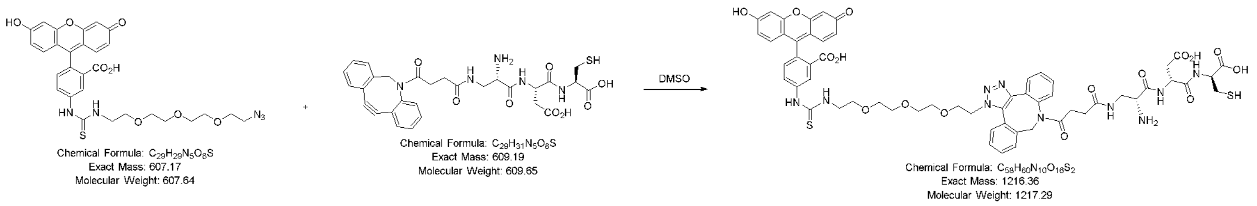


FITC-EC20

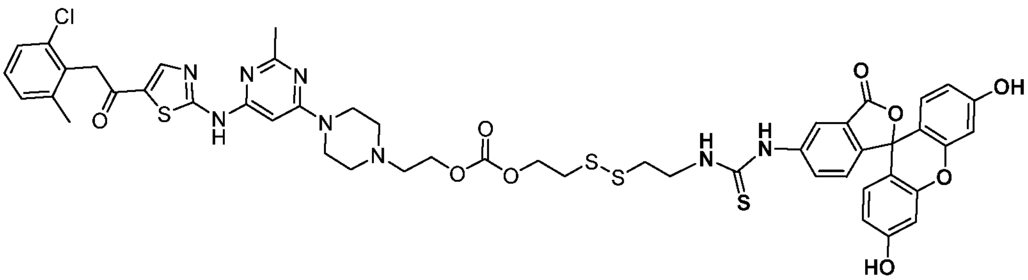


Chemical Formula: $C_{42}H_{50}N_6O_{16}S_2$
Exact Mass: 958.27
Molecular Weight: 959.01

Synthesis procedure:

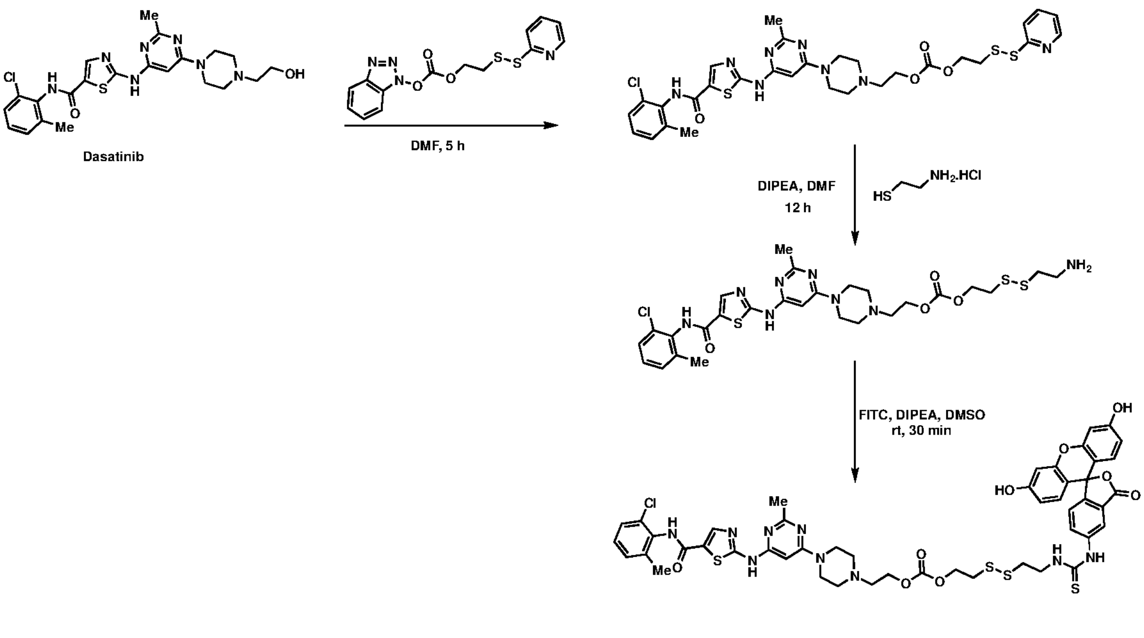


FITC-Dasatinib



Molecular Weight: 1055.65

Synthesis procedure:



The experiment procedure:

Cell culture

293TN cells were cultured in DMEM with 10% FBS, no antibiotic for lentivirus packaging. Raji and Jurkat cells were cultured in RPMI-1640 with 10% FBS, 10% Penicillin/Streptomycin. Primary human T cells were isolated from hPBMC using ficoll, enriched by negative selection with EasySep™ Human T Cell Enrichment Kit (19051, Stemcell Tech), activated by Dynabeads CD3/CD28 (11161D, Thermo Fisher) for 1 days, cultured with TexMACS medium supplemented with 30IU hIL2 (130-097-745, Miltenyi Biotec Inc.). T cells were cryopreserved in RPMI-1640 with 20% human AB serum (HP1022, Valley Biomedical) and 10% DMSO.

Lentivirus packaging

Pantropic VSV-G pseudotyped lentivirus was produced via transfection of 293TN cell with the transgene expression vector and packaging plasmid mix (CPCP-K2A, Cellecta) using Lipofectamine 2000. At 24h, viral supernatant was harvested, concentrated and then added to certain cell lines or the primary T cells that were thawed the same day. For T cell transduction, cells were centrifuged at 2500rpm for 90 mins, 37 degree after adding the virus supernatant and 8ug/ml polybrene.

Binding assay

For binding assay, cells were incubated with ligand-dye alone or with ligand-dye and free ligands (100x, preincubated for 30 min) for 30min, at 4 degree. Cells were washed 3 times after incubation, and re-suspended in 2% FBS PBS, 7-AAD were added to gate out the dead cells. FRET imaging of FK506-Rhodamine and FA-S0456: To understand the occupation of the fusion receptor, FKBP-FRa+ jurkat cells are incubated with FA/FA-S0456, FK506/FK506-Rhodamine at the indicated sequence and concentration. FRET is visualized by the loss of intensity of FA-Rhodamine as its energy transferred to FA-OTL38 on the same or nearby receptor, detected by BD Fortessa flow cytometer. Results were analyzed using FlowJo software.

PI-PLC treatment to release the GPI anchored protein

$1x10^5$ cells were incubated with 5mU or 50mU PI-PLC (P5542-5UN, Sigma) in digestion buffer (2% BSA) at 37 degree for 30 min; after incubation, cells were washed three times by PBS and then incubated with ligand-dye for 30min on ice.

Cell viability assay

Cells were seeded to 96 well plate and incubated with different concentration of certain ligand-cytotoxic drug for 2h, with or without 100x preincubation of the free ligand competition. After 2h incubation, cells were washed by warm medium 3 times and replenished with fresh medium. After 72h, cell number were tested by CellTiter-Glo® assay (G7570, Promega) or quantified by ligand-dye staining of receptor positive cell.

CAR T cell lysis effect

$1x10^5$ CAR T and certain number of Raji cell were co-incubated in 96 well plate, according to the E:T ratio, with or without the treatment drug. After 24h, 100ul supernatant were taken out for LDH assay. Lysis percentage were calculated as (treatment group – CAR T only)/(maximum lysis-CAR T only)%

Exhaustion of CAR T cell

$1x10^6$ Raji cell were repeated added to $1x10^6$ CAR T cell in 24 well plate every 12h, without changing the medium. Exhaustion status were characterized by lower lysis effect and higher expression of co-inhibitory molecules: PD-1, LAG-3 and Tim-3.

In vivo ablation of the fusion receptor positive CAR T by ligand-cytotoxic drug

$4x10^5$ luc+Raji cells were iv injected into the NSG mice. After 6 days, $1x10^7$ fusion receptor positive CAR T cells were iv injected. On Day 8, ligand-cytotoxic drug (0.5umole/kg, 1umole/kg) were iv injected once. IL2, INFr were measured by ELISA using serum sample taken every 3 days after the CAR T injection. CAR T cell in peripheral blood were counted by flow cytometry.

In vivo modulation of fusion receptor positive CAR T by ligand-drug

$4x10^5$ luc+Raji cells were iv injected into the NSG mice. After 6 days, $1x10^7$ fusion receptor positive CAR T cells were iv injected. On Day 7, ligand-drug drug (0.5umole/kg, 1umole/kg) were iv injected once. In the case of CAR T exhaustion model, $1x10^5$ CAR T were iv injection on Day 6, once the CAR T population were shown in peripheral blood and tumor burden is not stabilized and continue to increase, ligand-drug (0.5umole/kg, 1umole/kg) were iv injected.

In vivo imaging and tracking of fusion receptor positive CAR T

$2x10^6$ Raji were subcutaneously injected to the right should of NSG mice. 14 days later, $1x10^7$ fusion receptor positive CAR T were iv injected. On Day 16, animals were administrated 99mTc-bound conjugates (10 nmol, 150 μCi) by iv injection and imaged by SPECT imaging machine.

EXAMPLES

1. Design of fusion protein and its expression

1.1 Design of FKBP-FR fusion receptor (SEQ ID NO:2)

Synthesis of FK506 derivatives: FK506-Rhodamine and FK506-tubulysin Synthesis was described in materials and methods section.

hFRa is a GPI anchored membrane protein, which has 24 amino acids on the N terminal as a signal peptide. In order to preserve the membrane presentation and internalization property, we choose to use the full length of FRa, and incorporate the hFKBP sequence as well as a flexible peptide linker (SGGGS) between T24 and R25 of hFRa (Figure 4a). The flexible linker is chosen to be resistant to common enzyme digestions in human body. The whole sequence is then inserted into a pWPI lentivirus expression vector, with EF1a as the desired promoter for protein expression in transduced T cell.

1.2 Expression of FKBP-FR fusion receptor in transduced cells

Expression of FKBP-FRa is confirmed by western blot in lentivirus transduced K562. Transduced K562 cell lysis shows specific band around 50kDa compared to non-transduced cells against hFRa antibody (Figure 4b).

1.3 Construction of fusion protein of FKBPFR3GS (noted as FF3)

See Figure 4d (SEQ ID NO:12) . From N terminal to C terminal it has 1-24 aa of human FRa as the signal peptide, human FKBP protein, three Gly-Ser linker and then 2-258 aa of human FRa. In a construct design of FKBPFR1GS (noted as FF1), the three Gly-Ser linker of FF3 is substituted with one Gly-Ser linker with other parts unchanged. As will be shown in the binding assays, increasing linker length reduces the interference between the two components in the fusion protein.

1.4 Construction of fusion protein of FITC-svFv-FR with GS linker.

See Figure 4e. The construct is also named as 4M5.3 FR (SEQ ID NO:13). From N-terminal to C-terminal, it has hCD8 signal peptide, svFv of 4M5.3 (against FITC), GS linker, 25-258 aa of human FRa.

1.5. In vivo noninvasive tracking of FKBP-FRa/FKBPtFRa positive cells by FK506-99mTc

PET imaging

For CAR T cell model, 1x10^6 KB cells are subcutaneously implanted in NSG (Jackson laboratory). After the tumor reaches 100mm^3, 15 million anti-FITC CAR+ FKBP-FRa+ or anti-FITC CAR+ FKBP-FRa- human T cells are intravenously injected to the mice. FITC-FA is injected at the indicated days to induce the proliferation of the CAR T. Mice are imaged every two days after the CAR T implantation by the following procedure. At the day of imaging, FK506-EC20 head is formulated with 99mTc according to previous report. 200uCi 99mTc in 100ul solution is i.v. injected to each mouse and whole body image is taken by MiLab PET/CT, focusing on the tumor area, spleen and lymph nodes. 3D images are reconstructed by PMOD software. After the last imaging at around day 10 after CAR T implantation, mice are euthanized and 99mTc distribution in each organ are counted by gamma counter.

For Hematopoietic Stem Cell transplantation model, humanized NSG mice are generated as reported before. 10 million CD34+ FKBP-FRa+ hHSC are i.v infused into the humanized NSG mice. 4 months later, whole body image is taken using FK506-99mTc as mentioned above, focusing on the bone marrow and spine.

2. FKBP-FRa fusion receptor specifically binds and internalizes FK506-payload

2.1 FKBP-FRa fusion receptor specifically binds FK506-Rhodamine

For binding assay, cells were incubated with ligand-dye alone or with ligand-dye and free ligands (100x, preincubated for 30 min) for 30min, at 4 degree. Cells were washed 3 times after incubation, and re-suspended in 2% FBS PBS, 7-AAD were added to gate out the dead cells. BD Fortessa flow cytometer were used. Results were analyzed using FlowJo software.

2.2. Binding of FK506-Rhodamine by FKBP-FRa fusion receptor (FRET imaging of FK506-Rhodamine and FA-S0456)

To understand the occupation of the fusion receptor, FKBP-FRa+ jurkat cells are incubated with FA/FA-S0456, FK506/FK506-Rhodamine at the indicated sequence and concentration. FRET is visualized by the loss of intensity of FA-Rhodamine as its energy transferred to FA-OTL38 on the same or nearby receptor, detected by BD Fortessa flow cytometer.

Figure 5 shows binding of Folate acid in FKBPFR1GS fusion protein blocks the binding of FK506-Rhodamine at as low as 0.01nM and totally abolishes FK506 Rhodamine binding at 50nM.

Figure 6 shows FKBPFR1GS jurkat cells have decreased FK506-Rhodamine intensity after binding to OTL38, a folate receptor targeted dye. FRET from FK506-Rhodamine (donor) to OTL38 (FA-S0456, acceptor, ex/em:774/794nm) indicates the interaction between FR and FKBP within the fusion receptor.

Figure 7 shows increasing the linker length between FKBP and FR significantly lowers the interference between the two components of the fusion protein. Compare to FF1 (1GS between FKBP and FR), FF3 (3GS between FKBP and FR) preserves the binding of FK506-Rhodamine in the presence of 10nM FA, which is comparable to the physiological concentration of FA in human body.

2.3. Release of GPI anchored FF3 fusion receptor

Using PI-PLC treat T cells having FF3 fusion protein resulted the release of GPI anchored receptor FF3. Jurkat T cell with FF3 fusion receptor shows saturated binding with 20nM FA-FITC (EC17), while after 5mU PI-PLC or 50mU PI-PLC treatment, the FA-FITC loses binding to the cell, which indicates the release of the GPI anchored FF3 fusion receptor. See Figure 8.

