Supporting Information

Engineering Reversible Cell-Cell Interactions Using Enzymatically Lipidated Chemically Self-Assembled Nanorings

Yiao Wang,[a] Ozgun Kilic,[b] Clifford M. Csizmar,[b] Sudhat Ashok,[c] James L. Hougland,[c] Mark D. Distefano*[ab] and Carston R. Wagner*[ab]

[a]Department of Chemistry, University of Minnesota, Minneapolis, Minnesota, USA
[b]Department of Medicinal Chemistry, University of Minnesota, Minneapolis, Minnesota, USA
[c]Department of Chemistry, Syracuse University, Syracuse, NY, USA

* Address correspondence to: diste001@umn.edu and wagne003@umn.edu

University of Minnesota

Department of Medicinal Chemistry

2231 6th Street S.E.

Cancer & Cardiovascular Research Building

Minneapolis, Minnesota 55455, USA

Table of Contents

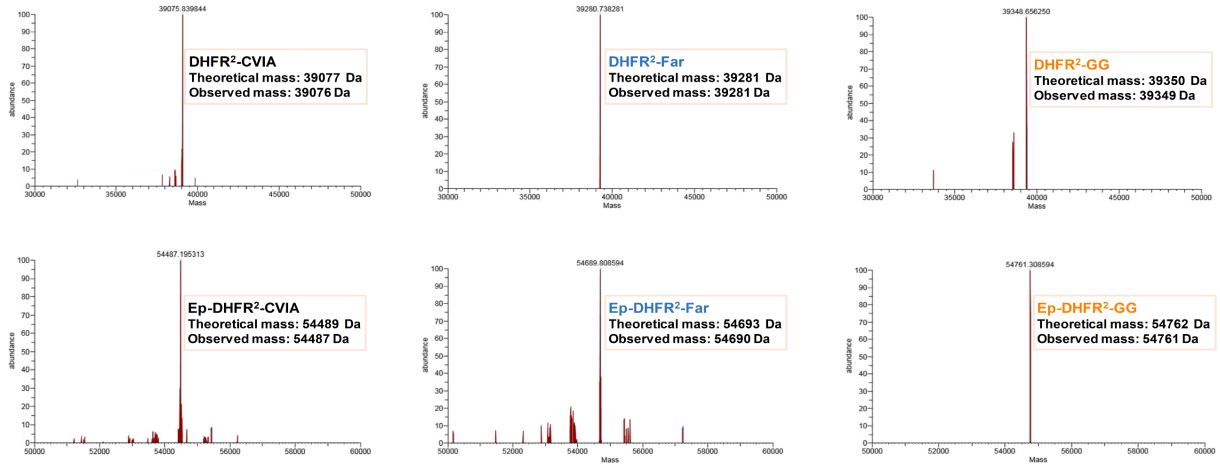

Fig. S1. LC-MS spectra of DHFR^2-CVIA proteins and Ep-DHFR^2-CVIA proteins. DHFR^2-CVIA protein and Ep-DHFR^2-CVIA protein were farnesylated or geranylgeranylated and characterized by LC-MS. All the constructs showed masses close to the target masses.

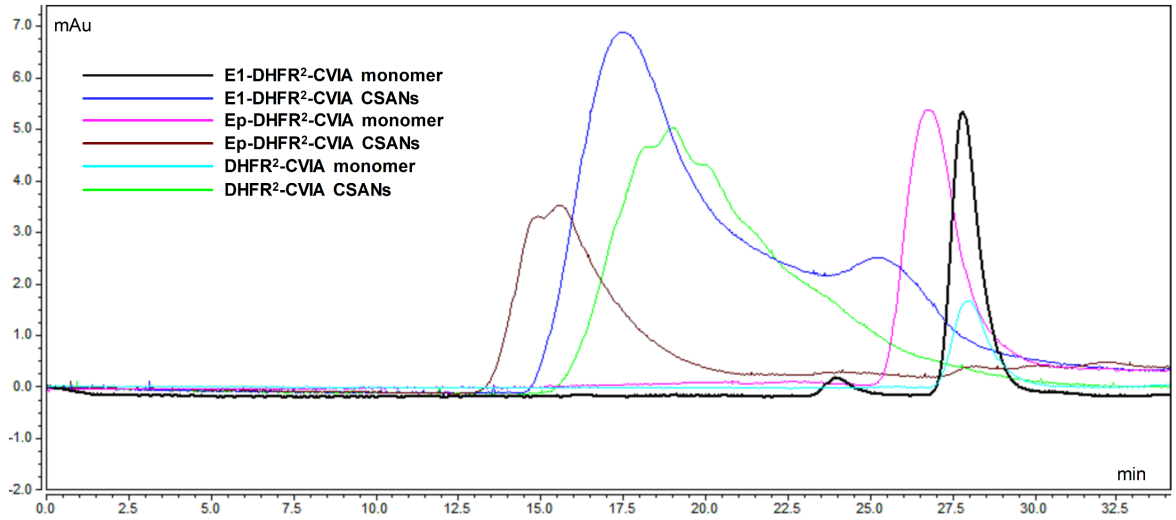

Fig. S2. CSANs formation was demonstrated by SEC. The E1-DHFR^2-CVIA protein, Ep-DHFR^2-CVIA protein or DHFR^2-CVIA protein was oligomerized by 2.5-fold molar excess of bisMTX. CSANs formed within 30 minutes and were characterized by SEC, which showed the chromatograms of the monomers shift left to an earlier elution time corresponding to the oligomeric CSAN species. Several oligomer species of 7-9 subunits were observed, with octameric nanorings as the predominant species, which was similar to the previously reported SEC analysis data[1].

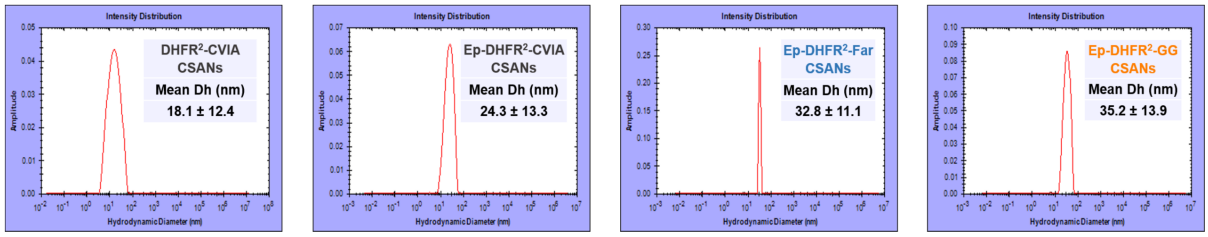

Fig. S3. Dynamic light scattering analysis of CSAN constructs. The hydrodynamic diameter was measured for DHFR^2-CVIA CSANs (18±12.4 nm), Ep-DHFR^2-CVIA CSANs (24.3±13.3nm), Ep-DHFR^2-Far CSANs (32.8±11.1 nm), and Ep-DHFR^2-GG CSANs (35 ± 13.9 nm) using dynamic light scattering.

