

Supporting Information

A Synthetic Hybrid Molecule for the Selective Removal of Human Pluripotent Stem Cells from Cell Mixtures

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Supplementary Contents

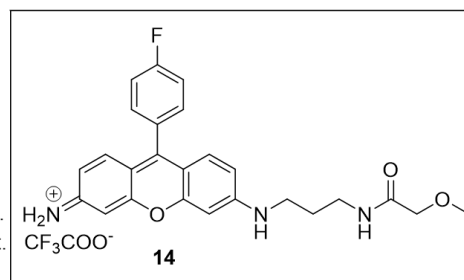
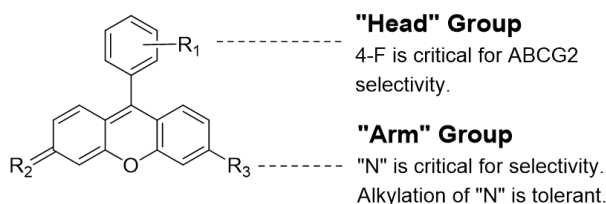
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Table S1. IC₅₀ values of 12 cytotoxic anticancer drugs with KB3-1, KB/ABCB1, KB/ABCC1 and KB/ABCG2 cells. Data are shown as mean $\pm$ SD. n=3.^a

IC ₅₀ /μM	KB3-1	ABCB1	ABCC1	ABCG2
SN38	0.30 $\pm$ 0.12	0.35 $\pm$ 0.17	0.72 $\pm$ 0.09	3.68 $\pm$ 1.24
Camptothecin	0.76 $\pm$ 0.30	0.45 $\pm$ 0.11	0.46 $\pm$ 0.10	11.85 $\pm$ 3.26
9-Aminocamptothecin	0.49 $\pm$ 0.12	0.28 $\pm$ 0.05	0.37 $\pm$ 0.03	3.96 $\pm$ 0.61
9-nitrocamptothecin	0.23 $\pm$ 0.08	0.37 $\pm$ 0.13	0.44 $\pm$ 0.01	0.61 $\pm$ 0.11
7-ethylcamptothecin	1.68 $\pm$ 0.13	1.28 $\pm$ 0.47	1.08 $\pm$ 0.53	2.05 $\pm$ 0.88
Doxorubicin	0.31 $\pm$ 0.15	2.45 $\pm$ 0.81	0.64 $\pm$ 0.17	0.30 $\pm$ 0.10
Mitoxantrone	0.25 $\pm$ 0.08	0.30 $\pm$ 0.10	1.16 $\pm$ 0.30	6.17 $\pm$ 1.04
Etoposide	7.7 $\pm$ 1.9	47.2 $\pm$ 12.5	31.4 $\pm$ 10.2	7.9 $\pm$ 2.7
Mitomycin C	1.06 $\pm$ 0.31	0.89 $\pm$ 0.18	0.41 $\pm$ 0.09	1.38 $\pm$ 0.18
Dactinomycin	0.008 $\pm$ 0.003	0.090 $\pm$ 0.001	0.007 $\pm$ 0.002	0.006 $\pm$ 0.002
Taxol	0.004 $\pm$ 0.001	0.44 $\pm$ 0.19	0.003 $\pm$ 0.001	0.005 $\pm$ 0.001
Vinblastin	0.008 $\pm$ 0.003	0.50 $\pm$ 0.05	0.005 $\pm$ 0.002	0.007 $\pm$ 0.003

^a The resistance is highlighted in red.

Table S2. Summary of structure-activity relationship studies and excitation and emission maxima of molecules 1-15 and conjugates 16-18.



	R ₁	R ₂	R ₃	KB3-1	ABCB1	ABCC1	ABCG2	Excitation max (nm)	Emission max (nm)
KP-1	4-F	NH ₂	NH ₂	81.4	0	58.7	0	502	518
1	3-F	NH ₂	NH ₂	103.3	5.7	70	83.8	504	522
2	2-F	NH ₂	NH ₂	83.9	3.2	78.7	45.7	508	525
3	4-Cl	NH ₂	NH ₂	86.2	0	67.2	46.2	503	520
4	4-Br	NH ₂	NH ₂	117.9	46.4	84.1	109	503	521
5	4-H	NH ₂	NH ₂	78.7	0	67.9	65.5	500	518
6	4-CH ₂ CH ₂ CH ₃	NH ₂	NH ₂	258.3	0	141.2	199.1	499	519
7	4-OH	NH ₂	NH ₂	115.7	0	87.4	67	498	516
8	2-OH	NH ₂	NH ₂	71.4	0	69.5	65.9	499	515
9	4-F-2-COOCH ₃	NH ₂	NH ₂	0	0	0	0	500	519
10	5-F-2-COOCH ₃	NH ₂	NH ₂	51.4	0	17.1	41.7	501	521
11	4-F	O	OH	3.9	4	4	4.4	497	511
12	4-F	NHCH ₃	NHCH ₃	69.7	3.4	37	3.3	517	542
13	4-F	NH(CH ₂) ₃ NH ₂	NH(CH ₂) ₃ NH ₂	61.3	0	53.7	33	512	528
14	4-F	NH ₂	NH(CH ₂) ₃ NHC(O)CH ₂ OCH ₃	58.7	0	33.2	0	514	531
15	4-F	NH ₂	NH(CH ₂) ₁₀ NH ₂	124.9	0	88.6	75.9	514	528
conjugate 16	4-F	NH ₂	NH(CH ₂) ₃ NHCA4	113.7	12.6	87.1	82.1	509	528
conjugate 17	4-F	NH ₂	NH(CH ₂) ₃ NHNS38	61	0	40.1	0	528	547
conjugate 18	4-F	NH ₂	NH(CH ₂) ₃ NHMitoxantrone	59.6	7.6	50.1	17.3	515	529

Blue bars show the average fluorescent intensity values in KB3-1, KB/ABCB1, KB/ABCC1 and KB/ABCG2 cells calculated from fluorescent images by ImageJ. Excitation and emission maxima were evaluated in PBS (pH=7.4) at 1 μM.

Table S3. Primer sequences for quantitative RT-PCR

Gene	Forward Sequence	Reverse Sequence
GAPDH	GCACCGTCAAGGCTGAGAAC	TGGTGAAGACGCCAGTGGA
NANOG	CCTGTGATTTGTGGGCCTGA	CTCTGCAGAAGTGGGTGTTTG
OCT3/4 ^[1]	TTCAGCCAAACGACCATCTG	CACGAGGGTTTCTGCTTTGC
SOX2 ^[1]	CCCACCTACAGCATGTCCTACTC	TGGAGTGGGAGGAAGAGGTAAC

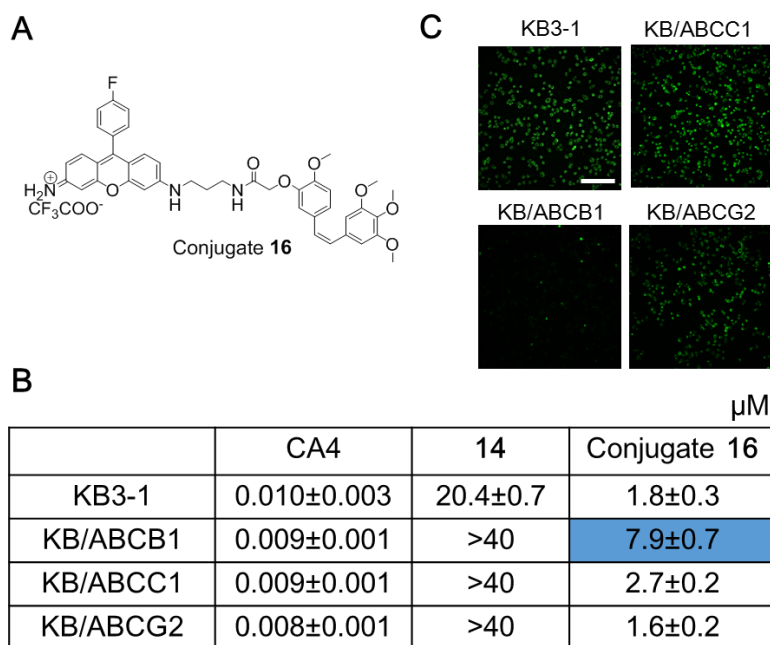


Figure S1. Selectivity of conjugate **16**. (A) Chemical structure of conjugate **16**. (B) IC₅₀ values of CA4, molecule **14**, and conjugate **16** for KB3-1, KB/ABCB1, KB/ABCC1, and KB/ABCG2 cells. The resistance is highlighted in blue. Data are shown as mean ± SD. n=3. (C) Fluorescence microscopic images of conjugate **16** (1 μM)-treated KB3-1, KB/ABCB1, KB/ABCC1, and KB/ABCG2 cells. The scale bar represents 200 μm.

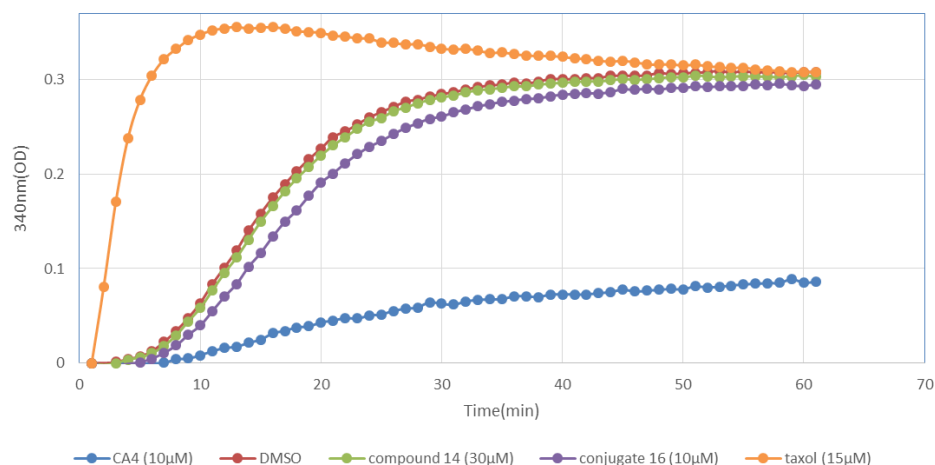


Figure S2. Effects of CA4 (10 μ M), compound **14** (30 μ M), conjugate **16** (10 μ M) and taxol (15 μ M) on tubulin polymerization. CA4 (microtubule destabilizer) and taxol (microtubule stabilizer) serve as positive controls. Compound **14** and DMSO are negative controls. Tubulin polymerization was detected by measuring UV absorbance at 340 nm. UV light at 340 nm is scattered by microtubules to an extent that is proportional to the concentration of microtubule polymer.