2.4 Fusion protein FKBPFR3GS in human T cell retains FR binding property

FA-Rhodamine binding curve is shown in FKBPFR3GS fusion receptor. FKBPFR3GS fusion receptor that stably expressed on human T cell can bind to folate acid derivative (FA-Rhodamine) with high affinity (Kd=0.95nM), which is comparable to the affinity of FA-Rhodamine in FR+ KB cell (Kd around 1nM). Therefore, the binding property of FR in the fusion receptor is preserved. See Figure 9.

2.5 Fusion protein FKBPFR3GS in human T cell retains FKBP binding property

FK506-Rhodamine binding curve in FKBPFR3GS fusion receptor. FKBPFR3GS fusion receptor that stably expressed on human T cell is able to bind to FK506 derivative (FK506-Rhodamine) with high affinity (Kd=3.93nM), which means the binding property of FKBP in the fusion receptor is preserved. See Figure 10.

2.6 SLF-FITC binds to FKBPFR3GS fusion receptor with relative high binding affinity (Kd=62nM)

Competition of free SLF (100, preincubation) blocks SLF-FITC binding. SLF, a mimic of FK506, presents a 10 times lower binding affinity to FKBPFR fusion receptor, compared to the parent ligand FK506. See Figure 11 and compare with Figure 10.

2.7 Binding curve of FA-Rhodamine in 4M5.3FR fusion receptor.

FA-Rhodamine can bind to 4M5.3FR fusion receptor that stably expressed on human T cell with high affinity (Kd=2.25nM), which is comparable to the affinity of FA-Rhodamine in FR+ KB cell (Kd around 1nM). Therefore, FR binding property is preserved in 4M5.3FR fusion receptor. See Figure 12.

2.8 FITC-AF647 binding curve in 4M5.3FR fusion receptor.

FITC-AF647 can bind to 4M5.3FR fusion receptor that stably expression on human T cell with high affinity (Kd=8.03nM). 100x comp indicates free FITC sodium. The binding property of scFv 4M5.3 with FITC is preserved in 4M5.3FR fusion receptor. See Figure 13.

3.1. FA-Tubulysin killing effect against FF3+ human T cell

FA-Tubulysin is able to mediate a receptor specific killing effect against FF3+ human T cell. Compensation with FA (100x preincubation with FA) blocks the effect. This implies the successful internalization and release of the free drug Tubulysin through the FF3 fusion receptor system. See Figure 14.

3.2 FA-Tubulysin killing effect against hFF3 + population in a mixed human T cell culture.

FA-Tubulysin specifically kills the hFF3+ population in a mixed human T cell culture. Percentage of hFF3+ cell decrease as the FA-Tub concentration increases. See Figure 15.

3.3 SLF-Tub specifically kill the hFF3+ Jurkat cells with a IC_{50} = 138nM.

2h incubation of SLF-Tub with hFF3 Jurkat cells is able to kill the receptor positive cells.This indicates the successful internalization of SLF-Tub by the FKBPFR3GS fusion receptor and the release of the Tubulysin inside the cell. See Figure 16.

3.4. FITC-DM4 and FITC-Tub killing effects against 4M5.3FR+ human T cells

Both FITC-DM4 and FITC-Tub can specifically kill the 4M5.3FR+ human T cells, while FITC-Tub has a higher IC_{50}. Compensation of free FITC sodium (100x preincubation) blocks the receptor mediated killing effect. This implies the successful internalization and release of FITC-cytotoxic drug into T cell through 4M5.3FR fusion receptor. See Figure 17.

3.5. FITC-Tubulysin specifically kill the 4M5.3FR+ population in a mixed human T cell culture.

Absolute number of 4M5.3FR+ cell decrease as the FITC-Tub concentration increases, while 4M5.3FR- cells are killed also through released drugs and bystander effect at high concentration. See Figure 18.

3.6. FITC-DM4 specifically kill the 4M5.3FR+ population in a mixed human T cell culture. Absolute number of 4M5.3FR+ cell decrease as the FITC-DM4 concentration increases, while 4M5.3FR- cells are killed also through released drugs and bystander effect at high concentration. See Figure 19.

3.7 Kinase inhibitors modulation effect on anti CD19 CAR T cells against CD19+ Raji

Dasatinib (Lck inhibitor) and Ibrutinib (ITK inhibitor) at 10nM concentration decrease the lysis effect of antiCD19 CAR T cell (FMC63 CAR T, Effector) against CD19+ Raji tumor cell (Target). Two Effector: Target ratio (E:T) have been tested. Normal T cell and antiCD19 CAR T with CD19- K562 cell were used as negative control. See Figure 20.

3.8. FITC-Dasatinib can decrease the lysis effect of FMC63+4MFR+hT cell towards Raji cell.

This implies the successful internalization and release of FITC-Dasatinib into T cell through 4M5.3FR fusion receptor and the release of Dasatinib into T cell. See Figure 21.

3.9 TC-PTP inhibitor at 100nM concentration decrease the co-inhibitor molecule population in exhausted antiCD19 CAR T cell

Exhausted antiCD19 CAR T cells are generated by 7 times of stimulation with CD19+Raji cells, see detailed procedure in material and methods section). PD-1 positive, LAG3 positive and double positive population decreases upon treatment. See Figure 22.

4. Other FK506-payload to control the activity of cell therapy

The technical advantageous feature of this drug payload delivery system is to have multi-functionality. The potential payloads and corresponding effects are listed below (Table 1). The small molecule payloads are selected based on the following parameters**:** 1. the functionality assay of the free drug, both in vitro and in vivo, has been confirmed by either published literature or work in our lab. 2. The chemical structure of the drug has relatively more accessible free amine for derivatization. 3. Any of the following will be preferable: FDA proved drug; commercially available for reasonable price. The FK506-payload will be tested first for in vitro experiments, T cell activation and stem cell cytokine release will be monitored by multiplex immunoassays. For in vivo disease models, we have well established CAR T therapy and bone fracture mouse models in our lab, and several potential collaborators for the neurodegenerative mouse models.

Disease model	Subtype	Cell type	Cell source	Payload type
stem cell therapy	HSC transplant	BMSC	Murine BM	GSK3b inhibitor
Tumor	tumor microenviroment	CAR T	hPBMC T cell	GSK3b inhibitor, HDAC inhibitor, MAPK inhibitor

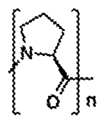
CLAIMS

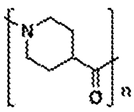
1. A drug delivery platform for cell therapy, comprising:
   a. an engineered protein on a target cell for transplant, wherein the engineered protein comprises a first component and a second component, the first component and the second component are connected by a peptide linker, the first component is a non-membrane protein, the second component is a membrane anchored peptide or protein;
   b. at least one small ligand conjugated to a linker, wherein the at least one small ligand has intrinsic high affinity to at least one component of the engineered protein; and
   c. at least one payload of drug conjugated to the linker, wherein the payload of drug is associated with the target cell when the small ligand binds to at least one component of the engineered protein.
2. The drug delivery platform according to claim 1, wherein the payload of drug is an imaging agent.
3. The drug delivery platform according to claim 1, wherein the payload of drug is a cytotoxic drug.
4. The drug delivery platform according to claim 1, wherien the payload of drug is a modulator of gene expression.
5. The drug delivery platform according to claim 1, wherien the payload of drug is a modulator of the cell's activity.
6. The drug delivery platform accordign to claim 2, wherein the imaging agent is selected from the group consisting of fluorescent dye rhodamine, fluorescein, and S0456.
7. The drug delivery platform according to claim 2, wherein imaging agent is selected from the group consisting of radioisotope chelating imaging moieties, EC 20 chelating head, NOTA and DOTA.
8. The drug delivery platform according to claim 3, wherein the cytoxic drug is selected from the group consisting of tubulysin, DM1, DM4, and an auristatin.

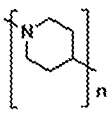
9. The drug delivery platform according to claim 4, wherein the modulator is selected from the group consisting of Dasatinib, MEK1/2 inhibitor, and PI3K inhibitor.
10. The drug delivery platform according to claim 4, wherein the modulator is selected from the group consisting of HDAC inhibitor, kinase inhibitor and metabolic inhibitor.
11. The drug delivery platform according to claim 4, wherein the modulator is selected from the group consisting of GSK3 beta inhibitor, MAO-B inhibitor and Cdk5 inhibitor.
12. The drug delivery platform according to claim 4, wherien the modulator is an RORγt agonist.
13. The drug delivery platform according to claim 4, wherein the payload of drug is siRNA mi181a1.
14. The drug delivery platform according to claim 4, wherein the payload of drug is a phosphatase inhibitor, including but not limited to inhibitors against SHP1/2, TC-PTP.
15. The drug delivery platform according to claim 1, wherien the payload of drug is further internalized by the target cell when the small ligand binds to at least one component of the engineered protein.
16. The drug delivery platform according to claim 1, wherein the linker to connect the small ligand and the payload drug is selected from the group consisting of

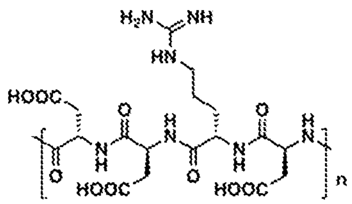

alkyl

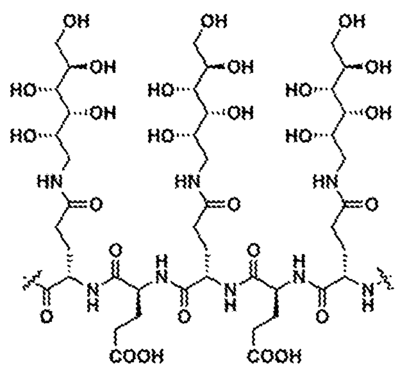

poly ethylene glycol (PEG)


polyproline


oligo-(4-piperidine carboxylic acid)


oligo piperidine


peptide


saccharo-peptide

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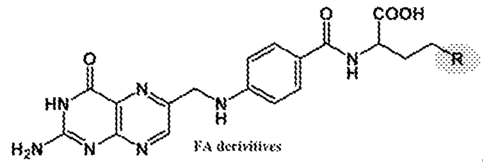
17. The drug delivery platform according to claim 1, wherein the engineered protein components are selected from the group consisting of Folate Receptor alpha (FRa), Folate Receptor beta (FRb), Urokinase receptor (uPAR), FK506 binding protein (FKBP), dihydrofolate reductase (DHFR), Single Chain Fragment Variable against Fluorescein isothiocyanate (scFv against FITC), and Single Chain Fragment Variable against dinitrophenol (scFv against DNP).
18. The drug delivery platform according to claim 1, wherein the small ligand is selected from the group consisting of 

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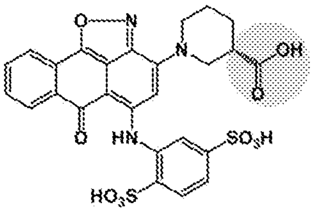
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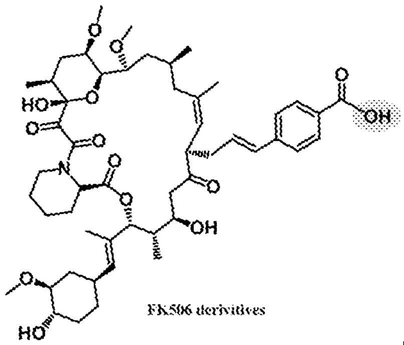

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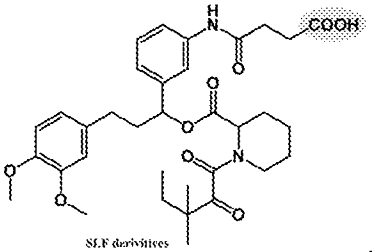

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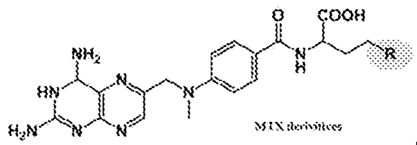

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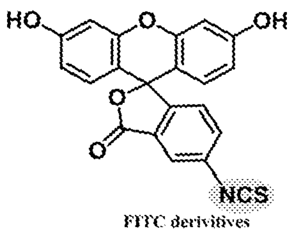
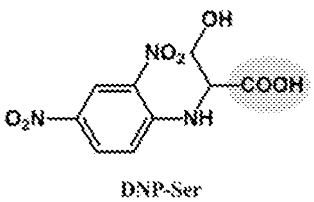


and 

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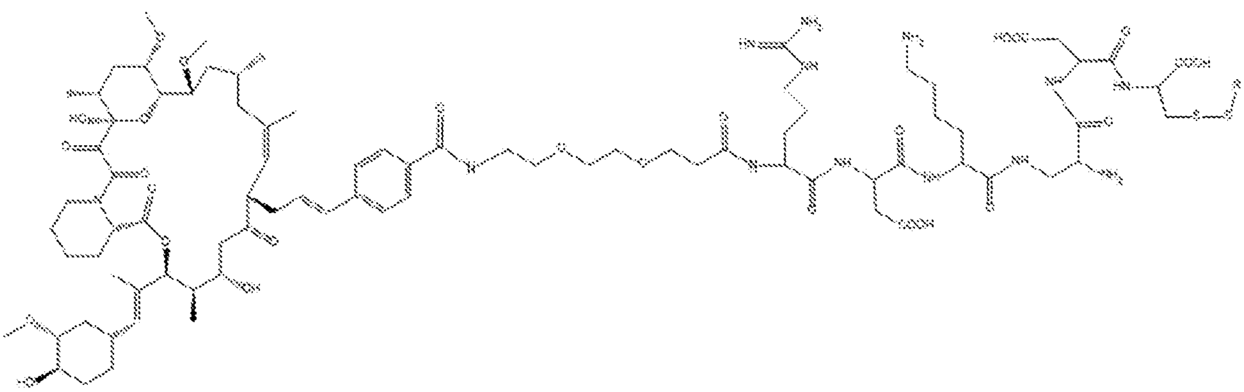
19. The drug delivery platform according to claim 1, wherien the first component is FKBP, the second component is a peptide that confers a glycosylphosphatidyl inositol (GPI) anchor on the first component, and the small ligand is FK506 or its derivative.
20. The drug delivery platform according to claim 18, wherien the second component is a full length or truncated Folate Receptor (FR).

21. The drug delivery platform according to claim 1, wherein the peptide linker is at least one segment of SGGGS.
22. The drug delivery platform according to claim 1, wherien the engineered protein is selected from the group consisting of SEQ ID NOS:1 -2.
23. The drug delivery platform according to claim 1, wherein the engineered protein is selected from the group consiting of SEQ ID NOS:12-15.
24. The drug delivery platform accoridng to claim 1, whererin the target cell for transplant is an immune cell.
25. The drug delivery platform according to claim 1, wherien the target cell for transplant is a CAR T cell expressing amino acid sequence selected from SEQ ID NOS: 3-4.
26. The drug delivery platform according to claim 1, wherien the small ligand conjugate has formula I.