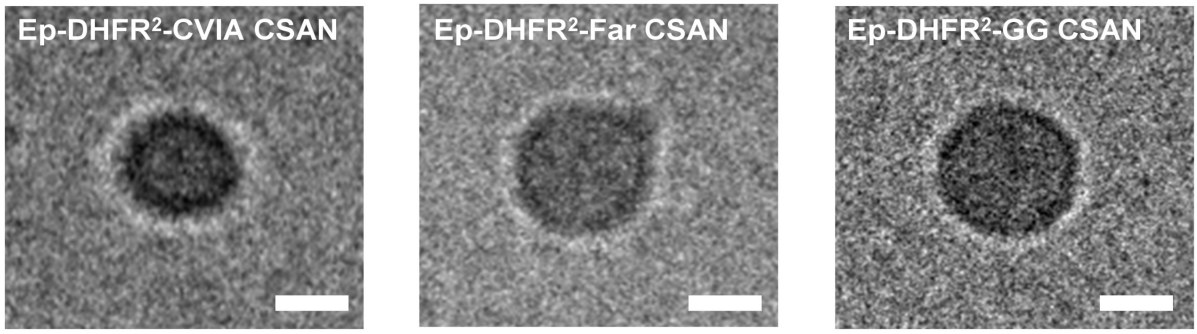

Fig. S4. Cryo-TEM imaging of anti-EpCAM CSANs. The Ep-DHFR^2-CVIA protein or prenylated Ep-DHFR^2-CVIA protein formed nanorings at the presence of bisMTX and the nanorings were imaged by cryo-TEM. The morphology of the nanorings exhibited the uniform circular conformation, and the sizes of the constructs are comparable to the DLS data (scale bar, 10□nm).

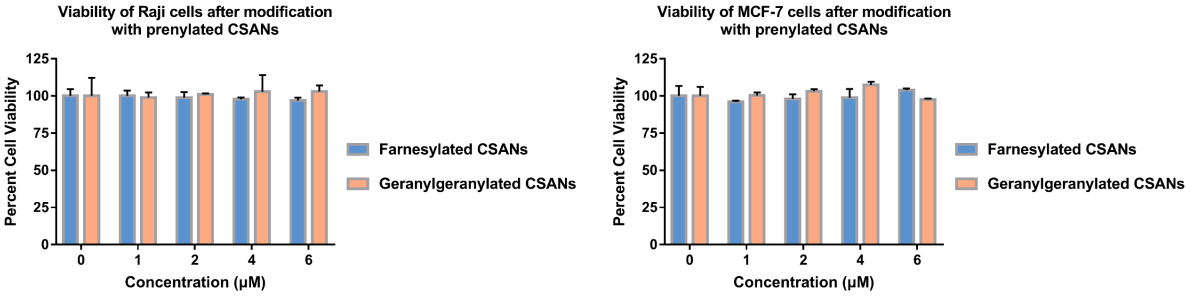

Fig. S5. Viability of cells modified with prenylated CSANs. The modification of cells with prenylated CSANs (DHFR^2-Far and DHFR^2-GG CSANs) was shown to be non-toxic, via trypan blue exclusion using a Bio-Rad TC20 automated cell counter.

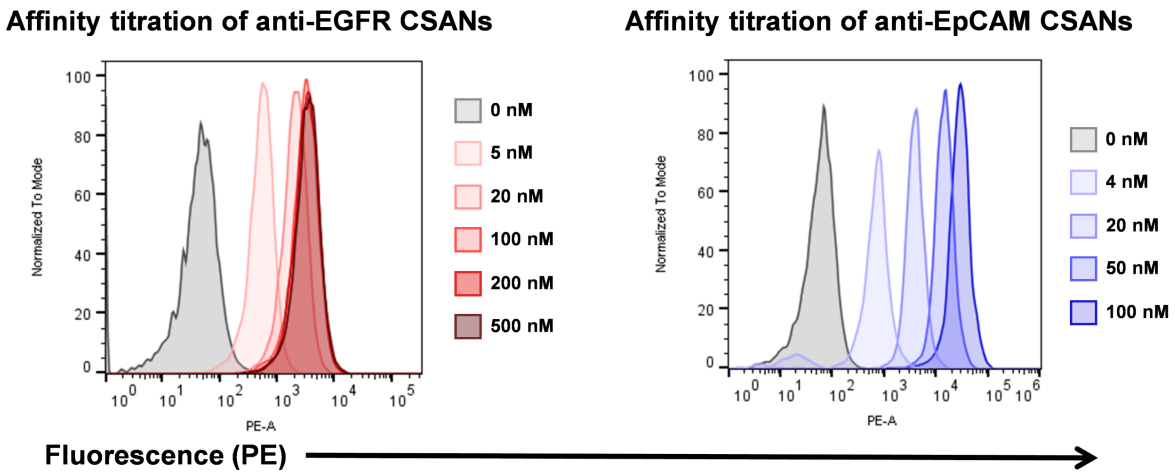

Fig. S6. Representative flow cytometry in the affinity titration assay for antigen-targeted CSANs. The binding affinity of E1-DHFR^2-CVIA CSANs and Ep-DHFR^2-CVIA CSANs was measured by flow cytometry. E1-DHFR^2-CVIA CSANs exhibited a K_d of 16 ± 2.4 nM towards EGFR$^+$ MDA-MB-231 cells while Ep-DHFR^2-CVIA CSANs demonstrated a K_d of 31 ± 11 nM towards EpCAM$^+$ MCF-7 cells.

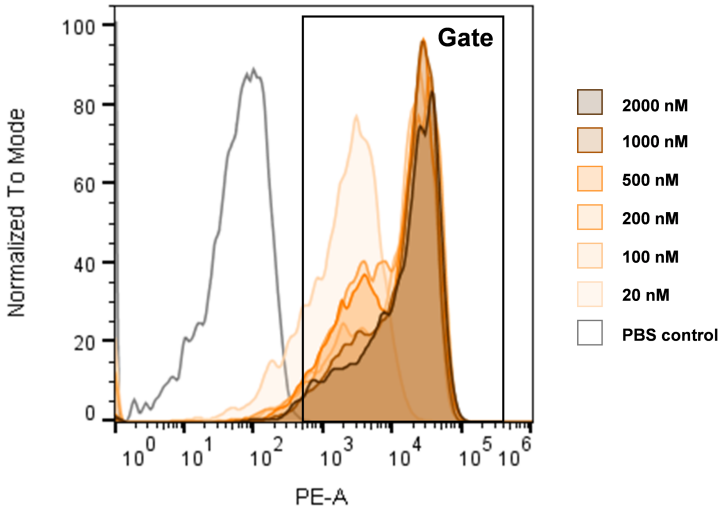

Fig. S7. Representative flow cytometry in the affinity titration assay for prenylated CSANs with gating strategy. The affinity titration assay was conducted for prenylated CSANs by flow cytometry. In this figure, the Raji cells were incubated with different concentrations of fully geranylgeranylated DHFR^2-CVIA CSANs, followed by staining with the anti-FLAG-PE antibody. The Raji cells that incubated with PBS was used as the negative control to define the lower bound of the PE signal.

