Electronic Supplementary Material (ESI) for ChemComm.

Chemical Decontamination of iPS Cell-Derived Neural Cell Mixtures

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Supplementary Contents

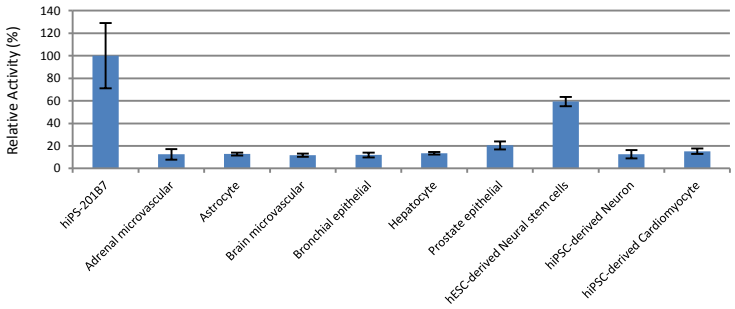

Figure S1. Quantification of alkaline phosphatase (ALP) activities of human iPSCs, human primary somatic cells, and cells derived from human pluripotent stem cells. The cells were stained with an Alkaline Phosphatase Substrate kit (SK-5300, Vector® Blue), and the cell images were captured by a microscope. The images were analyzed with imageJ. The ALP activity of hiPSCs was calculated as 100%.

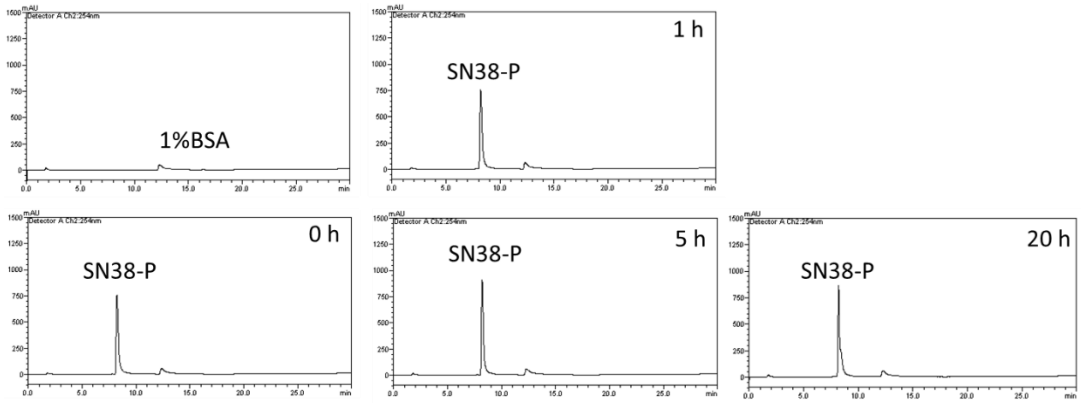

Figure S2. Stability of SN38-P in bovine serum albumin (BSA)-containing buffer. SN38-P (1 mM) was incubated with 1% BSA in PBS buffer at 37 ºC for 20 hours. The stability of SN38-P in buffer was analyzed with HPLC at 254 nm. No BSA-induced cleavage of SN38-P was observed even after 20h-incubation.

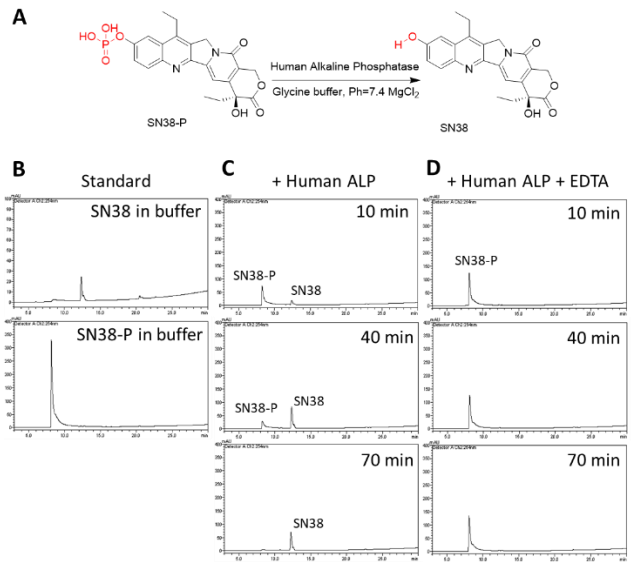

Figure S3. Enzymatic conversion of SN38-P to SN38 by human placental alkaline phosphatase in glycine buffer (pH = 7.4). (A) Chemical structures of SN38-P and SN38. (B) Standard peaks of SN38-P and SN38 in the buffer. (C) SN38-P was converted to SN38 in presence of alkaline phosphatase time-dependently. (D) The enzymatic conversion was inhibited by EDTA (80 mM), an alkaline phosphatase inhibitor. No production of SN38 was observed in presence of EDTA. The enzymatic reaction was traced by HPLC at 254 nm. The produced SN38 was also identified by mass spectroscopy.