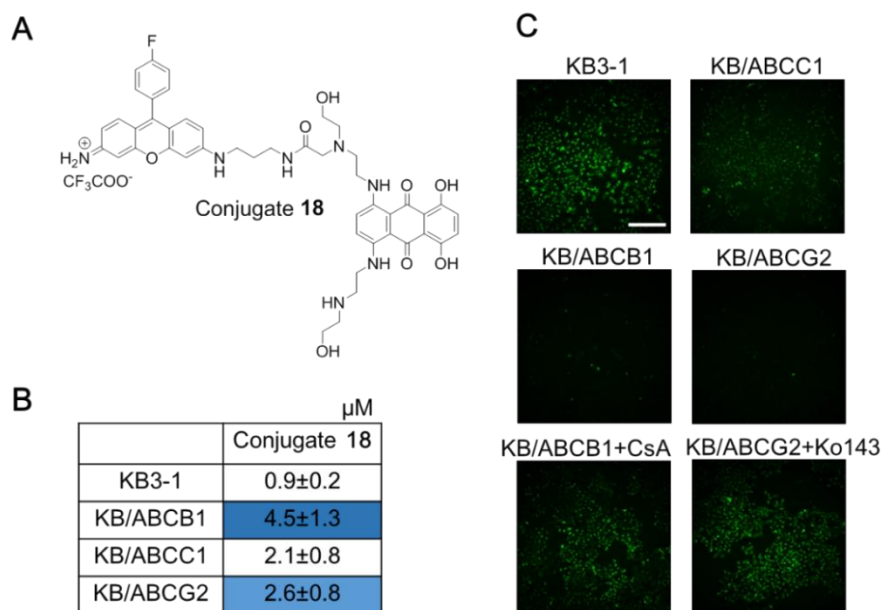


Figure S3. Selectivity of conjugate **18**. (A) Chemical structure of conjugate **18**. (B) IC₅₀ values of mitoxantrone and conjugate **18** for KB3-1, KB/ABCB1, KB/ABCC1, and KB/ABCG2 cells. The levels of resistance are highlighted in blue. Data are shown as mean $\pm$ SD. n=3. (C) Fluorescence microscopic images of conjugate **18** (10 μ M)-treated KB3-1, KB/ABCB1, KB/ABCC1, and KB/ABCG2 cells in the presence or absence of CsA (10 μ M) or Ko143 (1 μ M). The scale bar represents 200 μ m.

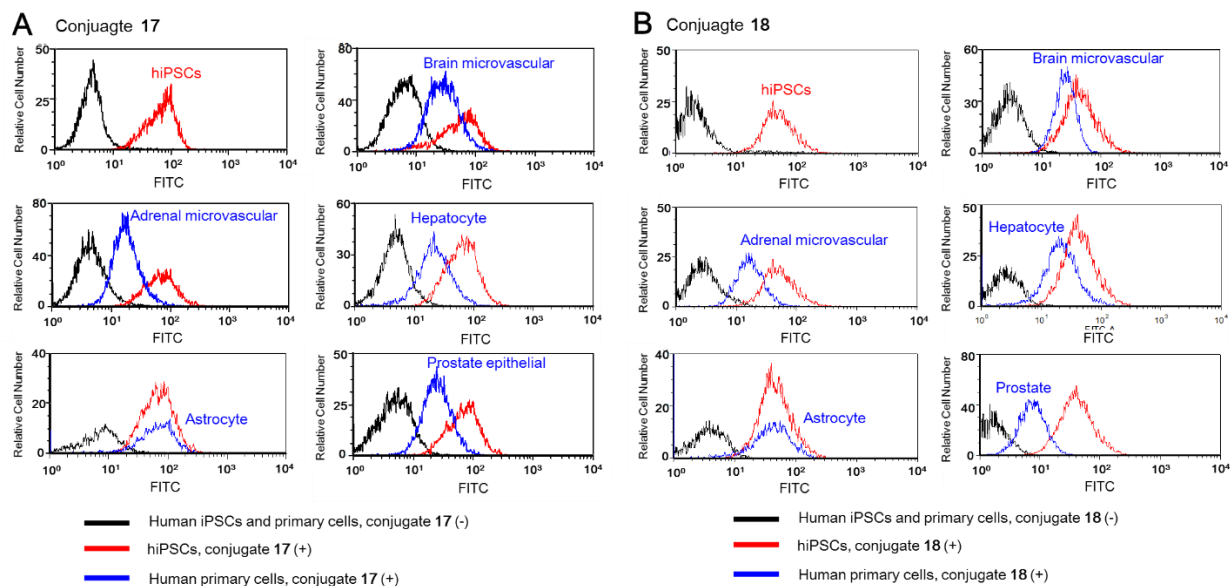


Figure S4. Staining patterns of conjugate **17** (A) and **18** (B) with human iPSC and somatic cells. The cells were incubated with 1 μ M conjugate **17** or 10 μ M conjugate **18** at 37°C for 2 h. The cells were then washed, disassociated, and analyzed with a flow cytometer on FITC channel. Conjugate **17** and **18** show similar staining pattern with KP-1^[6], although the fluorescent intensity of conjugate **17** and **18** are weaker than that of KP-1. Conjugate **17** is more selective than conjugate **18** in hepatocytes.

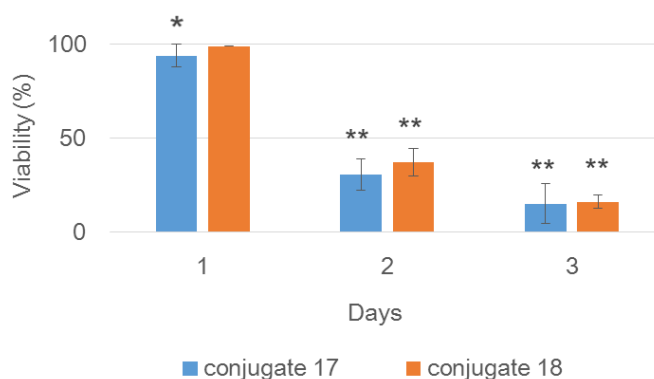


Figure S5. Time-course evaluation of cytotoxicities of conjugate **17** and **18** to hiPSCs at 5 μ M. Strong cytotoxicity was observed from day 2. Three-day incubation is desirable to ensure efficient removal of hiPSCs. * $p < 0.05$; ** $p < 0.01$. P values were calculated by comparing the viabilities of the conjugate-treated cells with a DMSO (0.1%) control at day 1, 2 and 3. Data are shown as mean $\pm$ SD. $n=3$.

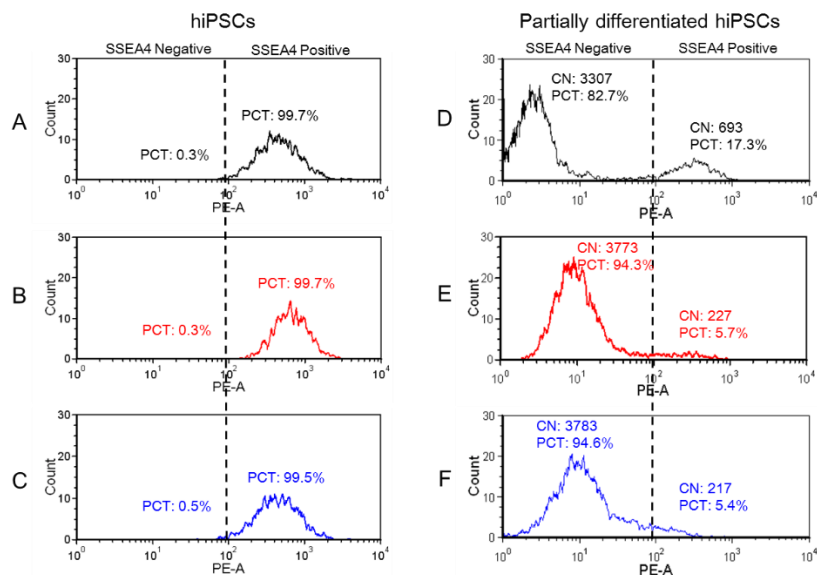


Figure S6. Fluorescence-activated cell sorting (FACS) analysis of partially differentiated hiPSCs after 72-h treatment with 0.1% DMSO (D), 5 μ M conjugate **17** (E), or 5 μ M conjugate **18** (F). There was a crossover of the fluorescence spectra between the KP-1 conjugates (**17** and **18**) and the PE-conjugated-anti SSEA4. To define the SSEA4-positive population, we performed FACS analysis of hiPSCs themselves after 0.5-h treatment with 0.1% DMSO (A), 5 μ M conjugate **17** (B), or 5 μ M conjugate **18** (C). The cells were immunostained by a PE-conjugated anti-SSEA4 antibody. The cell population was fixed as 4000 for analysis. It is evident that after treatment with conjugate **17** or **18**, the number of SSEA4-positive undifferentiated cells was reduced from 17.3% to 5.7% or 5.4%, respectively. CN: cell number; PCT: percentage.

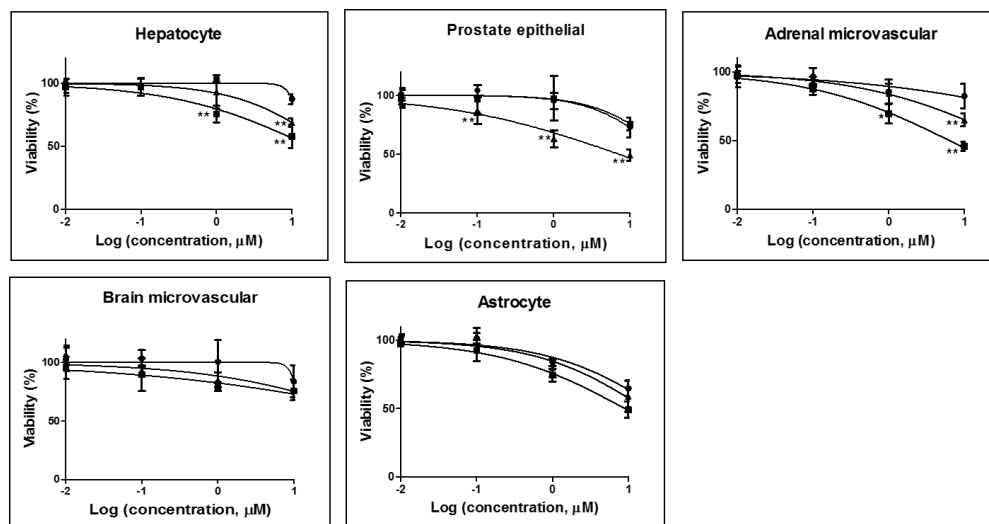


Figure S7. Viability of human primary cells when treated with conjugate **17** in the absence (●) or presence of CsA (■) or Ko143 (▲). Viability was determined by a WST-8-based colorimetric assay (Cell Counting Kit-8, Dojindo). Absorbance was measured at 450 nm. Transporter inhibitors, cyclosporine A (10 μ M) or Ko143 (1 μ M), were added 1 h prior to incubation with conjugate **17**. * p < 0.05; ** p < 0.01. P values were calculated by

comparing the viabilities of the inhibitor-treated cells with those of non-treated cells at each dosage. Data are shown as mean $\pm$ SD. n=3.

