Electronic Supplementary Material (ESI) for Chemical Communications.


Supplementary Information

# Golgi recruitment assay for visualizing small-molecule ligand–target engagement in cells

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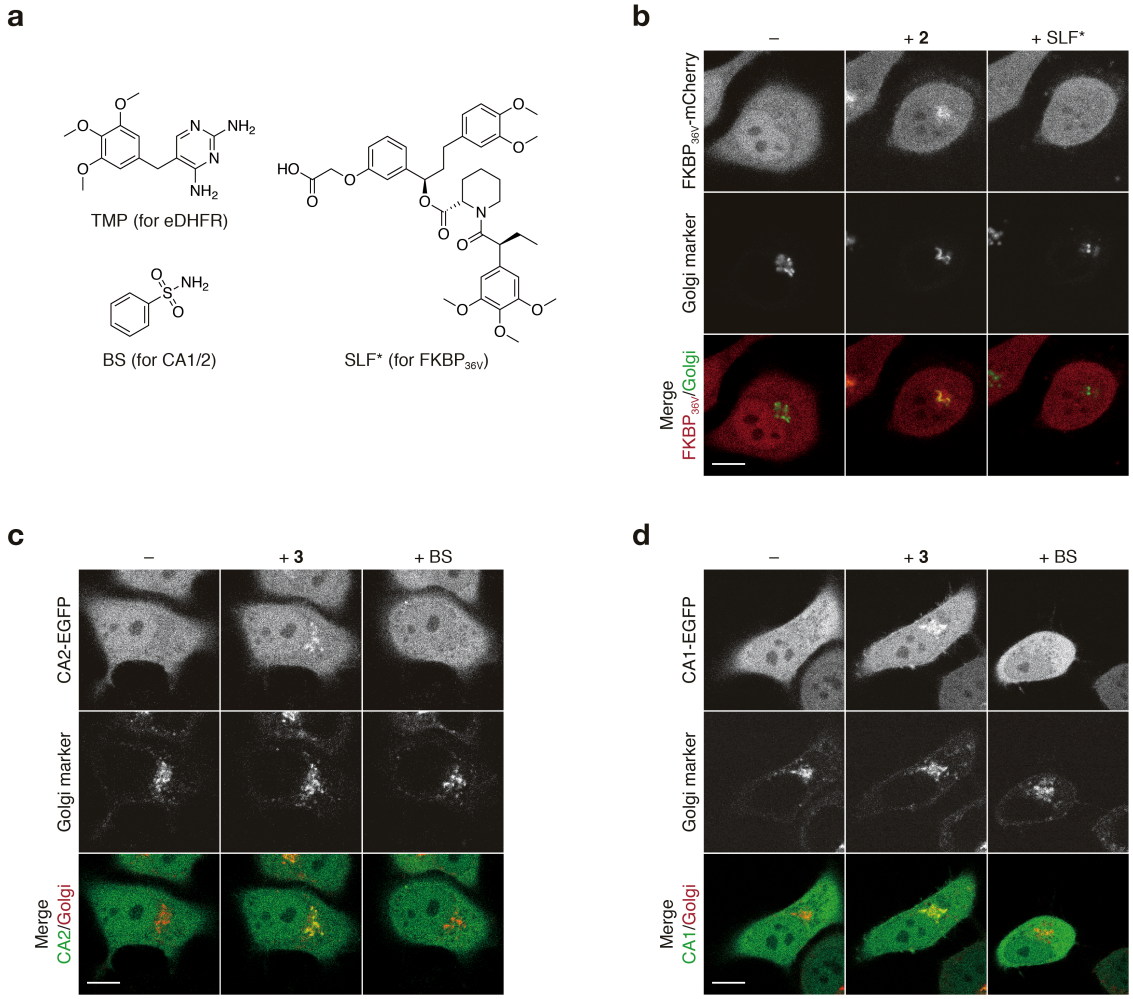


**Fig. S1** Visualization of various ligand–target interactions in cells by the G-REC assay. (**a**) Chemical structures of unmodified TMP, SLF*, and BS. (**b**) Detection of SLF*–$FKBP_{36V}$ interaction in cells. Confocal fluorescence images of HeLa cells coexpressing $FKBP_{36V}$-mCherry and AcGFP1-β(1,4)-GT (Golgi marker) were taken before (left), 60 min after incubation with 5 μM **2** (center), and 30 min after subsequent incubation with 50 μM SLF* (right). (**c**) Detection of BS–CA2 interaction in cells. Confocal fluorescence images of HeLa cells coexpressing CA2-EGFP and mCherry-giantin (Golgi marker) were taken before (left), 60 min after incubation with 10 μM **3** (center), and 30 min after subsequent incubation with 100 μM BS (right). (**d**) Detection of BS–CA1 interaction in cells. Confocal fluorescence images of HeLa cells coexpressing CA1-EGFP and mCherry-giantin (Golgi marker) were taken before (left), 60 min after incubation with 100 μM **3** (center), and 30 min after subsequent incubation with 1 mM BS (right). Scale bars, 10 μm.