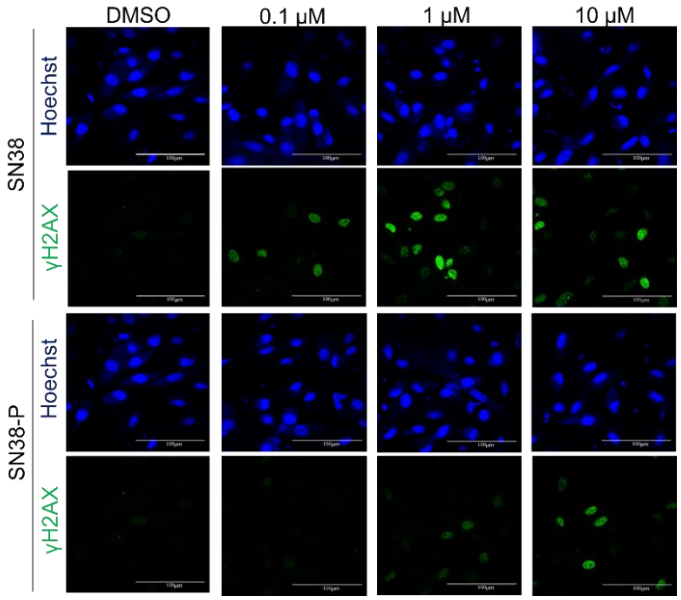

Figure S4. SN38-P exhibited less DNA damage than SN38 to ALP-negative astrocytes, due to the poor membrane permeability of SN38-P. γH2AX was used as an indicator of DNA damage. Hoechst 33258 (10 μg/mL) was used as a nuclear indicator. Scale bar: 100 μm.

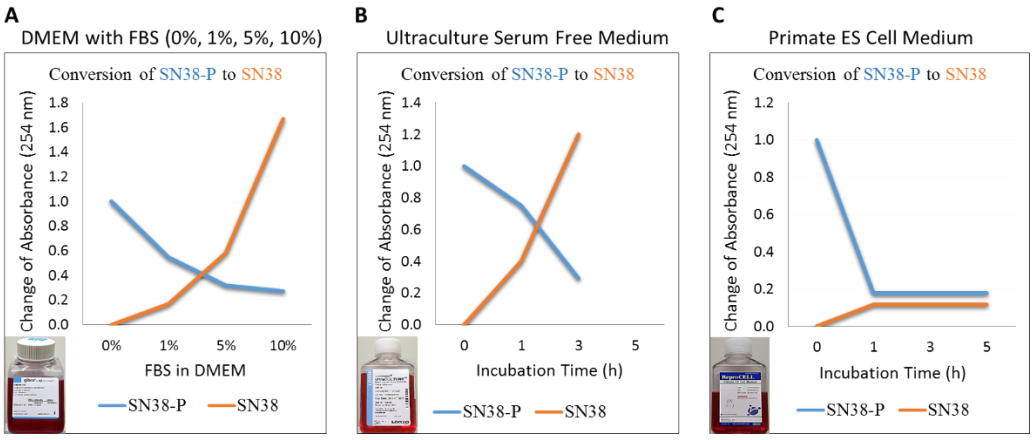

Figure S5. Effect of alkaline phosphatase in the serum on SN38-P. (A) DMEM did not induce dephosphorylation of SN38-P to SN38. Dephosphorylation was observed to be dose-dependent after the addition of 1% to 10% fetal bovine serum (FBS). (B) Commercially available serum-free medium (Ultraculture™ Serum Free Medium) also induced dephosphorylation of SN38-P to SN38 in a time-dependent manner. (C) Primate ES Cell Medium did not induce the dephosphorylation of SN38-P even after 5 h of incubation. The dephosphorylation reaction was monitored with HPLC at 254 nm. The absorbance of SN38-P at 0% FBS or 0 h was considered to be 1.0, and the absorbance of SN38 at 0% FBS or 0 h was considered to be 0.0.

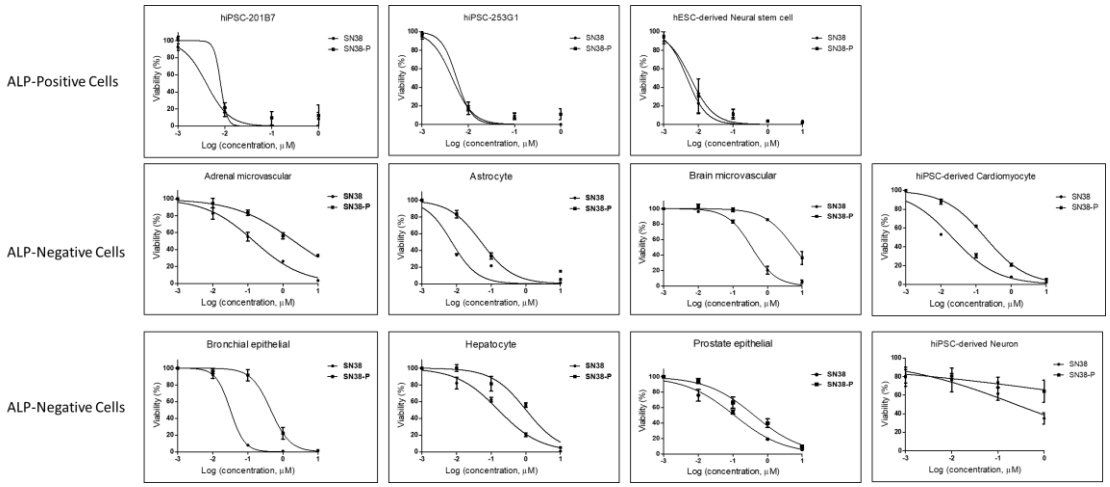

Figure S6. Viability of human iPSCs, somatic primary cells and pluripotent stem cell-derived cells when treated with SN38 or SN38-P. 0.01μM of SN38-P shows less cytotoxicity for ALP-negative cells (viability>80%), but strong cytotoxicity for ALP-positive cells (viability≈20%). Viability was determined by a WST-8-based colorimetric assay (Cell Counting Kit-8, Dojindo). Absorbance was measured at 450 nm. Data are shown as mean ±SD. n=3.