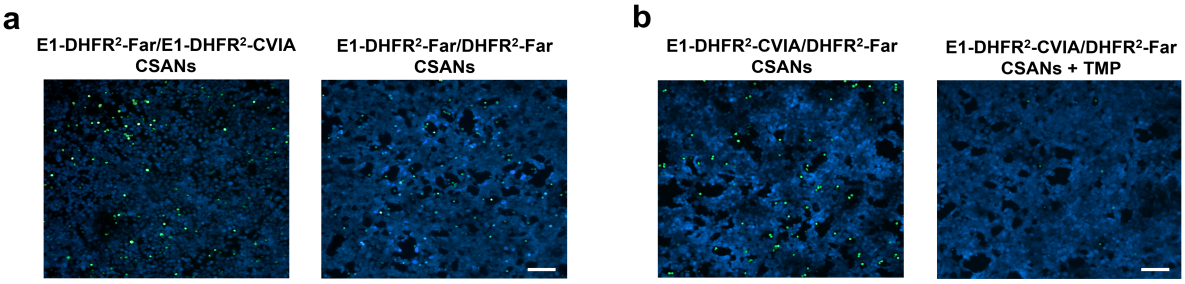

Fig. S8. Imaging of cell-cell interactions mediated by hybrid farnesylated anti-EGFR CSANs with reduced valency. (**a**) The hybrid farnesylated CSANs were prepared by mixing E1-DHFR^2-Far with either E1-DHFR^2-CVIA or DHFR^2-Far at 1:1 ratio, and the resulting CSANs have either 50% molar farnesyl groups or 50% molar EGFR-targeting elements in each ring. Both types of CSANs with reduced valency were able to mediate cell-cell interactions. (**b**) The hybrid farnesylated CSANs were prepared with E1-DHFR^2-CVIA and DHFR^2-Far proteins, and the CSANs were able to mediate cell-cell interactions. However, upon the addition of TMP, the cell-cell interactions were dissociated (scale bar, 100 μm).

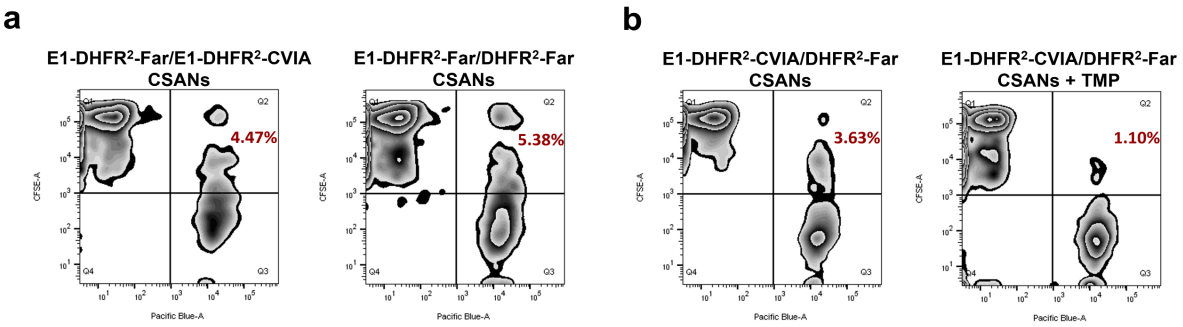

Fig. S9. Flow cytometry of cell-cell interactions mediated by hybrid farnesylated anti-EGFR CSANs with reduced valency. (**a**) The hybrid farnesylated CSANs were prepared by mixing E1-DHFR^2-Far with either E1-DHFR^2-CVIA or DHFR^2-Far at 1:1 ratio, yielding CSANs with either 50% farnesyl groups or 50% EGFR-targeting elements. Both types of reduced-valency CSANs induced fewer cell-cell interactions than the fully octavalent E1-DD-Far CSANs. (**b**) Hybrid farnesylated CSANs were prepared with E1-DHFR^2-CVIA and DHFR^2-Far proteins, and the CSANs were able to mediate cell-cell interactions. However, upon the addition of TMP, the cell-cell interactions were significantly diminished.

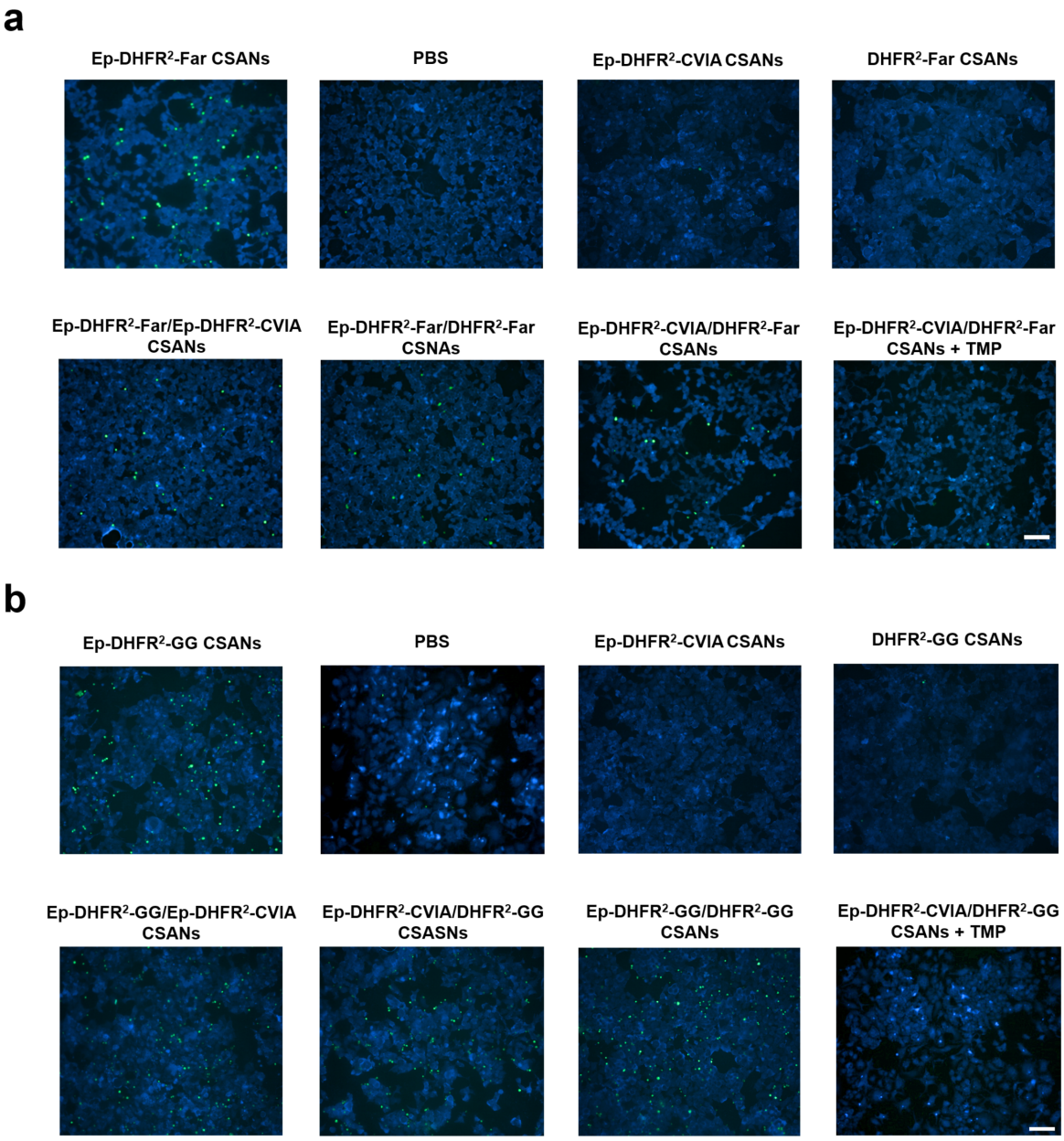

Fig. S10. Fluorescent microscopy imaging of cell-cell interactions mediated by prenylated anti-EpCAM CSANs. The cell-cell interactions mediated by (**a**) farnesylated anti-EpCAM CSANs or (**b**) geranylgeranylated anti-EpCAM CSANs were assessed via fluorescent microscopy. CFSE-stained Raji cells were modified with different CSANs constructs and interacted with CTV-stained EpCAM+ MCF-7 cells on the coverslips. The prenylated anti-EpCAM CSANs were shown to mediate cell-cell interactions, while TMP was shown to disassemble the hybrid Ep-DHFR^2-CVIA/DHFR^2-GG CSANs and efficiently reduce the cell-cell interactions (scale bar, 100 μm).