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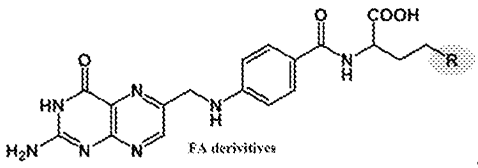
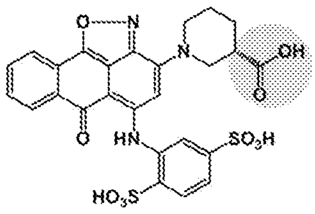
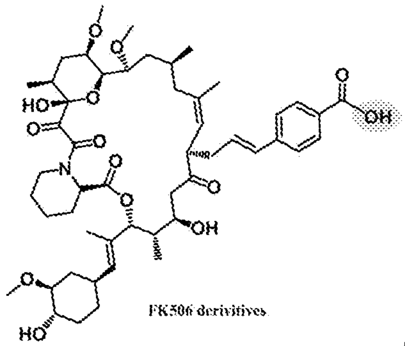
27. The drug delivery platform according to claim 1, wherein the target cell for transplant is a stem cell, a progenitor cell or a transplanted cell designed to synthesize a biochemical that is deficient in a patient.
28. The drug delivery platform according to claim 1, wherein the target cell for transplant is a chimeric antigen receptor (CAR) T cell.
29. The drug delivery platform according to claim 1, wherein the small ligand is further conjugated to a fluorescent dye or radioactive probe.
30. The drug delivery platform according to claim 1, wherein the small ligand is further conjugated to a regulator of endogenous gene expression.

31. The drug delivery platform according to claim 1, wherein the small ligand is further conjugated to a regulator of a transduced transgene expression.
32. A Chimeric Antigen Receptor T cell comprising a construct expressing amino acid sequences selected from SEQ ID NOS:12-15.
33. A DNA construct encoding an amino acid sequence selected from the group consisting of SEQ ID NOS:12-15.
34. A DNA construct encoding a FKBP-FRa fusion receptor comprising any of SEQ ID NOS:1-2 operably linked to an EF1a promoter in a expression vector.
35. The DNA construct of claim 34, wherein the expression vector is pWPI SEQ ID NO:5.
36. A DNA construct comprising any of SEQ ID NOS:6-8.
37. A transplanted cell comprising insert genes hFKBP-FR (SEQ ID NO:7) and human antiCD19 CAR (SEQ ID NO:9).
38. A transpant cell comprising insert genes mFKBP-FR(SEQ ID NO:8) and mouse antiCD19 CAR (SEQ ID NO:10).
39. A method to modulate cell therapy effect, comprising:
    a. Identifying a target cell for transplant, wherein the transplanted target cell has a cell therapy function;
    b. Providing an engineered protein on the surface of the target cell for transplant, the engineered protein comprises a first component and a second component, the first component and the second component are connected by a peptide linker, the first component is a non-membrane protein, the second component is a membrane anchored peptide or protein;
    c. Providing a payload of drug conjugate to the target cell, wherein the payload of drug is conjugated to a small ligand through a linker, and optionally conjugated to a fluorescent dye, wherein the small ligand binds to at least one component of the engineered protein with high affinity and is internalized by the target cell together with the payload of drug;
    d. releasing the payload drug within the target cell to modulate the target cell therapy function.
40. The method according to claim 39, wherein the cell therapy function is to provide optically guided surgery to a subject.

41. The method according to claim 39, wherein the cell therapy function is to control the target cell proliferation.
42. The method according to claim 39, wherein the cell therapy function is to execute cytotoxicty to the target cell engaged cancer cell.
43. The method according to claim 39, wharerin the target cell for transplant is an immune cell.
44. The method according to claim 39, wherien the target cell for transplant is a CAR T cell.
45. The method according to claim 39, wherein the target cell for transplant is a stem cell, a progenitor cell or a transplanted cell designed to synthesize a biochemical that is deficient in a patient.
46. The method according to claim 39, wherein the payload of drug is an imaging agent selected from flurescent dye of Rhodamine and FITC, or a radioisotope imaging agent selected from EC20 chelating head, NOTA and DOTA.
47. The method according to claim 38, wherein the payload of drug is a cytotoxic drug selected from the group consisting of Tubulysin, DM1, DM4 and auristatin.
48. The method according to claim 38, wherien the payload drug is a modulator of gene expression selected from kinase inhibitors consisting of Dasatinib, MEK1/2 inhibitor and PI3 Kinase inhibitor, or an siRNA of mi181a1.
49. The method according to claim 38, wherein the transplanted target cell comprises a fusion protein selected from the group consisting of SEQ ID NOS: 12-15.
50. The method according to claim 39, wherein the the engineered protein components are selected from the group consisting of FRa, FRb, uPAR, FKBP, DHFR, scFv against FITC, and scFv against DNP.
51. The method according to claim 39, wherein the small ligand is selected from the group consisting of  ,  ,



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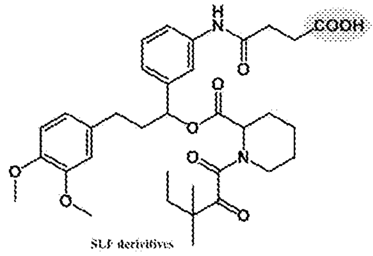

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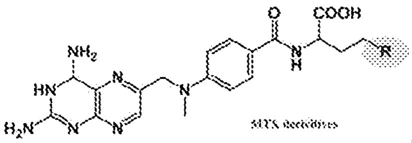

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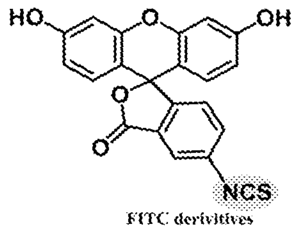

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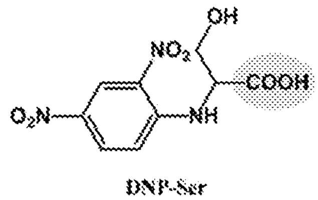

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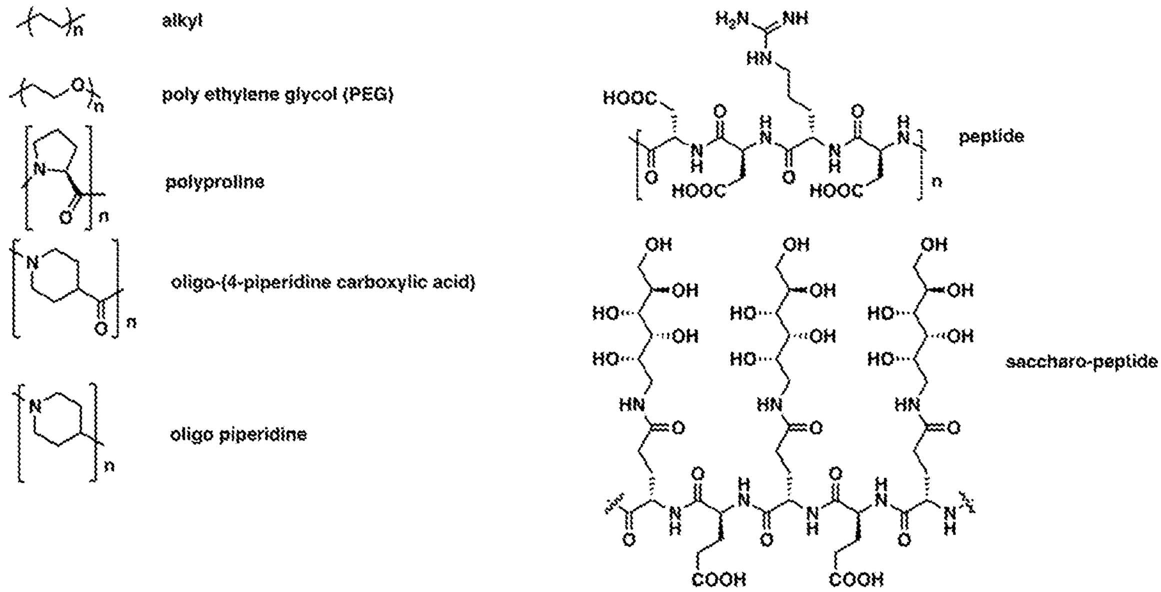
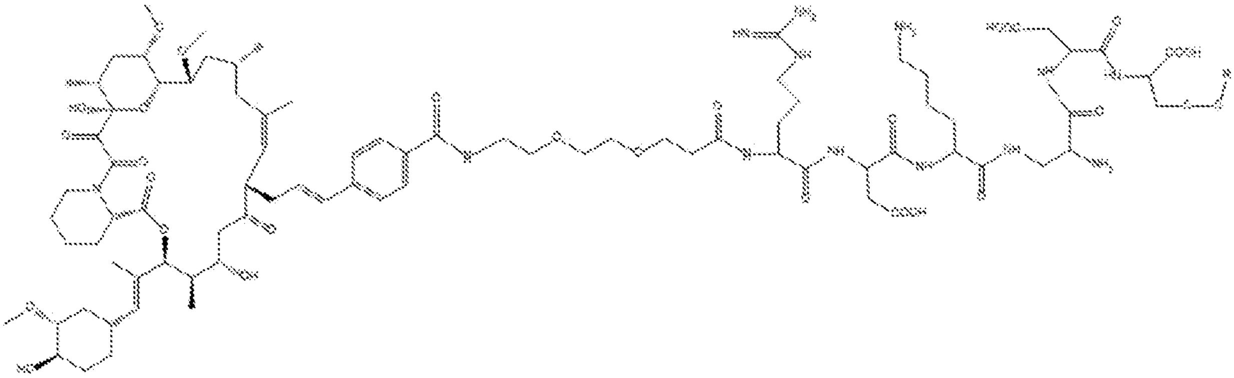
52. The method according to claim 39, wherein the linker to connect the small ligand and the payload drug is selected from the group consisting of



53. The method according to claim 39, wherein the transplanted target cell comprises an engineered FKBP-LINKER-FRa fusion protein selected from group consisting of SEQ ID NO:1 and SEQ ID NO:2.

54. The method according to claim 39 wherein the transplanted target cell is CAR T cell comprising an engineered antiCD19 CAR T construct selected from group consisting of SEQ ID NO:3 and SEQ ID NO:4.

55. The method according to claim 39, wherein the drug conjugate FK506-releasable linker comprises formula I, wherein the binding domain of FK506 has an affinity to FKBP of about 100pM.



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56. The method according to claim 39, wherein the transplanted target cell is a CAR T cell and the drug conjugate is designed to control a cytokine storm induced by the transplanted CAR T cell.
57. The method according to claim 39, wherein the transplanted target cell is a CAR T cell and the drug conjugate contains a modulator designed to control unwanted T cell proliferation.
58. The method according to claim 39, wherein the transplanted target cell is a stem cell, a progenitor cell or a transplanted cell designed to synthesize a biochemical that is deficient in a patient.
59. The method according to claim 39, wherein the transplanted target cell is a NK cell and the drug conjugate is a RORγt agonist to control Th17 cell mediated immune responses.
60. The method according to claim 39, wherein the payload of drug is a phosphatase inhibitor, including but not limited to inhibitors against SHP1/2, TC-PTP.

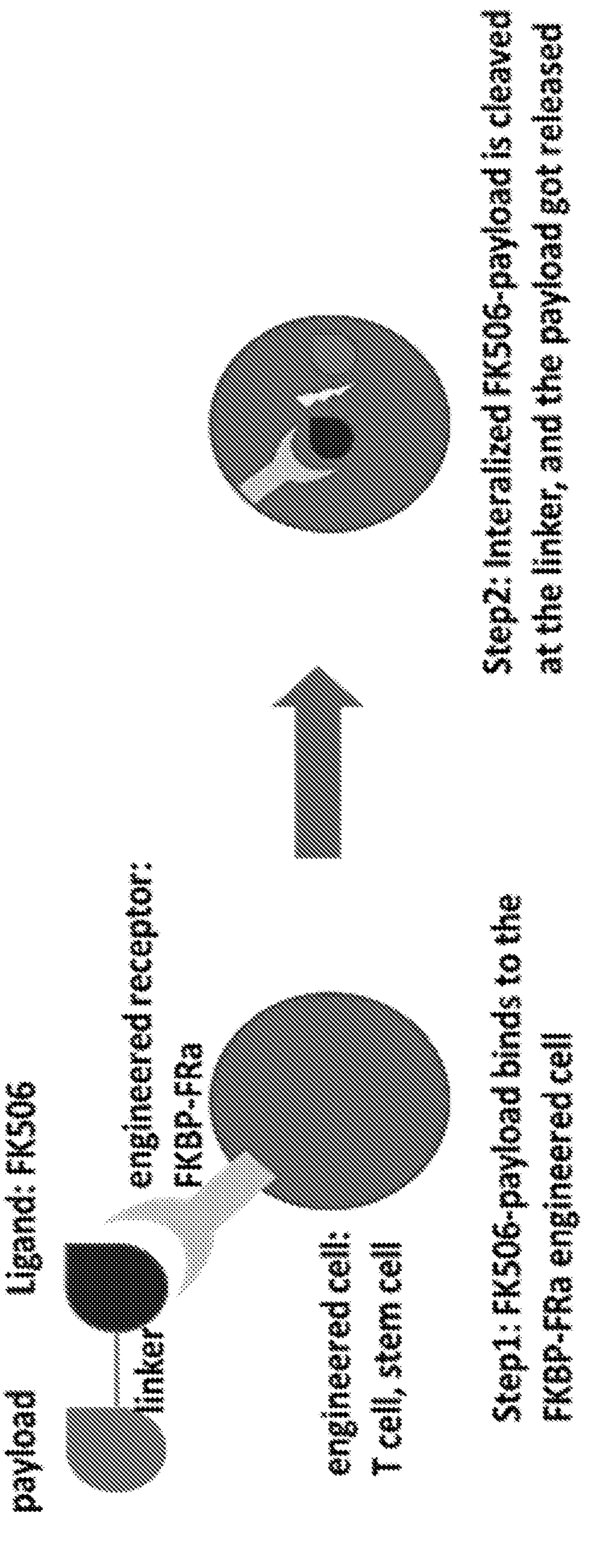

payload
linker
Ligand: FK506
engineered receptor:
FKBP-FRa
engineered cell:
T cell, stem cell
Step1: FK506-payload binds to the
FKBP-FRa engineered cell
Step2: Interalized FK506-payload is cleaved
at the linker, and the payload got released