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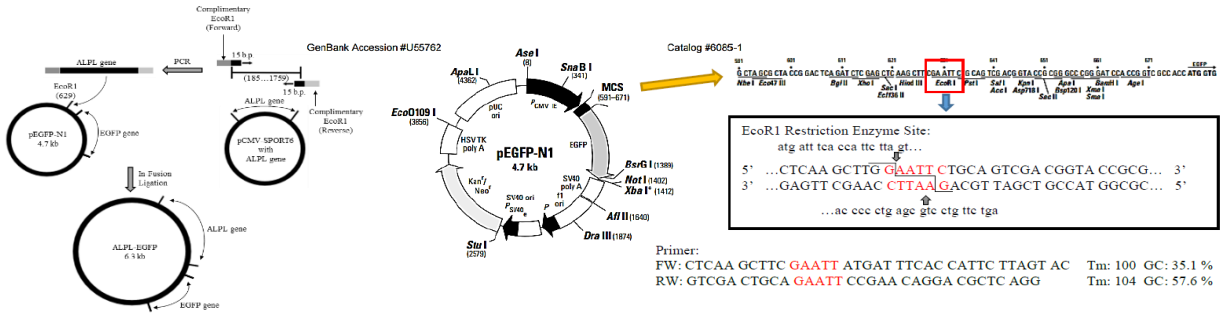

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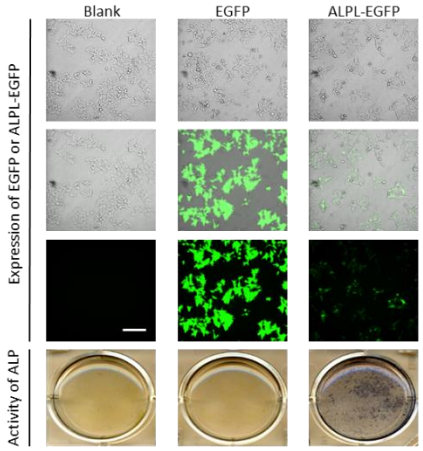


Figure S7. Design, sequencing, and transfection of the ALP-EGFP gene. (A) Schematic overview of recombinant DNA design of ALP-EGFP (6.3 kb), using an ALP gene-containing plasmid vector, pCMV-SPORT6 (1.6 kb), as donor plasmid, and pEGFP-N1 (4.7 kb) as recipient plasmid. Two primers with complementary EcoR1 restriction sites in pEGFP-N1 were designed and amplified with the donor plasmid. The amplified gene was ligated to pEGFP-N1 through In-Fusion® HD Cloning ligation protocol. PCR insert product and pEGFP-N1 vector were mixed in a 10:1 ratio, In-Fusion HD Enzyme Premix (5X, 1 μL) in sterilized water was added to make a total volume of 5 μL. The mixed solutions were then incubated (50ºC, 15 min), and transformed onto StellarTM competent cells following the manufacturer's instructions. (B) DNA sequencing of ALP-EGFP by Eurofins Genomics. Sequencing primers were designed as (i) pEGFP N1 (514-533) for forward reading: 5'-d(ATTGACGCAAATGGGCGGTA)-3', (ii) ALP (501-520) for forward reading: 5'-d(GAGAGTGAACCATGCCACCC)-3', and (iii) pEGFP-N1 (728-747) for reverse reading: 5'-d(TACGTCGCCGTCCAGCTCGA)-3'. ALP: red, EGFP: green. Primers were purchased from Invitrogen. (C) Transfection of Recombinant Plasmid ALP-EGFP into HEK293 cells. The expression and activity of transfected alkaline phosphatase was confirmed by fluorescence imaging of EGFP (green) and alkaline phosphatase staining (blue), respectively.

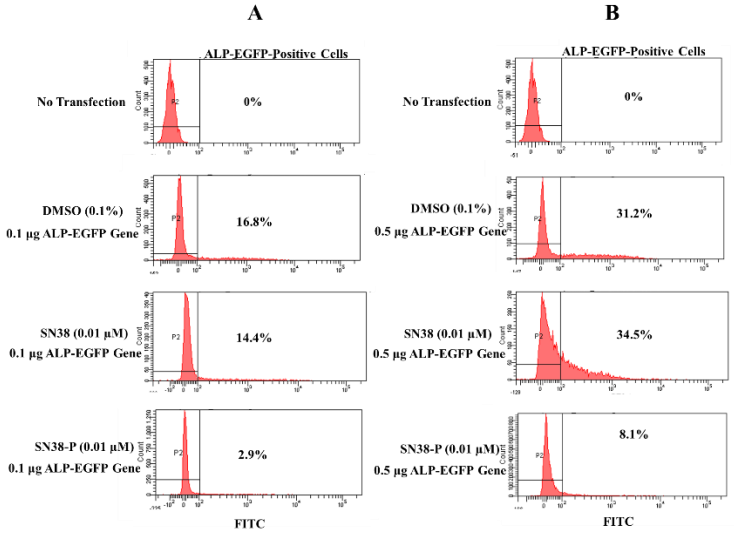


Figure S8. Selective elimination of transfected ALP-positive HEK293 cells from cell mixtures. HEK293 cells were transfected with 0.1 µg (A) or 0.5 µg (B) of a plasmid encoding EGFP-ALP in OPTI-MEM and FuGENE® HD. Approximately 10% (A) or 30% (B) of the cells were ALP-EGFP-positive after transfection. SN38 reduced the number of both ALP-EGFP-positive and ALP-EGFP-negative cells equally, while SN38-P selectively reduced the number of ALP-EGFP-positive cells from 16.8% to 2.9% (A) or from 31.2% to 8.1% (B). In the cells transfected with 0.1 µg of an expression vector encoding EGFP-ALP, SN38-P selectively eliminated 83% of the ALP-positive cells. However, in the cells transfected with 0.5 µg of the vector, SN38-P selectively eliminated 74% of the ALP-positive cells.