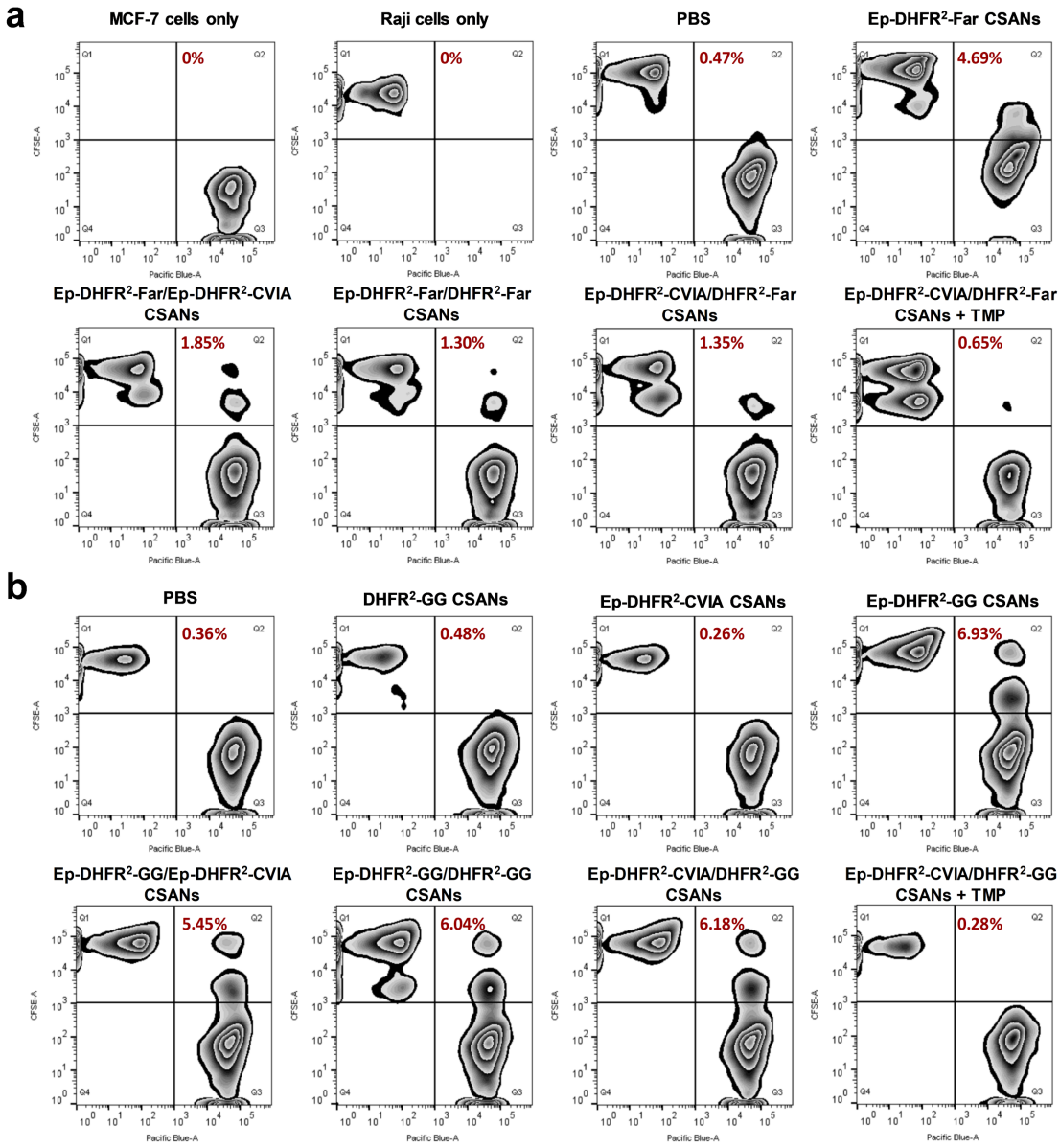

Fig. S11. Flow cytometry analysis of cell-cell interactions mediated by prenylated anti-EpCAM CSANs. The cell-cell interactions mediated by (**a**) farnesylated anti-EpCAM CSANs or (**b**) geranylgeranylated anti-EpCAM CSANs was assessed via flow cytometry. CFSE-stained Raji cells were modified with different CSANs constructs, followed by incubation with CTV-stained MCF-7 cells in PBS buffer. The cells were then analyzed on the flow cytometer. The $CFSE^+/CTV^+$ cell population was quantified as the indicator of Raji-MCF-7 cell interactions. Significantly more cell-cell interactions observed with geranylgeranylated CSANs compared to farnesylated CSANs. TMP was shown to disassemble the hybrid Ep-$DHFR^2$-CVIA/$DHFR^2$-GG CSANs and efficiently reduce the cell-cell interactions.

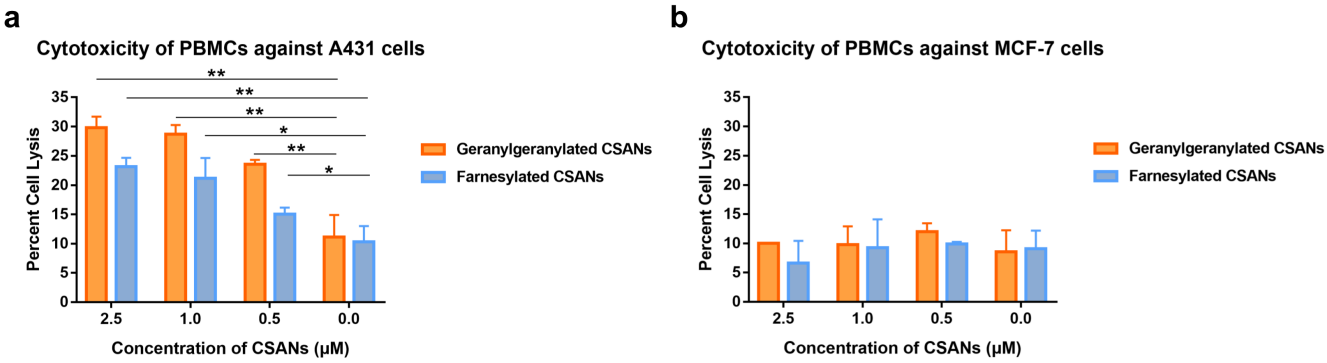

Fig. S12. Cytotoxicity of PBMCs modified with prenylated anti-EGFR CSANs against (a) EGFR$^+$ A431 cells and (b) EGFR$^-$ MCF-7 cells. PBMCs (Donor 28) were activated and modified with either farnesylated anti-EGFR CSANs (E1-DD-Far CSANs) or geranylgeranylated anti-EGFR CSANs (E1-DD-GG CSANs) at various concentrations. The CSANs-modified PBMCs were co-cultured with A431 cells or MCF-7 cells at the E/T ratio of 20:1 for the LDH release assay. The prenylated anti-EGFR CSANs significantly enhanced cancer-specific cytotoxicity of PBMCs against EGFR$^+$ A431 cells in a concentration-dependent manner, while they failed to enhance the cytotoxicity of PBMCs against EGFR$^-$ MCF-7 cells. Significance in a and b was tested using a two-tailed, unpaired t-test. and is indicated as **P <□0.01 or *P <□0.05. Error bars in a and b denote the standard error calculated from n=3 independent trials and each trial has n=3 independent samples.