FIG. 1A

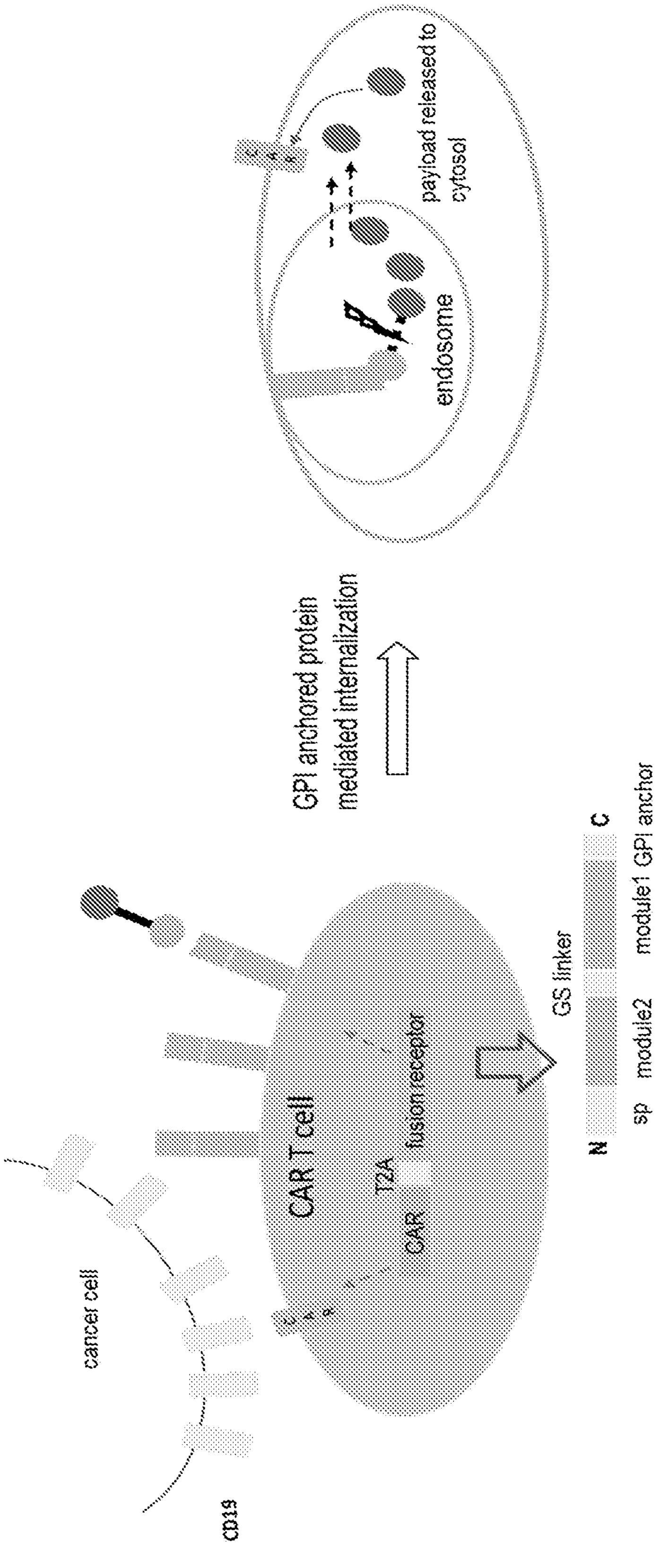

payload released to cytosol
endosome
GPI anchored protein mediated internalization
cancer cell
CD19
CAR T cell
CAR
T2A
fusion receptor
GS linker
N
sp
module2
module1
GPI anchor
C

FIG. 1B

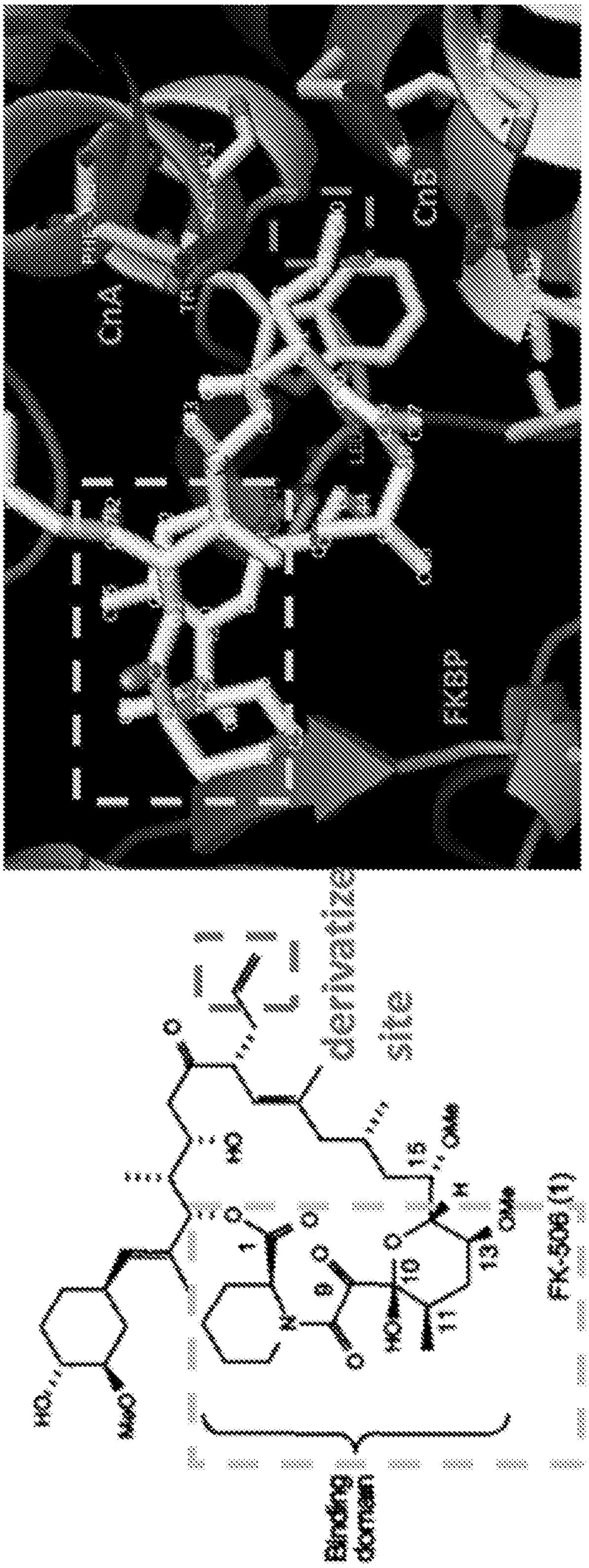


FIG. 2A

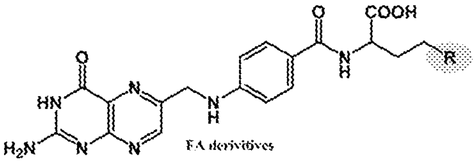
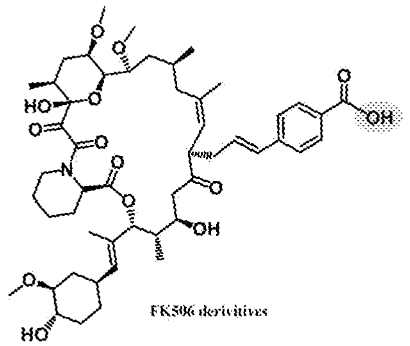
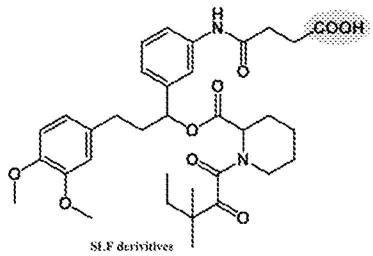
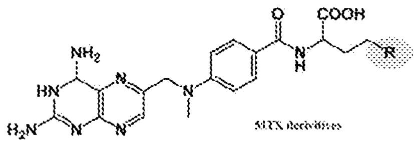
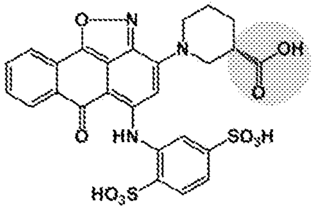
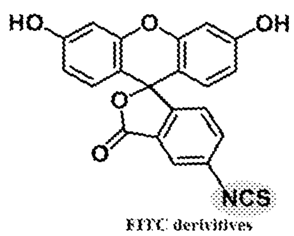
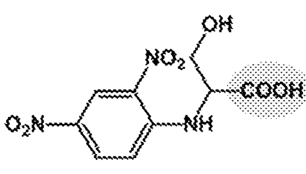
Module1: GPI anchored protein		Module 2	
Protein	**Ligand**	**Protein**	**Ligand**
FRa		FKBP	
			
FRb		DHFR	
uPAR		scFv against FITC	
		scFV against DNP	 DNP-Ser

FIG. 2B

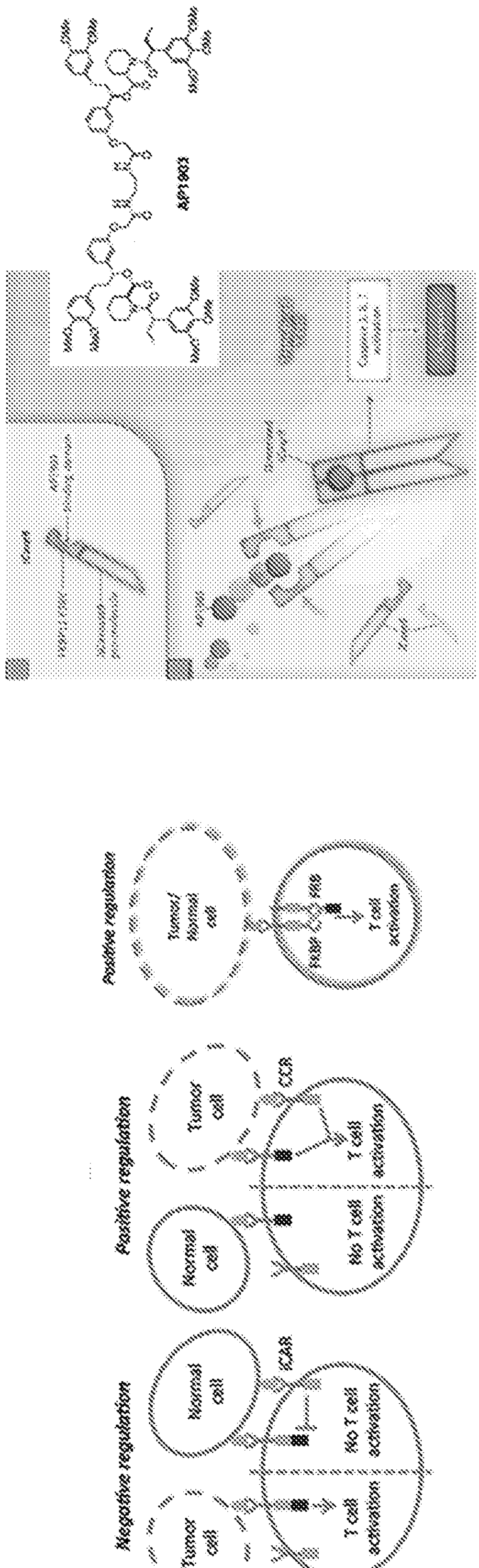

Negative regulation
Tumor cell
Normal cell
ICAR
T cell activation
No T cell activation
Positive regulation
Normal cell
Tumor cell
CCR
No T cell activation
T cell activation
Positive regulation
Tumor/ Normal cell
FKBP
FRB
T cell activation
AP1903