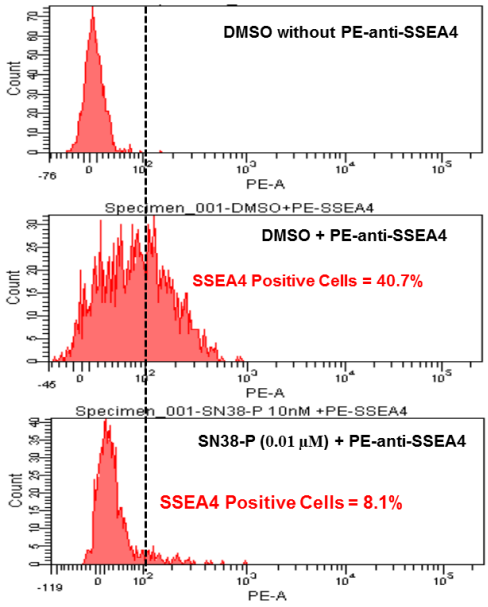


Figure S9. Flow cytometric analysis of partially differentiated hiPSCs with a PE-conjugated anti-SSEA4 antibody in the absence or presence of SN38-P. (A) DMSO control (0.1%) without the antibody treatment. (B) The percentage of SSEA4-positive cells was 40.7% in the DMSO control (0.1%) with the antibody treatment. (C) The percentage of SSEA4-positive cells was reduced to 8.1% in SN38-P treated cells (0.01 µM) with the antibody treatment.

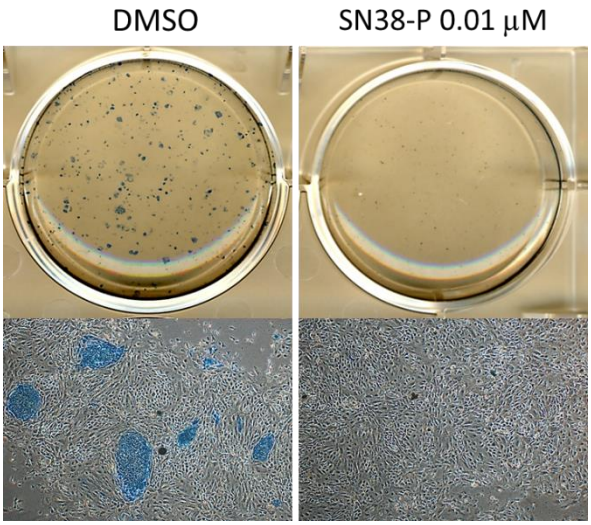


Figure S10. Colony formation by partially differentiated hiPSCs. The partially differentiated cell mixtures were treated with SN38-P (0.01 µM) or DMSO (0.1%) for 3 d. The surviving cells were washed, detached by cell scraper, re-seeded on a 6-well plate pre-coated with gelatin, and incubated in the growth medium for another 5 d. The cells were then fixed and stained with an Alkaline Phosphatase Substrate kit. SN38-P selectively eliminated the blue colony-forming cells from cell mixtures.

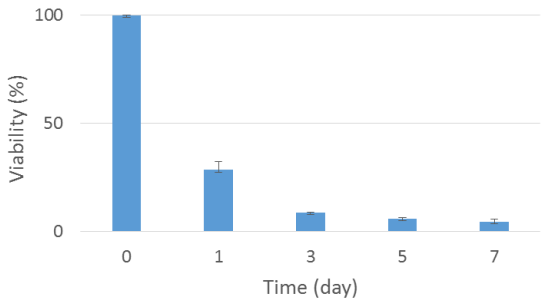


Figure S11. Viability of residual hiPSCs. hiPSCs were incubated with SN38-P (0.01 μM) for 72 h. After extensive washing with PBS, the residual iPSCs were incubated in growth medium for another 7 d. Cell viability was determined every 24 h by WST-8-based colorimetric assay. Cell viability on day 0 was considered as 100%. The residual iPSCs failed to proliferate, displaying delayed cell death. Data are mean±SD, n=3.

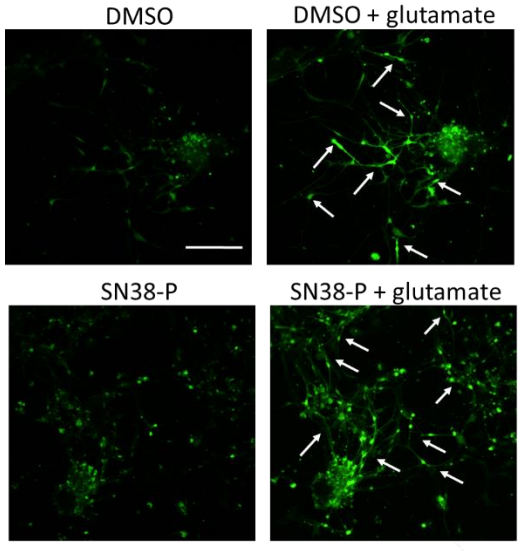
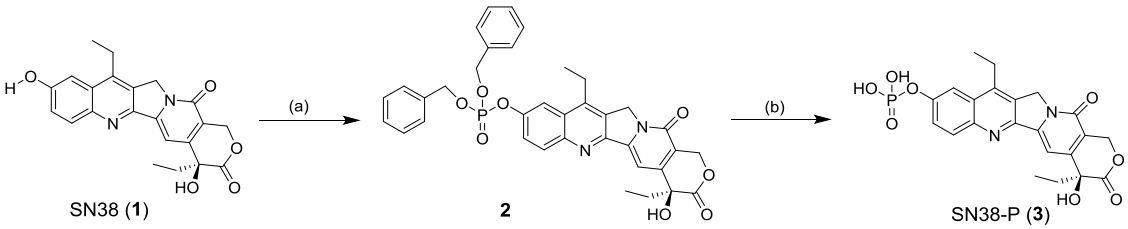