Apparent K_d values of prenylated CSANs

Valency	Farnesylated CSANs	Geranylgeranylated CSANs
8	154.3 ± 6.2 nM	47.9 ± 22.3 nM
4	616.5 ± 256.3 nM	224.4 ± 59.2 nM
2	2310.6 ± 881.5 nM	787.7 ± 147.3 nM

Table S1. Apparent K_d values of prenylated CSANs. Hybrid prenylated CSANs with different numbers of isoprenoids were prepared by cross-linking prenylated $DHFR^2$-Far or $DHFR^2$-GG protein with unprenylated $DHFR^2$-CVIA protein at different ratios. The apparent affinity of the prenylated CSANs was measured in the affinity titration assay by flow cytometry. The valency represents the number of isoprenoids groups. The standard errors in the table were calculated from n=3 independent samples.

Supplementary Note 1. Gene sequence of DHFR^2 fusion protein constructs.

DHFR^2-CVIA :

GGCATCAGTCTGATTGCGGCGTTAGCGGTAGATCGCGTTATCGGCATGGAAAACGCCATGCCGTGGA
ACCTGCCTGCCGATCTCGCCTGGTTTAAACGCAACACCTTAAATAAACCCGTGATTATGGGCCGCCAT
ACCTGGGAATCAATCGGTCGTCCGTTGCCAGGACGCAAAAATATTATCCTCAGCAGTCAACCGGGTAC
GGACGATCGCGTAACGTGGGTGAAGTCGGTGGATGAAGCCATCGCGGCGGCTGGTGACGTACCAGAA
ATCATGGTGATTGGCGGCGGTCGCGTTTATGAACAGTTCTTGCCAAAAGCGCAAAAACTGTATCTGAC
GCATATCGACGCAGAAGTGGAAGGCGACACCCATTTCCCGGATTACGAGCCGGATGACTGGGAATCG
GTATTCAGTGAATTCCACGATGCTGATGCGCAGAACTCTCACAGCTATAGCTTTGAGATTCTGGAGCG
GCGGGGCGGCATTAGCCTTATTGCCGCCTTAGCGGTTGATCGCGTGATCGGAATGGAGAACGCAATGC
CCTGGAATCTTCCGGCAGACCTTGCCTGGTTCAAACGCAACACTTTAAACAAGCCTGTCATTATGGGC
CGTCACACATGGGAGTCAATTGGTCGTCCCCTGCCTGGGCGCAAAAATATCATCTTGTCCTCGCAGCC
TGGGACAGATGATCGCGTTACATGGGTGAAGTCCGTAGACGAAGCGATTGCCGCTGCCGGCGATGTG
CCCGAGATTATGGTAATCGGGGGAGGGCGTGTTTACGAACAATTTCTGCCCAAAGCTCAGAAATTATA
CCTGACGCACATCGACGCGGAGGTCGAAGGTGACACACACTTTCCAGATTATGAGCCTGATGATTGG
GAATCCGTTTTCTCAGAATTTCATGACGCGGATGCTCAAAACTCGCACTCGTACTCTTTTGAAATTTTA
GAGCGCCGTGGCGGATCTGGAGGAAGTGGCGGTGACTACAAAGACGACGATGATAAGGGCGGCTCA
GGTGGTTCCGGTGGCAAAAAGAAAAAGAAAAAGACCTGTGTCATCGCCTAGTGA

E1-DHFR^2-CVIA:

ATGGACTACAAAGACGACGATGATAAGGGCGGATCTGGAGGAAGTGGCGGTATGGGTGTCTCTGACG
TCCCGCGTGACCTGGAGGTTGTTGCAGCGACCCCAACTAGCCTTCTTATCAGCTGGGATAGCGGTCGT
GGTTCTTATCAATACTACCGGATCACTTACGGAGAAACAGGAGGAAATAGCCCTGTTCAGGAGTTCAC
TGTGCCTGGTCCTGTACACACTGCTACCATCAGCGGCCTTAAACCTGGAGTAGATTATACCATCACTG
TGTATGCTGTCACTGACCATAAGCCTCATGCTGATGGACCTCACACTTATCATGAATCTCCAATTTCCA
TCAATTACCGTACAGATATTGATCGTCCTTCTCAAGGTGGTAGTGGCATCAGTCTGATTGCGGCGTTA
GCGGTAGATCGCGTTATCGGCATGGAAAACGCCATGCCGTGGAACCTGCCTGCCGATCTCGCCTGGTT
TAAACGCAACACCTTAAATAAACCCGTGATTATGGGCCGCCATACCTGGGAATCAATCGGTCGTCCGT
TGCCAGGACGCAAAAATATTATCCTCAGCAGTCAACCGGGTACGGACGATCGCGTAACGTGGGTGAA
GTCGGTGGATGAAGCCATCGCGGCGGCTGGTGACGTACCAGAAATCATGGTGATTGGCGGCGGTCGC
GTTTATGAACAGTTCTTGCCAAAAGCGCAAAAACTGTATCTGACGCATATCGACGCAGAAGTGGAAG
GCGACACCCATTTCCCGGATTACGAGCCGGATGACTGGGAATCGGTATTCAGTGAATTCCACGATGCT
GATGCGCAGAACTCTCACAGCTATAGCTTTGAGATTCTGGAGCGGCGGGGCGGCATTAGCCTTATTGC

CGCCTTAGCGGTTGATCGCGTGATCGGAATGGAGAACGCAATGCCCTGGAATCTTCCGGCAGACCTTG
CCTGGTTCAAACGCAACACTTTAAACAAGCCTGTCATTATGGGCCGTCACACATGGGAGTCAATTGGT
CGTCCCCTGCCTGGGCGCAAAAATATCATCTTGTCCTCGCAGCCTGGGACAGATGATCGCGTTACATG
GGTGAAGTCCGTAGACGAAGCGATTGCCGCTGCCGGCGATGTGCCCGAGATTATGGTAATCGGGGGA
GGGCGTGTTTACGAACAATTTCTGCCCAAAGCTCAGAAATTATACCTGACGCACATCGACGCGGAGGT
CGAAGGTGACACACACTTTCCAGATTATGAGCCTGATGATTGGGAATCCGTTTTCTCAGAATTTCATG
ACGCGGATGCTCAAAACTCGCACTCGTACTCTTTTGAAATTTTAGAGCGCCGTGGCGGCTCAGGTGGT
TCCGGTGGCCATCATCATCATCATCACGGCGGCTCAAAAAAGAAAAAGAAAAAGACCTGTGTCATCG
CCTAGTGA

