SUPPORTING INFORMATION

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1. Materials and Methods

Cell lines
NCI-60 panel ovarian cancer cell lines IGROV-1 and SKOV-3 were obtained from The Scripps Research Institute's Cell-based Screening Core and cultured in RPMI 1640 without folate supplemented with L-glutamine, 100 U/mL Penicillin-Streptomycin (all from Thermo Fisher Scientific), and 10% (v/v) fetal calf serum (BioFluid Technologies). IGROV-1 cells stably transfected with firefly luciferase (IGROV-1/ffluc) were previously described.[1] Expi293F cells were grown in Expi293 Expression Medium supplemented with 100 U/mL Penicillin-Streptomycin (all from Thermo Fisher Scientific).

Cloning, expression, and purification of Fabs and 52SR4 scFv-Fc
Gene fragments (Integrated DNA Technologies) encoding the humanized anti-β-diketone hapten mAb h38C2[2] in Fab format were PCR-amplified. The GCN4 14-aa peptide (NYHLENEVARLKKL) encoding sequence was introduced to the N- or C-termini of h38C2 Fab's light or heavy chain by overlap extension PCR using oligonucleotides (Integrated DNA Technologies). The resulting light and heavy chain encoding sequences were cloned into mammalian cell expression vector pCEP4 and transiently co-transfected into Expi293F cells using the ExpiFectamine 293 Transfection Kit (Thermo Fisher Scientific). One week later, supernatants were collected and loaded on a 1-mL CaptureSelect IgG-CH1 Affinity Matrix column (Thermo Fisher Scientific) in conjunction with an ÄKTA FPLC instrument (GE Healthcare Life Sciences). The purity of the Fabs was confirmed by SDS-PAGE followed by Coomassie Blue staining and their concentration was determined by measuring the absorbance at 280 nm. To generate the anti-GCN4 peptide mAb 52SR4 in scFv-Fc format, a codon-optimized DNA fragment of scFv 52SR4[3] in VL-$(G_4S)_3$-VH format was synthesized (Integrated DNA Technologies) and assembled with a human IgG1 Fc fragment encoding cDNA fragment by overlap extension PCR. The assembled expressing cassette was cloned into mammalian cell expression vector pCEP4 and transiently transfected into Expi293F cells as decribed above. The 52SR4 scFv-Fc was purified by 1-mL Protein A HiTrap HP columns in conjunction with an ÄKTA FPLC instrument (both from GE Healthcare Life Sciences).

ELISA
For coating, each well of a 96-well Costar 3690 plate (Corning) was incubated with 10 µg/mL Fabs in 25 µL PBS for 1 h at 37°C. After blocking with 150 µL 3% (w/v) BSA/PBS for 1 h at 37°C, the plate was incubated with 52SR4 scFv-Fc at 10 µg/mL for 2 h at 37°C. After washing with PBST (0.05% (v/v) Tween-20 in PBS; Thermo Fisher Scientific), the plate was incubated with 50 µL of Peroxidase AffiniPure $F(ab')_2$ Fragment Goat Anti-Human IgG Fcγ Specific pAbs (Jackson ImmunoResearch Laboratories) in 1% (w/v) BSA/PBS for 30 min at 37°C. The plate was washed with PBST as before, and colorimetric detection was performed using 2,2'-azino-bis(3-ethylbenzthiazoline)-6-sulfonic acid (ABTS; SurModics) as substrate according to the manufacturer's directions.

Generation of CAR-T cells
To build the CAR cassette, a codon-optimized DNA sequence of scFv 52SR4[3] in VL-$(G_4S)_3$-VH format was synthesized (Integrated DNA Technologies) and subcloned into lentiviral vector epHIV7 under control of the human elongating factor 1-α promoter, upstream of a mutated human IgG4 hinge segment (12 aa with S108P substitution), the 27-aa transmembrane segment of human CD28 and a signaling module comprising the 42-aa cytoplasmic domain of human 4-1BB and the 112-aa cytoplasmic domain of isoform 3 of human CD3ζ. Downstream of the CAR construct, a T2A ribosomal skip element and a truncated EGFR were encoded.[4] CAR lentivirus was produced in 293X cells (Clontech) using the packaging vectors pCHGP-2, pCMV-Rev2, and pCMV-G. Pan-T cells were isolated from fresh PBMC of healthy donors (Allcells) by negative selection (Pan T Cell Isolation Kit; Miltenyi Biotec), activated using Dynabeads Human T-Activator CD3/CD28 (Thermo Fisher Scientific), and transduced on day 2 by spinfection (800 × g for 1 h at 32°C) with concentrated lentivirus (MOI = 3:1) and 1 µg/mL polybrene (Millipore). CAR-Ts were expanded in RPMI 1640 supplemented with L-glutamine, 100 U/mL Penicillin-Streptomycin (all from Thermo Fisher Scientific), 10% (v/v) fetal calf serum (BioFluid Technologies) and 100 U/mL of IL-2 (Cell Sciences). Between 65-85% of the Pan-T cells expressed the CAR by day 8. CAR-Ts were maintained at a concentration of 0.5-3 × 10^6/mL at all times during expansion and used within 21 days.

Chemical programming of Fabs
The purified Fabs at 20 µM were chemically programmed by incubation with 4 equivalents (80 µM) of compounds **1**, **2**, **3**, **4** or **5** in PBS (pH 7.4) for 4 h at room temperature (RT), and purified with a 10-kDa MWCO Amicon ultrafiltration unit (Millipore).

Catalytic activity assay
Catalytic activity was analyzed using methodol.[5] The cp-Fabs were diluted to 1 µM in PBS (pH 7.4) and dispensed in 98-µL aliquots into a 96-well plate in triplicate. Then, 2 µL of 10 mM methodol in ethanol was added and the fluorescence was measured immediately using a SpectraMax M5 instrument (Molecular Devices) with SoftMax Pro software, a wavelength of excitation (λ ext) set to 330 nm, a wavelength of emission (λ em) set to 452 nm, and starting at 0 min using 5 min time points. The slopes were compared using linear regression analysis with GraphPad Prism 5.

Flow cytometry
Standard flow cytometry was performed on a FACSCanto (BD Biosciences) and data were analyzed with FlowJo (Tree Star). R-phycoerythrin conjugated goat anti-human IgG, $F(ab')_2$ fragment specific pAbs (Jackson ImmunoResearch Laboratories) were used for

the detection of Fab and cp-Fab binding to CAR-T, IGROV-1, and SKOV-3 cells. To detect T-cell activation, a PE-Cy7-conjugated mouse anti-human CD25 mAb (BD Biosciences) was used. T-cell transduction efficiency was detected indirectly by staining EGFRt using 5 µg/mL biotinylated cetuximab (R&D System) followed by R-phycoerythrin-conjugated streptavidin (BD Biosciences).

Liquid chromatography mass spectrometry (LC-MS)
Protein solutions in Dulbecco's phosphate-buffered saline (DPBS; Thermo Fisher Scientific) at 1 mg/mL were diluted with 0.1% (v/v) formic acid to 0.2 mg/mL and individual LC-MS analyses of 0.1 µg of each protein solution was subsequently carried out using a Q Exactive mass spectrometer (Thermo Fisher Scientific). Proteins were eluted from an Accucore 150-C4 µ-Precolumn (2.6 µm, 150 Å, 300 µm i.d x 5 mm; Thermo Fisher Scientific) inline with an Accucore 150-C4 nanoViper analytical column (2.6 µm, 150 Å, 75-µm ID × 15 cm; Thermo Fisher Scientific), using a hold of 5% solvent B (80/20 acetonitrile/water, 0.1% (v/v) formic acid) for 2 min, followed by a gradient of 5-100% (v/v) solvent B in 5 min, a hold of 100% solvent B for 10 min, a return to 5% solvent B in 2 min, and finally a hold of 5% solvent B for 1 min. All flow rates were 250 nL/min delivered using a Easy-nLC1000 nano liquid chromatography system (Thermo Fisher Scientific). Solvent A consisted of water and 0.1% (v/v) formic acid. Ions were created at 1.8 kV using the Nanospray Flex ion source (Thermo Fisher Scientific). The Q Exactive scanned the m/z range 550-2000 at 35,000 resolution, using an AGC target of 3e6 and a maximum injection time of 100 ms. In-source collision-induced dissociation (IS-CID) was also applied to the ions at 50 eV. Raw electrospray mass spectra summed over time were deconvoluted using MagTran version 1.02 (Amgen).

***In vitro* cytotoxicity assay**
Cytotoxicity was measured using CytoTox-Glo (Promega) following the manufacturer's protocol. CAR-T cells were incubated with target cells at an effector-to-target (E:T) ratio of 10:1 while maintaining a total cell concentration of 5×10^5/mL. cp-Fabs or unprogrammed Fabs were added to each well at a concentration of 32 pM-300 nM and the cells were incubated for 37°C for 24 h in complete RPMI 1640 medium. After centrifugation, 50 µL of the supernatant was transferred into a 96-well clear bottom white walled plate (Costar 3610; Corning) containing 25 µL/well CytoTox-Glo. After 15 min at RT, the plate was read using a SpectraMax M5 instrument with SoftMax Pro software. Maximum target cell lysis was determined by lysing the target cells with Cell Lysis Solution provided by the manufacturer. Target cell cytotoxicity was calculated using the following formula: Cytotoxicity = 100 × [(CAR-T cell + target cell + Fab) – (CAR-T cell + target cell)] / (maximum target cell lysis − target cells alone). The same supernatants (diluted 5 or 10-fold) used for the cytotoxicity assay were also used to determine IFN-γ, IL-2 and TNF-α secretion with Human IFN-γ, IL-2 or TNF-α ELISA MAX Deluxe kits (Biolegend), respectively, following the manufacturer's protocols.