FIG. 3

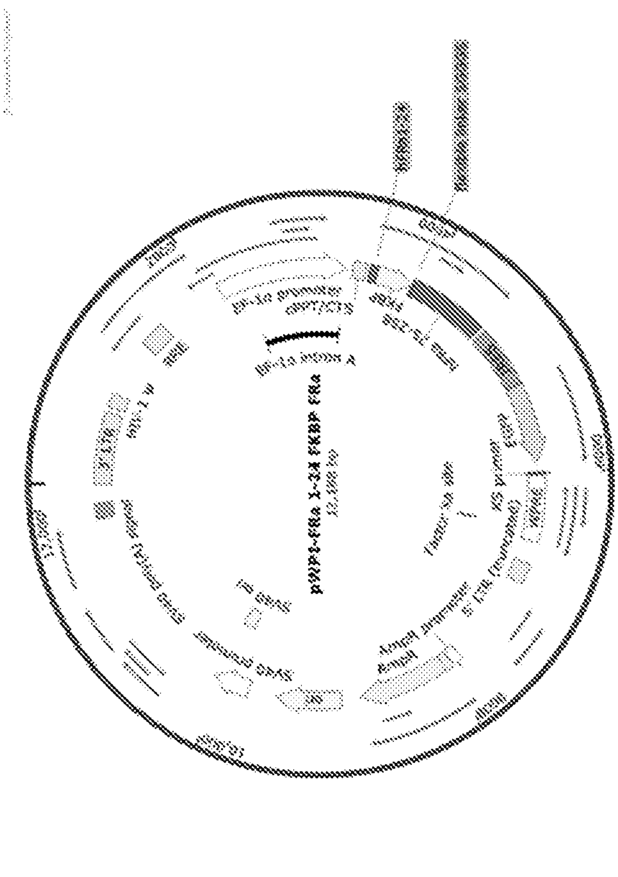


FIG. 4A

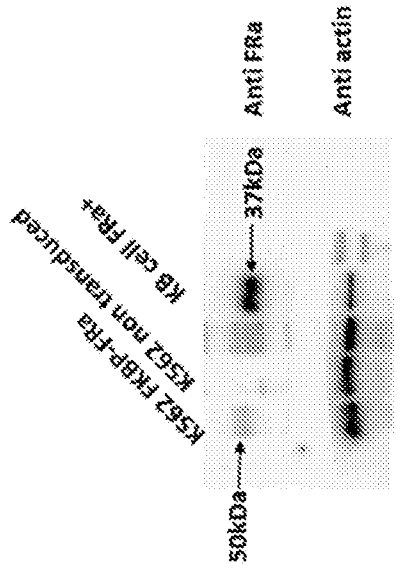

KB cell FRa+
K562 non transduced
K562 FKBP-FRa
37kDa
50kDa
Anti FRa
Anti actin

FIG. 4B

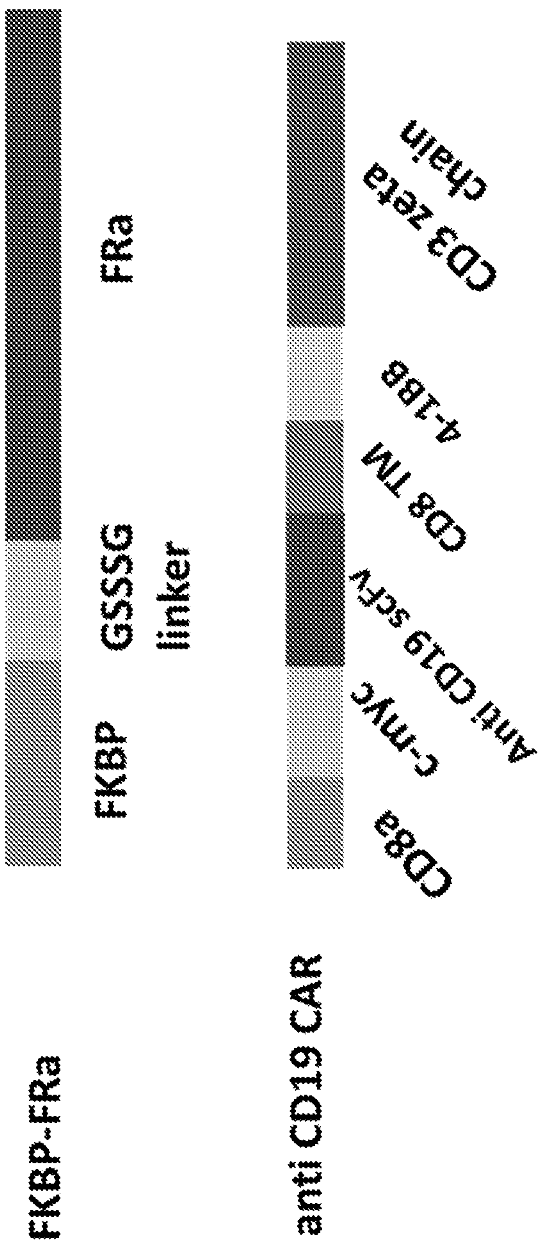
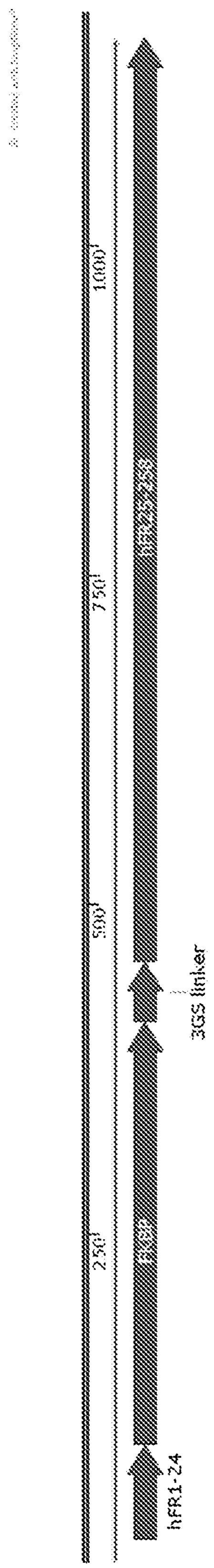
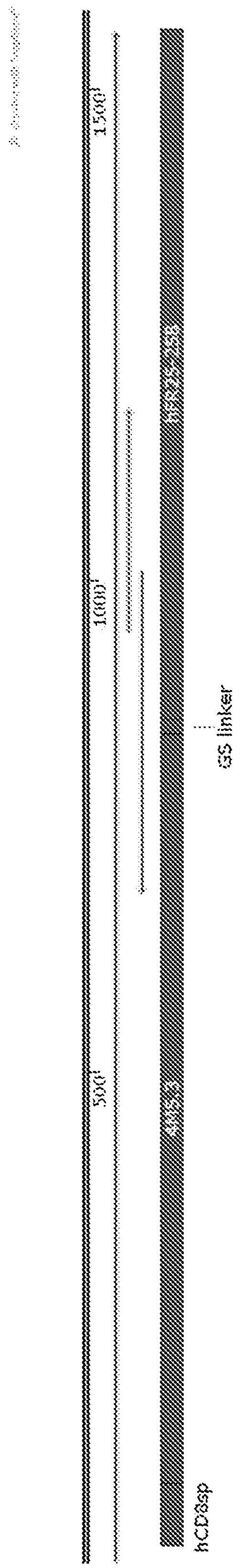

FKBP-FRa
FKBP
GSSSG linker
FRa
anti CD19 CAR
CD8a
C-myc
Anti CD19 scFv
CD8 TM
4-1BB
CD3 zeta chain

FIG. 4C



hFR1-24
FKBP
3GS linker
hFR25-258
250
500
750
1000

FIG. 4D



hCD8sp
4M5.3
GS linker
hFR25-258
500
1000
1500

FIG. 4E

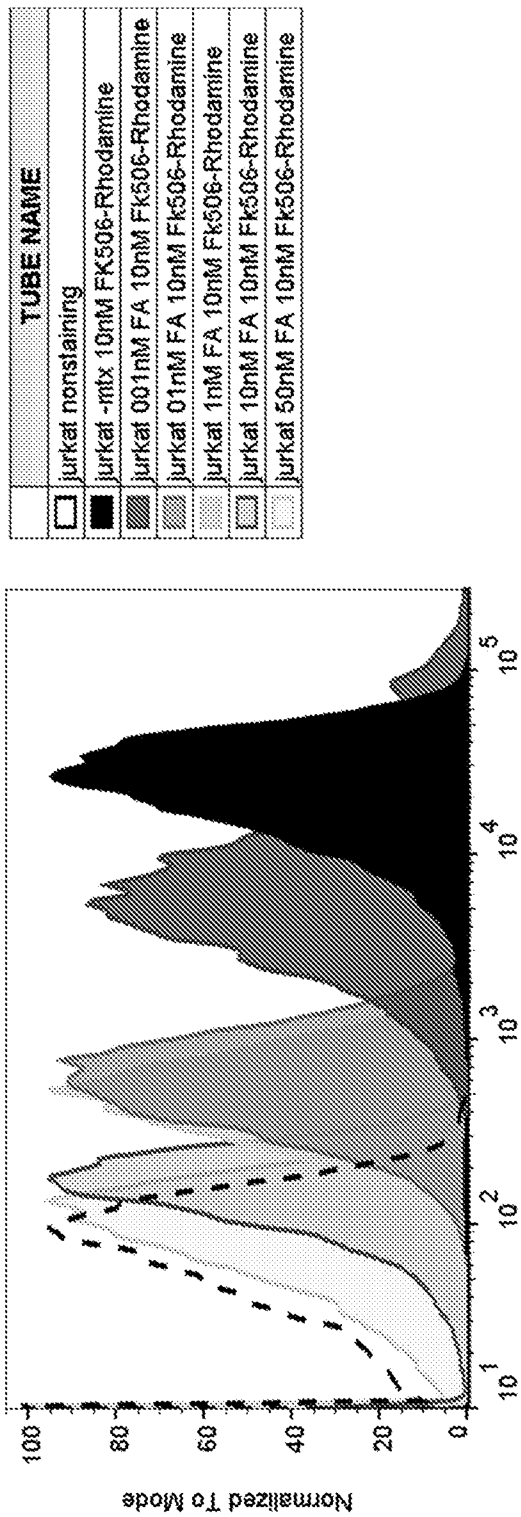

TUBE NAME
jurkat nonstaining
jurkat -mtx 10nM FK506-Rhodamine
jurkat 001nM FA 10nM Fk506-Rhodamine
jurkat 01nM FA 10nM Fk506-Rhodamine
jurkat 1nM FA 10nM Fk506-Rhodamine
jurkat 10nM FA 10nM Fk506-Rhodamine
jurkat 50nM FA 10nM Fk506-Rhodamine
Normalized To Mode
Comp-PE-A

FIG. 5

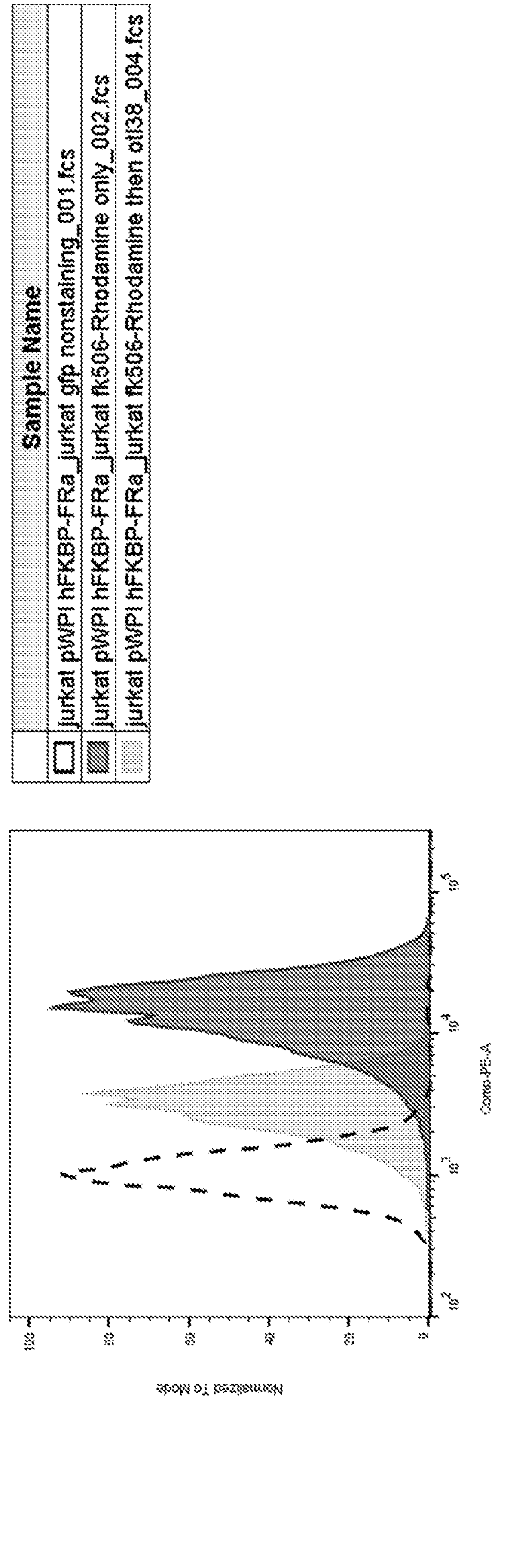

Sample Name
jurkat pWPI hFKBP-FRa_jurkat gfp nonstaining_001.fcs
jurkat pWPI hFKBP-FRa_jurkat fk506-Rhodamine only_002.fcs
jurkat pWPI hFKBP-FRa_jurkat fk506-Rhodamine then ol38_004.fcs
Normalized To Mode
Comp-PE-A

FIG. 6

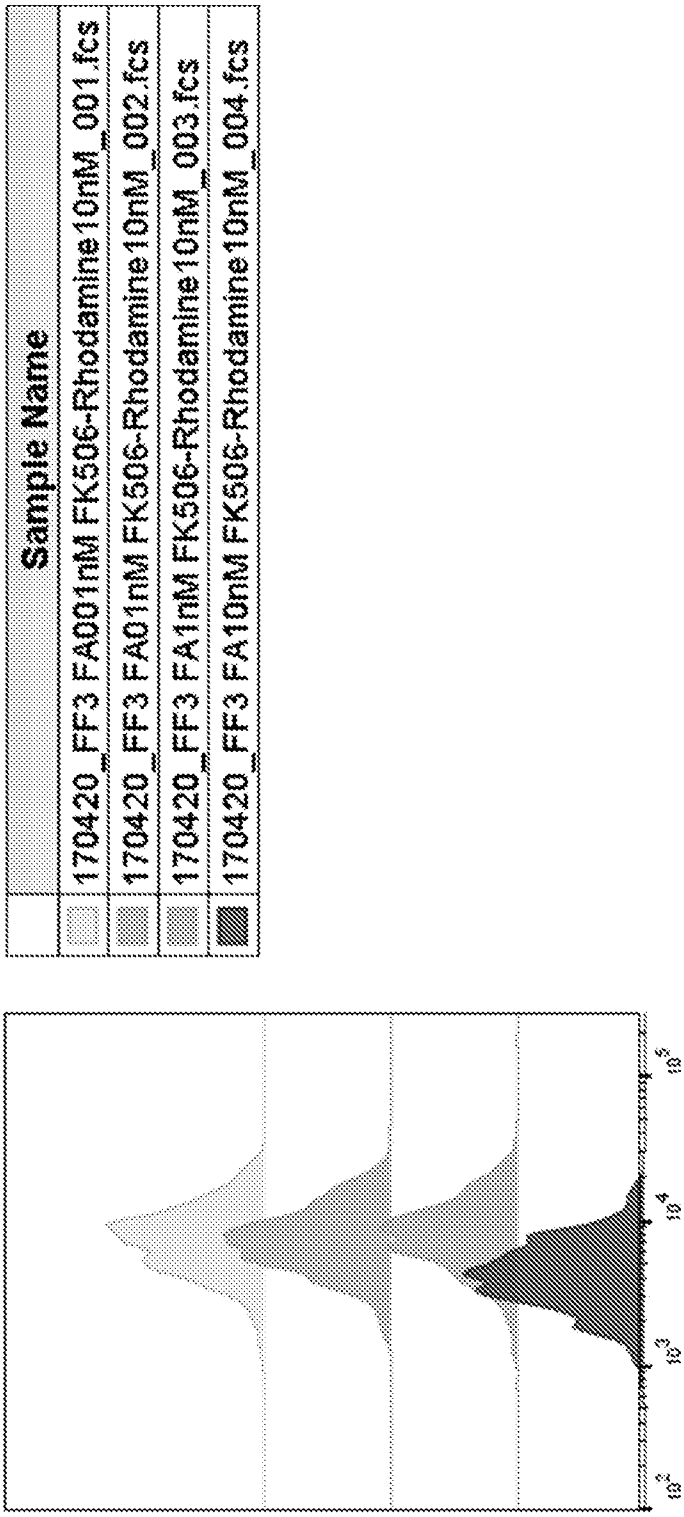

Sample Name
170420_FF3 FA001nM FK506-Rhodamine10nM_001.fcs
170420_FF3 FA01nM FK506-Rhodamine10nM_002.fcs
170420_FF3 FA1nM FK506-Rhodamine10nM_003.fcs
170420_FF3 FA10nM FK506-Rhodamine10nM_004.fcs
Comp-PE-A