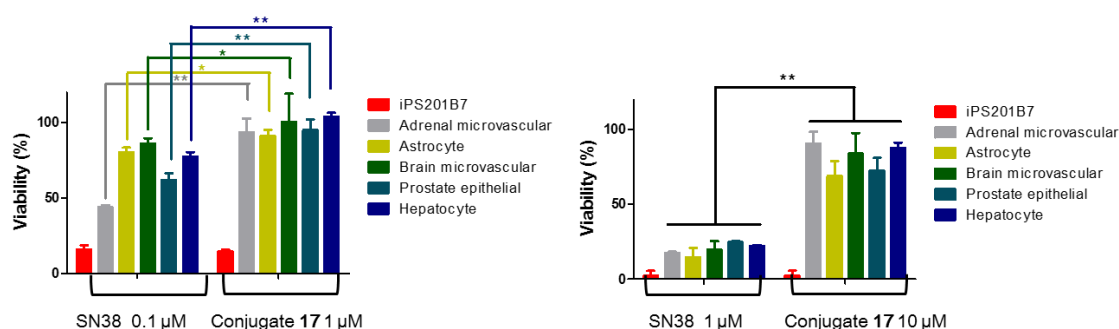


Figure S8. Comparison of selectivities of SN38 and conjugate **17**. hiPS or human primary somatic cells (adrenal microvascular, astrocyte, brain microvascular, prostate epithelial and hepatocyte) were treated with SN38 (0.1 or 1 μ M) or conjugate **17** (1 or 10 μ M) for 72 h. (A) Comparison of SN38 (0.1 μ M) with conjugate **17** (1 μ M), where viability levels of hiPSCs are ~20%; (B) Comparison of SN38 (1 μ M) with conjugate **17** (10 μ M), where viability levels of hiPSCs are ~5%. Viability was determined by WST-8-based colorimetric assay. It is evident that conjugate **17** exhibits higher selectivity than SN38. * p < 0.05; ** p < 0.01. Data are shown as mean $\pm$ SD. n=3.

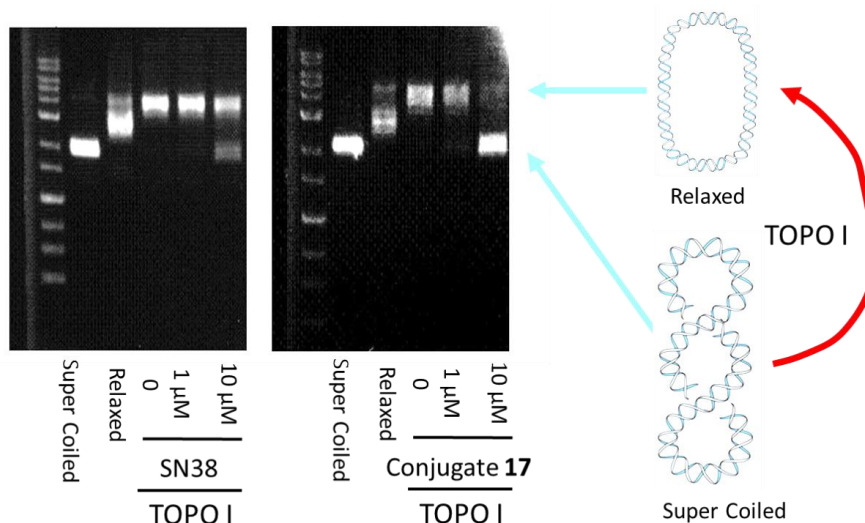


Figure S9. Inhibition of topoisomerase I by SN38 and conjugate **17**. Supercoiled DNA was incubated at 37°C for 30 min with recombinant human topoisomerase I, which had been premixed with SN38 (0, 1, or 10 μ M) or conjugate **17** (0, 1, or 10 μ M). S.C. DNA: supercoiled DNA; TOPO I: human recombinant topoisomerase I. The reaction mixture was loaded onto 1% agarose gel. After electrophoresis, the gels were stained with ethidium bromide in TAE buffer, and photographed under UV illumination.

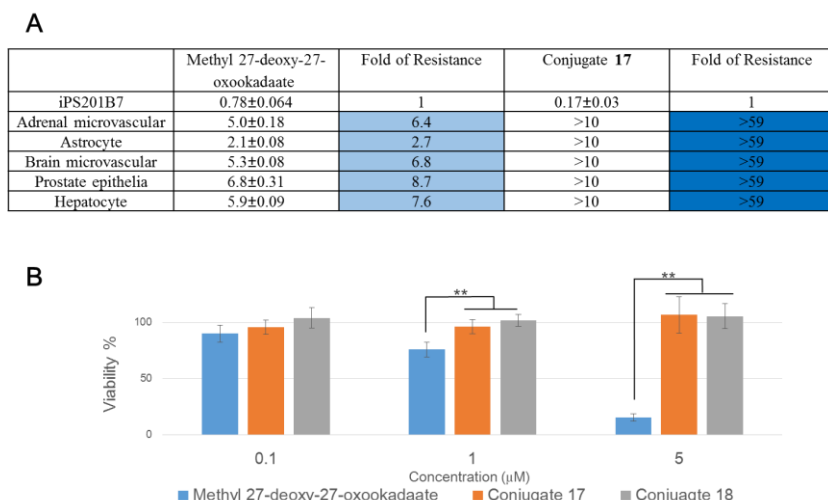


Figure S10. Comparison of selectivities of methyl 27-deoxy-27-oxookadaate, conjugate **17**, and conjugate **18**. (A) IC₅₀ values of methyl 27-deoxy-27-oxookadaate and conjugate **17** for human iPS and somatic cells. Human somatic cells exhibited more resistance to conjugate **17** than to methyl 27-deoxy-27-oxookadaate. The levels of resistance are highlighted in blue. Data are shown as mean ±SD. n=3. (B) Viabilities of hiPSC-derived fully differentiated cells after 72-h treatment with methyl 27-deoxy-27-oxookadaate, conjugate **17** or conjugate **18**. Methyl 27-deoxy-27-oxookadaate showed marked cytotoxicity at 1 and 5 μM. Both conjugate **17** and conjugate **18** exhibited little effects on the viability of hiPSC-derived fully differentiated cells even at 5 μM. ***p* < 0.01. Data are shown as mean ±SD. n=3.

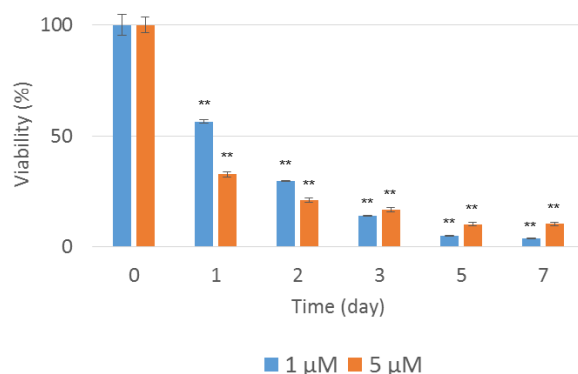


Figure S11. Viability of leftover hiPSCs. hiPSCs were incubated with conjugate **17** (1 or 5 μM) for 72 h. After extensive wash with PBS, the leftover iPSCs were further incubated in the growth medium for another 7 days. The viabilities of the cells were determined by WST-8-based colorimetric assay every 24 h. The viabilities of the initial leftover hiPSCs on day 0 were calculated as 100%. The residual iPSCs failed to proliferate, displaying delayed cell death. ***p* < 0.01. Data are shown as mean ±SD. n=3.

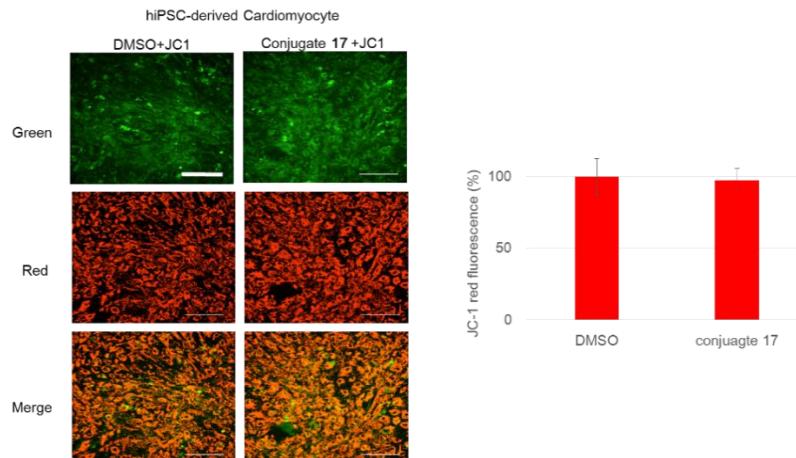


Figure S12. Mitochondria membrane potential analysis of hiPSC-derived cardiomyocytes after 72-h treatment with conjugate **17**. The cardiomyocytes were stained by JC-1, a mitochondria membrane potential probe. Mitochondria depolarization is indicated by a decrease in the red to green fluorescence intensity ratios. When mitochondria membrane potentials are high, the dye forms aggregates and stains the mitochondria bright red. After 72-h treatment with a DMSO control (0.1%) or 5 μ M of conjugate **17**, the cells were washed and stained by 10 μ g/mL JC-1 for 10 min. The cells were then washed by PBS and imaged with a fluorescent confocal microscopy. The fluorescence intensities of the J-aggregates (red) in the cells were recorded with a microplate reader (Ex/Em = 550/610 nm). The fluorescence intensity of the DMSO control was calculated as 100%. The scale bar represents 200 μ m. Data are shown as mean $\pm$ SD. n=3.