Mouse xenograft studies
Thirty-five 6-week old NOD-scid-IL2Rγnull (NSG) mice (The Jackson Laboratory) were each given 1×10^6 IGROV1/ffluc intraperitoneally (i.p.) on day 0. On day 6, the animals were i.p. injected with 150 mg/kg D-luciferin (Biosynth) and divided into 7 groups of 5 animals each by average bioluminescence. On day 6, each mouse was i.p. injected with 20×10^6 CAR-T, and 6 h later, with 10 µg or 1 µg cp-Fab (LCCT_**1**), 10 µg unprogrammed Fab (LCCT), PBS alone, 10 µg cp-Fab (WT Fab_**5**), an equimolar amount of compound **6** (0.8 µg), or 10 µg unprogrammed Fab (WT) (each in 100 µL PBS). The mice received one dose of CAR-T and 10 daily doses of cp-Fabs, unprogrammed Fabs, or PBS. Every 3-5 days, tumor growth was monitored by LagoX (Spectrum Instruments Imaging) and was quantified as radiance in the region of interest. The weight of the mice was measured every 3-4 days and euthanasia was performed when the mice gained more than 25% body weight due to tumor burden. All procedures were approved by the Institutional Animal Care and Use Committee of The Scripps Research Institute and were performed according to the NIH Guide for the Care and Use of Laboratory Animals.

PK study
Four 6-week old female CD-1 mice (~25 g; Charles River Laboratories) were injected i.p. with cp-Fab (LCCT_**1**) or unprogrammed Fab (LCCT) at 8 mg/kg. Blood was collected in heparinized capillary tubes at 5 min, 30 min, 1 h, 3 h, 6 h, 24 h, 48 h, 72 h, and 168 h after injection. Plasma was obtained by centrifuging the samples at 2,000 × g for 5 min in a microcentrifuge and stored at −80°C until analysis. The concentrations of Fabs in the plasma samples were measured by ELISA. For this, each well of a 96-well Costar 3690 plate (Corning) was incubated with 300 ng 52SR4 scFv-Fc in 25 µL carbonate/bicarbonate buffer (pH 9.6) at 37°C for 1 h. After blocking with 150 µL 3% (w/v) BSA/PBS solution for 1 h at 37°C, the prepared plasma samples were added. Peroxidase AffiniPure $F(ab')_2$ Fragment Goat Anti-Human IgG $F(ab')_2$ Fragment Specific pAbs (Jackson ImmunoResearch Laboratories) were used for detection as described above. The concentration of the Fabs in the plasma samples was extrapolated from a four-variable fit of the standard curve. PK parameters were analyzed by using Phoenix WinNonlin PK/PD Modeling and Analysis software (Pharsight).

Statistical analyses
Statistical analyses were performed using GraphPad Prism 5. The data shown in Figure 4, Figure 5b, Figure 6b, Supplementary Figure 1b, and Supplementary Figure 5 were subjected to an unpaired two-tailed t-test. Statistical analysis of survival (Figure 5c and Figure 6c) were done by log-rank (Mantel-Cox) testing. Results with a p-value of $p < 0.05$ were considered significant.

2. Amino Acid Sequences

GCN4 peptide:
NYHLENEVARLKKL

Heavy chain of WT h38C2 Fab:
EVQLVESGGGLVQPGGSLRLSCAASGFTFSNYWMSWVRQSPEKGLEWVSEIRLRSDNYATHYAESVKGRFTISRDNSKNTLYLQMN
SLRAEDTGIYYCKTYFYSFSYWGQGTLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAV
LQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKRVEPKSC

Light chain of WT h38C2 Fab:

ELQMTQSPSSLSASVGDRVTITCRSSQSLLHTYGSPYLNWYLQKPGQSPKLLIYKVSNRFSGVPSRFSGSGSGTDFTLTISSLQPEDFA
VYFCSQGTHLPYTFGGGTKVEIKRTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDST
YSLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC

Heavy chain of HCNT:
NYHLENEVARLKKLLVGEAAAKEAAAKAEVQLVESGGGLVQPGGSLRLSCAASGFTFSNYWMSWVRQSPEKGLEWVSEIRLRSDNY
ATHYAESVKGRFTISRDNSKNTLYLQMNSLRAEDTGIYYCKTYFYSFSYWGQGTLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCL
VKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKRVEPKSC

Heavy chain of HCCT:
EVQLVESGGGLVQPGGSLRLSCAASGFTFSNYWMSWVRQSPEKGLEWVSEIRLRSDNYATHYAESVKGRFTISRDNSKNTLYLQMN
SLRAEDTGIYYCKTYFYSFSYWGQGTLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAV
LQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKRVEPKSCGGGGSNYHLENEVARLKKL

Light chain of LCNT:
NYHLENEVARLKKLGGGGSELQMTQSPSSLSASVGDRVTITCRSSQSLLHTYGSPYLNWYLQKPGQSPKLLIYKVSNRFSGVPSRFS
GSGSGTDFTLTISSLQPEDFAVYFCSQGTHLPYTFGGGTKVEIKRTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDN
ALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC

Light chain of LCCT:
ELQMTQSPSSLSASVGDRVTITCRSSQSLLHTYGSPYLNWYLQKPGQSPKLLIYKVSNRFSGVPSRFSGSGSGTDFTLTISSLQPEDFA
VYFCSQGTHLPYTFGGGTKVEIKRTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDST
YSLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGECGGGGSNYHLENEVARLKKL

52SR4 scFv-Fc:
DAVVTQESALTSSPGETVTLTCRSSTGAVTTSNYASWVQEKPDHLFTGLIGGTNNRAPGVPARFSGSLIGDKAALTITGAQTEDEAIYF
CVLWYSDHWVFGGGTKLTVLGGGGGSGGGGSGGGGSGGGGSDVQLQESGPGLVAPSQSLSITCTVSGFLLTDYGVNWVRQSPGK
GLEWLGVIWGDGITDYNSALKSRLSVTKDNSKSQVFLKMNSLQSGDSARYYCVTGLFDYWGQGTTLTVSSEPKSSDKTHTCPPCPAP
ELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEY
KCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYS
KLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGA

Anti-GCN4 peptide CAR:
DAVVTQESALTSSPGETVTLTCRSSTGAVTTSNYASWVQEKPDHLFTGLIGGTNNRAPGVPARFSGSLIGDKAALTITGAQTEDEAIYF
CVLWYSDHWVFGGGTKLTVLGGGGGSGGGGSGGGGSGGGGSDVQLQESGPGLVAPSQSLSITCTVSGFLLTDYGVNWVRQSPGK
GLEWLGVIWGDGITDYNSALKSRLSVTKDNSKSQVFLKMNSLQSGDSARYYCVTGLFDYWGQGTTLTVSSESKYGPPCPPCPMFWV
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ELNLGRREEYDVLDKRRGRDPEMGGKPRRKNPQEGLYNELQKDKMAEAYSEIGMKGERRRGKGHDGLYQGLSTATKDTYDALHMQ
ALPPR

3. Chemical Syntheses

Methodol[5] and β-lactam-biotin-folate **1**[6] were synthesized as described. The syntheses of trifunctional β-lactam-GCN4-folate compounds, **2**, **3**, **4** and **5**, and bifunctional GCN4-folate compound **6** are described below.

