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COMMUNICATION

Chemical Decontamination of iPS Cell-Derived Neural Cell Mixtures

Received 00th January 20xx,
Accepted 00th January 20xx

DOI: 10.1039/x0xx00000x

www.rsc.org/

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This report describes the design and evaluation of phosphorylated 7-Ethyl-10-hydroxycamptothecin (SN38-P), which selectively eliminates tumor-forming proliferative stem cells, including human induced pluripotent stem cells (hiPSCs) and neural stem cells, from iPSC-derived neural cell mixtures. Results of the present study demonstrate that simple phosphorylation of an anticancer drug can provide a safe, cost-effective, and chemically-defined tool for decontaminating hiPSC-derived neuron.

Human induced pluripotent stem cells (hiPSCs) serve as a highly valuable resource for both basic research and regeneration therapy. However, there are a number of limitations for their clinical application, including the tumorigenic risk of undifferentiated cells during transplantation.[1, 2] Complete differentiation or selective elimination of the undifferentiated cells is required to ensure the safety of stem cell therapy. A number of biological strategies for selective elimination have been reported: antibody-mediated cell sorting and precipitation,[3, 4] specific cytotoxic antibodies,[5] cell culture conditions that limit the growth of iPSCs,[6, 7] a lectin-toxin fusion protein,[8] TRPV-1 activation,[9] and a microRNA switch,[10] and a synthetic peptide that selectively assembles on the surface of hiPSCs through alkaline phosphatase-mediated activation.[11]

Small molecule-based approaches are attractive alternatives to those strategies, due to easy handling and relatively low cost. Chemical elimination of hiPSCs has been achieved using a stearoyl-coA desaturase (SCD1) inhibitor,[12] chemical inhibitors of survivin,[13] DNA topoisomerase II inhibitors,[14, 15] a cytotoxic natural product that is a substrate for selective ABC transporters,[16] and an hiPSC-selective fluorescent probe that generates reactive oxygen species upon light irradiation.[17] Unfortunately, none of these approaches have reached the level of clinical application. We previously reported selective elimination of hiPSCs by a hybrid molecule of an iPSC-selective fluorescent probe and 7-Ethyl-10-hydroxycamptothecin (SN38), a clinically used topoisomerase I inhibitor. The mechanistic study indicated that the high selectivity stemmed from the synergistic effects of ABC-transporter-mediated efflux and the mode of action of SN38.[18] Although the SN38 hybrid molecule exhibited excellent selectivity for hiPSCs, it cannot be used for selective removal of iPSCs from neural cell mixtures, due to the lack of expression of efflux ABC transporters in neural cells.[19]

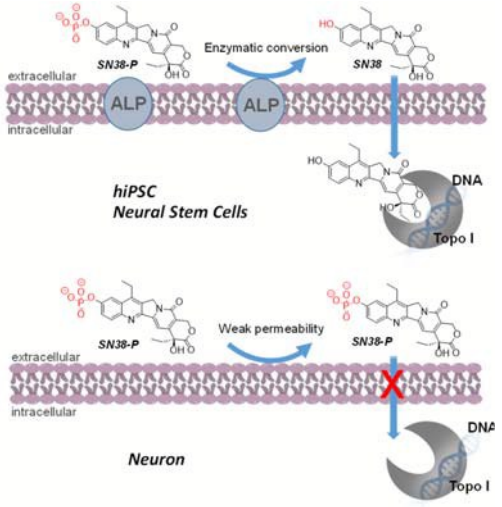

Fig. 1 Design of SN38-P. ALP: alkaline phosphatase; Topo I: topoisomerase I. hiPSC: Human induced pluripotent stem cell.

An increasing number of studies indicate that hiPSC-derived neurons or neural precursors have immediate promise for treatment of neural diseases and for modeling neurological diseases. [20-23] However, current differentiation protocols suffer from the residue of a few differentiation-resistant neural stem cells or hiPSCs. Their rapid proliferation poses a problem of tumor formation following *in vivo* engraftment, or of subsequently dominating the *in vitro* cell population.[24] Trials for addressing the problem have been proposed: cell sorting using antibodies for specific markers,[25-27] modified cell

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Electronic Supplementary Information (ESI) available: [details of any supplementary information available should be included here]. See DOI: 10.1039/x0xx00000x

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differentiation protocol,[28, 29] and a cell cycle promoter-driven suicide gene approach.[30] However, no small molecule-based approach has been developed. There is a pressing demand for a reliable, cost-effective, and safe chemical tool that eliminates hiPSCs or any highly proliferative stem cells from neural cell mixtures. Here, we report on the design and effectiveness of phosphorylated SN38 (SN38-P), which selectively eliminates proliferative stem cells, including human iPS and neural stem cells, from neural cell mixtures. The selectivity takes advantage of the high expression level of alkaline phosphatase on the membrane of human iPSCs and neural stem cells.[31] We hypothesized that ALP converts non-cell-permeable SN38-P to cell-permeable SN38 exclusively on the cell membrane of the stem cells, leading to selective cell death (Fig. 1).