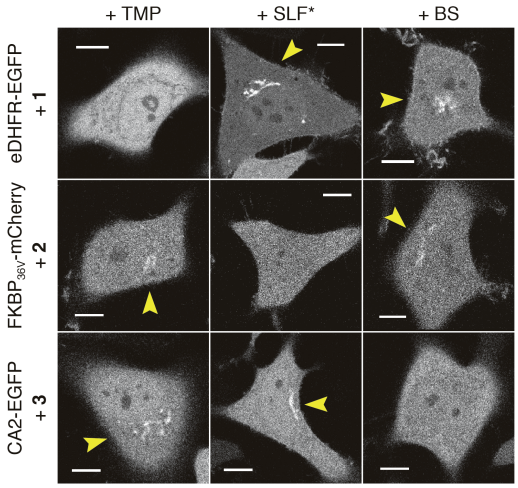


**Fig. S2** Competitive binding assay. HeLa cells expressing eDHFR-EGFP (top), $FKBP_{36V}$-mCherry (middle), or CA2-EGFP (bottom) were pre-treated with 5 μM **1**, 5 μM **2**, or 10 μM **3** for 30 min to localize the protein to the Golgi surface, respectively. After washing, confocal fluorescence images of the cells were taken 30 min after incubation with 50 μM TMP (left), 50 μM SLF* (center), or 100 μM BS (right). Scale bars, 10 μm.

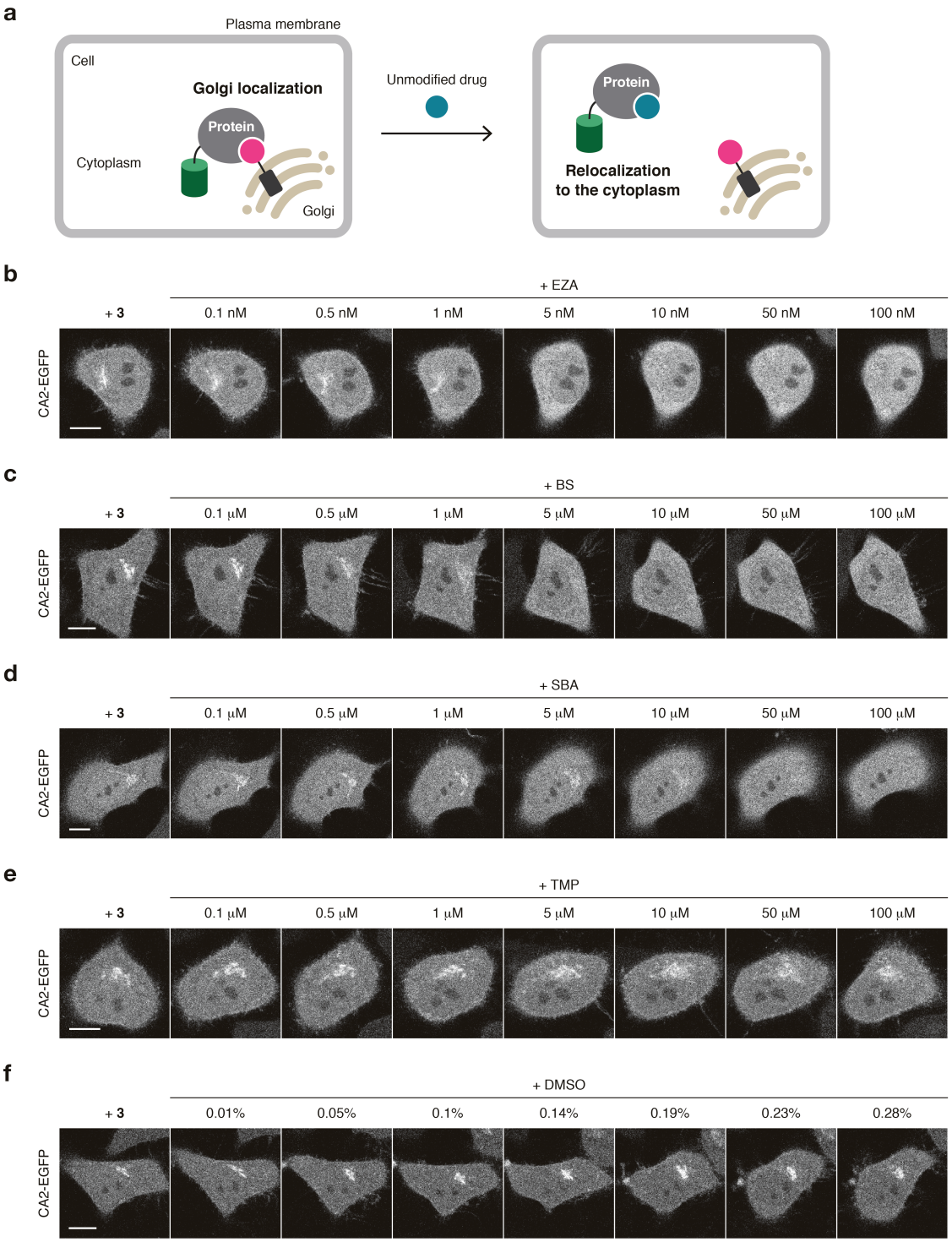


**Fig. S3** Representative confocal fluorescence images for the CA2 inhibitor titration experiments. (**a**) Schematic illustration of the unmodified drug–target engagement assay. (**b**–**f**) CA2-EGFP-expressing HeLa cells were pre-treated with 10 μM **3** for 30 min to localize the protein to the Golgi surface. After washing, confocal fluorescence images of the cells were taken before (left) and 15 min after every incubation with (**b**) EZA, (**c**) BS, (**d**) SBA, (**e**) TMP, and (**f**) DMSO (vehicle control) at the indicated concentration. Scale bars, 10 μm.