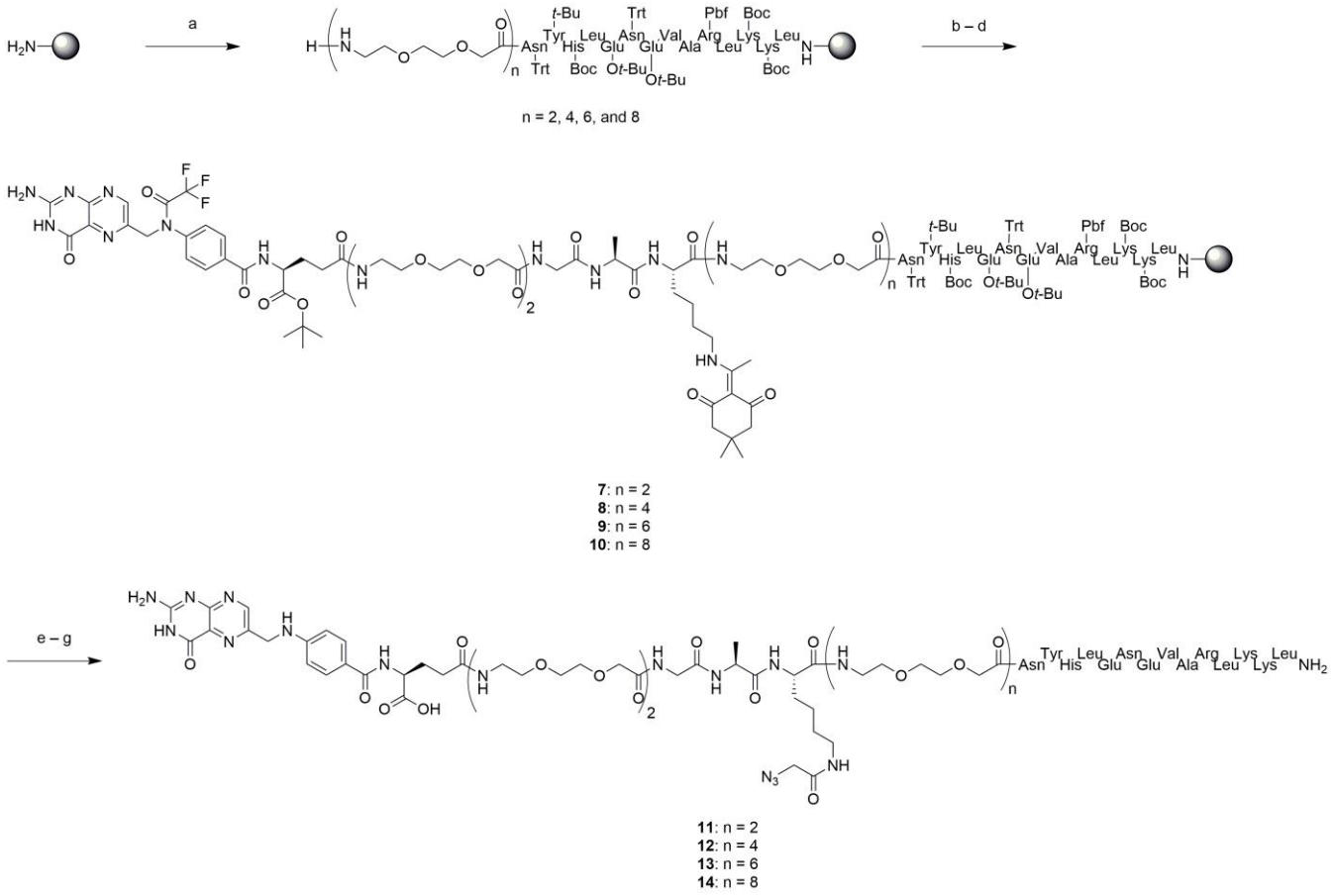
General methods. All experiments involving moisture-sensitive compounds were conducted under anhydrous conditions (positive argon pressure) using standard syringe, cannula, and septa apparatus. Commercial reagents were purchased from Sigma-Aldrich, TCI America, Acros, Chem-Impex, Novabiochem, CEM Corporation, Carbosynth, Kanto Chemical, and Watanabe Chemical.. All solvents were purchased in anhydrous form (Sigma-Aldrich) and used without further drying. HPLC-grade acetonitrile (MeCN) was used for HPLC purifications. Low-resolution mass spectra (ESI) were measured with an Agilent 260 1200 LC/MSD-SL system or a Bruker Daltonics micrOTOF focus (ESI-MS) spectrometer. Preparative HPLC purification was performed using a Waters 2545 binary pump (MeCN/H_2O gradient) with a Phenomenex Gemini-C18 perparative column (5 μm, 250 x 21 mm) or a JASCO PU-2086 plus (MeCN/H_2O gradient) with a Cosmosil $5C_{18}$-ARII column (10 x 250 mm; Nacalai Tesque), 10 mL/min flow rate, and UV detection at 210 nm. Analytical HPLC analyses of purified compounds were performed using an Agilent 1200 series quaternary pump (MeCN/H_2O gradient) with a Phenomenex Gemini-C18 analytical column (5 μm, 250 x 4 mm) or a JASCO PU-2089 plus (MeCN/H_2O gradient) with a Cosmosil $5C_{18}$-ARII column (4.6 x 250 mm; Nacalai Tesque), 1 mL/min flow rate, and UV detection at 210 nm.

General procedure for solid-phase peptide synthesis (SPPS). Syntheses of consensus sequence peptides [-(miniPEG)$_n$-NYHLENEVARLKKL-] were performed on Fmoc-Rink Amide PROTIDE resin (LL) (CEM Corporation, 0.19 mmol/g) using a Liberty Prime automatic peptide synthesizer (CEM Corporation) according to the manufacturer's instructions (Fmoc-miniPEG-OH: {2-[2-(Fmoc-amino)ethoxy]ethoxy}acetic acid). The resulting protected resins were subjected to further chain elongation. Fmoc-protected amino acids (2.0-4.0 equivalents based on resin loading) were dissolved in N-methyl-2-pyrrolidone (NMP) and pre-activated by the addition of 1-[bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxide hexafluorophosphate (HATU, 0.95 mole-equivalents relative to the amino acid) and *N*,*N*-diisopropylethylamine (DIEA, 2.0 mole-equivalents relative to the amino acid) with shaking (1 min).The resin was washed with NMP, and the solution of HATU-activated amino acid was added to the washed resin. Coupling reactions were shaken at RT and allowed to proceed from 2 h to overnight, depending on the equivalents used and the steric bulk of the amino acid. Coupling reactions were routinely checked for completion using a Kaiser test. Finished resin was filtered and washed with NMP, followed by Fmoc-deprotection using 20% piperidine in dimethylformamide (DMF) with shaking (10 min). A N-10-

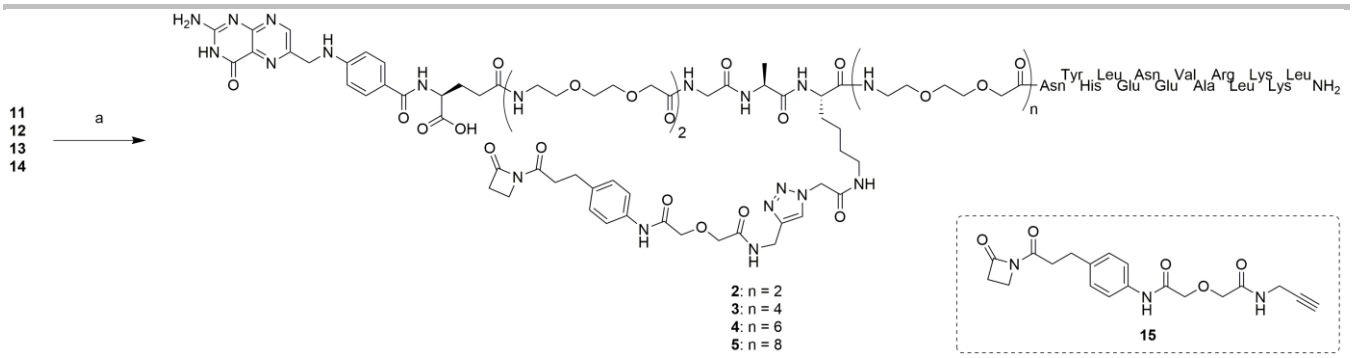
trifluoroacetic acid (TFA)-protected pteroic acid analog (either self-prepared or purchased from Carbosynth) was used with an α-protected glutamic acid residue.[1] Following removal of the Lys ε-(1-(4,4-dimethyl-2,6-dioxocyclohexylidene)ethyl) (Dde) protecting group with 2% hydrazine monohydrate in NMP (2 h, twice), an azido group was incorporated by coupling with azidoacetic acid (2.0 equivalents based on resin loading, twice). Cleavage of the finished resin with global deprotection was performed using a cocktail of TFA/triisopropylsilane (TIPS)/H_2O = 95:2.5:2.5 (4.0 mL, 2 h). The mixture was filtered and the filtrate was added to cold diethyl ether (Et_2O). The resulting precipitate was washed with cold Et_2O (three times). Crude peptides were dissolved in 0.1% TFA containing MeCN and H_2O and purified using preparative reverse-phase HPLC. HPLC eluents were A: 0.1% TFA in H_2O; B: 0.1% TFA in MeCN. Removal of the *N*-10-TFA protecting group on the pteroic acid moiety of **17** (4.4 mg, 1.13 μmol) was performed by treating **17** with 1 M NaOH aq. (750 μL) for 45 min at room temperature and then neutralizing the mixture by the addition of 2 M HCl aq. (650 μL). The crude peptide was purified by preparative reverse-phase HPLC to obtain desired product **6** (1.1 mg, 26%).

General Procedure for Cu-catalyzed azide-alkyne cycloaddition reactions. All Cu-catalyzed azide-alkyne cycloaddition reactions were performed according to the reported procedure.[7] Briefly, azide-containing peptide (1.0 equivalent) was dissolved in H_2O (5.0 mM) and mixed with β-lactam **15** (1.5 equivalents) in DMSO (5.0 mM based on the peptide). Separately, 4.0% (w/v) $CuSO_4 \cdot 5H_2O$ in H_2O (0.13 equivalents), 0.10 M tris[(1-benzyl-1H-1,2,3-triazol-4-yl)methyl]amine (TBTA) in DMSO (0.25 equivalents), and 0.50 M sodium ascorbate in H_2O (5.0 equivalents) were combined and the mixture was added to the peptide solution and incubated in the dark at RT (1 d). The resulting crude peptide was purified by preparative reverse-phase HPLC using gradients consisting of A: 0.1% TFA in H_2O; B: 0.1% TFA in MeCN.

