Redirection of Genetically Engineered CAR-T cells using Bifunctional Small Molecules

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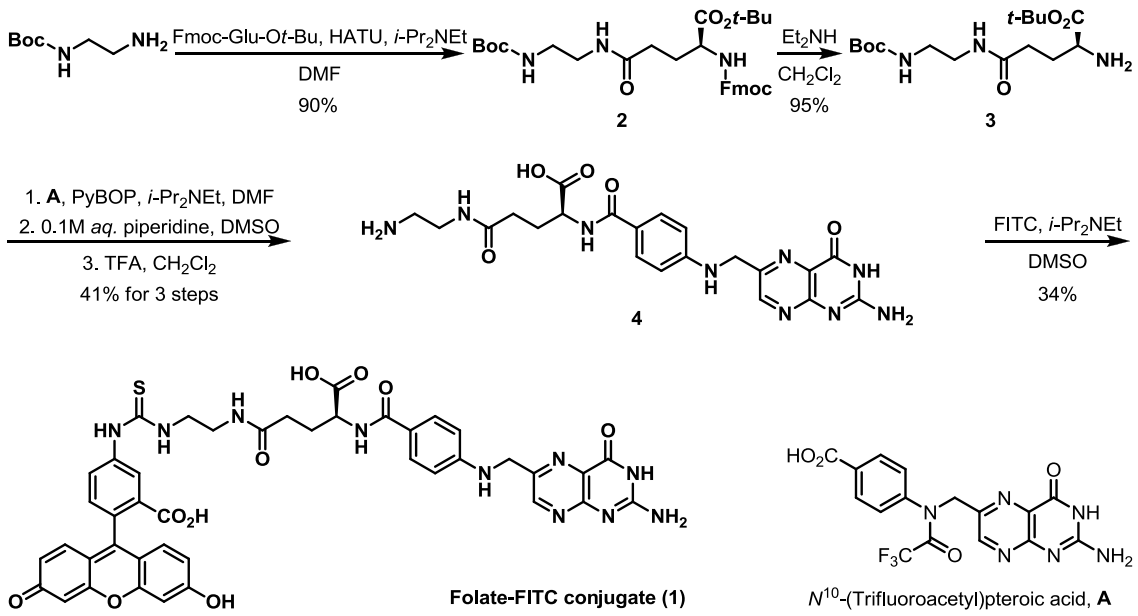
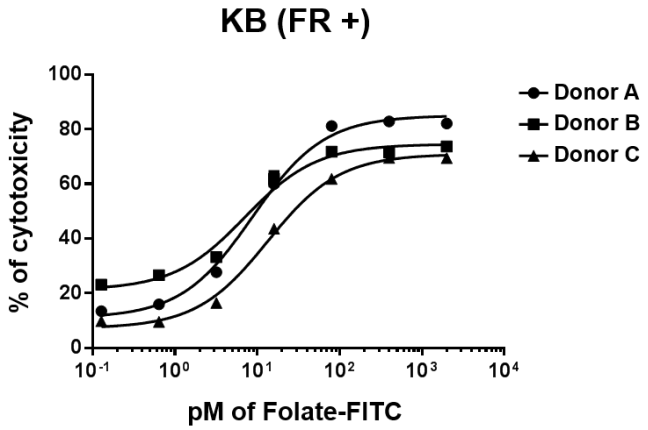
Supporting information