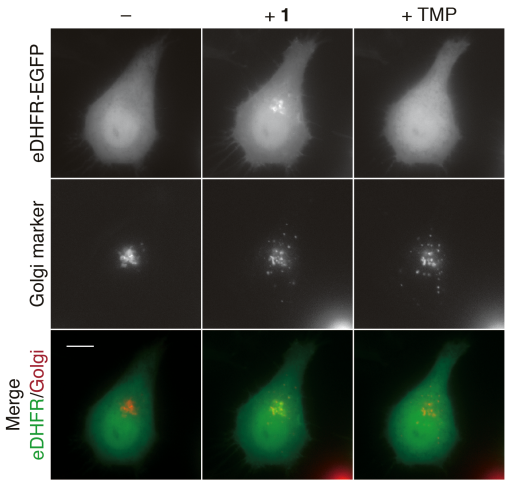


**Fig. S4** G-REC assay performed by epifluorescence microscopy. Epifluorescence images of HeLa cells coexpressing eDHFR-EGFP and mCherry-giantin (Golgi marker) were taken before (left), 30 min after incubation with 5 μM **1** (center), and 30 min after subsequent incubation with 50 μM TMP (right). Scale bar, 10 μm.

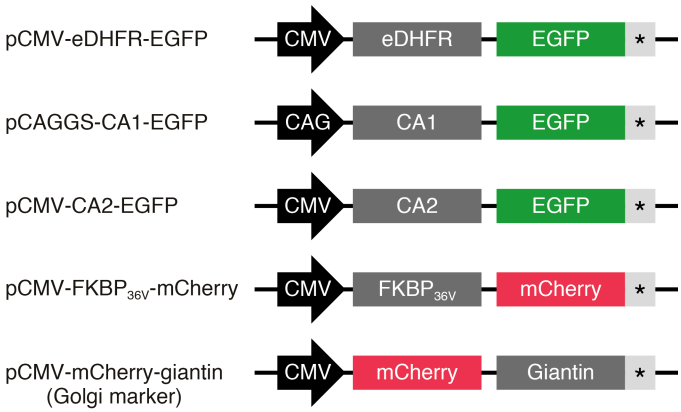


**Fig. S5** Schematic illustration of the domain structures of constructs used in this study.

**a**

pCMV-eDHFR-EGFP

>Amino acid sequence
MAISLIAALAVDRVIGMENAMPWNLPADLAWFKRNTLNKPVIMGRHTWESIGRPLPGRKNI
ILSSQPGTDDRVTWVKSVDEAIAACGDVPEIMVIGGGRVYEQFLPKAQKLYLTHIDAEVEG
DTHFPDYEPDDWESVFSEFHDADAQNSHSYCFEILERRAAASDPPVATMVSKGEELFTGVV
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NILGHKLEYNYNSHNVYIMADKQKNGIKVNFKIRHNIEDGSVQLADHYQQNTPIGDGPVLL
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>DNA sequence
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**b**

pCAGGS-CA1-EGFP

>Amino acid sequence

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>DNA sequence

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**c**

pCMV-CA2-EGFP

>Amino acid sequence

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>DNA sequence

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**d**

pCMV-FKBP$_{36V}$-mCherry

>Amino acid sequence
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>DNA sequence
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**e**

pCMV-mCherry-Giantin

>Amino acid sequence
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GACCCGAGTGCCACTTCTAGCAGCCATCTACTTTCTAATGATTCATGTCCTGCTCATTCTG
TGTTTTACGGGCCATCTATAG

**Fig. S6** DNA and amino acid sequences of constructs used in this study. (**a**) pCMV-eDHFR-EGFP: grey box, eDHFR; green, EGFP. (**b**) pCAGGS-CA1-EGFP: grey box, CA1; green, EGFP. (**c**) pCMV-CA2-EGFP: grey box, CA2; green, EGFP. (**d**) pCMV-FKBP$_{36V}$-mCherry: grey box, FKBP$_{36V}$; red, mCherry. (**e**) pCMV-mCherry-Giantin: red, mCherry; grey box, a Golgi targeting motif (residues 3131–3259)[S1] from human giantin.