FIG. 7

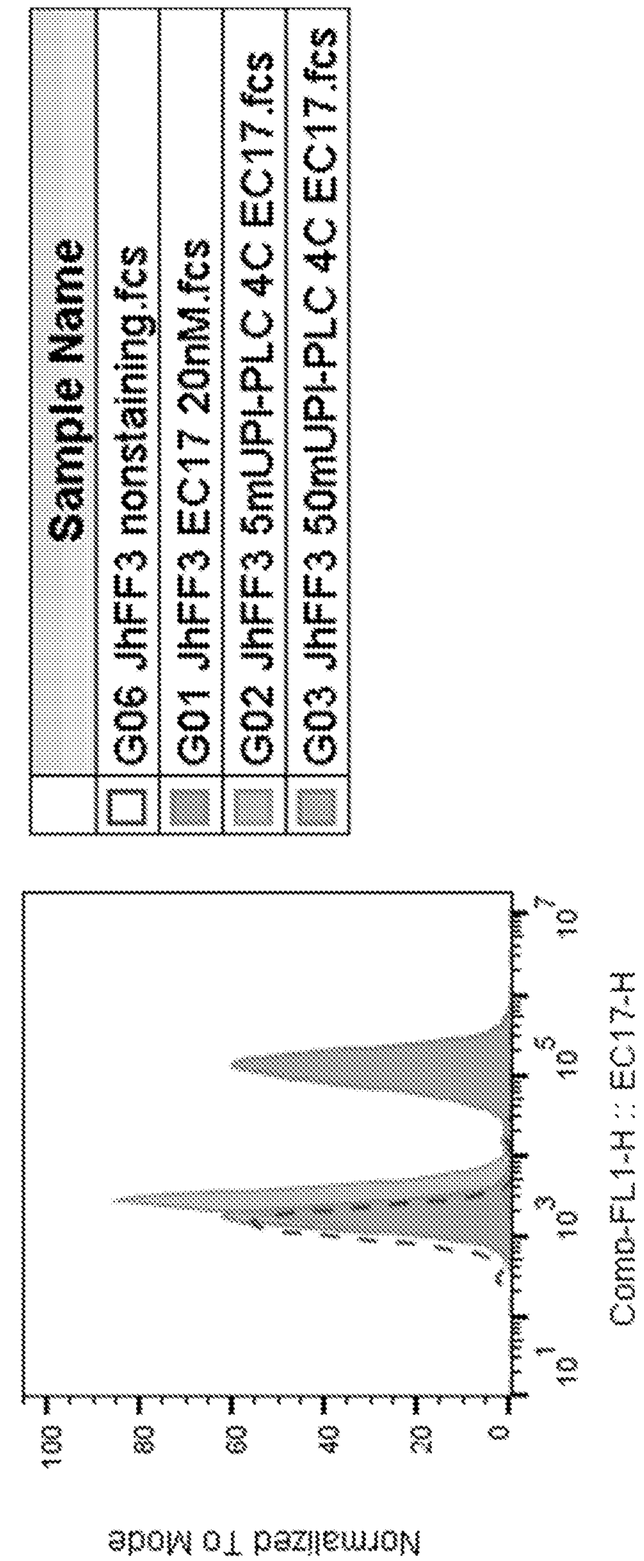

Sample Name
G06 JhFF3 nonstaining.fcs
G01 JhFF3 EC17 20nM.fcs
G02 JhFF3 5mUPI-PLC 4C EC17.fcs
G03 JhFF3 50mUPI-PLC 4C EC17.fcs
Normalized To Mode
0
20
40
60
80
100
Comp-FL1-H :: EC17-H
10^1
10^3
10^5
10^7

FIG. 8

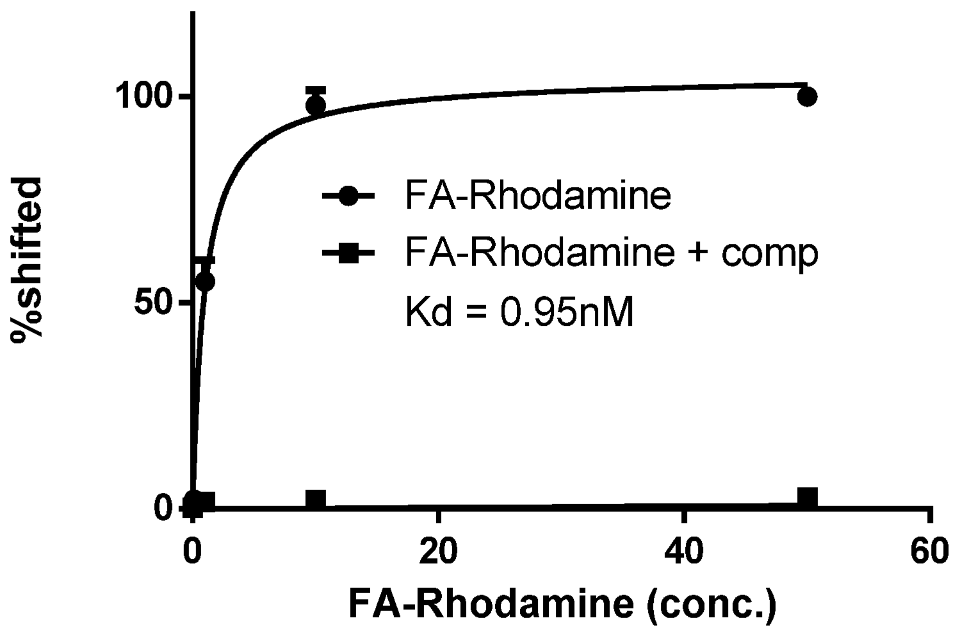

100
50
0
%shifted
FA-Rhodamine
FA-Rhodamine + comp
Kd = 0.95nM
0
20
40
60
FA-Rhodamine (conc.)

FIG. 9

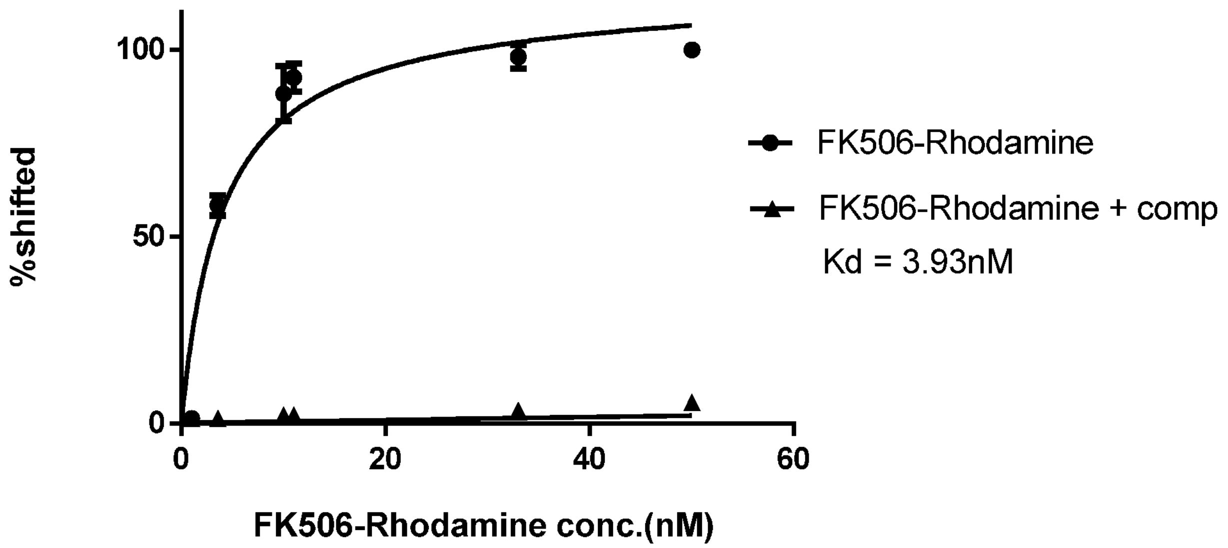

100
50
0
%shifted
0
20
40
60
FK506-Rhodamine conc.(nM)
FK506-Rhodamine
FK506-Rhodamine + comp
Kd = 3.93nM

FIG. 10

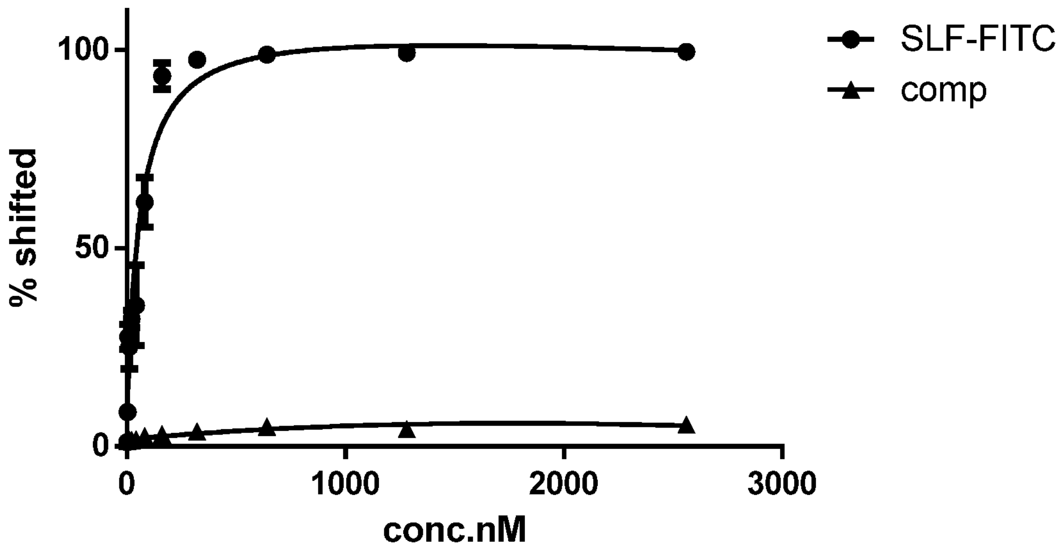


FIG. 11

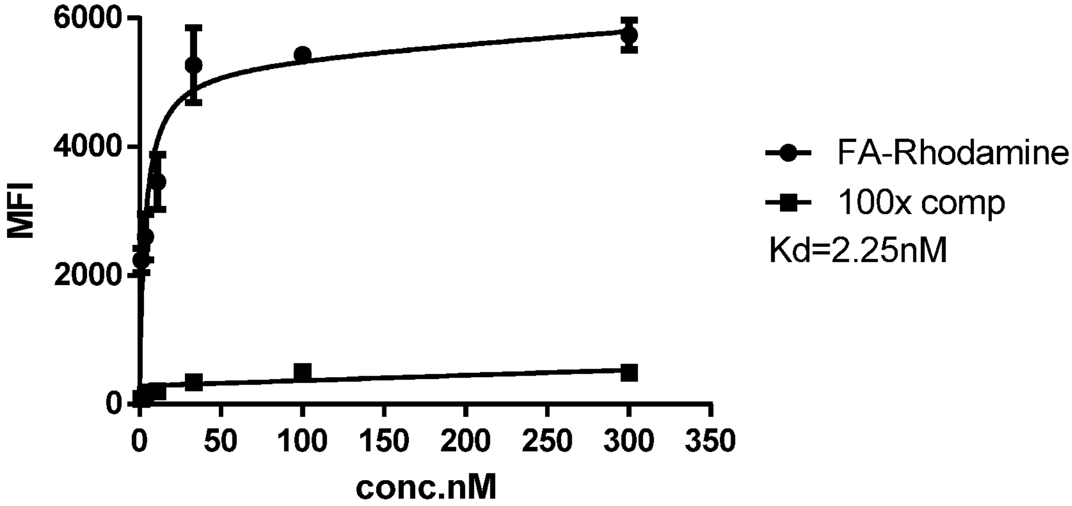

6000
4000
2000
0
MFI
0
50
100
150
200
250
300
350
conc.nM
FA-Rhodamine
100x comp
Kd=2.25nM

FIG. 12

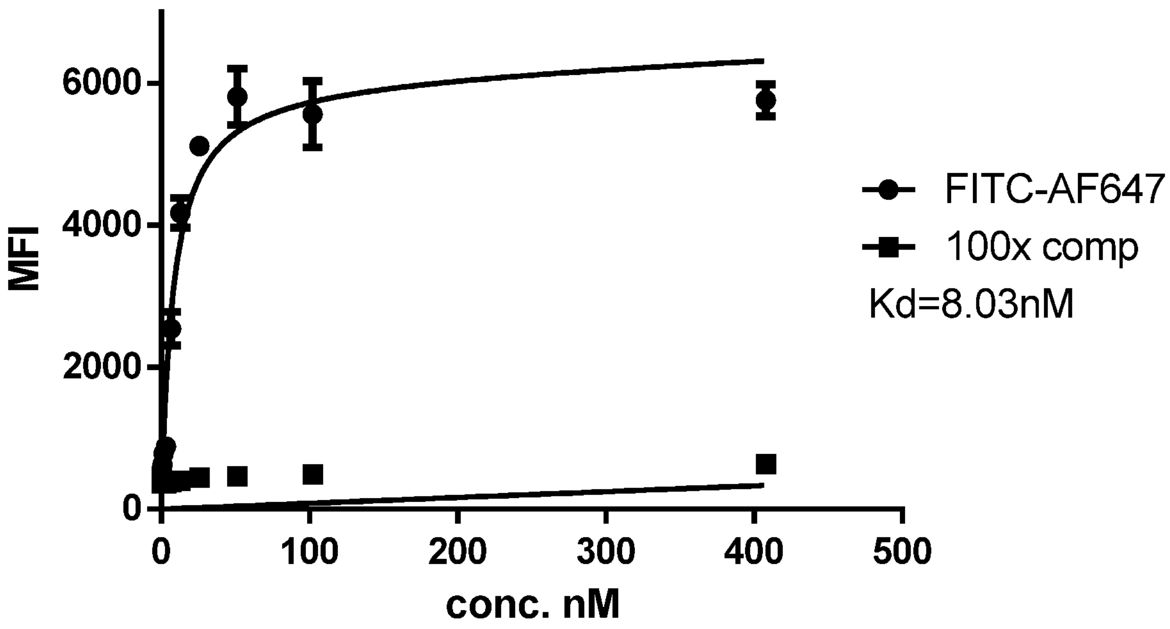

6000
4000
2000
0
MFI
0
100
200
300
400
500
conc. nM
FITC-AF647
100x comp
Kd=8.03nM