Synthesis of trifunctional peptides.

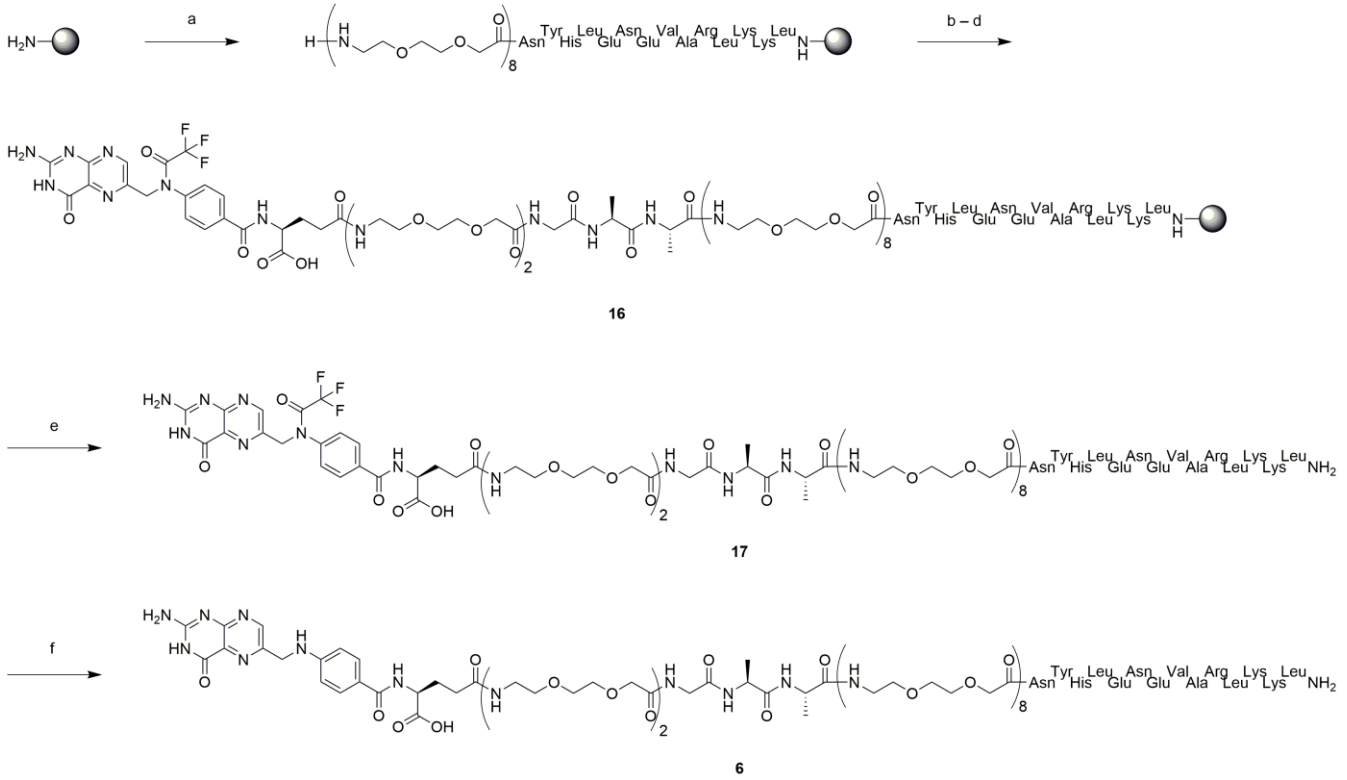


Scheme 1. Synthesis of azido-containing peptides **11** – **14**. (a) Fmoc-SPPS using an automatic peptide synthesizer (Liberty Prime, CEM Corporation); (b) manual Fmoc-SPPS sequentially using Fmoc-L-Lys(Dde)-OH, Fmoc- L-Ala-OH, Fmoc- L-Ala-OH, {2-[2-(Fmoc-amino)ethoxy]ethoxy}acetic acid, and {2-[2-(Fmoc-amino)ethoxy]ethoxy}acetic acid; (c) manual SPPS sequentially using Fmoc-L-Glu(OH)-O*t*-Bu, HATU, DIEA, NMP then 20% piperidine in DMF; (d) manual SPPS *N*-10-TFA-pteroic acid, HATU, DIEA, NMP; (e) 2% hydrazine monohydrate in NMP; (f) azidoacetic acid NHS ester, DIEA, NMP; (g) TFA/TIPS/H_2O/DODt = 95:2.5:2.5:2.5. Abbreviations: Dde; 1-(4,4-dimethyl-2,6-dioxocyclohexylidene)ethyl, DODt; 3,6-dioxa-1,8-octanedithiol.



Scheme 2. Synthesis of trifunctional peptides **2** – **5**. (a) **15**, $CuSO_4 \cdot 5H_2O$, TBTA, sodium ascorbate, DMSO/H_2O = 1:1, 36%, 38%, 41%, and 43% for **2** (n = 2), **3** (n = 4), **4** (n = 6), and **5** (n = 8), respectively.

*Synthesis of bifunctional peptide **6**.*



Scheme 3. Synthesis of bifunctional peptide **6**. (a) Fmoc-SPPS using an automatic peptide synthesizer (Liberty Prime, CEM Corporation); (b) manual Fmoc-SPPS sequentially using Fmoc-L-Ala-OH, Fmoc-L-Ala-OH, Fmoc-L-Ala-OH, {2-[2-(Fmoc-amino)ethoxy]ethoxy}acetic acid and {2-[2-(Fmoc-amino)ethoxy]ethoxy}acetic acid; (c) manual SPPS using Fmoc-L-Glu(OH)-O*t*-Bu, HATU, DIEA, NMP, then 20% piperidine in DMF; (d) manual SPPS using *N*-10-TFA-pteroic acid, HATU, DIEA, NMP; (e) TFA/TIPS/H_2O/DODt = 95:2.5:2.5:2.5; (f) 1 M NaOH aq.

SUPPORTING INFORMATION

Compound data for synthesized peptides **2** – **6**, **11** – **14** and **17**.

Compound	Calculated *m/z*	Found	Preparative HPLC gradient (% B in A)
11	1023.7 $[M + 3H]^{3+}$	1023.5	10 to 45% over 30 min
12	1120.2 $[M + 3H]^{3+}$	1120.6	10 to 45% over 30 min
13	1217.0 $[M + 3H]^{3+}$	1217.3	10 to 45% over 30 min
14	1313.7 $[M + 3H]^{3+}$	1314.1	10 to 45% over 30 min
2	1147.2 $[M + 3H]^{3+}$	1147.6	15 to 45% over 30 min
3	1244.0 $[M + 3H]^{3+}$	1244.3	15 to 45% over 30 min
4	1340.7 $[M + 3H]^{3+}$	1341.1	15 to 45% over 30 min
5	1437.4 $[M + 3H]^{3+}$	1437.8	15 to 45% over 30 min
17	1299.0 $[M + 3H]^{3+}$	1299.3	15 to 45% over 30 min
6	951.0 $[M + 4H]^{4+}$	951.0	15 to 35% over 30 min