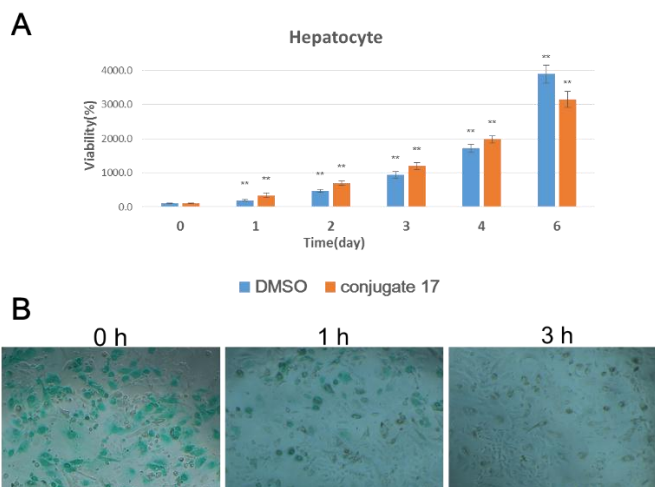


Figure S13. Functional analysis of human primary hepatocytes after 72-h treatment with 5 μ M of conjugate **17**. (A) Proliferation levels of the hepatocytes after 72-h treatment with DMSO (0.1%) or 5 μ M of conjugate **17**. The conjugate **17**-treated hepatocytes exhibited proliferation comparable to that of non-treated cells. $**p < 0.01$. Data are shown as mean $\pm$ SD. n=3. (B) Cellular uptake and excretion of indocyanine green (ICG) in hepatocytes. After 1-h treatment with 1 mg/mL ICG at 37°C, the cells were then washed by PBS and incubated in a growth medium. The staining pattern of ICG was observed under a microscope after 0, 1 and 3 h. The cellular uptake of ICG was clearly observed at 0 h, while the excretion of ICG was evident after 1 and 3 h.

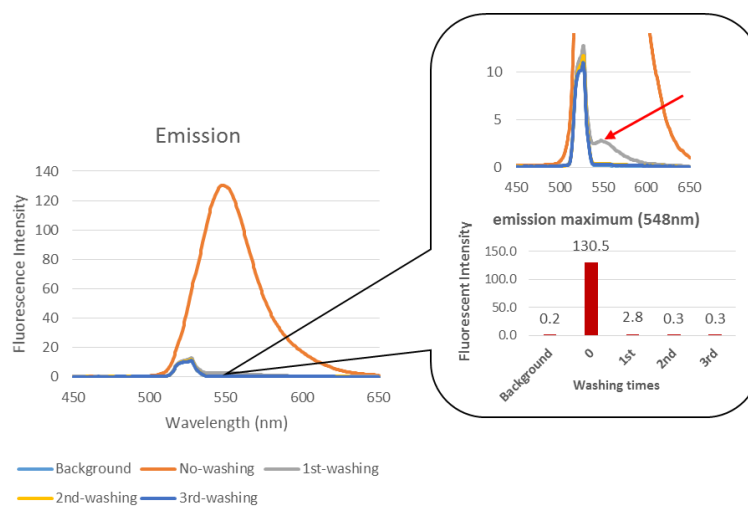
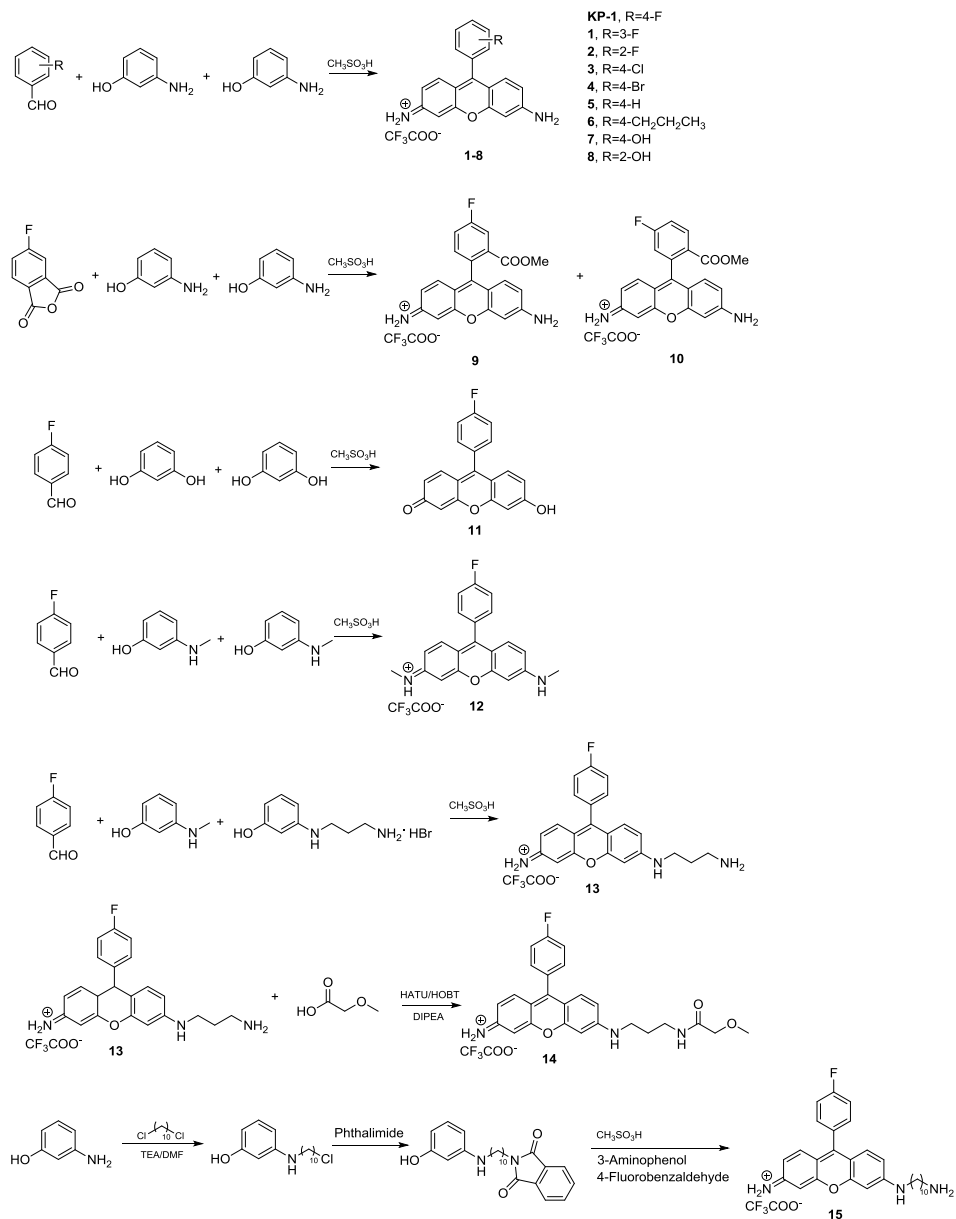
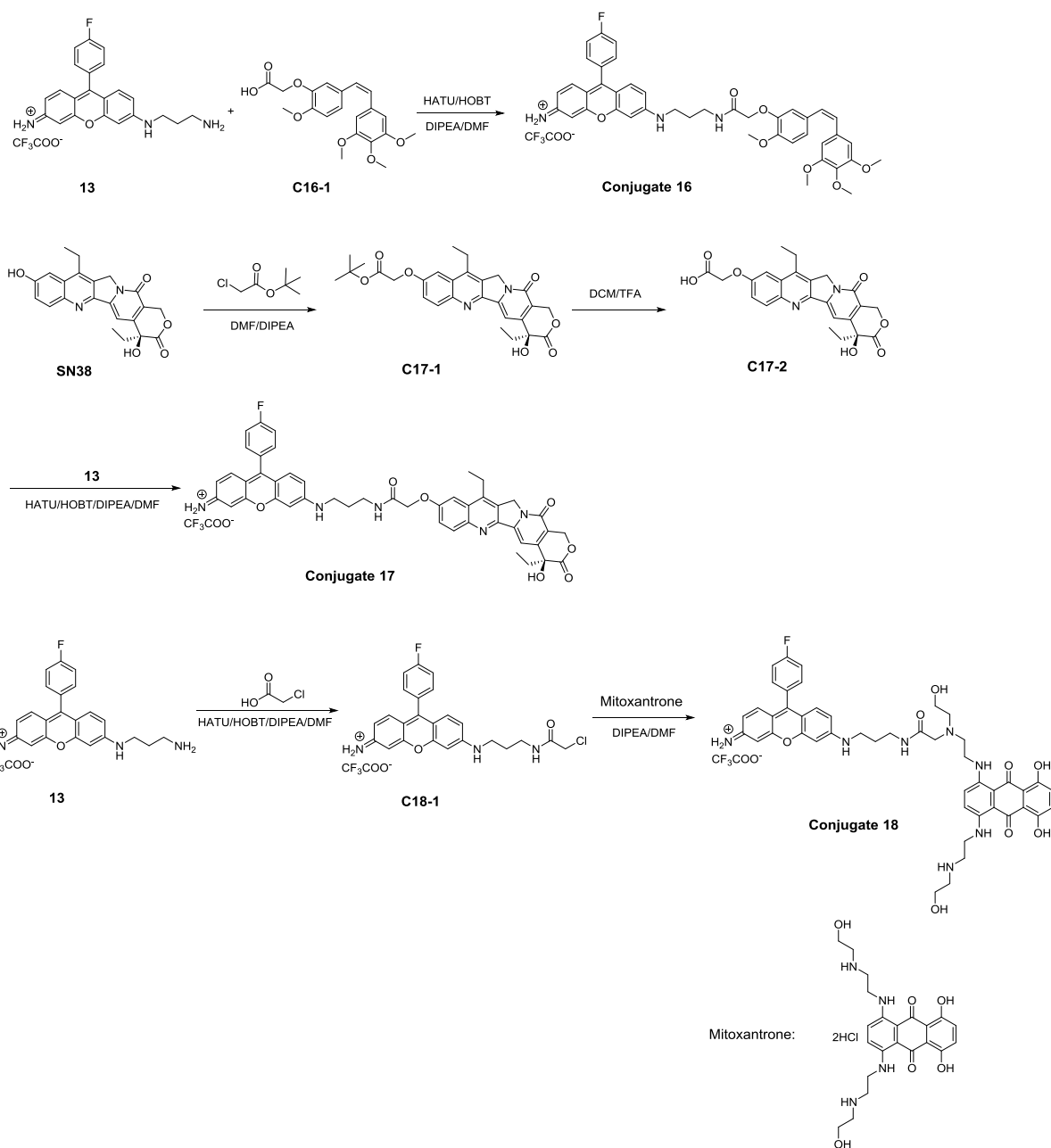


Figure S14. Removal of conjugate **17**. Partially differentiated hiPSCs were prepared by incubation for 48 h with all-trans retinoic acid (0.5 μM), then treated with conjugate **17** (5 μM) for 72 h. After 3 d of cell treatment with conjugate **17**, the medium was collected. The cells were then washed three times with 2 mL fresh ES medium, and the washing medium was collected. The medium samples were centrifuged and analyzed for fluorescence intensity (excitation at 528 nm).



Scheme S1. Synthesis of compounds **1-15**.



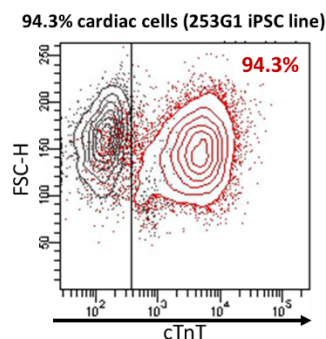
Scheme S2. Synthesis of conjugates **16**, **17** and **18**.