**Table S1** Quantitative analysis of the specific ligand–target binding assay shown in **Fig. 2b**. The percentages of cells with Golgi-localized fluorescence signals (with a G/C ratio of >1.2) are shown (n > 24 cells).

| | **1** | **2** | **3** |
|---|---|---|---|
| eDHFR-EGFP | 100% | 0% | 0% |
| $FKBP_{36V}$-mCherry | 0% | 96% | 0% |
| CA2-EGFP | 0% | 0% | 100% |

## Supplementary Methods: Chemical Synthesis

### General materials and methods

All chemical reagents and solvents were purchased from commercial suppliers (Watanabe Chemical Industries, Tokyo Chemical Industry, FUJIFILM Wako Pure Chemical Corp. and Kanto Chemical) and used without further purification. Reverse-phase HPLC was performed on a Hitachi LaChrom Elite system with UV detection at 220 nm using a YMC-Pack ODS-A column (10 × 250 mm or 20 × 250 mm) or YMC-Pack C4 column (10 × 250 mm). $^{1}$H NMR spectra were recorded on a Bruker AVANCE III HD400SJ (400 MHz) spectrometer. $^{1}$H NMR chemical shifts were referenced to tetramethylsilane (0 ppm). MALDI-TOF mass analysis was performed on a JEOL JMS-S3000 mass spectrometer. High-resolution mass spectra were measured on a Thermo Scientific Extractive Plus Orbitrap mass spectrometer.

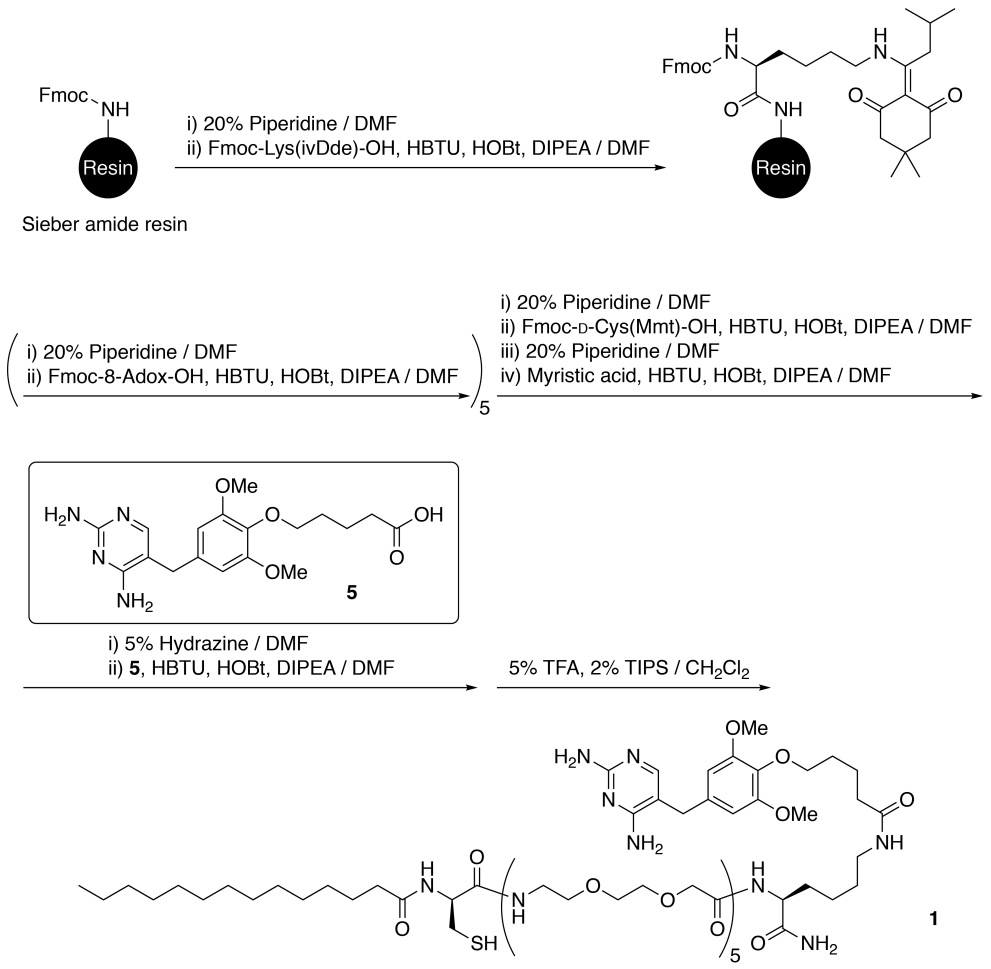
### Reagent abbreviations

DIPEA: *N*,*N*-diisopropylethylamine
DMF: *N*,*N*-dimethylformamide
Fmoc-Adox-OH: Fmoc-8-amino-3,6-dioxaoctanoic acid
HBTU: *O*-(benzotriazole-1-yl)-*N*,*N*,*N*',*N*'-tetramethyluronium hexafluorophosphate
HOBt: 1-hydroxybenzotriazole (monohydrate)
TFA: trifluoroacetic acid
TIPS: triisopropylsilane
TMS: tetramethylsilane

### General methods for solid-phase synthesis

Compounds **1**–**3** were synthesized manually on Sieber amide resin by standard Fmoc-based solid-phase peptide synthesis protocols. Fmoc deprotection was performed with 20% piperidine in DMF at room temperature for 15 min. Amino acid coupling reactions were performed at room temperature with a mixture of Fmoc-protected amino acid (3.1 eq.), HBTU (3.0 eq.), HOBt (3.0 eq.), and DIPEA (6.0 eq.) in DMF. All Fmoc deprotection and coupling steps were monitored by the Kaiser test.[S2] Unless otherwise stated, all washing procedures were performed with DMF. Compound **5** (TMP-COOH)[S3] and **6** (SLF*-COOH)[S4] were synthesized as described previously.