Ep-DHFR^2-CVIA:

ATGGACTACAAAGACGACGATGATAAGGCTAGCTCCTCCGACTCTCCGCGTAACCTGGAGGTTACCA
ACGCAACTCCGAACTCTCTGACTATTTCTTGGGACAATTCTAACTATGCTTCGTATTACCGTATCACCT
ACGGCGAAACCGGTGGTAACTCCCCGAGCCAGGAACTCACTGTTCCGGGAAGTACTTATAATGCGAC
CATCAGCGGTCTGAAACCGGGCCAGGATTATATCATTACCGTGTACGCTGTAACCTATCGTGACAATT
ATTCCTATTCAAATCTAATCAGCATCAATTATCGCTCCGAAATCGACAAACCGTCTCAGGGATCCGGA
GGTTCCGGCGGGGGCGGAAGCGGAGGTGGAGGCTCAGGGGGCGGAGGGTCGGGCGGTGGAGGTTCG
GGGGGAGGCGGGAGCGGTGGCGGTGGTTCAGGAGGAGGGGGTTCCGGGGGTGGTGGATCGGGCGGT
GAGCTCGGCGGCATCAGTCTGATTGCGGCGTTAGCGGTAGATCGCGTTATCGGCATGGAAAACGCCAT
GCCGTGGAACCTGCCTGCCGATCTCGCCTGGTTTAAACGCAACACCTTAAATAAACCCGTGATTATGG
GCCGCCATACCTGGGAATCAATCGGTCGTCCGTTGCCAGGACGCAAAAATATTATCCTCAGCAGTCAA
CCGGGTACGGACGATCGCGTAACGTGGGTGAAGTCGGTGGATGAAGCCATCGCGGCGGCTGGTGACG
TACCAGAAATCATGGTGATTGGCGGCGGTCGCGTTTATGAACAGTTCTTGCCAAAAGCGCAAAAACTG
TATCTGACGCATATCGACGCAGAAGTGGAAGGCGACACCCATTTCCCGGATTACGAGCCGGATGACT
GGGAATCGGTATTCAGTGAATTCCACGATGCTGATGCGCAGAACTCTCACAGCTATAGCTTTGAGATT
CTGGAGCGGCGGGGCGGCATTAGCCTTATTGCCGCCTTAGCGGTTGATCGCGTGATCGGAATGGAGA
ACGCAATGCCCTGGAATCTTCCGGCAGACCTTGCCTGGTTCAAACGCAACACTTTAAACAAGCCTGTC
ATTATGGGCCGTCACACATGGGAGTCAATTGGTCGTCCCCTGCCTGGGCGCAAAAATATCATCTTGTC
CTCGCAGCCTGGGACAGATGATCGCGTTACATGGGTGAAGTCCGTAGACGAAGCGATTGCCGCTGCC
GGCGATGTGCCCGAGATTATGGTAATCGGGGGAGGGCGTGTTTACGAACAATTTCTGCCCAAAGCTCA
GAAATTATACCTGACGCACATCGACGCGGAGGTCGAAGGTGACACACACTTTCCAGATTATGAGCCT
GATGATTGGGAATCCGTTTTCTCAGAATTTCATGACGCGGATGCTCAAAACTCGCACTCGTACTCTTTT
GAAATTTTAGAGCGCCGTGGCGGTTCAGGTGGTGGCTCGGGAGGCCATCATCATCATCATCACGGCGG
ATCTGGAGGAAGTGGCGGTAAAAAGAAAAAGAAAAAGACCTGTGTCATCGCCTAGTGA

Experimental Methods

1. Cell lines and cell culture

The human cancer cell lines, MCF-7, MDA-MB-231, A431, and Raji cells were previously purchased from the American Type Culture Collection (ATCC) and were validated via the STR fingerprinting service at the Cytogenetics and Cell Authentication Core (CCAC) in the Department of Genetics at MD Anderson Cancer Center. The cell lines were tested and certified as mycoplasma-free using PCR Mycoplasma Detection Kit (Applied Biological Materials Inc., Cat: G238). MCF-7 and A431 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) with 4.5 g/L glucose, L-glutamine, and supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 μg/mL streptomycin at 37 ºC with 5.0% CO_2. Raji cells were cultured in Roswell Park Memorial Institute (RPMI) medium with L-glutamine and supplemented with 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin at 37 ºC with 5.0% CO_2.

Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood leukoreduction filters (LRS, Innovative Blood Resources, St. Paul, MN) following standard procedures[2] and cultured in ImmunoCult™-XF T Cell Expansion Medium supplemented with 30 U/ml IL-2, 100 U/mL penicillin, and 100 μg/mL streptomycin at 37 ºC with 5.0% CO_2. The healthy donors blood samples (Donor 15 or Donor 28) were purchased from Memorial Blood Centers, Saint Paul, MN. Donor 15 was used for the LDH release assay unless specified otherwise.

2. Expression plasmids

gBlock Gene Fragments coding for the EpCAM-$DHFR^2$-CVIA and EGFR-$DHFR^2$-CVIA fusion proteins were ordered from Integrated DNA Technologies (IDT) and cloned into the Novagen pET28a(+) vector (EMD Millipore, Cat: 69864-3) via NcoI and XhoI restriction sites. The gene fragment for the $DHFR^2$-CVIA protein was generated via site-directed mutagenesis of the gene of EpCAM-$DHFR^2$-CVIA protein using a New England Biolabs Q5 Site-Directed Mutagenesis Kit. Notably, the EpCAM-$DHFR^2$-CVIA and EGFR-$DHFR^2$-CVIA fusion proteins have a FLAG tag and poly-histidine (His6) tag to facilitate detection via flow cytometry and purification via immobilized metal affinity chromatography (IMAC), respectively. Similarly, the $DHFR^2$-CVIA protein has a FLAG tag to enable flow cytometric detection. The sequences of the protein constructs are listed in Supplementary Note 1.

3. Protein expression and purification

The EpCAM-$DHFR^2$-CVIA, EGFR-$DHFR^2$-CVIA and $DHFR^2$-CVIA fusion proteins were produced in T7 Express Competent *E. coli* cells (New England Biolabs). The *E. coli* cells were cultured at 37 ºC (250 rpm) to the point when the OD_{600} reached 0.6-0.8, and then the protein was expressed at 37 ºC for 6 hours by the addition of IPTG (0.5 mM). The EpCAM-$DHFR^2$-CVIA and EGFR-$DHFR^2$-CVIA fusion proteins were purified from the soluble fractions of the cell lysate via immobilized metal affinity chromatography (IMAC) using the cobalt column (Thermo Fisher Scientific, Cat: 89964) according to previously reported methods[3]; meanwhile, the $DHFR^2$-CVIA fusion proteins were purified from the soluble fractions of the cell lysate via methotrexate affinity chromatography and DEAE ion-exchange chromatography, also according to the previously reported methods[3]. Purified protein was analyzed by gel electrophoresis using

NuPAGE Bis-Tris protein gels (Thermo Fisher Scientific, Cat: NP0321PK2). DTT (5mM) was added to the protein samples for gel electrophoresis. Yeast farnesyl transferase (yFTase) and rat geranylgeranyl transferase type □ (rGGTase-□) were expressed and purified following the previously reported procedures[4,5].