Experimental Procedures

Materials and Methods. Chemical reagents were purchased from Sigma-Aldrich Japan, Wako Pure Chemical Industries, Ltd. (Japan), and Tokyo Chemical Industry (TCI), and used as received. The solvents used for chemical synthesis were dried prior to use. High performance liquid chromatography was performed with a Shimadzu LC-2010C and a Hitachi HPLC system (L-7260 autosampler, L-7150 pump, D-7600 interface, L-7410 UV detector). Mass spectra were recorded by a Shimadzu LCMS-2010 in ESI mode. High resolution mass spectra of three conjugates were obtained using a JEOL JMS LG-2000 in FAB mode. Solution ^1H NMR spectra were collected on JEOL JNM-ECP 300 MHz or JEOL JNM-ECA 600MHz spectrometers. Fluorescence spectra were recorded using a LS55 Fluorescence Spectrometer (Perkin Elmer).

Cell Cultures. KB3-1 and KB3-1-derived cell lines (KB/ABCB1, KB/ABCC1, and KB/ABCG2) were cultured in Dulbecco's modified Eagle's Medium (DMEM, Gibco) with 10% heat-inactivated fetal bovine serum (Equitech-Bio, Inc., Kerrville, TX) and 1% antibiotics (Sigma Aldrich, Inc., St. Louis, MO). SNL feeder cells were cultured in Dulbecco's modified Eagle's Medium containing 4.5 g/L glucose (DMEM-high glucose, Nacalai tesque, Japan) with 7% heat-inactivated fetal bovine serum (Equitech-Bio, Inc., Kerrville, TX), 1% L-glutamine (200 mM, Gibco) and 1% antibiotics. Human somatic primary cells were cultured in MSCM (bronchial epithelial cells), BEGM (adrenal microvascular cells), or CSC (astrocytes, brain microvascular cells, prostate epithelial cells; and hepatocytes) from Cell Systems Corporation. For hiPSCs, gelatin-precoated dishes seeded with SNL feeder cells, and ES primate medium (ReproCELL) containing 4 ng/mL bFGF (ReproCELL) were used for regular maintenance. For hiPSC-derived cardiomyocytes, the cells were cultured on a laminin (iMatrix-511, nippi)-precoated 96-well plate with a cardiomyocyte culture medium containing 20% FBS. All cells were maintained in a humidified incubator with 5% CO_2 at 37°C.

Preparation of hiPSC-derived cardiomyocytes. The hiPSC-derived cardiomyocytes were derived from hiPSCs (253G1) as reported protocol.^[2] Fluorescence-activated cell sorting (FACS) analysis indicated that 94.3% of the cells were cardiac troponin T positive.



Representative flow cytometry data for cardiac Troponin T (cTnT)-positive cells of iPSCs-derived cardiomyocytes. Red indicates cTnT-positive cardiomyocytes. Gray indicates negative controls without the primary antibody. A total of 30,000 cells were measured in each sample by FACSCanto II. For flow cytometry, single cells from hPSC-derived cardiac colonies were stained by primary antibodies against cTnT (1:100) and Alexa488-conjugated anti-mouse IgG second antibody. Cells were analyzed using a FACSCanto II Flow Cytometer (BD Biosciences). Data was analyzed using FACSDiva software (BD Biosciences).

Preparation of Partially Differentiated hiPSCs. Twenty-four hours after subculture of hiPSCs, 0.5 μM all-trans retinoic acid was added, and the cells were incubated for another 72 h. The partially differentiated hiPSCs were then subjected to treatment with a selected compound for 72 h. For hiPSCs cultured under a feeder-free

condition (colony formation assay), 0.2 μ M all-trans retinoic acid was used, and the cells were incubated for another 48 h.

Cell-based Assays. Test cells were plated on 96-well plates at 5000 per well. A selected compound was added at varied concentrations, 24 h after plating. After 24 or 72 h of incubation, the cells were washed with PBS. Viability was determined by WST-8-based colorimetric assay (Cell Counting Kit-8, Dojindo). Absorbance was measured at 450 nm, and the IC₅₀ was calculated based on the dose-response values. For experiments with transporter inhibitors, cyclosporine A (10 μ M) or Ko143 (1 μ M) was added 1 h prior to incubation with the selected compound.

Evaluation of viability of hiPSC. Human ESCs or hiPSCs were plated on 6-well plates and grown for 72 h, then treated with a selected compound for 72 h. Cells were fixed for 1 h with 4% paraformaldehyde, washed twice with PBS, and stained for 15 min with 1% crystal violet solution (Sigma Aldrich). Plates were then washed with PBS and distilled water for 3-5 times, and air-dried before taking images. For quantification, 1% SDS solution was added to each well and incubated for 15 min. Absorbance of the elution was measured at 570 nm.

Fluorescence Microscope Imaging of KB3-1 Cell Lines and hiPSC Colonies. KB3-1 and KB3-1-derived cell lines were plated on 96-well plates (Nunc 165305, Thermo Fisher Scientific) at 5000 per well. Selected compounds (1 μ M or 10 μ M) were added 24 h after plating, and the cells were incubated for 2 h at 37°C. The treated cells were rinsed with PBS and observed in DMEM containing 10% FBS, using a confocal microscope (Cell Voyager 1000, Yokogawa Electric Corporation) with laser excitation at 405, 488, and 561 nm. For quantitative analysis, fluorescence intensities were measured and analyzed using NIH ImageJ, version 1.30. The hiPSCs (clone #201B7) were plated on SNL feeder cells in μ -Dish (35 mm high, ibidi). Donut-shaped colonies of iPSCs were obtained after 6 d of incubation. The colonies were then incubated with KP-1 (1 μ M), conjugate **17** (1 μ M), or conjugate **18** (10 μ M) for 2 h at 37°C. The treated cells were rinsed with PBS and observed in ES primate medium containing 4 ng/mL bFGF. Fluorescence images were taken using a confocal microscope (Cell Voyager 1000, Yokogawa Electric Corporation) with laser excitation at 405, 488, and 561 nm.

Tubulin Polymerization Assay. A Tubulin Polymerization Assay Kit was purchased from Cytoskeleton (BK006P), and the assay was performed according to the manufacturer's instructions. The final concentration of each component was 80 mM PIPES (pH 6.9), 2 mM MgCl₂, 0.5 mM EGTA, 10.2% glycerol, 1 mM GTP and 3mg/mL tubulin. The reaction system was pre-warmed at 37°C without tubulin, then initiated by the addition of tubulin. Absorption at 340 nm was measured immediately after addition of tubulin and at 1 min intervals for 60 min, with a SpectraMax M5 (Molecular Devices). CA4 and Taxol were used as positive controls; molecule **14** and DMSO alone were used as negative controls.

Alkaline Phosphatase Assays. The alkaline phosphatase activity of hiPSCs was measured with an Alkaline Phosphatase Substrate kit (SK-5300, Vector® Blue). hiPSC colonies and partially differentiated hiPSCs were prepared as described above. The cells were then treated with SK-5300, incubated for 1 h at room temperature, rinsed, and finally maintained in PBS buffer.

Quantitative Polymerase Chain Reaction (QPCR). Partially differentiated hiPSCs were prepared by

incubation for 48 h with all-trans retinoic acid (0.5 μ M), then treated with the selected compound for 72 h. Total mRNA was isolated (ISOGEN), and cDNA was synthesized (PrimeScript, TaKaRa). QPCR was carried out on a 7500 Fast Real-Time PCR System (Applied Biosystems), using Fast SYBR®Green Master Mix. The primer sequences are shown in **Table S4**.

Fluorescence-Activated Cell Sorting (FACS) Analysis. Partially differentiated hiPSCs were treated with DMSO or each conjugate for 72 h. The residual cells were then washed by PBS, dissociated by accutase, and fixed by methanol. The fixed cells were then stained by a PE-anti-SSEA4 antibody (BD Biosciences) and analyzed on a BD FACSAria II flow cytometer.

Colony Formation Assay of Partially Differentiated hiPSCs. hiPSCs (253G1) were seeded in the presence of Y-27632, a ROCK inhibitor, on a 6-well plate that had been pre-coated by laminin (iMatrix-511). The cells were cultured with a feeder free medium (complete mTeSR1, StemCell Technologies) for 2 days. The medium was then replaced with ES primate medium without bFGF and the cells were treated with all-trans retinoic acid (0.2 μ M) for 2 days to yield a mixture of partially differentiated cells. The partially differentiated cell mixture was treated with conjugate **17** (1 μ M) or DMSO (0.1%) for another 3 days. The surviving cells were washed and further cultured in the growth medium (complete mTeSR1) for another 7 days. The cells were next fixed and immunostained by a monoclonal anti-Oct4 antibody (Santa Cruze Biotechnology sc-5279). The Oct4 positive cells were subsequently visualized by a horseradish peroxidase-conjugated goat anti-mouse immunoglobulins polyclonal antibody and 3,3'-diaminobenzidine (DAB)^[3]. The whole cell populations were visualized by crystal violet.

Topoisomerase I Inhibition Assays. The topo I inhibitory activity of conjugate **17** was tested by supercoiled DNA relaxation assay. Supercoiled DNA (TopoGEN) was incubated at 37°C for 30 min with recombinant human topoisomerase I (wild type protein, TopoGEN), which had been premixed with conjugate **17** (0, 1, or 10 μ M) or with SN38 (0, 1, or 10 μ M) as a control. The reaction was terminated by stop buffer (TopoGEN), and the reaction mixture was loaded onto 1% agarose gel. After electrophoresis, the gel were stained with ethidium bromide in TAE buffer, and photographed under UV illumination.

Mitochondria Membrane Potential Assay. After 72-h treatment with conjugate **17** (5 μ M), hiPSC-derived cardiomyocytes were washed and incubated in a growth medium containing 10 μ g/mL JC-1 (invitrogen) for 10 min. The cells were then washed by PBS and analyzed under a confocal microscope (Cell Voyager 1000, Yokogawa Electric Corporation) with laser excitation at 488 and 561 nm. For quantification, the fluorescence intensities of JC-1 aggregates (red) in the cells were recorded by a microplate reader (MTP-880) with excitation wavelength at 550 nm and emission wavelength at 610 nm.

Proliferation of primary hepatocytes and leftover hiPSCs. After 72-h treatment with conjugate **17** (5 μ M), human primary hepatocytes (ACBRI 3716; passage 4) were washed, collected, and re-seeded on a gelatin-precoated 96-well plate. Their viability was determined by a WST-8-based colorimetric assay (Cell Counting Kit-8, Dojindo) at day 0, 1, 2, 3, 4, and 6. For hiPSCs (253G1), after 72-h treatment with conjugate **17** (1 or 5 μ M), the leftover hiPSCs were washed by PBS and further

incubated in the growth medium (ES primate medium containing 4 ng/mL bFGF, ReproCELL) for another 7 days. The viabilities of the cells were determined by WST-8-based colorimetric assay every 24 h.