## Synthesis of Compound 1




**Scheme S1** Synthetic route of **1**

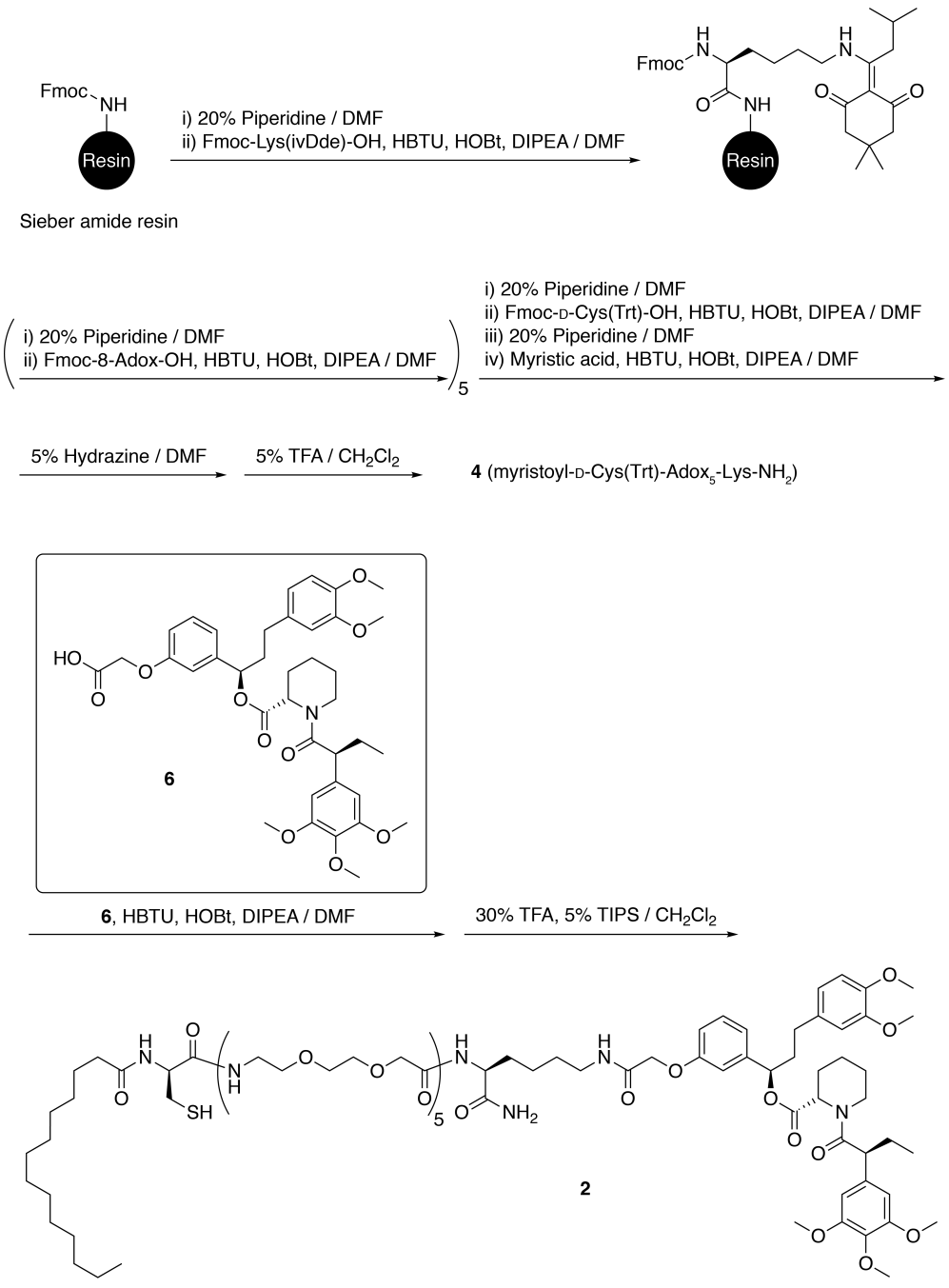
Compound **1** was synthesized manually on Sieber amide resin (0.65 mmol/g) (31 mg, 20 μmol). First, Fmoc-Lys(ivDde)-OH, Fmoc-Adox-OH (×5), and Fmoc-D-Cys(Mmt)-OH were coupled to the resin. The N-terminus was then myristoylated using a mixture of myristic acid (3.1 eq.), HBTU (3.0 eq.), HOBt (3.0 eq.), and DIPEA (6.0 eq.) in DMF. After washing the resin with DMF, the ivDde group was deprotected by treatment with DMF containing 5% hydrazine monohydrate. The resin was then washed with DMF. **5** (TMP-COOH) was coupled to the side chain of the lysine with a mixture of **5** (3.1 eq.), HBTU (3.0 eq.), HOBt (3.0 eq.), and DIPEA (6.0 eq.) in DMF. After washing with DMF, MeOH, and $CH_2Cl_2$, the resin was dried *in vacuo*. Deprotection and cleavage from the resin was performed with $CH_2Cl_2$ containing 5% TFA and 2% TIPS. The crude product

was purified by reversed-phase HPLC using a semi-preparative C18 column (a linear gradient of MeCN containing 0.1% TFA and 0.1% aqueous TFA) to afford **1** as a white solid [12.94 mg, 39% (as a mono-TFA salt)].