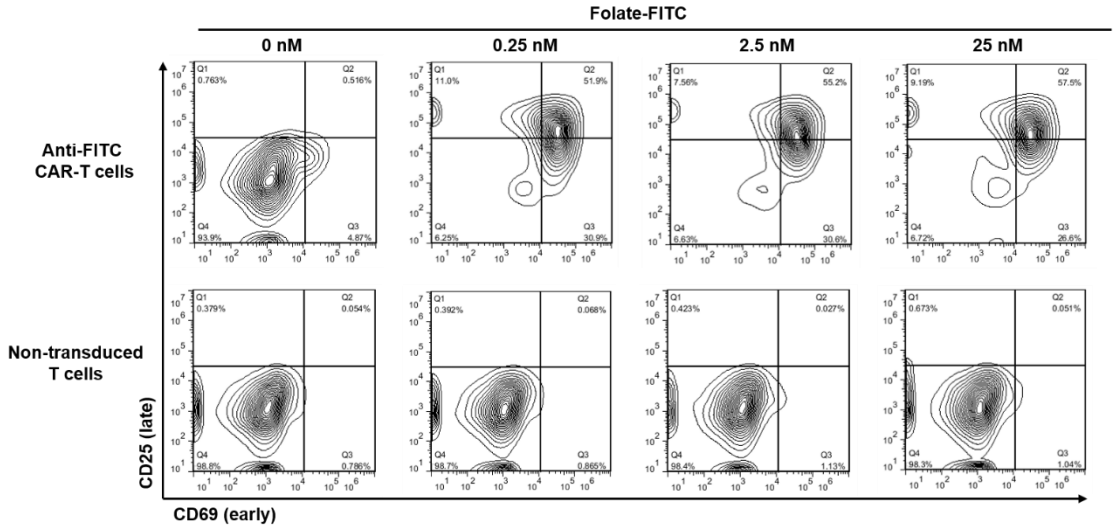


Supplementary Figure 1. Synthetic scheme of folate-FITC

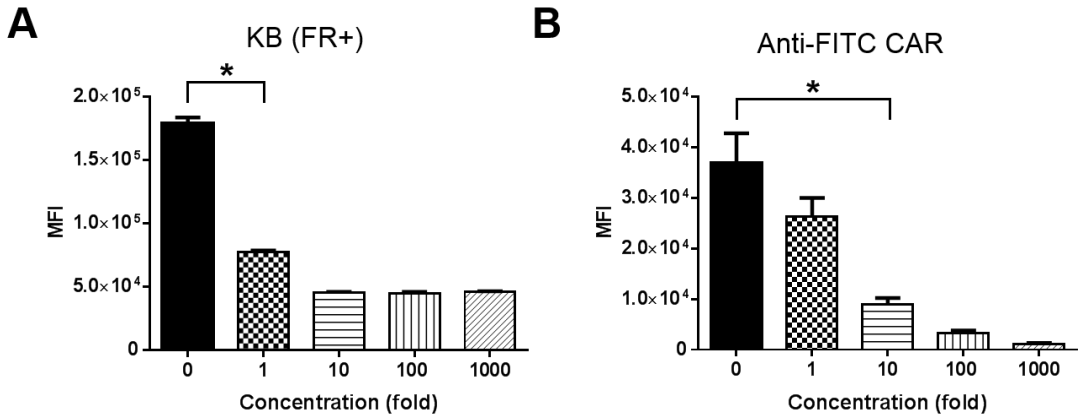


Donor	Transduction efficiency	EC_{50}	Max. Killing
A	48 %	8.7 pM	85 %
B	54 %	7.1 pM	75 %
C	40 %	13.4 pM	71 %
Average	**47.3(±7.0) %**	**9.7(±3.2) pM**	**77(±7.2) %**

Supplementary Figure 2. Transduction efficiency and cytotoxicity of anti-FITC CAR-T cells from different donors. Donor A is illustrated in the main text.



Supplementary Figure 3. CD25 and CD69 expression on anti-FITC CAR-T cells or non-transduced CAR-T cells in the presence of the folate-FITC switch and FR-positive tumor cells.



Supplementary Figure 4. Competitive binding assay of 50 nM folate-FITC to KB (FR+) (A) and anti-FITC CAR-T cells (B) in the presence of varied concentrations of free-folate and free-fluorescein, respectively. Each column represents a mean of triplicates and error bars represent SEMs. * $P \leq 0.05$ was determined by 2-paired Student t test.

General Experimental

Synthesis

Unless noted otherwise, all starting materials and reagents were obtained from commercial suppliers and were used without further purification. Reaction flasks were dried at 100 °C. Air and moisture sensitive reactions were performed under argon atmosphere. Flash column chromatography was performed using silica gel 60 (230-400 mesh, Merck) with the indicated solvents. Thin-layer chromatography was performed using 0.25 mm silica gel plates (Merck). ^{1}H and ^{13}C NMR spectra were obtained on a Varian INOVA-399 (400MHz) spectrophotometer. Chemical shifts are expressed in parts per million (ppm, δ) downfield from tetramethylsilane and are referenced to the deuterated solvent ($CHCl_3$). ^{1}H NMR data were reported in the order of chemical shift, multiplicity (s, singlet; d, doublet; t, triplet; m, multiplet and/or multiple resonance), number of protons and coupling constant in hertz (Hz).