Figure S12. Imaging of glutamate-induced calcium influx in hiPSC-derived neurons. Cells were treated with DMSO (0.1%) or SN38-P (0.01 μM) for 72 h. The residual cells were then washed with HBSS, and the intracellular Ca^{2+} was observed by Fluo-8® AM (4 μM in HBSS). The images were taken before and after the addition of glutamate (100 μM). hiPSC-derived neurons treated with SN38-P maintained their glutamate-induced calcium influx. Scale bar: 200 μm.

Table S1. PAMPA assay for investigation of the effective membrane permeability of SN38 and SN38-P. SN38-P is 16.9 times less permeable than SN38. Data are shown as mean±SD, n=4.[a]

Compound	Pe (10^{-6} cm s^{-1})
Atenolol	0.0436±0.0268
Verapamil	17.8±5.36
SN38	0.125±0.0196
SN38-P	0.0074±0.00384

[a] Atenolol: Negative Control; Verapamil: Positive Control; Pe: Effective Permeability Coefficient. Pe >10 is defined as high permeability. Pe <10 is defined as low permeability.



Conditions and reagents: (a) NaH, Dibenzyl chlorophosphate, pyridine, rt, 2.5h, 25.1%. (b) H_2, Pd/C, EtOH/1,4-Dioxane, 1 atm, rt, 2h, 73.7%.

Scheme S1. Synthesis of SN38-P (**3**).

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 were obtained using a JEOL JMS LG-2000 in FAB mode. ^{1}H NMR and ^{31}P NMR spectra were collected on JEOL JNM-ECP 300 MHz and BRUKER AVANCE III 600MHz spectrometers.

Cell Cultures. 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 were seeded with SNL feeder cells. ES primate medium (ReproCELL) containing 4 ng/mL bFGF (ReproCELL) was used for regular maintenance. hiPSC-derived cardiomyocytes were cultured on laminin (iMatrix-511, nippi)-precoated 96-well plates with a cardiomyocyte culture medium containing 20% FBS. hESC-derived neural stem cells (Gibco) were cultured on CELLStart (Gibco)-precoated 60-mm dishes with NSC SFM complete medium (Gibco). hiPSC-derived neurons were obtained with Neurobasal medium containing B27 supplement (Gibco) from hiPSC-derived neurospheres, which were cultured with KBM Neural Stem Cell medium with supplement (KOHJIN BIO). For the measurement of ALP activity and IC_{50} values, we used commercially available, qualified, adherent hESC-derived neural stem cells instead of hiPSC-derived neurospheres, aggregates suspended in the medium. All cells were maintained in a humidified incubator with 5% CO_2 at 37°C.

Evaluation of Intercellular SN38 and SN38-P. When the cells reach 90% confluency in a 6-well plate, they were incubated with SN38 (10 μM in PBS) or SN38-P (10 μM in PBS) for 1 h. The cells were then collected and extracted in 1 mL of MeOH. The extract was concentrated, and the residual powder was dissolved in 200 μL MeOH. Intercellular SN38 and SN38-P were analyzed by HPLC, with an injection of 15 μL the MeOH solution for each sample.

Preparation of Partially Differentiated hiPSCs. After 48 h of subculture, hiPSCs were incubated in ES primate medium without bFGF for another 7-10 d. The partially differentiated hiPSCs were then subjected to treatment with a selected compound for 72 h.

Effect of Serum on Stability of SN38-P. Serum is known to contain alkaline phosphatase that will dephosphorylate SN38-P to SN38. The alkaline phosphatase in serum could disrupt the results of cell-based assays with SN38-P. To investigate the effect of alkaline phosphatase in the serum, we incubated SN38-P with DMEM containing various percentages of serum (0, 1, 5, and 10%), and with other two types of serum-free medium (UltracultureTM Serum-Free Medium and Primate ES Cell Medium). The alkaline phosphatase in the serum dephosphorylated SN38-P to SN38 dose-dependently (Figure S5). Therefore, we used serum-free Primate ES Cell Medium as a medium for the cell-based assays.

Cell Viability Assays. We used serum-free Primate ES Cell Medium as a medium for the cell viability assays. Test cells were plated on 96-well plates at 5000 per well. A selected compound was added at various concentrations, 24 h after plating. After 72 h 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 IC_{50} was calculated based on the dose-response values.

Alkaline Phosphatase Staining Assays. The alkaline phosphatase activity of hiPSCs, somatic cells, and hPSC-derived cells was measured with an Alkaline Phosphatase Substrate kit (SK-5300, Vector® Blue). hiPSC-derived cardiomyocytes and hiPSC-derived neurons were prepared as previously described.[1,2] The cells were first fixed for 2 min with 4% paraformaldehyde, washed with PBS, treated with SK-5300, incubated for 1 h at room temperature, rinsed, and finally maintained in PBS buffer.