4. Prenylation reactions and characterization

Prenylation reactions were conducted following the previously reported methods[4]. Specifically, a reaction cocktail (typically 500 uL) was prepared with Tris· HCl buffer (50mM, pH 7.5) containing KCl (20 mM), $MgCl_2$ (10 mM), $ZnCl_2$ (10 μM), DTT (5 mM), and the protein of interest (2.5 μM). The mixture was incubated on ice for 0.5 h and the reaction was initiated by the addition of FPP (10 μM) or GGPP (10 μM) with yFTase (200 nM) or rGGTase-□ (250 nM) respectively and allowed to proceed for 6-12 h in a 32 ºC water bath. The prenylated protein was subsequently purified by buffer exchange with PBS using an Amicon Ultra-0.5 centrifugal filter (10 kDa cutoff, Millipore) for characterization or cell surface modification purposes. The prenylated and unprenylated proteins were characterized by LC-MS using an Orbitrap Elite Hybrid Mass Spectrometer. The LC-MS data was analyzed by Thermo Scientific™ Protein Deconvolution 3.0.0.

5. CSAN formation and characterization

CSANs were formed by the addition of a 1.1-3.0-fold molar excess of dimerizer, bisMTX, to a solution of the targeted $DHFR^2$ fusion protein monomers in PBS (1-2 mL, unless specified otherwise). The oligomerization occurs within minutes after adding bisMTX. CSAN formation was characterized by SEC using a Superdex 200 Increase 10/300 gel filtration column (GE Healthcare Life Sciences, Cat: 28990944). The retention times were compared to those of commercial molecular weight standards (Sigma-Aldrich, Cat: MWGF1000-1KT). The hydrodynamic diameters of CSANs were measured with a Punk Dynamic Light Scattering unit (Unchained Laboratories) and presented as mean value ± standard deviation of at least three measurements.

The CSAN samples for cryo-TEM were prepared at 1 μM concentrations in PBS buffer. The CSAN solutions (2.5 μL) were applied to a lacey Formvar/carbon grid (Ted Pella, Inc.; Cat: 01883) in the humidified chamber of a Vitrobot Mark IV (FEI), blotted for 13 seconds, and plunged into liquid ethane for vitrification. Grids were imaged on a Tecnai Spirit G2 BioTWIN (FEI) equipped with an Eagle 2k CCD camera (FEI) under a high tension of 120 kV.

6. Binding selectivity and apparent affinity of antigen-targeted CSANs.

The binding specificity of the CSANs was studied by flow cytometry, where MCF-7 cells were chosen as the $EpCAM^+$ cell line, and A431 as the $EGFR^+$ cell line. Raji cells were selected as the $EpCAM^-/EGFR^-$ cell line. The cells were harvested and washed with Dulbecco's phosphate-buffered saline (DPBS), and aliquots of 10^5 cells were then resuspended in DPBS solutions containing 2.5 μM of anti-EpCAM CSANs or anti-EGFR CSANs and incubated for 1 h at 4 ºC. The cells were then pelleted, washed, and resuspended in 50 μL of Alexa Fluor® 647 anti-His-tag antibody solution and incubated for ≥ 30 min on ice in the dark, after which they were then washed three times with 1 mL of cold DPBS and analyzed using an LSR II flow cytometer (BD Biosciences) at the University Flow Cytometry Resource (UFCR).

The binding affinity of the antigen-targeted CSANs was also studied via a titration assay by flow cytometry. The $EpCAM^+$ MCF-7 cells were harvested and washed with DPBS. Aliquots of 10^5 cells were then resuspended in DPBS solutions containing different concentrations of anti-EpCAM CSANs and incubated for 1 h at 4 ºC. The cells were then pelleted, washed, and resuspended in 50 µL of anti-FLAG-PE antibody solution and incubated for ≥ 30 min on ice in the dark, after which they were washed three times with 1 mL of cold DPBS and analyzed using an LSR II flow cytometer (BD Biosciences). The affinity of anti-EGFR CSANs was studied using MDA-MB-231 cells as the target cells following the same procedures.

7. Cell surface modification with prenylated CSANs and membrane affinity of prenylated CSANs.

The Raji cells or PBMCs were collected from cell culture, pelleted at 350 g for 5 min and washed with 1 mL DPBS. Aliquots of 10^5 cells were then incubated in DPBS solutions containing the desired concentrations of prenylated CSANs for at least 1 h at room temperature with rotation and washed three times with 1 mL cold DPBS to remove unbound CSANs. The modified cells were directly used for subsequent applications.

For the membrane affinity studies, Raji cells were harvested and washed with DPBS, and then incubated with different concentrations of prenylated CSANs prepared from prenylated $DHFR^2$-CVIA protein for 1 h at room temperature with rotation. After wash steps, the cells were resuspended in 50 µL of anti-FLAG-PE antibody solution and incubated for ≥ 30 min in the dark. The cells were then washed thrice with 1 mL of cold DPBS and analyzed with an LSR II flow cytometer (BD Biosciences). $DHFR^2$-CVIA protein was mixed with prenylated $DHFR^2$-CVIA protein for the formation of hybrid CSANs with different valency, and the subsequent hybrid CSANs were also studied in an analogous fashion.

8. Viability of cells after CSANs modification

The Raji and MCF-7 cells were collected from cell culture, pelleted at 350 g for 5 min and washed with 1 mL DPBS. Aliquots of 10^5 cells were modified with desired concentrations of prenylated CSANs for 1 h at room temperature with rotation and washed three times with 1 mL cold DPBS to remove unbound CSANs. After modification, the cells were stained by trypan blue and the percentage of live cells for each sample was quantified using a Bio-Rad TC20 automated cell counter. The percentage of live cells for the sample incubated with PBS was used as a standard to calculate the relative viability for the cells modified with prenylated CSANs.

9. Trimethoprim-induced CSANs dissociation and kinetics

Raji cells or PBMCs were modified with 2.5 µM of prenylated CSANs, washed with DPBS, resuspended in DPBS solution containing 50 µM of trimethoprim (Fisher Scientific, Cat: AAJ66646MD), and incubated for 1 h. For the kinetic study of CSAN disassembly on the cell surface, Raji cells were modified with E1-$DHFR^2$-CVIA/$DHFR^2$-GG hybrid CSANs (2.5 µM) at room temperature for 1 h, and then the cells were washed with DPBS and resuspended in DPBS solution containing 50 µM of trimethoprim. The samples were incubated for 0 min, 5 min, 10 min, 20 min or 40 min and washed with DPBS. Since the $DHFR^2$-GG protein does not contain a His6-tag, the disassembly of CSANs on the cell membrane was quantified by incubating the cells on ice for 1 h with Alexa Fluor 647 anti-His-tag

antibody conjugate that only detects the remaining E1-DHFR^2-CVIA protein on the cell surface. The cells were then washed and analyzed by flow cytometry as described above.

10. Fluorescent imaging of membrane-bound prenylated CSANs

The Raji cells were modified with FITC-labelled prenylated CSANs (2.5 μM) according to the procedures described above. The cells were washed three times with 1 mL cold DPBS and stained with Alexa Fluor 594-conjugated cholera toxin subunit B (Thermo Fisher Scientific, Cat: C34777) according to manufacturer's protocol. After washing with cold DPBS, the cells were fixed with 4% paraformaldehyde in DPBS at room temperature for 20 min before washing thrice with 1 mL DPBS. The cells were resuspended in DPBS at a concentration of 10^6 cells/mL and then were added on to the coverslip. The coverslips were mounted on glass slides using ProLong Diamond Antifade Mountant with DAPI (Invitrogen, Cat: P36970) after the excess liquid was removed. The slides were kept in dark overnight for curing and were then imaged using an Eclipse Ti-E Wide Field Deconvolution Inverted Microscope (Nikon Instruments, Inc.).