(*S*)-*tert*-butyl 2-(((9*H*-fluoren-9-yl)methoxy)carbonylamino)-5-(2-(*tert*-butoxycarbonylamino)ethylamino)-5-oxopentanoate (2).

To a stirred solution of *t*-butyl-*N*-(2-aminoethyl)carbamate (1.87 g, 11.7 mmol) and Fmoc-Glu-O*t*-Bu (4.99 g, 11.7 mmol) in DMF (30 mL) were added HATU (6.69 g, 17.6 mmol) and DIPEA (2.04 mL, 11.7 mmol). After stirring for 16 hr, the reaction mixture was diluted with EtOAc, and then washed with H_2O (x 3). The organic layer was dried over $MgSO_4$ and concentrated *in vacuo*. The residue was purified by flash column chromatography on silica gel (EtOAc : *n*-hexane = 4 : 1) to afford 5.96 g (90%) of the amide **2** as a colorless oil: ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.75 (d, 2H, *J* = 7.6 Hz), 7.60 (d, 2H, *J* = 7.4 Hz), 7.39 (t, 2H, *J* = 7.5 Hz), 7.30 (m, 2H), 6.47 (s, 1H), 5.70 (d, 1H, *J* = 8.2 Hz), 5.26 (s, 1H), 4.40 (m, 2H), 4.20 (m, 2H), 3.41 (m, 1H), 3.24 (s, 3H), 2.21 (m, 3H), 1.86 (m, 1H), 1.46 (s, 9H), 1.38 (s, 9H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 172.6, 171.2, 156.8, 156.5, 144.0, 143.7, 141.4, 141.4, 127.8, 127.1, 125.2, 120.1, 82.6, 79.5, 67.0, 53.9, 47.3, 40.6, 40.3, 32.5, 29.0, 28.4, 28.1; LR-MS (ESI+) *m*/*z* 568 ($M + H^+$); HR-MS (ESI+) calcd for $C_{31}H_{42}N_3O_7$ ($M + H^+$) 568.3017; found 568.3022.

(*S*)-*tert*-butyl 2-amino-5-(2-(*tert*-butoxycarbonylamino)ethylamino)-5-oxopentanoate (3).

To a stirred solution of the amide **2** (5.96 g, 10.5 mmol) in CH_2Cl_2 (70 mL) was added Et_2NH (70.0 mL). After stirring for 14 hr, the solvent was removed and the residue was purified by flash column

chromatography on silica gel (MeOH : EtOAc = 1 : 10) to afford 3.45 g (95%) of the amine **3** as a colorless oil: ^{1}H NMR ($CDCl_3$, 400 MHz) δ 6.95 (m, 1H), 5.43 (m, 1H), 3.26 (m, 3H), 3.17 (m, 2H), 2.25 (t, 2H, *J* = 7.5 Hz), 1.99 (m, 1H), 1.82 (s, 2H), 1.75 (m, 1H), 1.38 (s, 9H), 1.35 (s, 9H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 174.8, 173.3, 156.7, 81.3, 79.3, 54.5, 40.3, 40.2, 32.9, 30.3, 28.4, 28.0; LR-MS (ESI+) *m*/*z* 346 ($M + H^+$); HR-MS (ESI+) calcd for $C_{16}H_{32}N_3O_5$ ($M + H^+$) 346.2336; found 346.2349.

(*S*)-2-(4-((2-amino-4-oxo-3,4-dihydropteridin-6-yl)methylamino)benzamido)-5-(2-aminoethylamino)-5-oxopentanoic acid (4).