$^1$H NMR (400 MHz, $CD_3OD$): δ 7.21 (1H, s), 6.56 (2H, s), 4.41–4.47 (2H, m), 4.00–4.03 (10H, m), 3.91 (2H, t, $J$ = 6.0 Hz), 3.80 (6H, s), 3.66–3.68 (20H, m), 3.56–3.59 (10H, m), 3.43–3.47 (10H, m), 3.15–3.18 (2H, t, $J$ = 6.0 Hz), 2.56–2.58 (2H, m), 2.23–2.28 (4H, m), 1.53–1.82 (10H, m), 1.28–1.31 (22H, m), 0.89 (3H, t, $J$ = 6.0 Hz).

HRMS (ESI): calculated for $[M+H]^+$, 1542.8660; found, 1542.8633.

## Synthesis of Compound 2




**Scheme S2** Synthetic route of **2**

Compound **4**

Compound **4** was synthesized on Sieber amide resin (0.65 mmol/g) (62 mg, 40 μmol). First, Fmoc-Lys(ivDde)-OH, Fmoc-Adox-OH (×5), and Fmoc-D-Cys(Trt)-OH were

coupled to the resin. The N-terminus was then myristoylated using a mixture of myristic acid (3.1 eq.), HBTU (3.0 eq.), HOBt (3.0 eq.), and DIPEA (6.0 eq.) in DMF. After washing the resin with DMF, the ivDde group was deprotected by treatment with DMF containing 5% hydrazine monohydrate. After washing with DMF, MeOH, and $CH_2Cl_2$, the resin was dried *in vacuo*. Cleavage from the resin (without deprotecting the Trt group) was performed with $CH_2Cl_2$ containing 5% TFA. The crude product was purified by reversed-phase HPLC using a semi-preparative C18 column (a linear gradient of MeCN containing 0.1% TFA and 0.1% aqueous TFA) to afford **4** as a white solid [6.67 mg, 11% (as a mono-TFA salt)].

$^1$H NMR (400 MHz, $CD_3OD$): δ 7.21 (1H, s), 6.56 (2H, s), 4.41–4.47 (2H, m), 4.00–4.03 (10H, m), 3.91 (2H, t, $J$ = 6.0 Hz), 3.80 (6H, s), 3.66–3.68 (20H, m), 3.56–3.59 (10H, m), 3.43–3.47 (10H, m), 3.15–3.18 (2H, t, $J$ = 6.0 Hz), 2.56–2.58 (2H, m), 2.23–2.28 (4H, m), 1.53–1.82 (10H, m), 1.28–1.31 (22H, m), 0.89 (3H, t, $J$ = 6.0 Hz).

MALDI-TOF-MS: calculated for $[M+H]^+$, 1426.8159; found, 1426.4748.