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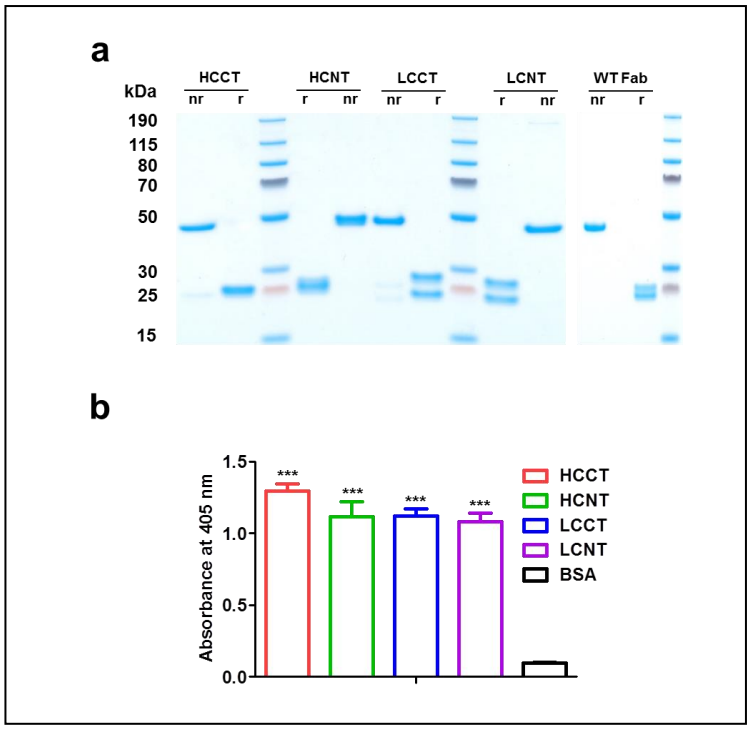
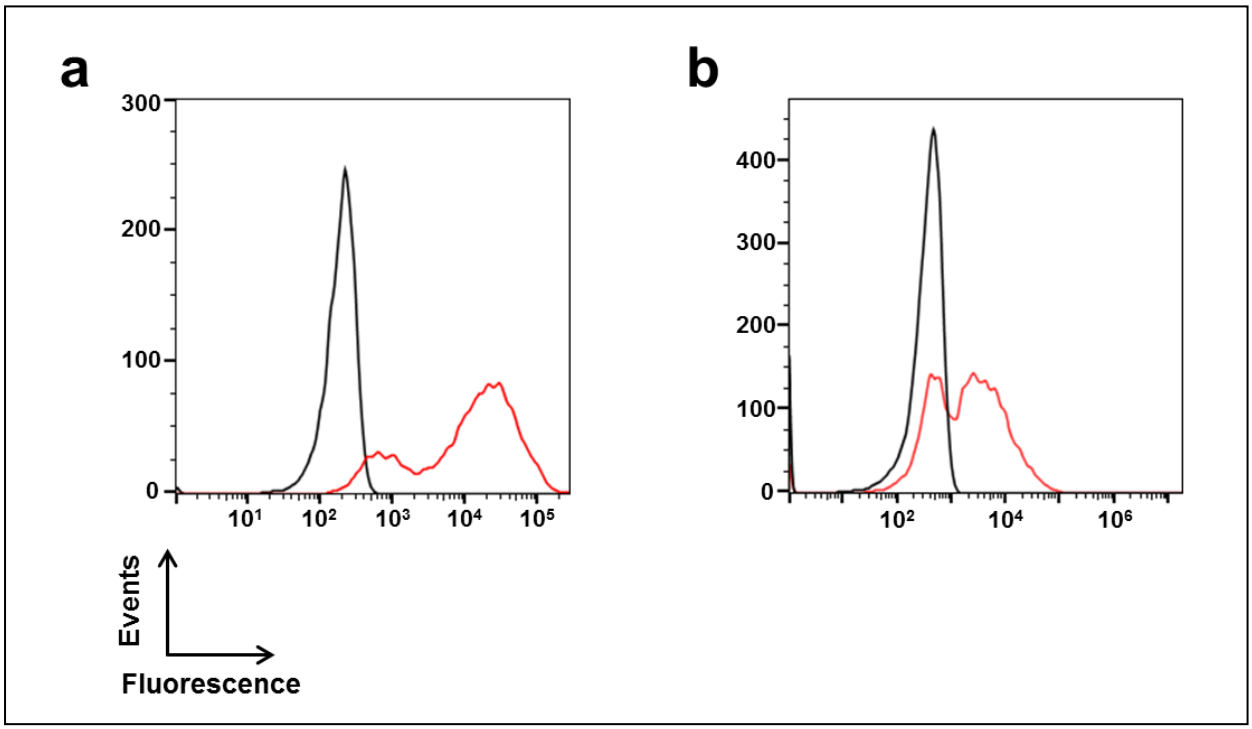
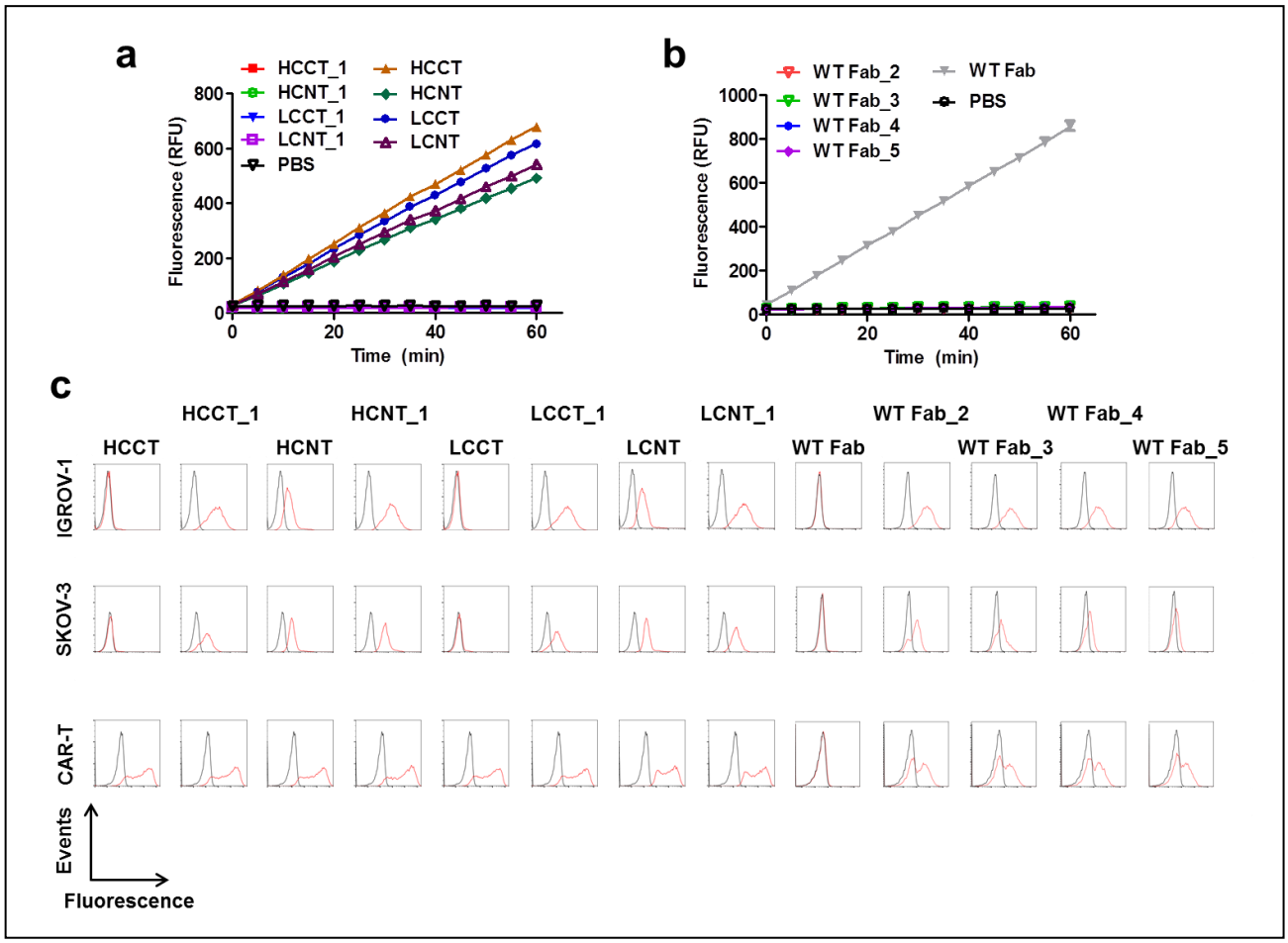
4. Supplementary Figures



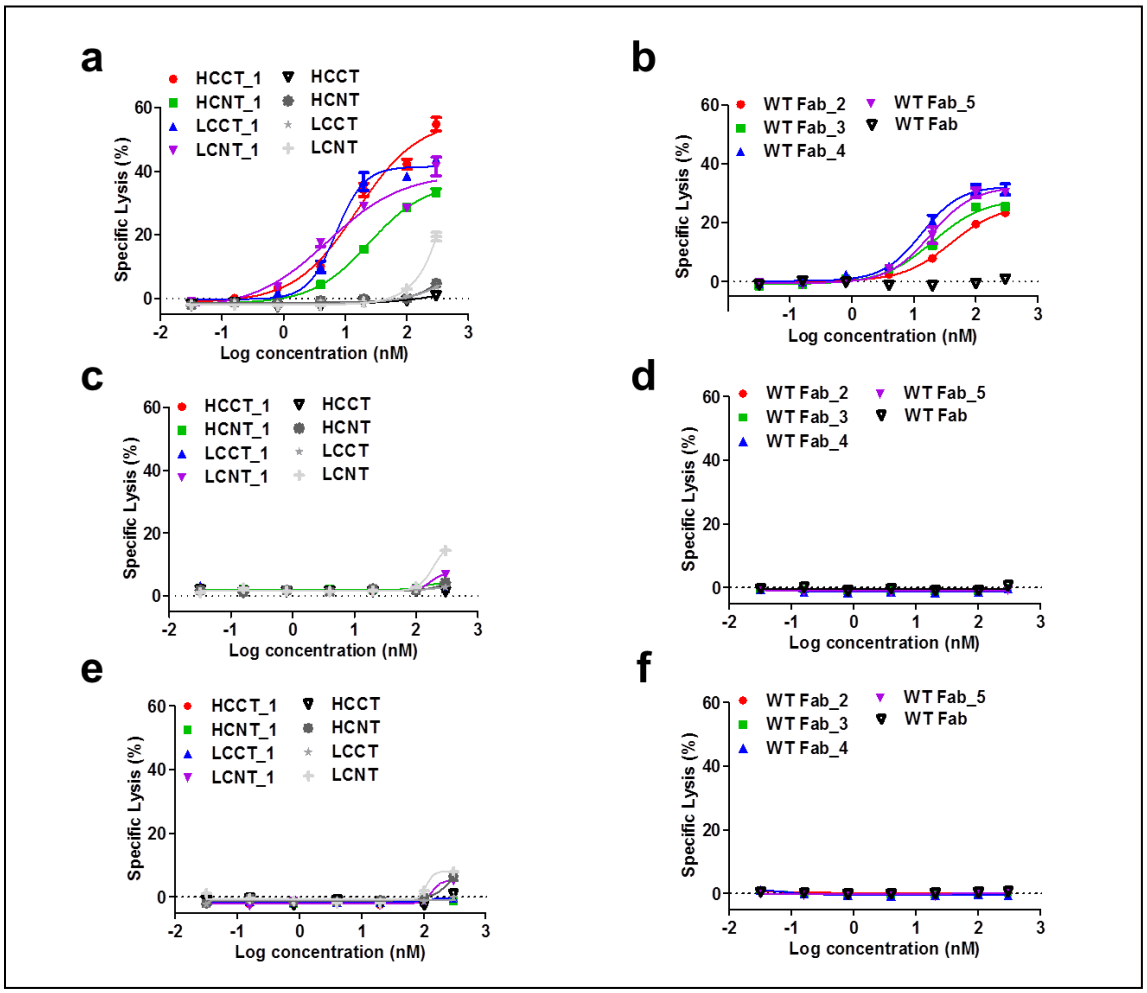
Supplementary Figure 1. Analysis of purified cp-Fabs. (a) SDS-PAGE and Coomassie Blue staining analysis of purified cp- Fabs based on tagged and WT h38C2 Fabs showing the expected single band at ~50 kDa under nonreducing (nr) and the expected double bands at ~25 kDa under reducing (r) conditions. (b) For ELISA, the indicated cp-Fabs were coated at a concentration of 10 μg/mL and detected with 10 μg/mL 52SR4 scFv-Fc followed by HRP-conjugated goat anti-human IgG Fcγ pAbs.