To a stirred solution of the amine **3** (504 mg, 1.46 mmol) and N^{10}-(trifluoroacetyl)pteroic acid **A** (497 mg, 1.22 mmol) in DMF (10 mL) were added PyBOP (760 mg, 1.46 mmol) and DIPEA (0.50 mL, 2.68 mmol). After stirring for 4 hr, the yellow reaction mixture was precipitated with Et_2O (640 mL) and the resulting precipitate was washed with Et_2O (2 x 80 mL). The residue was concentrated *in vacuo* and used for next step without further purification. To a stirred solution of the amide in DMF (5 mL) was added aqueous piperidine (10 mL, 0.1 M). After stirring for 1 hr, the reaction mixture was diluted with THF (45 mL) and precipitated with Et_2O (400 mL). The yellow precipitate was washed with Et_2O (2 x 10 mL) and concentrated *in vacuo*. The crude mixture was used for next step without further purification. To a stirred solution of the amine in CH_2Cl_2 (3 mL) was added TFA (3 mL). After stirring for 12 hr, the reaction mixture was concentrated *in vacuo*. The residue was purified by preparative HPLC and lyophilized to afford 277 mg (41%) of the amine **4** as yellow solid: LR-MS (ESI+) *m*/*z* 484 ($M + H^+$).

(*S*)-5-(3-(2-(4-(4-((2-amino-4-oxo-3,4-dihydropteridin-6-yl)methylamino)benzamido)-4-carboxybutanamido)ethyl)thioureido)-2-(6-hydroxy-3-oxo-3*H*-xanthen-9-yl)benzoic acid (1).

To a stirred solution of the amine **4** (134 mg, 0.277 mmol) in DMSO (5 mL) were added FITC (108 mg, 0.277 mmol) and DIPEA (0.10 mL, 0.554 mmol). After stirring for 6 hr, the reaction mixture was purified by preparative HPLC and lyophilized to afford 82.2 mg (34%) of the Folate-FITC **1** as yellow solid: ^{1}H NMR (DMSO-d_6, 400 MHz) δ 10.00 (s, 1H), 8.66 (s, 1H), 8.20 (d, 1H, *J* = 7.6 Hz), 8.17 (m, 1H), 8.08 (s, 1H), 8.00 (m, 1H), 7.69 (d, 2H, *J* = 8.8 Hz), 7.61 (d, 2H, *J* = 4.6 Hz), 7.14 (d, 2H, *J* = 8.3 Hz), 6.57 (m, 8H), 4.49 (s, 3H), 4.26 (m, 2H), 3.52 (s, 3H), 3.23 (m, 3H), 2.46 (m, 1H), 2.19 (m, 2H), 2.04 (m, 1H),

1.88 (m, 1H); LR-MS (ESI+) *m/z* 873 ($M + H^+$); HR-MS (ESI+) calcd for $C_{42}H_{37}N_{10}O_{10}S$ ($M + H^+$) 873.2409; found 873.2404.

T cell transduction

For lentiviral production, the HEK293FT cell line was cultured in Dulbecco's modified Eagle's medium (DMEM, Invitrogen) supplemented with 10% fetal bovine serum (FBS, Invitrogen) and 1% penicillin and streptomycin. 5 x 10^6 cells were plated on 100 mm culture dish pre-coated with poly-D-lysine and transfected with lentiviral vectors using lipofectamine 2000 (Invitrogen). Following 48 hr of culture, supernatant containing lentivirus was harvested and cryopreserved at -80°C until ready for use. PBMCs were isolated from healthy human donor blood (from The Scripps Research Institute normal blood donor service) by conventional Ficoll-Paque density gradient method (GE healthcare). PBMCs were cultured in AIM-V (Invitrogen) supplemented with 5% human AB serum and 300 IU/mL of IL-2 (R&D systems) and stimulated with anti-CD3 and anti-CD28 mAbs coated beads (Invitrogen) as described in the manufacturer's instructions. 24 hr thereafter, 1 x 10^6 cells of PBMCs were transduced with 1.5 mL of lentivirus at a multiplicity of infectivity (MOI) of 10~15 with 1 ug/mL of protamine sulfate (Sigma) and incubated overnight at 37°C, 5 % CO_2. The next day, cells were initially seeded at 0.125 x 10^6 cells/mL and later maintained at 0.5-1 x 10^6 cells/mL in AIM-V media supplemented with 5% human AB serum and 300 IU/mL IL-2. Seventy-two hr after transduction, cells were harvested for flow cytometry analysis of CAR expression (described below).