Fluorescence-Activated Cell Sorting (FACS) Analysis. We used serum-free Primate ES Cell Medium as a medium for the FACS analysis. Partially differentiated hiPSCs were treated with DMSO (0.1%) or SN38-P (0.01 μM) for 72 h. The residual cells were then washed with PBS and dissociated using Accutase (Gibco). The cells were then stained using a PE-anti-SSEA4 antibody (BD Biosciences), and analyzed with a BD FACSAria II flow cytometer.

Colony Formation Assay. hiPSCs (253G1) were seeded on 6-well plates pre-coated with gelatin (ReproCELL). After 24 h incubation, the cells were treated with all-trans retinoic acid (1 μM) for 3 d to yield a mixture of partially differentiated cells. The cell mixtures were treated with SN38-P (0.01 μM) or DMSO (0.1%) for another 3 d. The surviving cells were washed, detached by cell scraper, re-seeded on gelatin-coated 6-well plates and incubated in growth medium for another 4 d. The cells were then fixed with 4% formaldehyde and stained with an Alkaline Phosphatase Substrate kit (SK-5300, Vector® Blue).

Proliferation of Residual hiPSCs. After 72 h treatment with SN38-P (0.01 μM), the residual hiPSCs were washed with PBS and further incubated in growth medium (ES primate medium containing 4 ng mL^{-1} bFGF, ReproCELL) for another 7 d. The viability of the cells was determined every 24 h by WST-8-based colorimetric assay.

Immunocytochemistry. Human primary astrocytes were treated with SN38 or SN38-P for 6 h to induce DNA damage. The cells were then washed with PBS, fixed with 4% paraformaldehyde, and subjected to immunostaining with an anti-γH2A.X (phospho S139) antibody as a primary antibody (mouse, invitrogen) and a Alexa Fluor® 488 Goat Anti-mouse IgG H&L (abcam) as a secondary antibody. hiPSC-derived neural cells were treated with SN38-P (0.01 μM) for 72 h, washed with PBS, fixed with 4% paraformaldehyde, and subjected to multicolor immunostaining with primary antibodies (anti-beta III Tubulin antibody (rabbit, abcam), anti-Ki-67 (mouse, BD)) and secondary antibodies (Alexa Fluor® 488 Goat Anti-Rabbit IgG H&L (abcam), (Alexa Fluor® 568 Goat Anti-Mouse IgG H&L (abcam)). Fluorescence images were taken using a confocal microscope (Cell Voyager 1000, Yokogawa Electric Corporation) with laser excitation at 405, 488, and 561 nm.

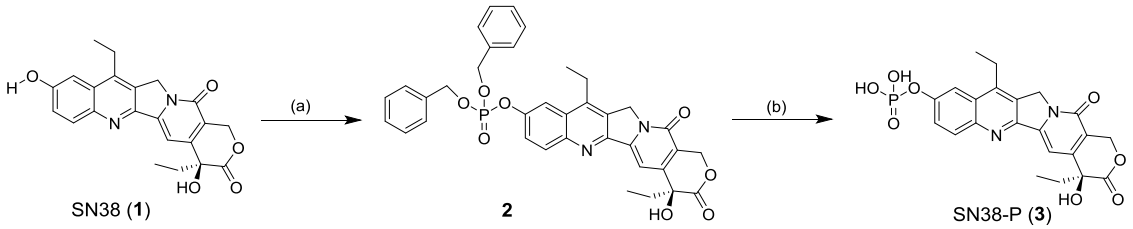
Live Cell Calcium Imaging. hiPSC-derived neurons were treated for 72 h with DMSO (0.1%) or SN38-P (0.01 μM), washed with HBSS, and then incubated with Fluo-8® AM (4 μM in the HBSS) for 30 min. Glutamate (100 μM) was then added, and the cells were imaged.

Parallel Artificial Membrane Permeability Assay (PAMPA). The membrane permeability of SN38 and SN38-P was evaluated using the PAMPA assay by Cyprotex. Atenolol was used as a negative control. Verapamil was used as a positive control. The Pe value indicated the effective permeability coefficient. Pe >10 was defined as high permeability. Pe <10 was defined as low permeability.

Recombinant DNA Design of ALP-EGFP. Tissue non-specific ALP, also known as liver/bone/kidney ALP (TNALP or ALPL; here on ALP) was purchased from RIKEN DNA Bank (Clone name: IRAK110G05). This 2561bp cDNA clone contained human ALP (185-1759 bp) inserted in pCMV-SPORT6 vector. pEGFP-N1 (Clontech, GenBank Accession No. U55762), encoding a red-shifted variant of wild type GFP (max λ ex = 488 nm; max λ em = 507 nm), was ligated with the insert DNA, using In-Fusion® HD Cloning technology (Clontech). EcoR1 from the multiple cloning site of pEGFP-N1 was designed as the ligation point, and primers were designed following instructions from Clontech (Figure S7A). Complementary primers were designed and used in PCR amplification of the ALP gene from pCMV-SPORT6. After gel electrophoresis separation, ethidium bromide-stained gel was visualized with UV, and the desired PCR band was cut and purified. Purified PCR product was then ligated with pEGFP-N1 vector, using In-Fusion® HD Cloning protocol, and transformed onto StellarTM competent cells (Clonetech). Colonies were randomly selected for colony PCR amplification quick analysis. Colonies with correct DNA size were liquid cultured overnight in Lysogeny broth (LB) containing 0.1% kanamycin, and purified. Collected recombinant plasmid, labelled EGFP-ALP, was confirmed with DNA sequencing (Figure S7B), diluted with Tris-EDTA (TE) buffer, and stored at -30°C.