To examine the expression levels of ALP on the membrane of different cell types, we used colormetric alkaline phosphatase staining with hiPSCs (201B7), six types of commercially

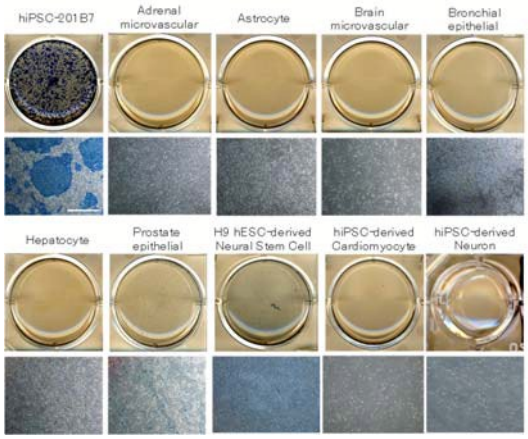

Fig. 2 Colorimetric alkaline phosphatase staining (blue) of living human iPSCs, human primary somatic cells, and cells derived from human pluripotent stem cells. hiPSCs and hESC-derived neural stem cells exhibited highest expression levels of ALP. Prostate epithelial cells had a lower expression level of ALP. Other types of cells had no significant expression level of ALP. Alkaline Phosphatase: ALP; ESC: embryonic stem cell; NSC: neural stem cell. Scale bar: 100 µm.

available human primary somatic cells (astrocytes, adrenal microvascular cells, brain microvascular cells, bronchial epithelial cells, hepatocytes, and prostate epithelial cells), and three types of human pluripotent stem cell-derived cells (hESC-derived neural stem cells, hiPSC- derived neurons, and hiPSC-derived cardiomyocytes). hiPSCs and hESC-derived neural stem cells had the highest expression levels of ALP. The other cell types had no significant expression level of ALP, with the exception of prostate epithelial cells which had a lower, but detectable, expression level (Fig. 2, Fig. S1).

The synthetic route of SN38-P is shown in scheme S1. We initially examined whether SN38-P is, in fact, a substrate for ALP and is significantly less cell-permeable than SN38. When SN38-P was incubated with purified human placental alkaline phosphatase, it was almost completely converted to SN38 within 70 min. In contrast, incubation with bovine serum albumin (BSA) failed to convert SN38-P (Fig. S2). The enzymatic

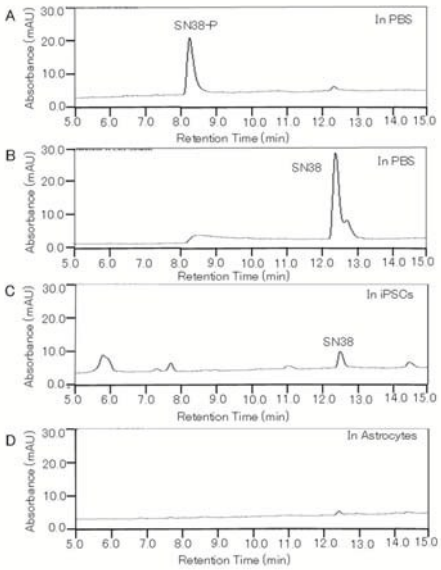

Fig. 3 Conversion and membrane permeability of SN38-P in living cells. The amounts of SN38 and SN38-P in cell lysates were analyzed with an HPLC at 254 nm and the molecular weights were confirmed with mass spectrometry. Retention times of SN38-P and SN38 in PBS are shown in (A) and (B), respectively. hiPSCs converted SN38-P to SN38, which was detected in the cell lysates (C), whereas human primary astrocytes failed to do so (D).

Table 1. IC_{50} values of SN38 and SN38-P in human stem cells and primary somatic cells.

Cell type	SN38 (µM)	SN38-P (µM)	Fold
iPSC-201B7	0.005±0.001	0.007±0.001	1.4
iPSC-253G1	0.007±0.001	0.005±0.001	0.7
Astrocyte	0.003±0.001	0.048±0.015	16
Adrenal microvascular	0.106±0.033	1.794±0.166	16.9
Brain microvascular	0.387±0.090	5.092±0.946	13.2
Bronchial epithelial	0.003±0.001	0.067±0.019	22.3
Hepatocyte	0.134±0.037	0.940±0.154	7.0
Prostate epithelial	0.077±0.045	0.234±0.069	3.0
hESC-derived neural stem cell	0.007±0.001	0.008±0.003	1.1
hiPSC-derived neuron	0.365±0.318	>10	>27.4
hiPSC-derived cardiomyocyte	0.015±0.002	0.181±0.004	12.1

Cells were treated with SN38 or SN38-P for 72 h. Data are mean ± SD, n=3. Fold increase was calculated as IC_{50} value of SN38-P / IC_{50} value of SN38. Significantly increased resistances are shown in red.

conversion was inhibited by EDTA, a well-known alkaline phosphatase inhibitor (Fig. S3). These results collectively demonstrate that SN38-P is a substrate for the alkaline phosphatase.