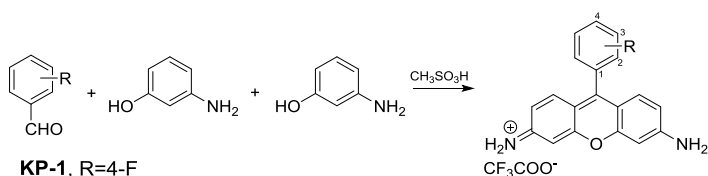
Cellular Uptake and Excretion of Indocyanine Green (ICG). After 72-h treatment with conjugate **17** (5 μ M), human primary hepatocytes were washed and incubated in a growth medium containing 1 mg/mL ICG (wako) at 37°C for 1 h. The cells were then washed by PBS and incubated with a fresh grow medium. The uptake levels of ICG were examined by a microscopy immediately (0 h), while the excretion levels of ICG were examined after 1 or 3 h.

Removal of Conjugate 17. Partially differentiated hiPSCs were prepared by incubation for 48 h with all-trans retinoic acid (0.5 μ M), then treated with conjugate **17** (5 μ M) for 72 h. After 3 d of cell treatment with conjugate **17**, the medium was collected. The cells were then washed three times with 2 mL fresh ES medium, and the washing medium was collected. The medium samples were centrifuged and analyzed for fluorescence intensity (excitation at 528 nm).

Cytotoxicity of Methyl 27-deoxy-27-oxookadaate, Conjugate 17 and Conjugate 18 to hiPSC-Derived Fully Differentiated Cells. hiPSCs were incubated with all-trans retinoic acid (0.5 μ M) for 10 days to achieve fully differentiation.^[4] The hiPSC-derived fully differentiated cells were re-seeded on a gelatin-precoated 24-well plate. After 72-h incubation with varied concentrations of methyl 27-deoxy-27-oxookadaate, conjugate **17** or conjugate **18**, cell viabilities were evaluated by a WST-8-based colorimetric assay.

Chemical Synthesis

Synthesis of compounds 1-8



KP-1, R=4-F

1, R=3-F

2, R=2-F

3, R=4-Cl

4, R=4-Br

5, R=4-H

6, R=4- $\text{CH}_2\text{CH}_2\text{CH}_3$

7, R=4-OH

8, R=2-OH

General procedure for the synthesis of compounds 1-8. A suspension of 3-aminophenol (3.0 eq) and substituted benzaldehyde (1.0 eq) in methanesulfonic acid was stirred at 130°C for 24 h. The reaction mixture was then cooled to ambient temperature, diluted with ice water, adjusted to pH $\approx$ 6 by K_2CO_3 , and filtered. The precipitate was washed with water, dissolved in methanol, and concentrated under reduced pressure. The residue was purified by HPLC (Inertsil ODS-3, methanol: 0.1% (v/v) TFA- H_2O = 0:1 to 1:0).

Compound 1. Purple oil. Yield: 0.5%. ^1H -NMR (300 MHz, CD_3OD) δ 7.72-7.64 (m, 1H), 7.44-7.39 (m, 1H), 7.29-7.24 (m, 4H), 6.86 (dd, J = 9.1, 2.2 Hz, 2H), 6.82 (d, J = 2.2 Hz, 2H); ESIMS m/z =305 $[\text{M}]^+$

Compound 2. Purple oil. Yield: 0.6%. $^1\text{H-NMR}$ (300 MHz, CD_3OD) δ 7.76-7.69 (m, 1H), 7.51-7.40 (m, 3H), 7.23 (dd, $J = 6.3, 1.1$ Hz, 2H), 6.87 (dd, $J = 9.1, 2.2$ Hz, 2H), 6.83 (d, $J = 2.2$ Hz, 2H); ESIMS $m/z=305$ $[\text{M}]^+$

Compound 3. Purple oil. Yield: 1.8%. $^1\text{H-NMR}$ (300 MHz, CD_3OD) δ 7.68 (dd, $J = 5.5, 1.9$ Hz, 2H), 7.46 (dd, $J = 6.5, 2.1$ Hz, 2H), 7.28 (d, $J = 9.1$ Hz, 2H), 6.87 (dd, $J = 9.1, 2.2$ Hz, 2H), 6.82 (d, $J = 9.1$ Hz, 2H); ESIMS $m/z=321$ $[\text{M}]^+$

Compound 4. Purple oil. Yield: 1.5 %. $^1\text{H-NMR}$ (300 MHz, CD_3OD) δ 7.84 (dd, $J = 6.6, 1.9$ Hz, 2H), 7.39 (dd, $J = 6.6, 1.9$ Hz, 2H), 7.28 (d, $J = 9.1$ Hz, 2H), 6.86 (dd, $J = 9.1, 2.2$ Hz, 2H), 6.81 (d, $J = 2.2$ Hz, 2H); ESIMS $m/z=365$ $[\text{M}]^+$

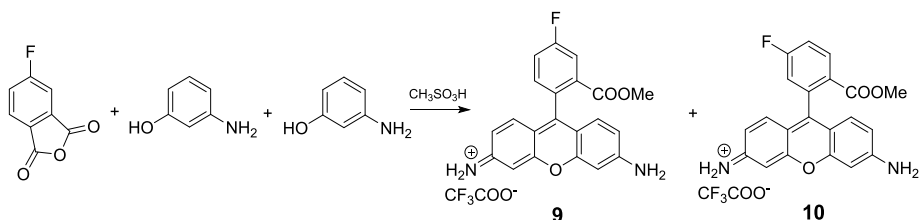
Compound 5. Purple oil. Yield: 1.3%. $^1\text{H-NMR}$ (300 MHz, CD_3OD) δ 7.67-7.64 (m, 3H), 7.47-7.44 (m, 2H), 7.29 (d, $J = 9.1$ Hz, 2H), 6.86 (dd, $J = 9.1, 2.2$ Hz, 2H), 6.82 (d, $J = 1.9$ Hz, 2H); ESIMS $m/z=287$ $[\text{M}]^+$

Compound 6. Purple oil. Yield: 0.7%. $^1\text{H-NMR}$ (300 MHz, CD_3OD) δ 7.49 (d, $J = 8.3$ Hz, 2H), 7.38 (dd, $J = 8.3, 1.9$ Hz, 2H), 7.29 (d, $J = 9.1$ Hz, 2H), 6.85 (dd, $J = 9.1, 1.9$ Hz, 2H), 6.82 (d, $J = 2.2$ Hz, 2H), 2.76 (t, $J = 7.7$ Hz, 2H), 1.82-1.70 (m, 2H), 1.03 (t, $J = 7.4$ Hz, 3H); ESIMS $m/z=329$ $[\text{M}]^+$

Compound 7. Purple oil. Yield: 0.2%. $^1\text{H-NMR}$ (300 MHz, CD_3OD) δ 7.44 (d, $J = 9.1$ Hz, 2H), 7.31 (dd, $J = 6.6, 2.2$ Hz, 2H), 7.05 (dd, $J = 6.6, 1.9$ Hz, 2H), 6.86 (dd, $J = 9.1, 1.9$ Hz, 2H), 6.80 (d, $J = 2.2$ Hz, 2H); ESIMS $m/z=303$ $[\text{M}]^+$

Compound 8. Purple oil. Yield: 0.2%. $^1\text{H-NMR}$ (300 MHz, CD_3OD) δ 7.42 (d, $J = 8.7$ Hz, 2H), 7.31 (dd, $J = 6.6, 1.9$ Hz, 2H), 7.05 (dd, $J = 6.6, 1.9$ Hz, 2H), 6.85 (dd, $J = 9.1, 2.2$ Hz, 2H), 6.78 (d, $J = 2.2$ Hz, 2H); ESIMS $m/z=303$ $[\text{M}]^+$

Synthesis of compounds 9 and 10



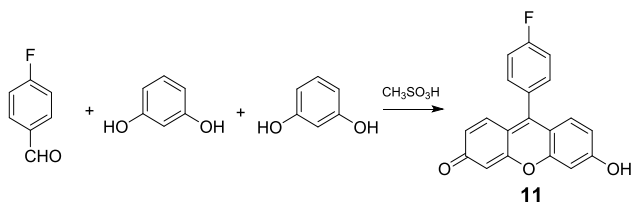
A suspension of 5-fluoro-1, 3-isobenzofurandione (498 mg, 3 mmol) and 3-aminophenol (982 mg, 9 mmol) in methanesulfonic acid (9 mL) was stirred at 130°C for 24 h. After cooling down to ambient temperature, MeOH (25 mL) was added to the reaction and refluxed overnight. The reaction mixture was then cooled to ambient temperature, diluted with ice water, adjusted to $\text{pH}\approx 6$ by K_2CO_3 , and then filtered. The precipitate was washed with water, dissolved in methanol and concentrated under reduced pressure. The residue was purified by HPLC (Inertsil ODS-3, methanol: 0.1% (v/v) TFA- $\text{H}_2\text{O} = 0:1$ to $1:0$) to give 0.9 mg of compound **9** and 0.8 mg of compound **10** as purple oil.

Compound 9. Purple oil. Yield: 0.8%. $^1\text{H-NMR}$ (300 MHz, CD_3OD) δ 8.06 (dd, $J = 9.6, 2.1$ Hz, 1H), 7.66 (ddd, $J = 8.1, 8.1, 2.8$ Hz, 1H), 7.49 (dd, $J = 8.9, 5.5$ Hz, 1H), 7.22 (d, $J = 8.9$ Hz, 2H), 7.15 (d, $J = 2.1$ Hz, 1H), 6.99 (dd, $J = 9.0, 2.0$ Hz, 1H), 6.96 (dd, $J = 9.6, 2.0$ Hz, 1H), 6.92 (d, $J = 2.0$ Hz, 2H), 3.65 (s, 3H); ESIMS $m/z=363$ $[\text{M}]^+$

Compound 10. Purple oil. Yield: 0.7%. $^1\text{H-NMR}$ (300 MHz, CD_3OD) δ 8.40 (dd, $J = 8.9, 5.5$ Hz, 1H), 7.57 (ddd, $J = 8.3, 8.3, 2.8$ Hz, 1H), 7.32 (dd, $J = 8.3, 2.8$ Hz, 1H), 7.23 (d, $J = 9.6$ Hz, 2H), 7.16 (d, $J = 2.1$ Hz, 1H), 6.99