Transfection of ALP-EGFP into HEK293 cells. HEK293 cells were suspended at a cell density of 1×10^5 cells mL^{-1}, and 2 mL per well seeded onto 6-well plates. Transfection mixtures were prepared with 100 µL OPTI-MEM® Reduced Serum Media, 0.5 µg plasmid DNA (for both ALP-EGFP and EGFP), and 6 µL FuGENE® HD transfection reagent per well. Negative control mixtures contained 2 µL of OPTI-MEM® Reduced Serum Media in place of plasmid DNA. The transfection mixtures were mixed well and incubated for 10 min prior to transfection. After 24 h incubation at 37°C, cells were treated with 100 µL of the prepared transfection mixtures and further incubated for another 24 h at 37°C. After incubation, transfection efficiency was first confirmed by fluorescence imaging and then subjected to alkaline phosphatase staining (Figure S7C).

Chemical Synthesis



Conditions and reagents: (a) NaH, Dibenzyl chlorophosphate, pyridine, r.t., 2.5 h, 25.1%. (b) H_2, Pd/C, EtOH/1,4-Dioxane, 1 atm, r.t., 2 h, 73.7%.

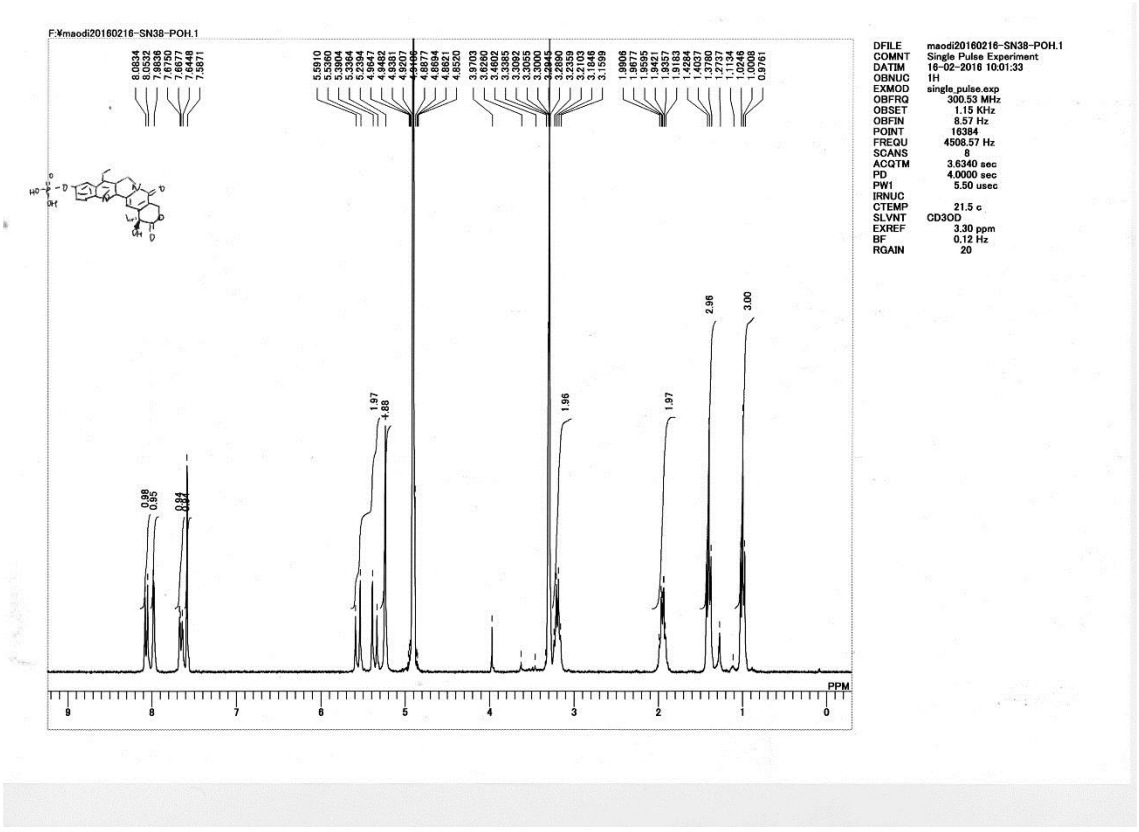
10-*O*-dibenzylphosphoryl-SN38 (2)