FIG. 13

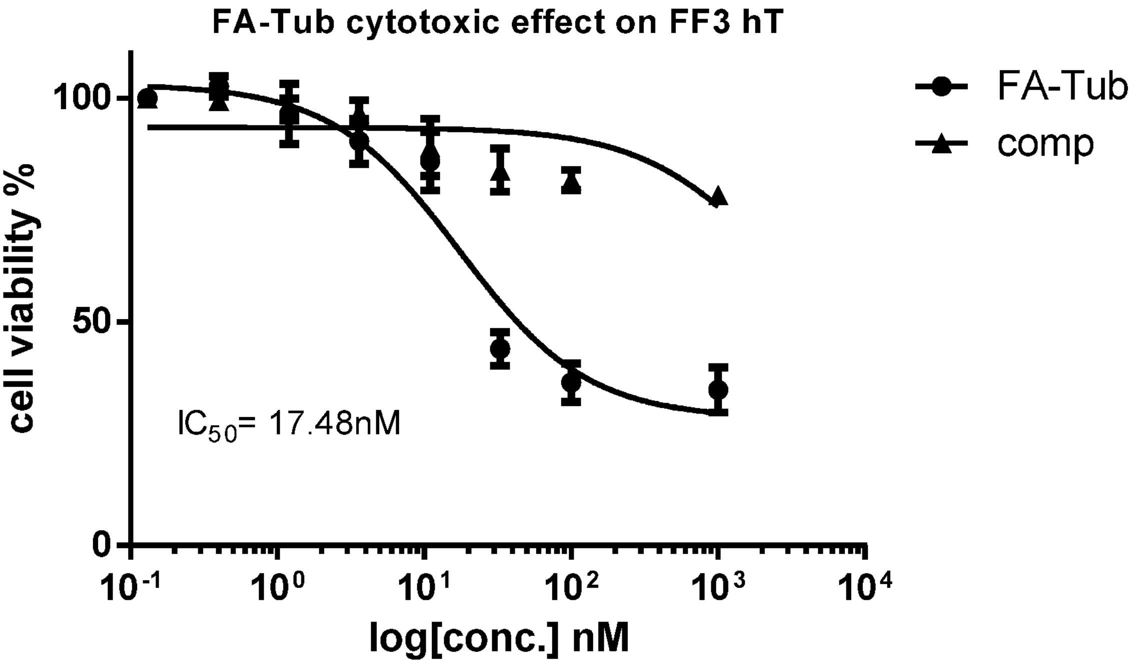

FA-Tub cytotoxic effect on FF3 hT
cell viability %
100
50
0
IC50= 17.48nM
10-1
100
101
102
103
104
log[conc.] nM
FA-Tub
comp

FIG. 14

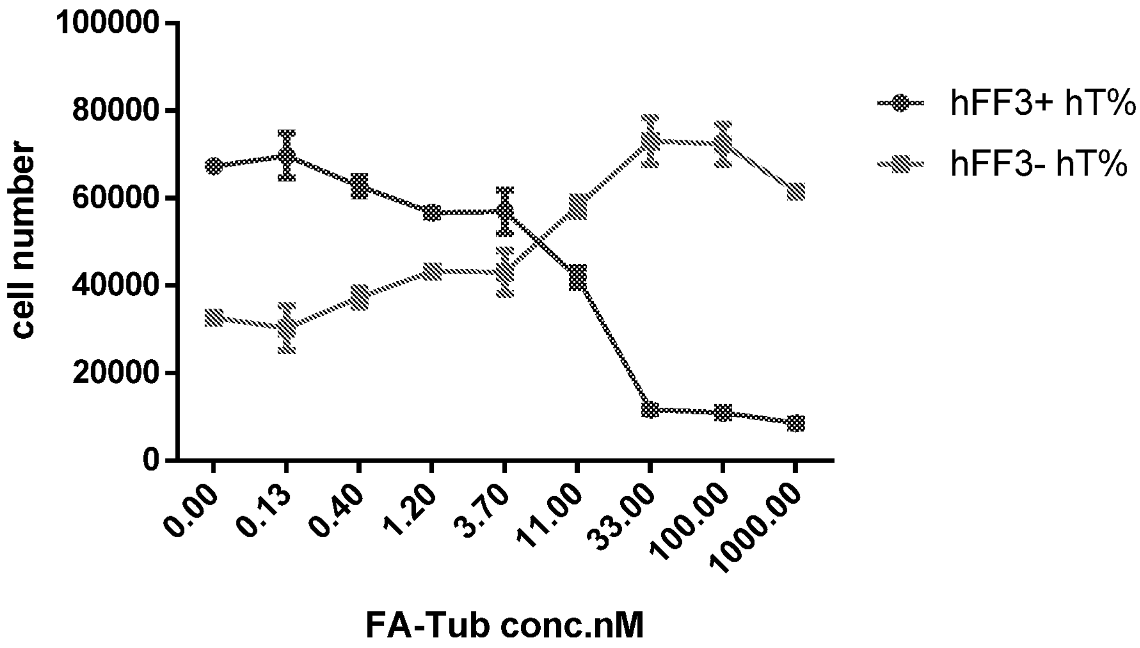

100000
80000
60000
40000
20000
0
cell number
0.00
0.13
0.40
1.20
3.70
11.00
33.00
100.00
1000.00
FA-Tub conc.nM
hFF3+ hT%
hFF3- hT%

FIG. 15

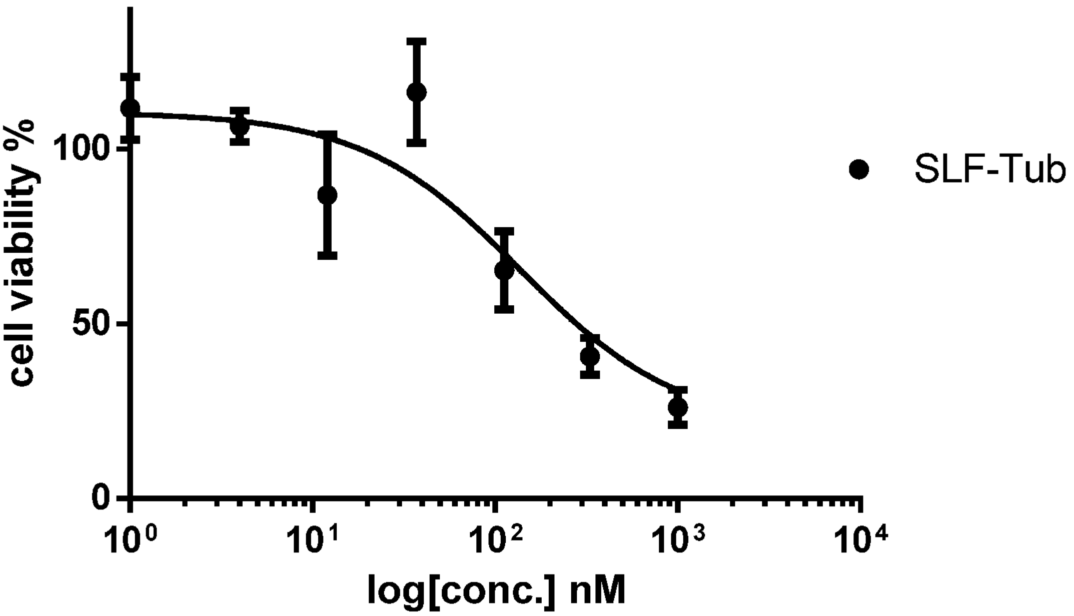


FIG. 16

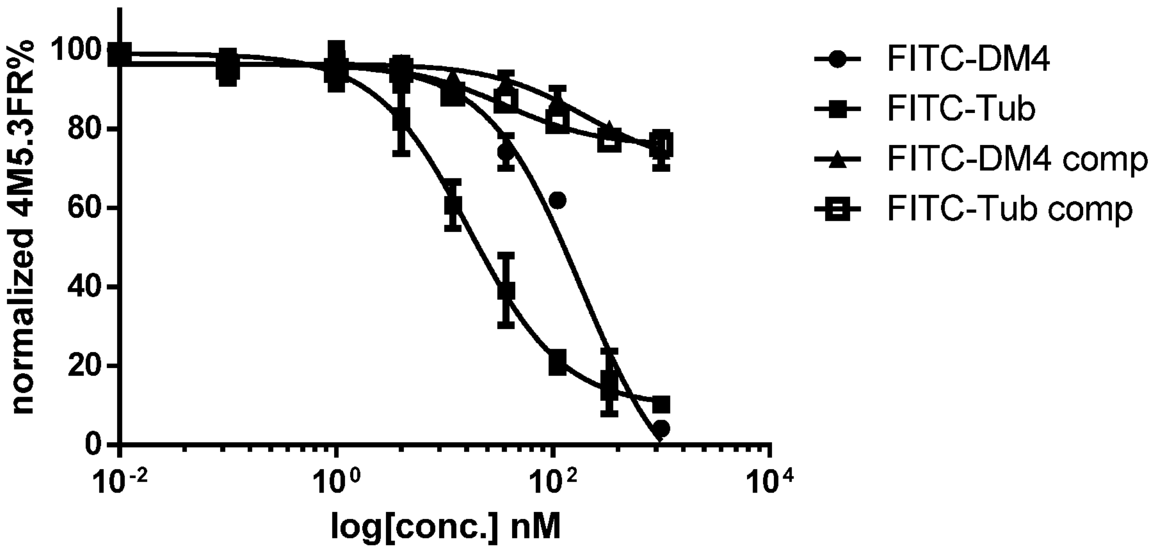

normalized 4M5.3FR%
100
80
60
40
20
0
10^{-2}
10^{0}
10^{2}
10^{4}
log[conc.] nM
FITC-DM4
FITC-Tub
FITC-DM4 comp
FITC-Tub comp

FIG. 17

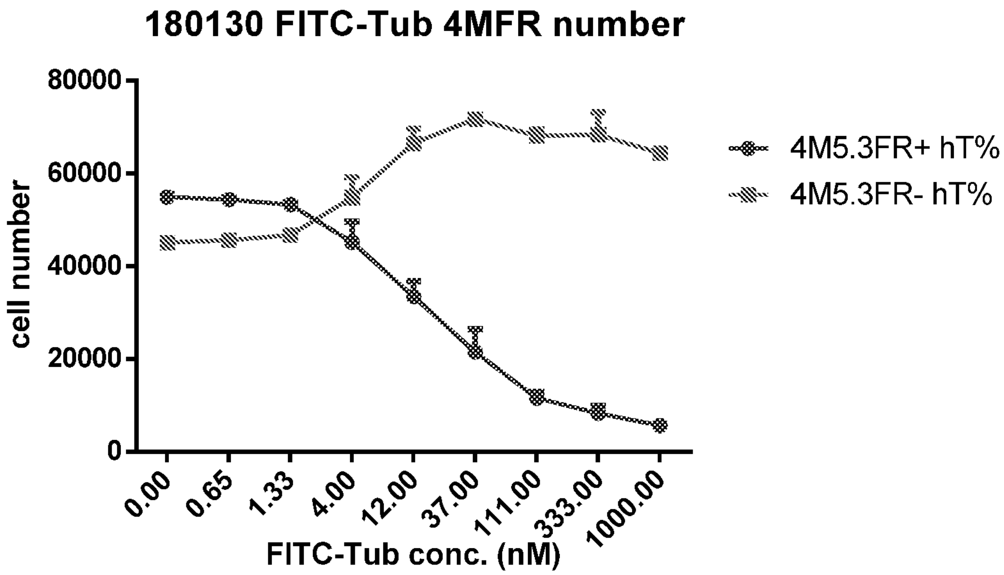

180130 FITC-Tub 4MFR number
80000
60000
40000
20000
0
cell number
0.00
0.65
1.33
4.00
12.00
37.00
111.00
333.00
1000.00
FITC-Tub conc. (nM)
4M5.3FR+ hT%
4M5.3FR- hT%

FIG. 18

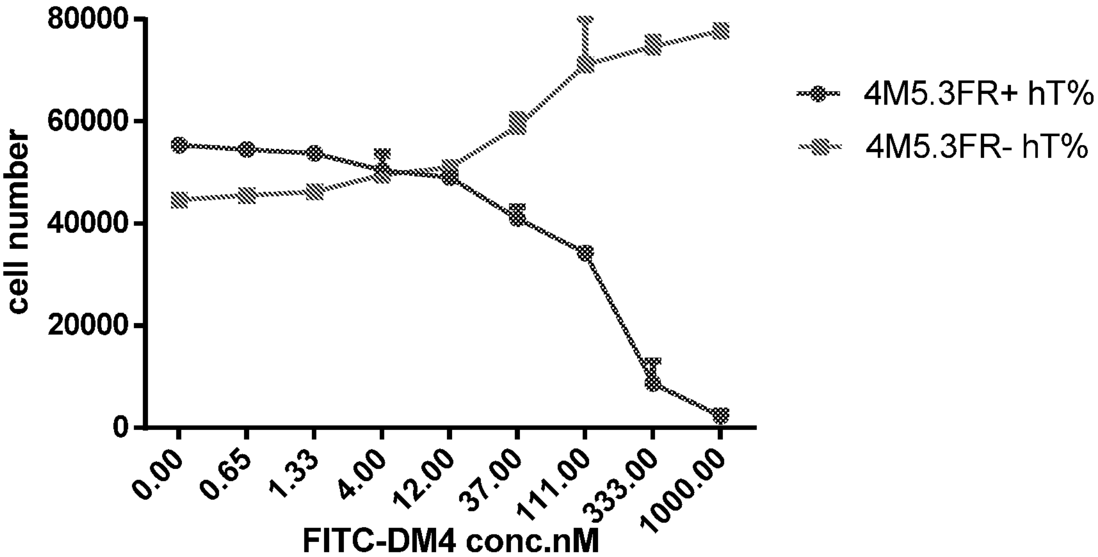

80000
60000
40000
20000
0
cell number
0.00
0.65
1.33
4.00
12.00
37.00
111.00
333.00
1000.00
FITC-DM4 conc.nM
4M5.3FR+ hT%
4M5.3FR- hT%

FIG. 19

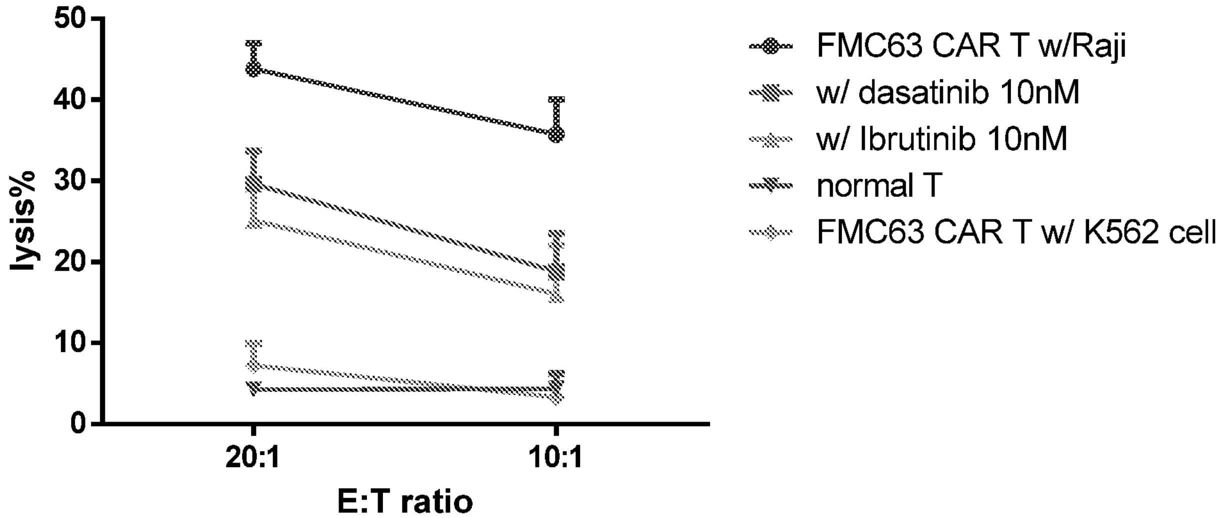

50
40
30
20
10
0
lysis%
20:1
10:1
E:T ratio
FMC63 CAR T w/Raji
w/ dasatinib 10nM
w/ Ibrutinib 10nM
normal T
FMC63 CAR T w/ K562 cell