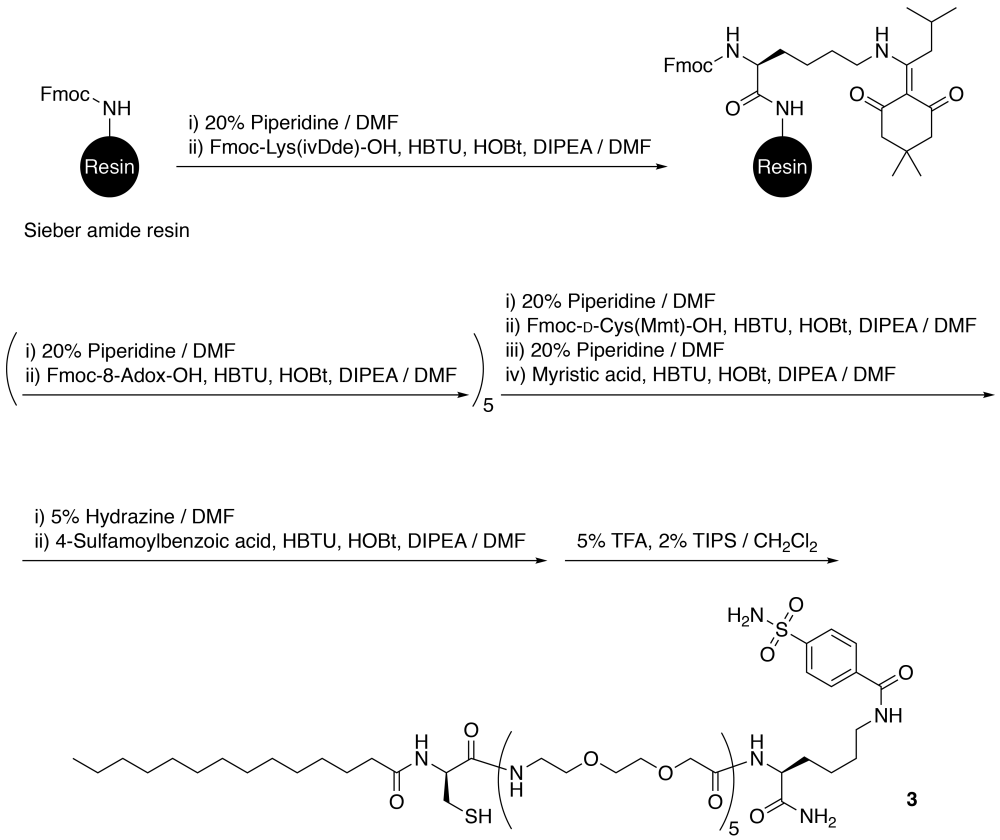
Compound **2**

To a solution of **4** (3.31 mg, 2.15 μmol, 1.0 eq.) in DMF (50 μL) was added **6** (1.0 eq.), HBTU (1.3 eq.), HOBt (1.3 eq.), and DIPEA (3.0 eq.), and the mixture was stirred for 48 h at room temperature. After evaporation, the deprotection of the Trt group was performed in $CH_2Cl_2$ containing 30% TFA and 5% TIPS for 30 min. The mixture was co-evaporated with toluene (×2). The crude product was purified by reversed-phase HPLC using a semi-preparative C4 column (a linear gradient of MeCN containing 0.1% TFA and 0.1% aqueous TFA) to afford **2** as a white solid (1.06 mg, 27%).

$^1$H NMR (400 MHz, $CD_3OD$): δ 7.19 (1H, m), 6.84–6.87 (2H, m), 6.79 (1H, s), 6.73 (1H, d, $J$ = 2.0), 6.68 (1H, d, $J$ = 8.0), 6.58 (2H, s), 6.54 (1H, d, $J$ = 8.0), 5.58 (1H, m), 5.39 (1H, m), 4.46–4.52 (4H, m), 3.80–3.82 (10H, m), 3.57–3.69 (39H, m), 3.42–3.46 (10H, m), 2.69–2.85 (3H, m), 2.50 (2H, m), 2.26–2.37 (4H, m), 1.59–2.10 (16H, m), 1.25–1.38 (20H, m), 0.83–0.91 (6H, m).

HRMS (ESI): calculated for $[M+H]^+$, 1860.0102; found, 1860.0090.

**Synthesis of Compound 3**




**Scheme S3** Synthetic route of **3**

Compound **3** was synthesized on Sieber amide resin (0.65 mmol/g) (31 mg, 20 μmol). First, Fmoc-Lys(ivDde)-OH, Fmoc-Adox-OH (×5), and Fmoc-D-Cys(Mmt)-OH were coupled to the resin. The N-terminus was then myristoylated using a mixture of myristic acid (3.1 eq.), HBTU (3.0 eq.), HOBt (3.0 eq.), and DIPEA (6.0 eq.) in DMF. After washing the resin with DMF, the ivDde group was deprotected by treatment with DMF containing 5% hydrazine monohydrate. The resin was then washed with DMF. 4-sulfamoylbenzoic acid was coupled to the side chain of the lysine with a mixture of 4-sulfamoylbenzoic acid (3.1 eq.), HBTU (3.0 eq.), HOBt (3.0 eq.), and DIPEA (6.0 eq.) in DMF. After washing with DMF, MeOH, and $CH_2Cl_2$, the resin was dried *in vacuo*. Deprotection and cleavage from the resin was performed with $CH_2Cl_2$ containing 5% TFA and 2% TIPS. The crude product was purified by reversed-phase HPLC using a semi-preparative C18 column (a linear gradient of MeCN containing 0.1% TFA and 0.1% aqueous TFA) to afford **3** as a white solid (8.42 mg, 31%).

$^{1}$H NMR (400 MHz, $CD_3OD$): δ 7.94–7.99 (4H, m), 4.43–4.48 (2H, m), 3.99–4.02 (10H, m), 3.65–3.67 (20H, m), 3.54–3.61 (10H, m), 3.37–3.46 (12H, m), 2.73–2.88 (2H, m), 2.26 (2H, t, *J* = 8.0 Hz), 1.47–1.67 (7H, m), 1.28–1.31 (22H, m), 0.88–0.91 (3H, t, *J* = 6.0 Hz).

HRMS (ESI): calculated for $[M+H]^+$, 1367.7009; found, 1367.6996.

## Supplementary Methods: Molecular and Cell Biology Experiments

### Plasmid construction

All of the cDNA and amino acid sequences of the constructs used in this study are listed in **Fig. S5** and **Fig. S6**. We used pCAGGS (provided by Dr. Jun-ichi Miyazaki, Osaka University),[S5] pEGFP-N1 (Clontech), pmCherry-C1 (Clontech), and pmCherry-N1 (Clontech) as vector backbones. We used pBAD-DHFR (provided by Dr. Teruyuki Nagamune, The University of Tokyo),[S6] $pC_4$-$F_V$1E (provided by ARIAD Pharmaceuticals), and pFRB-ECFP(W66A)-Giantin (Addgene #67903: provided by Dr. T. W. J. Gadella, University of Amsterdam)[S7] as PCR templates. The CA1 and CA2 genes were amplified by PCR from a human cDNA library (Takara Bio). All PCR amplified sequences were verified by DNA sequencing. All expression plasmids were generated using standard cloning procedures.