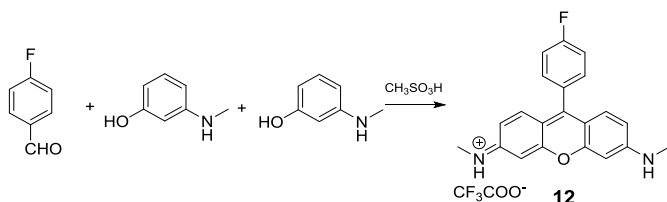
(dd, $J = 9.6, 2.1$ Hz, 1H), 6.97 (dd, $J = 9.6, 2.1$ Hz, 1H), 6.92 (d, $J = 2.1$ Hz, 1H), 3.62 (s, 3H); ESIMS $m/z = 363$ $[M]^+$

Synthesis of compound 11



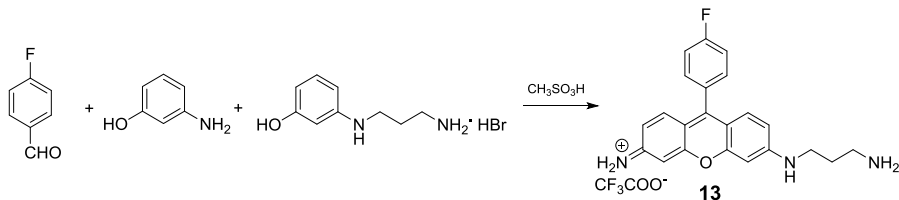
A suspension of resorcinol (1.32 g, 12 mmol) and 4-fluorobenzaldehyde (422 μ l, 4 mmol) in methanesulfonic acid (12 mL) was stirred at 130°C for 24 h. The reaction mixture was then cooled to ambient temperature, diluted with ice water, adjusted to pH $\approx$ 6 by K_2CO_3 and then filtered. The precipitate was washed with water, dissolved in methanol and concentrated under reduced pressure. The residue was purified by HPLC (Inertsil ODS-3, methanol: 0.1% (v/v) TFA- H_2O = 0:1 to 1:0) to give 3 mg compound **11** as purple oil. Yield: 0.1%. 1H -NMR (300 MHz, CD_3OD) δ 7.70-7.61 (m, 4H); 7.51-7.48 (m, 2H), 7.24-7.15 (m, 4H); ESIMS $m/z = 307$ $[M+H]^+$

Synthesis of compound 12



A suspension of 3-(methylanilino)phenol (411 mg, 3.3 mmol) and 4-fluorobenzaldehyde (176 μ l, 1.67 mmol) in methanesulfonic acid (11 mL) was stirred at 130°C for 24 h. The reaction mixture was then cooled to ambient temperature, diluted with ice water, adjusted to pH $\approx$ 6 by K_2CO_3 and then filtered. The precipitate was washed with water, dissolved in methanol and concentrated under reduced pressure. The residue was purified by HPLC (Inertsil ODS-3, methanol: 0.1% (v/v) TFA- H_2O = 0:1 to 1:0) to give 23 mg compound **12** as purple oil. Yield: 3.1%. 1H -NMR (300 MHz, CD_3OD) δ 7.48-7.36 (m, 2H); 7.21 (d, $J = 9.1$ Hz, 2H), 7.04-6.90 (m, 2H), 6.83 (dd, $J = 9.3, 1.7$ Hz, 2H), 6.69 (s, 2H), 2.99 (s, 6H); ESIMS $m/z = 333$ $[M]^+$

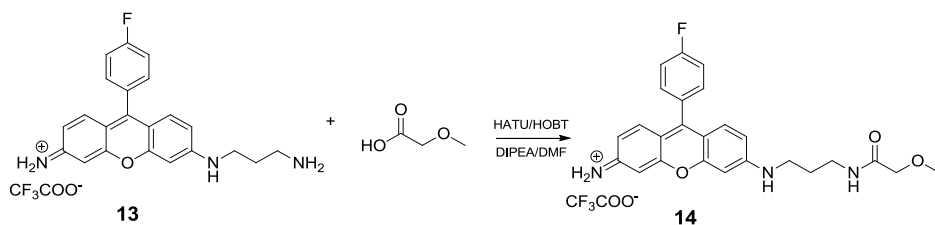
Synthesis of compound 13



A suspension of 3-((3-aminopropyl)amino)phenol hydrobromide (2.5 g, 10 mmol)^[5], 3-aminophenol (1.1 g, 10

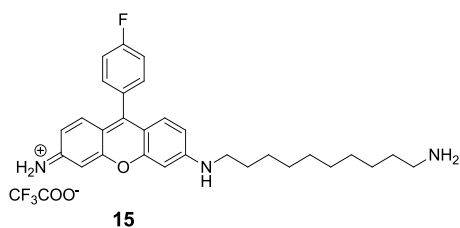
mmol), 4-fluorobenzaldehyde (710 μ l, 6.7 mmol) in methanesulfonic acid (25 mL) was stirred at 130°C for 24 h. The reaction mixture was then cooled to ambient temperature, diluted with ice water, adjusted to pH $\approx$ 6 by K₂CO₃ and then filtered. The precipitate was washed with water, dissolved in methanol and concentrated under reduced pressure. The residue was purified by HPLC (Inertsil ODS-3, methanol: 0.1% (v/v) TFA-H₂O = 0:1 to 1:0) to give 150 mg Compound **13** as purple oil. Yield: 4.7%. ¹H-NMR (300 MHz, CD₃OD) δ 7.53-7.48 (m, 2H); 7.45-7.39 (m, 2H), 7.31 (dd, J = 9.1, 2.2 Hz, 2H), 6.93-6.90 (m, 2H), 6.86 (dd, J = 10.4, 2.2 Hz, 2H), 3.51 (t, J = 7.1 Hz, 2H), 3.09 (t, J = 7.7 Hz, 2H), 2.12-2.00 (m, 2H); ESIMS m/z =362 [M]⁺

Synthesis of compound 14



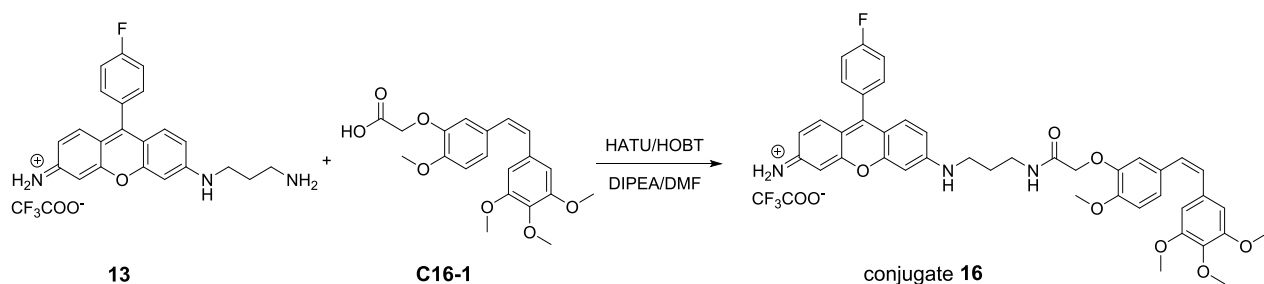
To a solution of compound **13** (20 mg, 42 μ mol) in dried DMF (600 μ l) was added 2-methoxyacetic acid (3.8 mg, 42 μ mol), HATU (19 mg, 54 μ mol), HOBT (7 mg, 54 μ mol) and DIPEA (20 mg, 155 μ mol). The reaction mixture was stirred at room temperature for 3 h. After reaction finished, the mixture was diluted by MeOH and purified by HPLC (Inertsil ODS-3, methanol: 0.1% (v/v) TFA-H₂O = 0:1 to 1:0) to give 8.8 mg compound **14** as purple oil. Yield: 38.3%. ¹H-NMR (300 MHz, CD₃OD) δ 7.52-7.48 (m, 2H); 7.44-7.38 (m, 2H), 7.29 (dd, J = 9.1, 1.1 Hz, 2H), 6.88-6.82 (m, 4H), 3.89 (s, 2H), 3.44-3.33 (m, 7H), 1.87-1.97 (m, 2H); ESIMS m/z =434 [M]⁺

Synthesis of compound 15



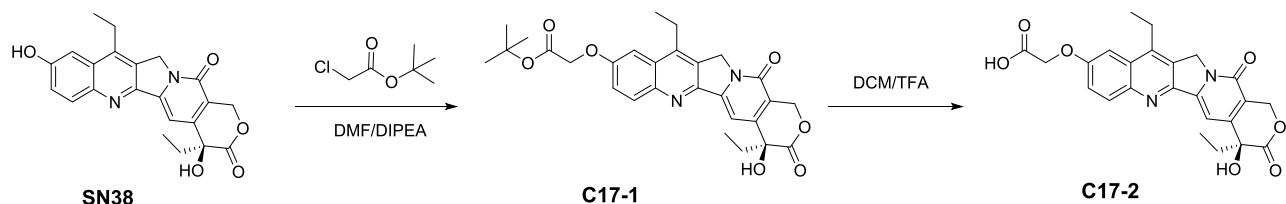
Compound **15** was synthesized by reported method^[6]. Purple oil. Yield: 2.4%. ¹H-NMR(600 MHz, acetic acid-*d*4) δ 7.51 (dd, J = 8.9, 5.5 Hz, 2H), 7.40 (dd, J = 8.9, 8.9 Hz, 2H), 7.31 (d, J = 8.9 Hz, 2H), 6.98–6.93 (m, 4H), 3.40 (m, 2H), 3.04 (m, 2H), 1.74-1.67 (m, 2H), 1.44-1.32 (m, 14H); ESIMS m/z =460 [M]⁺

Synthesis of conjugate 16

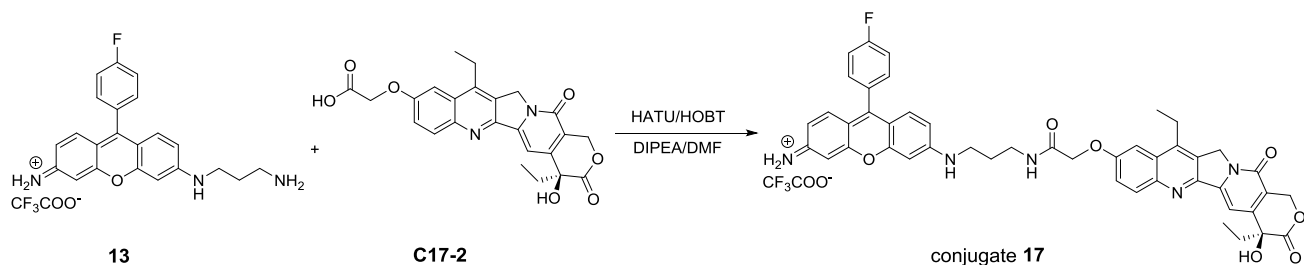


To a solution of compound **13** (18 mg, 37 μmol) in dried DMF (600 μl) was added **C16-1**^[7] (14 mg, 37 μmol), HATU (16 mg, 41 μmol), HOBT (6 mg, 41 μmol) and DIPEA (16 mg, 124 μmol). The reaction mixture was stirred at room temperature for 3 h. After reaction finished, the mixture was diluted by MeOH and purified by HPLC (Inertsil ODS-3, methanol: 0.1% (v/v) TFA-H₂O = 0:1 to 1:0) to give 14 mg conjugate **16** as purple oil. Yield: 44.4%. ¹H-NMR (300 MHz, CD₃OD) δ 7.50-7.37 (m, 4H), 7.27 (d, J = 9.1 Hz, 1H), 7.24-7.15 (m, 1H), 6.92 (s, 2H), 6.89-6.82 (m, 3H), 6.78 (dd, J = 8.2, 2.2 Hz, 2H), 6.50-6.43 (m, 4H), 4.35 (s, 2H), 3.83 (s, 3H), 3.69 (s, 3H), 3.59 (s, 6H), 3.41-3.34 (m, 4H), 1.94-1.89 (m, 2H); ESIMS m/z = 718 [M]⁺; HRMS (FAB) calcd for [M]⁺ m/z = 718.2923, found 718.2946.