FIG. 20

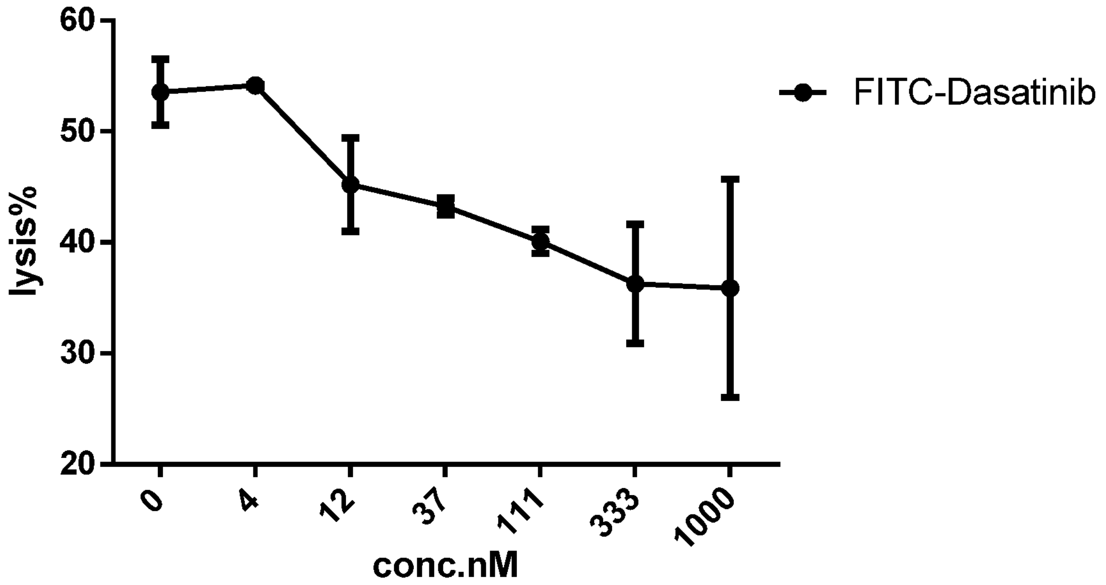

60
50
40
30
20
lysis%
FITC-Dasatinib
0
4
12
37
111
333
1000
conc.nM

FIG. 21

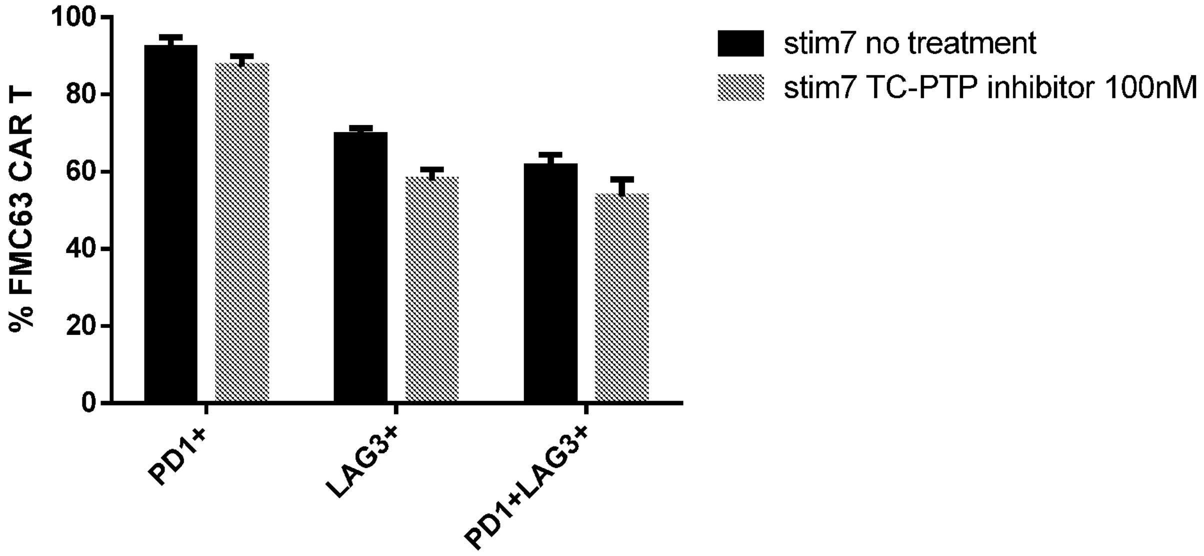

% FMC63 CAR T
100
80
60
40
20
0
PD1+
LAG3+
PD1+LAG3+
stim7 no treatment
stim7 TC-PTP inhibitor 100nM

FIG. 22

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 18/18557

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - C12N 15/62, C12N 15/63 (2018.01)
CPC - C12N 15/62, C12N 15/63, C07K 2319/09, C07K 2319/70, C07K 2319/81

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.
Y -- A	WO 2016/098078 A2 (NOVARTIS AG) 23 June 2016 (23.06.2016) pg 7, ln 11-25, pg 32, ln 20-24, pg 41, ln 5-13, pg 70, ln 5-15, pg 98, ln 23 to pg 99, ln 4, Figures 6A	1-2, 6-7, 15-18, 20-21, 24, 27, 29 ------ 22, 34, 35
Y -- A	SHILLINGFORD et al., "Folate-Conjugated Rapamycin Slows Progression of Polycystic Kidney Disease" J. Am Soc Nephrol, 28 September 2012, Vol 23, No 10; abstract, Figure 2 (A)	1-2, 6-7, 15-18, 20-21, 24, 27, 29 ------ 22, 34, 35
Y	US 2007/0077197 A1 (WEDEKING et al.) 05 April 2007 (05.04.2007) abstract, para [0032], [0042], [0274]	2, 6, 7, 15, 29
Y	US 2012/0076728 A1 (WU et al.) 29 March 2012 (29.03.2012) para [0059]	21
A	US 2003/0185840 A1 (IOANNIDES et al.) 2 October 2003 (02.10.2003) para [0096], SEQ ID NO: 45	22, 34, 35
A	JAMES et al., "Biophysical mechanism of T-cell receptor triggering in a reconstituted system" Nature, Vol 487, No 7405, pg 65-69; 05 July 2012; absract, Fig. 5a	1

☐ 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
11 June 2018	02 JUL 2018

Name and mailing address of the ISA/US	Authorized officer:
Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-8300	Lee W. Young PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 18/18557

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.:
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 for Box No. III Observations where unity of invention is lacking -

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.:
1-2, 6-7, 15-18, 20-22, 24, 27, 29, 34, 35 limited to imaging agent, folate receptor alpha, FA derivatives

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

International application No.
PCT/US 18/18557

Continuation of:
Box NO 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 1-38, drawn to a drug delivery platform for cell therapy comprising: an engineered protein on a target cell, at least one small ligand conjugated to a linker and at least one payload of drug conjugated to the linker. The drug delivery platform will be searched to the extent that the payload of drug encompasses an imaging agent; and the engineered protein encompass folate receptor alpha (FRa) comprising SEQ ID NO: 1 (mouse FKBP-Fra, see instant Specification, pg 5) and the small ligand encompasses FA derivatives. It is believed that claims 1-2, 6-7, 15-18, 20-22, 24, 27, 29, 34, 35 encompass this first named invention, and thus these claims will be searched without fee to the extent that they encompass imaging agent, folate receptor alpha, FA derivatives, SEQ ID NO: 1. Additional payload(s), engineered protein(s) and small ligand(s) will be searched upon the payment of additional fees. Applicants must specify the claims that encompass any additionally elected payload(s), engineered protein(s) and small ligand(s). Applicants must further indicate, if applicable, the claims which encompass the first named invention, if different than what was indicated above for this group. Failure to clearly identify how any paid additional invention fees are to be applied to the "+" group(s) will result in only the first claimed invention to be searched. An exemplary election would be a drug delivery platform encompasses cytotoxic drug, FK506 binding protein (FKBP) comprising SEQ ID NO: 2, and FK506 derivatives (Claims 1, 3, 8, 16-22, 24, 26, 27, 34, 35).

Group II+: Claims 39-60, drawn to a method to modulate cell therapy effect, comprising: providing an engineered protein on the surface of the target cell, a payload of drug conjugated to a small ligand through a linker. Group II+ will be searched upon payment of additional fees. The method may be searched, for example, to the extent that the cell therapy function encompasses providing optically guided surgery to a subject, the target cell encompasses immune cell, the payload of drug encompasses an imaging agent; and the engineered protein encompass SEQ ID NO: 12 (FKBP-3SG-FR with GPI anchor amino acid sequence, see SEQUENCE LISTING) and the small ligand encompasses FA derivatives. It is believed that claims 39, 40, 43, 46, 49-52 encompass this first named invention, and thus these claims will be searched without fee to the extent that they encompass optically guided surgery, immune cell, imaging agent, SEQ ID NO: 12 and FA derivatives. Additional cell therapy function(s), target cell(s), payload(s), engineered protein(s) and small ligand(s) will be searched upon the payment of additional fees. Applicants must specify the claims that encompass any additionally elected cell therapy function(s), payload(s), engineered protein(s) and small ligand(s). Failure to clearly identify how any paid additional invention fees are to be applied to the "+" group(s) will result in only the first claimed invention to be searched. An exemplary election would be a method to modulate cell therapy effect encompasses control the target cell proliferation, the target cell encompasses a stem cell, the payload encompasses cytotoxic drug, SEQ ID NO: 13 (4M5.3-FR with GPI anchor amino acid sequence, see SEQUENCE LISTING), and FK506 derivatives (Claims 39, 41, 45, 47, 49-52, 55).

Special Technical Features

Group I+ includes the special technical feature of a composition comprising a drug delivery platform, a DNA constrcut, and a transplanted cell, not required by Groups II+.

Groups II+ includes the special technical feature of a method to modulate cell therapy effect, comprising releasing the payload drug with the target cell, not required by Groups I+.
The technical feature of each of the inventions listed as Groups I+ and II+ is the specific engineered proteins and the ligand-linker-drug conjugates, recited therein. Each invention requires a specific engineered protein and ligand-linker-drug conjugate, not required by any of the other inventions.

Common Technical Features

The inventions of Groups I+ and II+ share the technical feature of an engineered proteins and the ligand-linker-drug conjugates. However, these shared technical features do not represent a contribution over prior art in view of the article "Biophysical mechanism of T -cell receptor triggering in a reconstituted system" by James et al. (hereinafter 'James') (Nature vol 487 pg 65-69; 05 July 2012) and the article "Folate-Conjugated Rapamycin Slows Progression of Polycystic Kidney Disease" by Shillingford et al. (hereinafter 'Shillingford') (J. Am Soc Nephrol 23: 1674?1681, 2012).

James teaches an an engineered protein on a target cell for transplant, wherein the engineered protein comprises a first component and a second component, the first component and the second component are connected by a peptide linker, the first component is a non-membrane protein, the second component is a membrane anchored peptide or protein (pg 67, col 2, para 4, we engineered a chemically controlled, cell-surface-receptor system consisting of a transmembrane protein with extracellular FKBP and the intracellular CD3.zeta. ITAM domains expressed in the HEK cell (FKBP.sup.(Ex.zeta.Int); mimicking the TCR) and a transmembrane protein with an extracellular FRB expressed in the APC (mimicking the pMHC) (Fig. 5a). FKBP.sup.(Ex.zeta.Int) and FRB.sup.(Ex) will only interact in the presence of rapamycin, forming a complex that spans a similar distance to TCR?pMHC.; abstract, An artificial, chemically controlled receptor system generates the same effect as TCR - pMHC, demonstrating that the binding energy of an extracellular protein?protein interaction can drive the spatial segregation of membrane proteins without a transmembrane conformational change.). James further teach activating said engineered protein with ligand, rapamycin (pg 67, col 2, para 4), but does not specifically teach a ligand-linker-drug conjugate.

- Please see extra sheet for continuation -

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 18/18557

Continuation of:
Box NO III. Observations where unity of invention is lacking

Shillingford teaches a a ligand-linker-drug conjugate comprising folate-conjugated rapamycin (abstract, we synthesized a folate-conjugated form of rapamycin (FC-rapa) that is taken up by folate receptor - mediated endocytosis and cleaved intracellularly to reconstitute the active drug......Treatment of a PKD (polycystic kidney disease) mouse model with FC-rapa inhibited mTOR in the target tissue, strongly attenuated proliferation and growth of renal cysts and preserved renal function. Figure 2 (A) Chemical structure of EC0371 showing the modular components folate (black), hydrophilic spacer (blue), biologically cleavable linker (green), and rapamycin (red).). Given that Fc-rapa of Shillingford can be used to bind to the FKBP.sup.(Ex.zeta.Int) (mimicking the TCR) in a T cell of James, it would have been obvious to one of ordinary skill in the art to have modified the drug payload to, for example, an imaging agent, and thus, can be used to image TCR activation. In addition, such a drug delivery platform can be applied to adoptive cell therapy with transplanted CAR T cells, because James further teaches that "This model provides a plausible mechanism to explain how Cars trigger T cells to kill cancerous cells and may apply to other cell types that signal using membrane bound receptors and brigands." (pg 69, col 1, para 2).

James further a chimeric antigen receptor T cell (pg 69, col 1, para 2) and a DNA construct (suppl. Info. pg 6, HEK cells were transfected as described above in 6-well plates, using equal amounts of the lentiviral backbone vector (derived from pHRSIN-CSGW).).

As said technical features were known in the art at the time of the invention, these cannot be considered special technical features that would otherwise unify the groups.

Groups I+ and II+ therefore lack unity under PCT Rule 13 because they do not share a same or corresponding special technical feature.