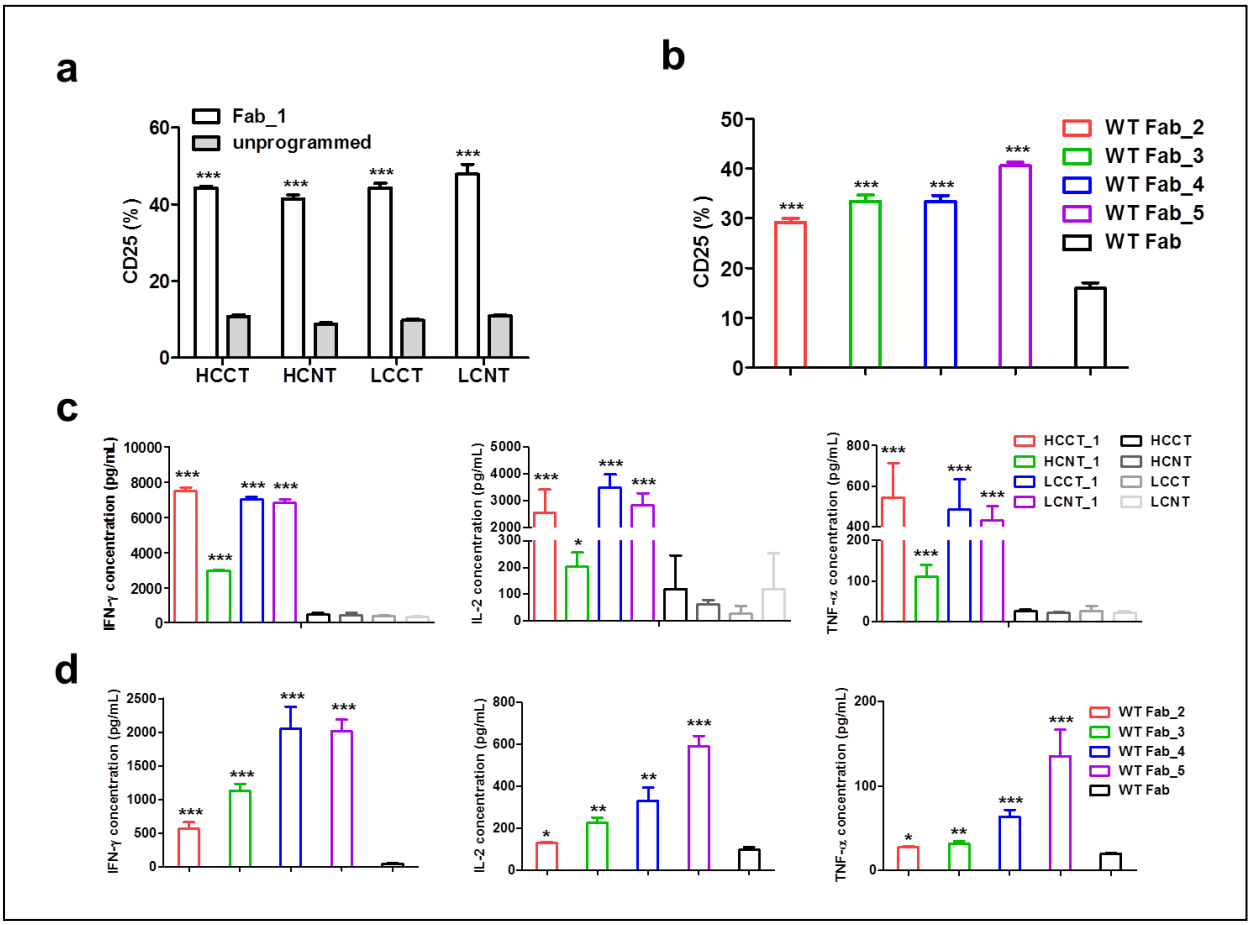
Supplementary Figure 2. Characterization of lentivirally transduced CAR-T. (a) The transduction efficiency of the CAR-T used in the assays with tagged h38C2 Fabs was measured with 5 μg/mL biotinylated cetuximab followed by R-phycoerythrin-conjugated streptavidin. (b) The transduction efficiency of the CAR-T used in the assays with WT h38C2 Fab was measured with 5 μg/mL Alexa Fluor 647-conjugated cetuximab.



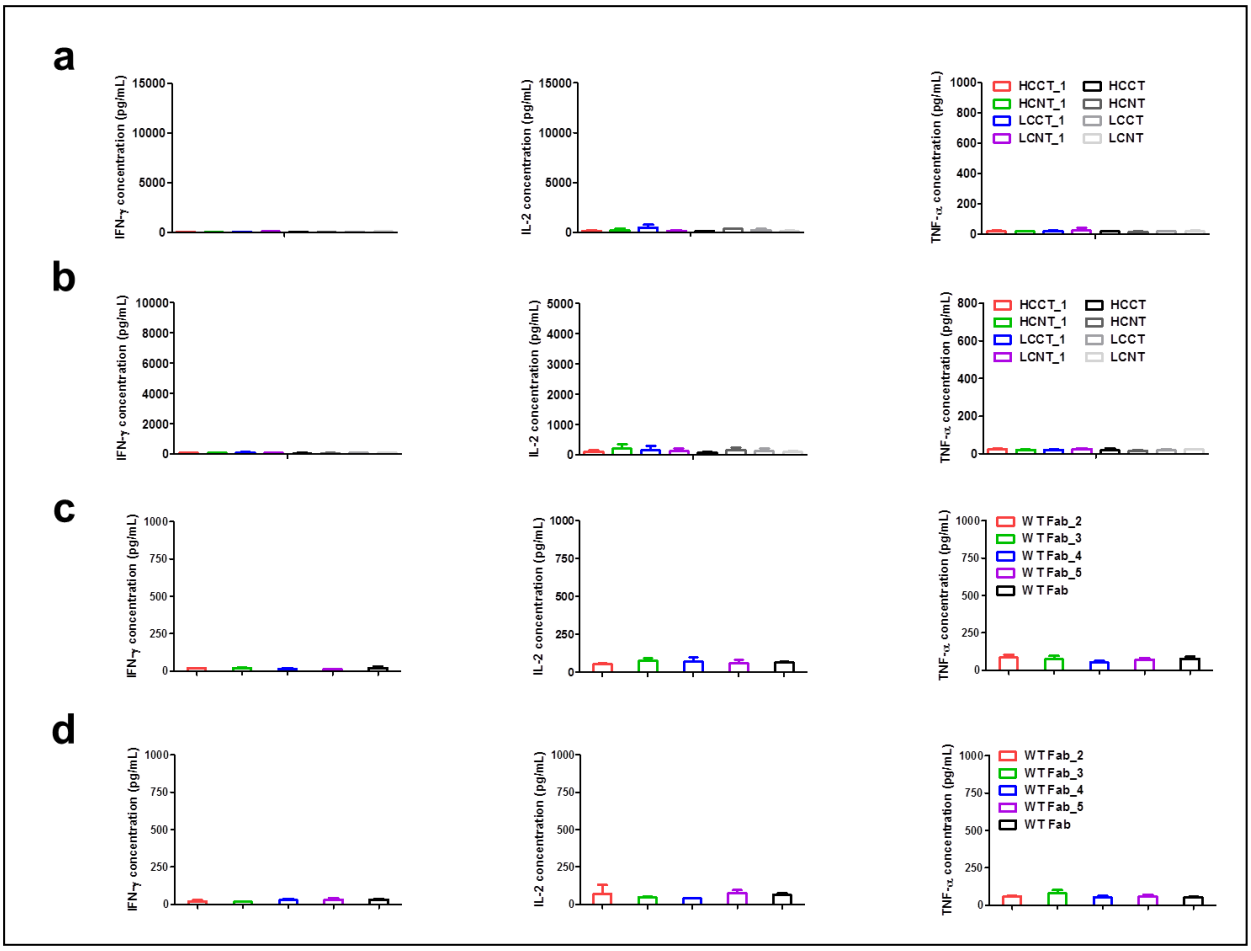
Supplementary Figure 3. Chemical programming. The catalytic retro-aldol activities of the four tagged h38C2 Fabs before and after chemical programming with compound **1** (a) and WT h38C2 Fab before and after chemical programming with compounds **2**, **3**, **4**, and **5** (b) were measured using methodol as a substrate. The signal is reported in relative fluorescent units (RFU; mean ± SD of triplicates). PBS was used as negative control (black). (c) The cp-Fabs and corresponding unprogrammed Fabs (all at 10 μg/mL) were analyzed by flow cytometry for binding to FOLR1-expressing human ovarian cancer cell lines IGROV-1 (top panel) and SKOV-3 (middle panel) along with the scFv 52SR4-based CAR-T (bottom panel). R-phycoerythrin-conjugated goat anti-human Fab pAbs were used for detection.