Synthesis of conjugate 17

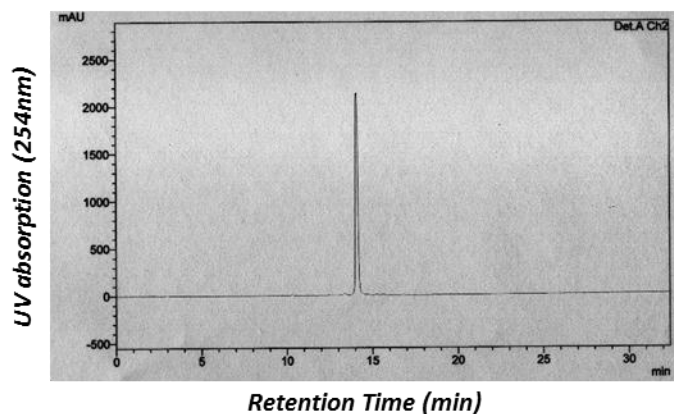


A suspension of SN38 (30 mg, 76 μmol), tert-butyl chloroacetate (46 mg, 307 μmol) and DIPEA (65 mg, 504 μmol) in DMF (1.5 mL) was stirred at 70°C for 48 h. The solvent was removed by vacuum and the residue was purified by flash column (DCM:MeOH=20:1) to give 30 mg **C17-1** as yellow solid. To a solution of **C17-1** (11 mg, 22 μmol) in DCM (500 μl) was added trifluoroacetic acid (500 μl) dropwise. The reaction mixture was stirred at room temperature overnight and then diluted by MeOH and purified by HPLC (Inertsil ODS-3, methanol: 0.1% (v/v) TFA-H₂O = 0:1 to 1:0) to give 5.5 mg **C17-2** as yellow solid. Yield: 43.3% in 2 steps. ¹H-NMR (300 MHz, CD₃OD) δ 7.99 (d, J = 9.1 Hz, 1H), 7.54 (s, 1H), 7.49 (dd, J = 9.4, 2.8 Hz, 1H), 7.34 (d, J = 2.5 Hz, 1H), 5.55 (d, J = 16 Hz, 1H), 5.35 (d, J = 16 Hz, 1H), 5.16 (s, 2H), 4.86 (s, 2H), 3.14 (q, J = 7.7 Hz, 2H), 1.94 (q, J = 7.1 Hz, 2H), 1.36 (t, J = 7.4 Hz, 3H), 1.00 (t, J = 7.4 Hz, 3H); ESIMS m/z = 451 [M+H]⁺

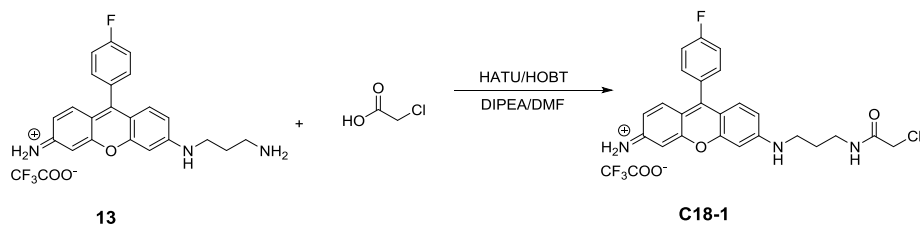


To a solution of compound **13** (10 mg, 21 μmol) in dried DMF (600 μl) was added **C17-2** (12 mg, 21 μmol),

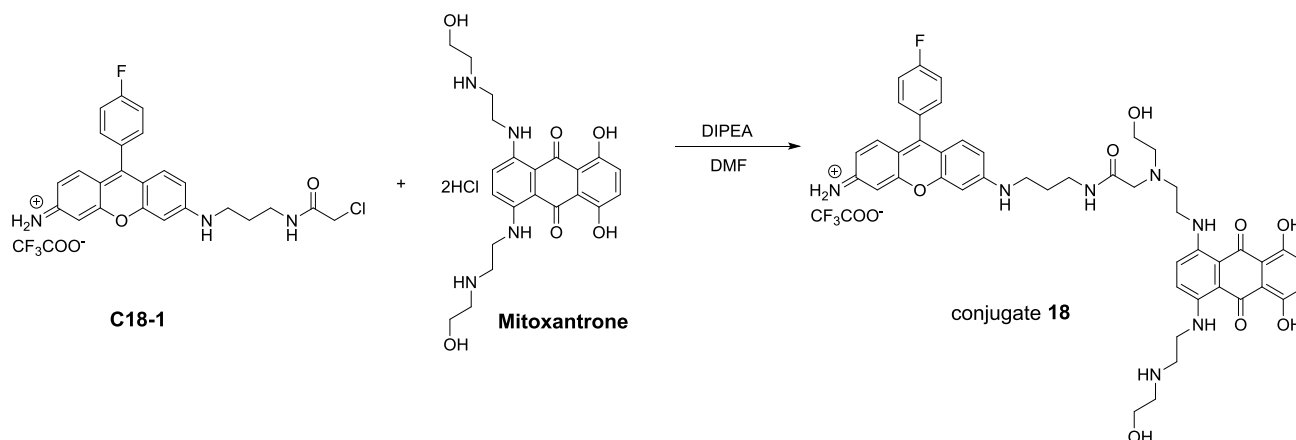
HATU (8.8 mg, 23 μ mol), HOBT (4 mg, 23 μ mol) and DIPEA (13.5 mg, 105 μ mol). The reaction mixture was stirred at room temperature for 3 h. After reaction finished, the mixture was diluted by MeOH and purified by HPLC (Inertsil ODS-3, methanol: 0.1% (v/v) TFA-H₂O = 0:1 to 1:0) to give 5 mg conjugate **17** as purple oil. Yield: 26.2%. ¹H-NMR (300 MHz, CD₃OD) δ 8.03 (d, J = 9.1 Hz, 1H), 7.57 (d, J = 9.1 Hz, 2H), 7.38 (brs, 4H), 7.28 (s, 2H), 7.09 (d, J = 9.3 Hz, 2H), 6.67 (d, J = 8.8 Hz, 2H), 6.55 (d, J = 1.7 Hz, 2H), 5.42 (brs, 1H), 5.26 (brs, 1H), 5.16-5.05 (m, 2H), 4.83 (s, 2H), 3.64 (brs, 2H), 3.13-2.85 (m, 6H), 1.93 (brs, 4H), 1.38 (t, J = 7.4 Hz, 3H), 0.89 (brs, 3H); ESIMS m/z = 794 [M]⁺; HRMS (FAB) calcd for [M]⁺ m/z = 794.2985, found 794.2962.



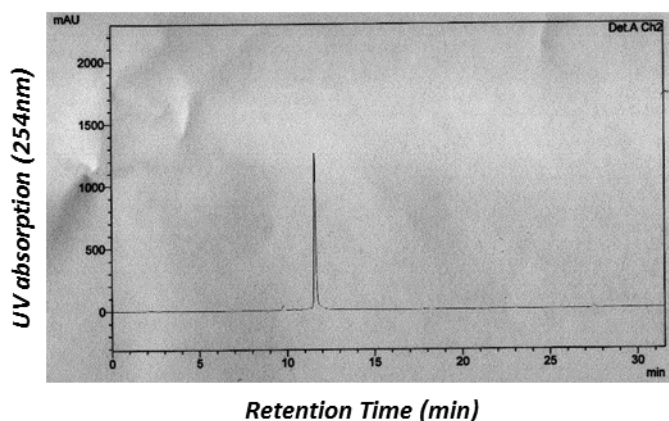
Synthesis of conjugate **18**



To a solution of compound **13** (30 mg, 63 μ mol) in dried DMF (600 μ l) was added chloroacetic acid (6 mg, 63 μ mol), HATU (26.3 mg, 69 μ mol), HOBT (9.4 mg, 71 μ mol) and DIPEA (26.7 mg, 207 μ mol). The reaction mixture was stirred at room temperature for 3 h. After reaction finished, the mixture was diluted by MeOH and purified by HPLC (Inertsil ODS-3, methanol: 0.1% (v/v) TFA-H₂O = 0:1 to 1:0) to give 12 mg **C18-1** as purple oil. Yield: 34.6%. ¹H-NMR (300 MHz, CD₃OD) δ 7.56-7.48 (m, 2H), 7.44-7.38 (m, 2H), 7.29 (dd, J = 9.1, 2.5 Hz, 2H), 6.89-6.82 (m, 4H), 4.06 (s, 2H), 3.49-3.32 (m, 4H), 1.95-1.91 (m, 2H); ESIMS m/z = 438 [M]⁺



A suspension of **C18-1** (12 mg, 22 μmol), mitoxantrone dihydrochloride (22.8 mg, 44 μmol) and DIPEA (56.4 mg, 440 μmol) in DMF (300 μl) was stirred at 40°C for 24h. The reaction mixture was then cooled to ambient temperature, diluted with MeOH and purified by HPLC (Inertsil ODS-3, methanol: 0.1% (v/v) TFA-H₂O = 0:1 to 1:0) to give 9.9 mg conjugate **18** as dark purple oil. Yield: 46.9%. ¹H-NMR (300 MHz, CD₃OD) δ 7.48-7.35 (m, 6H), 7.16-6.94 (m, 4H), 6.74-6.66 (m, 2H), 6.49 (s, 1H), 6.42 (s, 1H), 4.13 (s, 2H), 3.97 (s, 2H), 3.92 (brt, J = 4.7 Hz, 2H), 3.82-3.79 (m, 6H), 3.72 (brs, 2H), 3.53 (brs, 2H), 3.47 (brs, 2H), 3.38 (brs, 2H), 1.93 (brs, 2H); ESIMS m/z = 846 [M]⁺; HRMS (FAB) calcd for [M]⁺ m/z = 846.3621, found 846.3611.



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