We next compared the cell membrane permeability of SN38 and SN38-P, using a parallel artificial membrane permeability (PAMPA) assay (Table S1). SN38 showed weak cell permeability, but SN38-P was 16.9 times less permeable than

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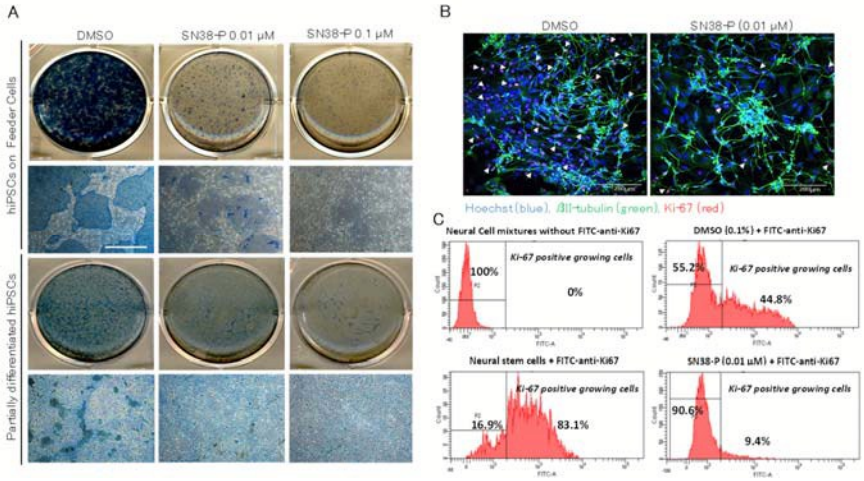

Fig. 4 Selective elimination of hiPSCs and hiPSC-derived Ki-67-positive cells by SN38-P. (A) Effects of SN38-P (0.01 μM or 0.1 μM), or a DMSO control (0.1%), on hiPS colonies and partially differentiated hiPSCs. The cells were treated with SN38-P for 72 h. Colonies of hiPSCs were visualized by colorimetric alkaline phosphatase staining (blue). Scale bar: 100 μm. (B, C) Selective elimination of Ki-67-positive cells from hiPSC-derived neural cell mixtures. The hiPSC-derived neural cell mixtures were incubated with DMSO (0.1%) or SN38-P (0.01 μM) for 72 h. The residual cells were fixed and co-immunostained with an anti-βIII-tubulin antibody (green) for neurons and an anti-Ki-67 antibody (red) for proliferating cells, and then analyzed by fluorescent microscopy (B) or flow cytometry (C). Hoechst 33258 (blue, 10 μg/mL) was used as a nuclear indicator. The proliferating cells (Pink) were indicated with arrowheads. Scale bar: 200 μm.

SN38, indicating that the phosphate group of SN38-P reduced its membrane permeability.

The poor membrane permeability of SN38-P was further confirmed by cell-based experiments. When hiPSCs were incubated with SN38-P for 1 h, SN38, but not SN38-P, was detected in the cell lysate (Fig. 3C). In contrast, when SN38-P was incubated with astrocytes in which ALP was not detectable (Fig. 3D), a high level of SN38-P was observed in the extracellular buffer, but not in the cell lysate (Fig. 3A).

SN38 is a topoisomerase inhibitor that induces DNA damage. DNA damage induced by SN38 and SN38-P was subsequently compared in ALP-negative astrocytes by measuring γH2AX, a marker for DNA damage.[32] After a 3 h incubation with SN38 or SN38-P, the cells were fixed and immunostained with an anti-γH2AX antibody. As expected, SN38 induced DNA damage at lower concentrations than SN38-P (Fig. S4). These results indicate that the negatively charged phosphate group reduces the penetration of SN38-P through the cell membrane of ALP-negative cells.