11. In vitro stability of prenylated CSANs

Raji cells were harvested and washed with 1 mL DPBS. Aliquots of 0.5×10^6 cells were stained with CellTrace Violet (CTV; Thermo Fisher Scientific, Cat: C34571) according to manufacturer's protocol to determine the number of cell divisions throughout the experiment. The cells were then incubated with prenylated CSANs (2.5 μM) or prenylated protein monomers (2.5 μM) for 1 h at room temperature with rotation. After washing with DPBS, the Raji cells were returned to cell culture for 0-72 h. At 24 h intervals, aliquots of 10^5 cells were taken, labeled with an anti-FLAG-PE antibody (1 μg/mL in PBS) to detect cell surface CSANs, and analyzed with an LSR II flow cytometer, as described above. The media was refreshed every 24 h. For data analysis, the Mean Fluorescence Intensity (MFI) of the samples at 0 h was normalized to 1.0, representing maximum labeling, and the MFI on subsequent days was scaled relative to this value. Because cell division reduces the MFI value through dilution of the CSANs/monomers across the daughter cell surface and not because of loss of the protein constructs, the MFI values of subsequent analyses were corrected for the number of cell divisions, as measured by the CTV labeling.

To study the impact of trimethoprim on the *in vitro* stability of CSANs, a similar study was conducted. Briefly, the Raji cells were labeled with 2.5 μM of prenylated CSANs in the same way described above and returned to cell culture for 0-48 h. At 24 h, a 20-fold molar excess of trimethoprim was added to the cell culture. The cells were collected at 24 h intervals and the surface-bound CSANs were quantified following the same procedures described above.

12. Fluorescent imaging of reversible cell-cell interactions

Adherent MCF-7 or A431 cells were plated on glass coverslips in a 6-well plate and labeled with CTV according to the manufacturer's protocol. Meanwhile, aliquots of 5×10^5 Raji cells were labeled with CFSE (Thermo Fisher Scientific, Cat: C34554) according to the manufacturer's protocol and treated with different prenylated anti-cancer CSANs or control solutions following previously described methods. The Raji cells were resuspended in DPBS and added to the wells containing the adherent cancer cells on

the glass coverslips. The two cell populations were co-incubated in the dark at 4 ºC for 1 h. The cell layers were gently washed three times with 1 mL cold DPBS and unbound Raji cells were removed through aspiration. Then, 1 mL of DMEM with or without 50 μM trimethoprim was added to the wells and the cells were incubated in the dark at 4 ºC for another 1 h. The cell layers were again washed three times with 1 mL cold DPBS and unbound Raji cells were removed through aspiration. The cells were fixed with 4% paraformaldehyde in DPBS at room temperature for 20 min before washing thrice with 1 mL DPBS. After the excess liquid was removed, the coverslips were mounted on glass slides using SlowFade Diamond Antifade Mountant (Invitrogen, Cat: S36967). The slides were kept in the dark overnight for curing and were then imaged using an Eclipse Ti-E Wide Field Deconvolution Inverted Microscope (Nikon Instruments, Inc.).

13. Flow cytometry study of reversible cell-cell interactions

Target cells (MCF-7 or A431) were harvested from cell culture and labeled with CTV while Raji cells were labeled with CFSE according to the manufacturer's protocol. The Raji cells were then modified with different prenylated anti-cancer CSANs or control solutions following previously described methods. The Raji cells were then resuspended in DPBS solution containing the target MCF-7 or A431 cells, combined at a 1:1 ratio, and incubated in the dark at 4 ºC for 1 h. The cells were then pelleted, washed with 1 mL cold DPBS, and resuspended in DPBS with or without 50 μM trimethoprim. After incubating in the dark at 4 ºC for another 1 h, the cells were washed thrice with DPBS and analyzed with an LSR II flow cytometer to ascertain the number of $CTV^+/CFSE^+$ cell clusters.

14. LDH release assay for cytotoxicity study

Two days prior to performing the LDH assay, PBMCs were activated by ImmunoCult Human CD3/CD28/CD2 T Cell Activator beads (STEMCELL Technologies). Target A341cells were plated in a 96-well plate at a concentration of 2,500 cells per well and cultured overnight. The activated PBMCs were harvested and modified with prenylated CSANs at different concentrations following previously described methods. The aliquots of PBMCs were resuspended in 200 μL of ImmunoCult-XF T Cell Expansion Media and added to a 96-well plate containing target cells at different E/T ratios ranging from 10:1 to 40:1. The cells were cultured for 20 h and 50 μL of the supernatant in each well was collected for the LDH assay at room temperature using the CytoTox 96 Non-Radioactive Cytotoxicity Assay kit (Promega, Cat: G1780) according to manufacturer's protocol . Each group was triplicated in three wells and each experiment was triplicated in three plates.

15. Statistical considerations.

Data analysis and data visualization were performed in GraphPad Prism8. For the affinity titration assays, data were fit using the "One site -- Specific binding" option in GraphPad Prism8 software, which uses the equation $Y = B_{max} \times X/(K_d + X)$. All K_d derived include the standard error reported for these curve fits. For studying the Affinity–Valency relationship of prenylated CSANs, the data were fit in GraphPad Prism8 software using the equation $K_{d,N} = K_{d,1}/Valency^2$. For the kinetic study of CSANs disassembly on cell surface, data were fit to a "one phase decay" model using the equation $Y = (Y_0 - Plateau) \times \exp(-KX) + Plateau$. Information about error bars, statistical tests and n values are reported in

each figure legend. Unless otherwise stated, experiments were conducted in triplicate and data are presented as the mean ± standard deviation of three independent trials. Differences between means were compared using the unpaired two-tailed Student's t-tests, and a *P*-value <0.05 is denoted in graphics with an (*), $P < 0.01$ is denoted with (**), and $P < 0.001$ is denoted with (***).

16. Safety Statement

No unexpected or unusually high safety hazards were encountered.

Supplementary References

1 O. Kilic, M. R. de Souza, A. A. Almotlak, Y. Wang, J. M. Siegfried, M. D. Distefano and C. R. Wagner, *J. Med. Chem.*, 2020, **63**, 10235–10245.

2 S. Néron, L. Thibault, N. Dussault, G. Côté, É. Ducas, N. Pineault and A. Roy, *Transfusion*, 2007, **47**, 1042–1049.

3 C. M. Csizmar, J. R. Petersburg, A. Hendricks, L. A. Stern, B. J. Hackel and C. R. Wagner, *Bioconjug. Chem.*, 2018, **29**, 1291–1301.

4 Y. Zhang, M. J. Blanden, C. Sudheer, S. A. Gangopadhyay, M. Rashidian, J. L. Hougland and M. D. Distefano, *Bioconjug. Chem.*, 2015, **26**, 2542–2553.

5 S. A. Gangopadhyay, E. L. Losito and J. L. Hougland, *Biochemistry*, 2014, **53**, 434–446.