### Cell culture and transfection

HeLa cells were obtained from the Cell Resource Center for Biomedical Research, Institute of Development, Aging and Cancer, Tohoku University. Cells were cultured in DMEM (Wako) supplemented with 10% heat-inactivated FBS (Biowest), penicillin (100 U/mL), and streptomycin (100 μg/mL) [DMEM(+)] at 37°C under a humidified 5% $CO_2$ atmosphere. For transient expression experiments, cells were transfected using Polyethyleneimine MAX (Polysciences) in accordance with the manufacturer's protocol.

### Live cell imaging

Confocal fluorescence imaging was performed with either (i) a LSM880 confocal laser-scanning microscope (Zeiss) equipped with a Plan-Apochromat 63×/1.40 NA oil objective (Zeiss), a Definite Focus.2 module (Zeiss), and an incubation chamber (Incubator PM 2000 RBT, Pecon), or (ii) an IX83/FV3000 confocal laser-scanning microscope (Olympus) equipped with a PlanApo N 60×/1.42 NA oil objective (Olympus), a Z drift compensator system (IX3-ZDC2, Olympus), and a stage top incubator (Tokai Hit). Lasers used for excitation were as follows: 488 nm for EGFP and AcGFP1 (i/ii), 543 nm for mCherry (i), and 561 nm for mCherry (ii). Live cell imaging was performed at 37°C. Fluorescence images were analyzed using the Fiji distribution of ImageJ.[S8]

For epifluorescence imaging, cells were imaged with an IX83 inverted microscope

(Olympus) equipped with a UPlanSApo 60×/1.35 NA oil objective (Olympus), an ORCA-Flash4.0 V3 CMOS camera (Hamamatsu Photonics), an U-HGLGPS mercury light sources (Olympus), an IX2-ZDC laser-based autofocusing system (Olympus), and a stage top incubator (Tokai Hit). The filters and dichroic mirrors used for time-lapse imaging were as follows: two excitation filters (FF01-470/22-25 for EGFP and FF01-556/20-25 for mCherry), an FF493/574-Di01-25.8x37.8 dichroic mirror, and two emission filters (FF01-514/30-25 for EGFP and FF02-617/73-25 for mCherry) (Semrock). The imaging system was controlled by MetaMorph software (Universal Imaging). Live cell imaging was performed at 37°C. Fluorescence images were analyzed using the Fiji distribution of ImageJ.[S8]

**G-REC assay**

The G-REC assay was conducted by coexpressing protein targets (as a fluorescent protein fusion) and the Golgi marker. HeLa cells were plated at $1.2 \times 10^5$ cells in 35 mm glass-bottomed dishes (Iwaki Glass) and cultured for 24 h at 37°C in 5% $CO_2$. The cells were cotransfected with pCMV-mCherry-giantin and either pCMV-eDHFR-EGFP, pCAGGS-CA1-EGFP, or pCMV-CA2-EGFP at a 1:1 ratio. For the assay of $FKBP_{36V}$-mCherry, the cells were cotransfected with pAcGFP1-Golgi (Clontech) and pCMV-$FKBP_{36V}$-mCherry. Twenty-four hours after transfection, the medium was changed to serum-free DMEM supplemented with penicillin (100 U/mL) and streptomycin (100 μg/mL) [DMEM(–)]. The cells were observed by time-lapse imaging before and after addition of the indicated compound (5–100 μM) dissolved in DMSO (final DMSO concentration < 0.1% v/v).

**Affinity comparison assay for CA isoforms**

HeLa cells were plated at $1.2 \times 10^5$ cells in 35 mm glass-bottomed dishes and cultured for 24 h at 37°C in 5% $CO_2$. The cells were transfected with pCAGGS-CA1-EGFP or pCMV-CA2-EGFP. Twenty-four hours after transfection, the medium was changed to DMEM(–). The cells were then imaged before and 60 min after addition of **3** (1–100 μM). Cells with similar expression levels were used for analysis.

**Unmodified drug–target engagement assay**

HeLa cells were plated at $1.2 \times 10^5$ cells in 35 mm glass-bottomed dishes coated with collagen type I-C (Nitta gelatin). The cells were cotransfected with pCMV-mCherry-

giantin and pCMV-CA2-EGFP and cultured for 24 h at 37°C in 5% $CO_2$. The medium was changed to DMEM(–) and the cells were incubated with **3** (10 μM) in DMEM(–) for 30 min at 37°C. After washing with DMEM(–), the cells were treated in DMEM(–) with an increasing concentration of the indicated unmodified drug: EZA, 0.1–100 nM; BS, SBA, and TMP, 0.1–100 μM. Fluorescence images were taken 15 min after every drug addition.

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