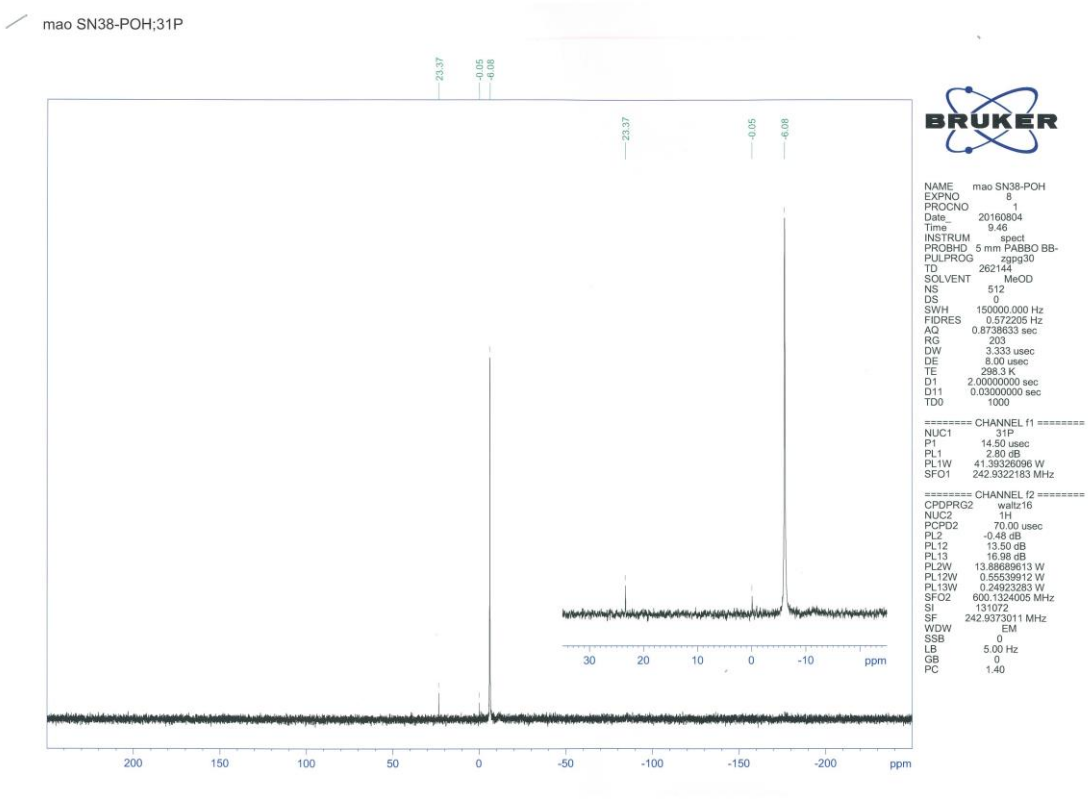
To an ice-cooled solution of SN38 (**1**) (36 mg, 91.7 µmol) in dry pyridine (4 ml) was added NaH (60%, 18.4 mg, 458.5 µmol) portion wise and the mixture was stirred at room temperature for 30 min. Dibenzyl chlorophosphate (54.4 mg, 183.6 µmol) was then added to the mixture portion wise and stirred at room temperature for 1 h. The solvent was then removed by vacuum and the residue was purified by HPLC to give **2**

as light yellow powder (15 mg, yield: 25.1%). ^{1}H-NMR (CD_3OD): δ 8.06 (d, *J*=9.1 Hz, 1H), 7.79 (s, 1H), 7.60 (s, 1H), 7.36 (d, *J*=5.2 Hz, 1H), 7.33 (m, 10H), 5.55 (d, *J*=16.2 Hz, 1H), 5.37 (d, *J*=16.2 Hz, 1H), 5.24 (d, *J*=5.2 Hz ,4H), 5.20 (s, 2H), 3.05 (q, *J*=7.7 Hz, 2H), 1.95 (q, *J*=9.3, 2H), 1.27 (t, *J*=7.4 Hz, 3H), 1.00 (t, *J*=7.4 Hz, 3H); ESIMS *m/z*=653 $[M+H]^+$.

10-*O*-phosphoryl-SN38 (3)

To a solution of **2** (15 mg, 23 μmol) in 1:1 mixture of EtOH and 1, 4-dioxane (2 ml) was added Pd/C (3 mg, 10%) and the mixture was stirred under H_2 at room temperature for 2 h. The mixture was then filtered through celite and the filtrate was concentrated by the vacuum. The residue was finally purified by HPLC to give **3** as light yellow powder (8 mg, yield: 73.7%). ^{1}H-NMR (CD_3OD): δ 8.07 (d, *J*=9.1 Hz, 1H), 7.98 (s, 1H), 7.66 (d, *J*=9.1 Hz, 1H), 7.59 (s, 1H), 5.56 (d, *J*=16.2 Hz, 1H), 5.36 (d, *J*=16.2 Hz, 1H), 5.24 (s, 2H), 3.20 (q, *J*=7.7 Hz, 2H), 1.95 (q, *J*=7.7 Hz, 2H), 1.40 (t, *J*=7.4 Hz, 3H), 1.00 (t, *J*=7.1 Hz, 3H); ^{31}P-NMR (CD_3OD) δ -6.08 (ppm from H_3PO_4); ESIMS *m/z*=473 $[M+H]^+$, 471$[M-H]^-$; HRMS (FAB) calcd for $[M+H]^+$ m/z=473.1108, found 473.1083; calcd for $[M-H]^-$ m/z=471.0963, found 471.0914 .





Reference

[1] Mao, D.; Ando, S.; Sato, SI.; Qin, Y.; Hirata, N.; Katsuda, Y.; Kawase, E.; Kuo, TF.; Minami, I.; Shiba, Y.; Ueda, K.; Nakatsuji, N.; Uesugi, M. *Angew. Chem. Int. Ed.* **2017**, 56, 1765-1770.

[2] Matsumoto, T.; Fujimori, K.; Andoh-Noda, T.; Ando, T.; Kuzumaki, N.; Toyoshima, M.; Tada, H.; Imaizumi, K.; Ishikawa, M.; Yamaguchi, R.; Isoda, M.; Zhou, Z.; Sato, S.; Kobayashi, T.; Ohtaka, M.; Nishimura, K.; Kurosawa, H.; Yoshikawa, T.; Takahashi, T.; Nakanishi, M.; Ohyama, M.; Hattori, N.; Akamatsu, W.; Okano, H. *Stem Cell Rep.* **2016**, 6, 422-35.