Flow cytometry analysis

To assess anti-FITC CAR expression, CAR-T cells were washed with PBS and resuspended with 1% BSA in PBS (FACS buffer). Then, cells were incubated with APC-conjugated anti-mouse IgG F(ab')2 antibody for 30 min at 4°C. After washing twice, cells were analyzed using BD Accuri™ C6 Flow Cytometer (Beckton Dickinson Immunocytometry Systems) (shown in Figure 1D). To determine the availability of the folate molecule on our folate-FITC conjugate, we first stained T cells with folate-FITC and then with an anti-FITC antibody which was labeled with Alexa Fluor® 647 dye. Dye labeling was carried out using an Alexa Fluor® antibody labeling kit (Invitrogen) following the manufacturer's protocol. Briefly, anti-

folate antibody and isotype antibody were mixed with 1/10 volume of the 1M sodium bicarbonate solution and transferred into a vial of Alexa Flour® 647 dye. After incubating for 1 hr at room temperature, the labeled antibodies were purified using purification columns. In folate-FITC binding experiments, KB and A549 cells were trypsinized and washed. Cells were incubated with 100 nM folate-FITC and incubated for 30 min at 4°C. After washing twice, cells were analyzed using BD Accuri™ C6 Flow Cytometer. Isotype matched antibodies were used in all experiments. Flow cytometry data were analyzed by FlowJo software.

Competitive binding assay

KB (1×10^5) and anti-FITC CAR-T (1×10^5) cells were incubated with 50 nM of folate-FITC and varied concentrations of free-folate or free-fluorescein, respectively. After 30 min incubation at 4°C, cells were washed three times with FACS buffer. KB cells were immediately analyzed using BD Accuri C6, and binding of folate-FITC conjugate was determined by FITC emission. Anti-FITC CAR-T cells were further stained with anti-folate antibody conjugated with Alexa Fluor® 647 dye for 30 min at 4°C. After washing twice, cells were analyzed using BD Accuri™ C6 Flow Cytometer. Flow cytometry data was analyzed by FlowJo software.

Cytotoxicity assay

Target cells (1×10^4) were mixed with anti-FITC CAR-T cells (1×10^5) at a 10:1 E:T ratio in 100 ul of folate-deficient RPMI media supplemented with 10% FBS and incubated with different concentrations of folate-FITC for 24 hr at 37°C. Cytolytic activity was determined by the amount of LDH (lactate dehydrogenase) released into cultured media using CytoTox 96 Non-radioactive cytotoxicity assay kit (Promega). The absorbance at 490nm was measured using a SpectraMax 250 plate reader (Molecular Devices Corp.). % Cytotoxicity= (experimental – effector spontaneous – target spontaneous) / (target maximum – target spontaneous) x 100

T cell proliferation assay

For proliferation assays, tosyl-activated beads (4×10^7 beads in 100ul, Dynabeads® M-450, Invitrogen)

were coupled with 20 ug of anti-folate (Novus Biologicals) or isotype (mouse IgG1, R&D) antibodies as described in manufacturer's instructions, and resuspended in 100 uL PBS supplemented with 0.1% bovine serum albumin (BSA) and 2 mM EDTA, pH 7.4. Ten microliters of the conjugated beads was added to anti-FITC CAR-T cells (1×10^5) in 100 ul of media in the presence of 100 nM of folate-FITC. After 72 hr incubation at 37^{o}C, proliferation of CAR-T cells was assessed by levels of reduced MTS using the CellTiter 96 Aqueous One Solution Assay Kit (Promega) following the manufacturer's protocol. The absorbance at 490 nm was recorded using a SpectraMax 250 plate reader. Each experiment was carried out in triplicate.

Cytokine release assay

1×10^5 CAR-T cells were co-cultured with 1×10^4 target cells in 100 ul cultured media per well in 96-well round bottom plates for 24 hr. Cultured supernatants were harvested and assayed for the presence of IFN-gamma and IL-2 using an ELISA kit, according to manufacturer's instructions (R&D systems). Values represent the mean of triplicate wells and error bars represent SEMs derived from triplicate samples.

Statistical Analysis

2-paired Student *t* test was used to calculate *p*-values. *P*-values less than 0.05 were considered significant. All graphs and statistics were generated using Graphpad Prism 6.0.