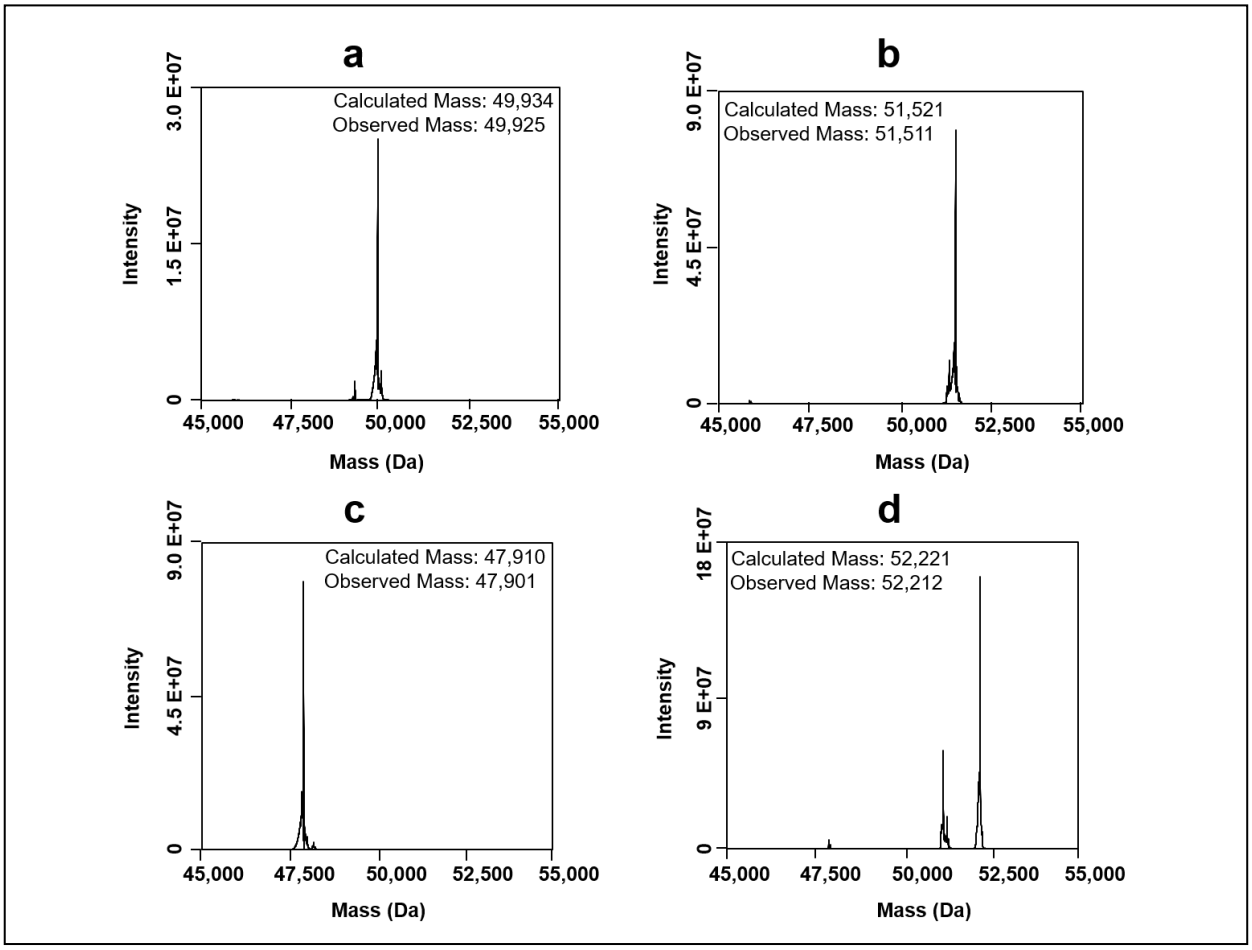
Supplementary Figure 4. *In vitro* activity of cp-Fab/CAR-T. The cytotoxicity of the FOLR1-targeting cp-Fab/CAR-T against SKOV-3 cell with titration of cp-Fabs based on tagged (a) or WT (b) h38C2 Fabs were tested at an E:T ratio of 10:1 and measured after 24 h incubation. The cytotoxicity mediated by corresponding unprogrammed tagged and WT h38C2 Fabs were also investigated. Shown are mean ± SD values from independent triplicates. The cytotoxicity of untransduced T cells against IGROV-1 (c, d) and SKOV-3 cells (e, f) in the presence of cp-Fabs based on tagged (c, e) or WT (d, f) h38C2 Fabs was tested at an E:T ratio of 10:1 and measured after 24 h incubation. Shown are mean ± SD values from independent triplicates.



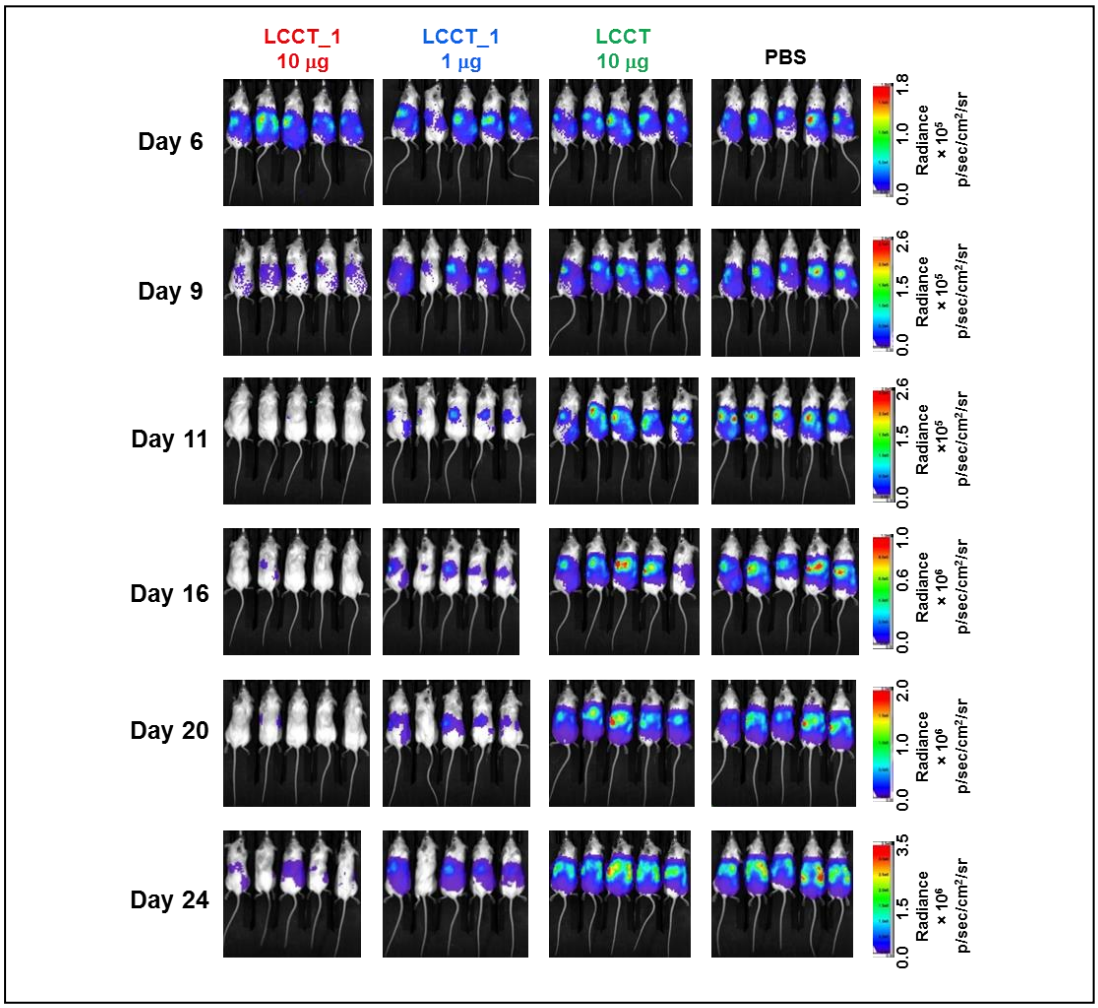
Supplementary Figure 5. CAR-T activation mediated by cp-Fabs. The CAR-T was incubated with 20 nM of the indicated FOLR1-targeting cp-Fabs or the corresponding unprogrammed Fabs in the presence of SKOV-3 cells at an E:T ratio of 10:1 for 24 h. The percentage of activated T cells based on CD25 expression after incubation with cp-Fabs based on tagged (a) or WT (b) h38C2 Fabs was measured by flow cytometry. Cytokines released from the T cells in the presence of cp-Fabs based on tagged (c) or WT (d) h38C2 Fabs were measured by ELISA. Shown are mean ± SD values for independent triplicates. An unpaired two-tailed t-test was used to analyze significant differences (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$).



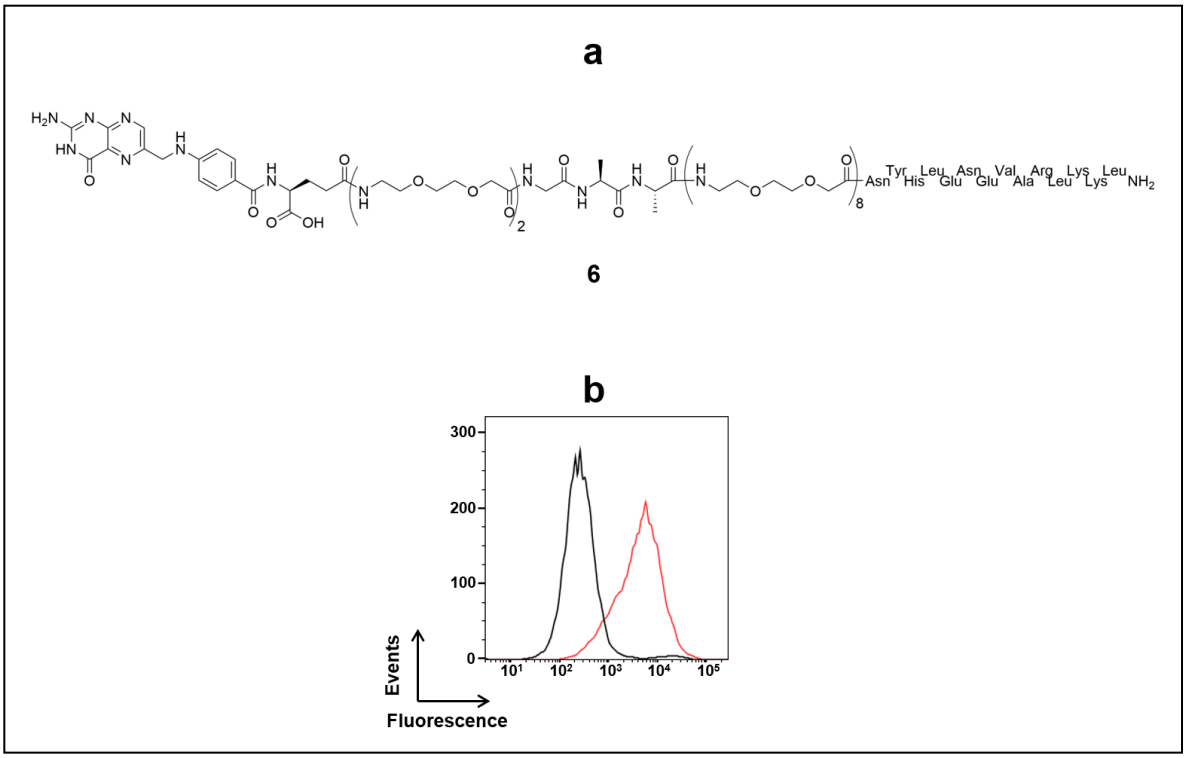
Supplementary Figure 6. Control experiment for CAR-T activation mediated by cp-Fabs. Untransduced T cells were incubated with 20 nM of the cp-Fabs based on tagged (a, b) or WT (c.d) h38C2 Fabs in the presence of IGROV-1 (a, c) or SKOV-3 (b, d) cells at an E:T ratio of 10:1 for 24 h. Cytokines released from the T cells were measured by ELISA. Shown are mean ± SD values for independent triplicates.



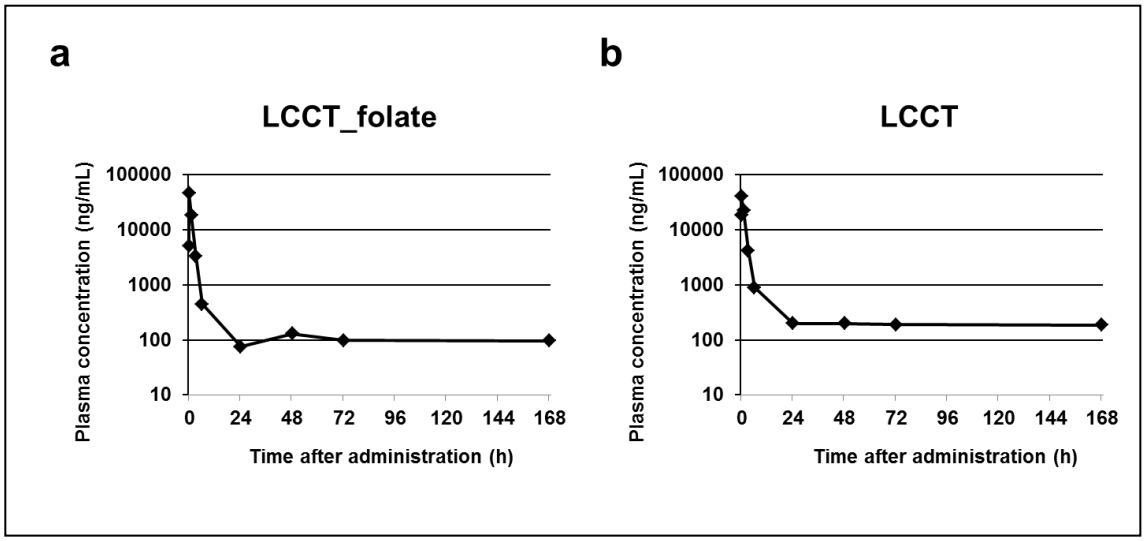
Supplementary Figure 7. Liquid chromatography mass spectrometry (LC-MS) analysis of cp-Fabs. Deconvolution was manually performed by averaging the molecular masses obtained for all detectable peaks in the spectrum. The deconvoluted spectra of LCCT (a), LCCT_**1** (b), WT Fab (c), WT Fab_**5** (d) are shown.



Supplementary Figure 8. *In vivo* activity of cp-Fab/CAR-T. The preparation and treatment of four cohorts of NSG mice (n = 5) is described in Figure 5. Bioluminescence images of all 20 mice were taken from day 6 (before treatment) to day 24 (after treatment) at the indicated time points.



Supplementary Figure 9. Bifunctional FOLR1-targeting compound. (a) Structure of GCN4-folate compound **6**. (b) The functionality of both folate and GCN4 peptide moieties of compound **6** were analyzed by flow cytometry for binding to FOLR1-expressing IGROV-1 cell. 20 nM compound **6** was incubated with IGROV-1 cells, followed by 10 µg/mL 52SR4 scFv-Fc and detected by Alexa Fluor 647-conjugated goat anti-human Fcγ specific pAbs.



Supplementary Figure 10. PK of cp-Fab. Four female CD-1 mice each were injected i.p. with 8 mg/kg LCCT_**1** (a) or LCCT (b). The plasma concentrations of the cp-Fab and unprogrammed Fab at the indicated time points were quantified with a sandwich ELISA, using 52SR4 scFv-Fc for capture and HRP-conjugated goat anti-human Fab pAbs for detection. Shown are mean ± SD values for each time point. The calculated PK parameters are listed in Table 1.

5. Supplementary Table

Supplementary Table 1. Half maximal killing (EC_{50}) of target cells mediated by cp-Fab/CAR-T.

	HCCT_**1**	HCNT_**1**	LCCT_**1**	LCNT_**1**	WT Fab_**2**	WT Fab_**3**	WT Fab_**4**	WT Fab_**5**
EC_{50} (IGROV-1)	4.2 nM	15.9 nM	3.7 nM	2.8 nM	7.4 nM	5.0 nM	4.5 nM	2.6 nM
EC_{50} (SKOV-3)	15.4 nM	24.5 nM	7.1 nM	5.2 nM	39.7 nM	20.5 nM	13.3 nM	18.6 nM