To evaluate the effect of phosphorylation on selectivity, we measured IC_{50} values of SN38 and SN38-P with two human iPSC lines, six types of primary human somatic cells, and three cell types derived from human pluripotent stem cells. Fetal bovine serum (FBS) is known to contain alkaline phosphatase (Fig. S5). Thus, serum-free primate ES cell medium was selected for the cell-based assays. SN38-P exhibited highly potent cytotoxcity for ALP-positive cells, including hiPSCs (201B7 and 253G1) and hESC-derived neural stem cells (Table 1, Fig. S6). For ALP-negative cells, SN38-P was 3.0 to >27.4-fold less cytotoxic than SN38. The fold differences and ALP expression levels are well correlated (Fig. 2). The ALP-dependent selectivity was further confirmed using HEK293 cells that lack ALP expression. HEK293 cells were transfected with an ALP-EGFP gene to yield a mixture of ALP-positive and ALP-negative cells (Fig. S7). The cell mixture was then treated with SN38 or SN38-P, followed by FACS analysis at the FITC channel. As expected, SN38-P selectively reduced the number of ALP-EGFP-positive cells (Fig. S8). These results support the hypothesis that the selectivity of SN38-P depends on the ALP expression. However, increasing number of ALP-EGFP positive cells tends to lower the selectivity, possibly due to bystander effects on ALP-negative cells, posing a precaution for future application.

To investigate the ability of SN38-P to eliminate hiPSCs from cell mixtures, we first incubated SN38-P (0.01 and 0.1 μM) with hiPSCs or partially differentiated hiPSCs for 3 d. Treatment with SN38-P removed hiPSCs (stained blue in Fig. 4A) in a dose-dependent manner, but had little effect on ALP-negative SNL feeder cells (Fig. 4A, *top*) or differentiated cells (Fig. 4A, *bottom*). Selective elimination was further confirmed by flow cytometric analysis with an antibody against SSEA4, a cell-surface marker for human pulripotent stem cells. The partially differentiated hiPSC mixtures treated with DMSO alone had 40.7% SSEA4-positive cells. Treatment with SN38-P decreased the number of SSEA4-positive cells to 8.1% (Fig. S9). When the residual SSEA4-positive cells were incubated in growth medium for another 5 d, no detectable ALP-positive cell colonies (blue in Fig. S10) were formed, suggesting that delayed cytotoxicity of SN38 completely eliminated ALP-positive undifferentiated cells in the cell mixtures (Fig. S11).[33, 34]

SN38-P showed potent cytotoxicity for the hiPSCs and hESC-derived neural stem cells, but >1000-times weaker cytotoxicity for hiPSC-derived neurons (Table 1). This encouraged us to test SN38-P for elimination of proliferating hiPSCs and neural stem cells in hiPSC-derived neural mixtures. A mixture of hiPSC-derived neural cells was treated with SN38-P (0.01 μM) for 72 h. The cells were then fixed and immunostained with an antibody against βIII-tubulin, a marker for neurons, and with an antibody against Ki-67, a marker for proliferative cells. As expected, treatment with SN38-P significantly reduced the number of Ki-67-positive proliferative cells, but had little effect on the βIII-tubulin-positive hiPSC-derived neurons (Fig. 4B). Flow cytometric analysis was carried out for further quantitative evaluation. Following treatment with DMSO alone, more than 80% of the hESC-derived neural stem cells and 44.8% of the hiPSC-derived neural cells were Ki-67-positive. Treatment of the hiPSC-derived neural cell mixtures with SN38-P reduced the Ki-67-positive cells to 9.4% (Fig. 4C). These results collectively demonstrate that SN38-P

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decontaminates hiPSC-derived neurons by selectively eliminating proliferating stem cells. However, the potential risk of the leftover Ki-67-positive cells can not be ignored although those cells express relatively low levels of Ki-67.

For clinical application, it is also important to ensure that the decontaminated neurons are alive and functional after SN38-P treatment. The hiPSC-derived neurons displayed healthy morphology after 3 d treatment with SN38-P (Fig. 4B). Their functional activity was assessed by measuring glutamate-induced calcium influx.[35] Comparable levels of Ca^{2+} influx were observed after addition of glutamate (100 μM) in DMSO-treated and SN38-P-treated hiPSC-derived neurons (Fig. S12). Thus, 3 d treatment with 0.01 μM of SN38-P is within an acceptable range for maintaining the function of hiPSC-derived neurons.

In conclusion, simple phosphorylation of the clinically-used anticancer drug, SN38, provides a cost-effective, chemically-defined tool for decontaminating hiPSC-derived neurons. Neural precursor cells have been reported to express higher levels of ALP than terminally differentiated neural cells. SN38-P may also be useful for purification of other types of the terminally differentiated neural and glail cells such as mature oligodendrocytes.[36] Clinical application of SN38-P requires further safety evaluations due to potential mutagenesis risks of topoisomerase inhibitors. Nevertheless, similar phosphorylation strategies might be applied to other chemical iPSC eliminators to improve their selectivity and safety profiles, with the ultimate goal of clinical application.

This research is supported by the project, "Development of Cell Production and Processing Systems for Commercialization of Regenerative Medicine," from the Japan Agency for Medical Research and Development, AMED. This work is also supported in part by JSPS (26220206 to M.U.). This work was inspired by JSPS CORE-to-CORE “Asian Chemical Biology Initiative.”

Conflicts of interest

There are no conflicts to declare.

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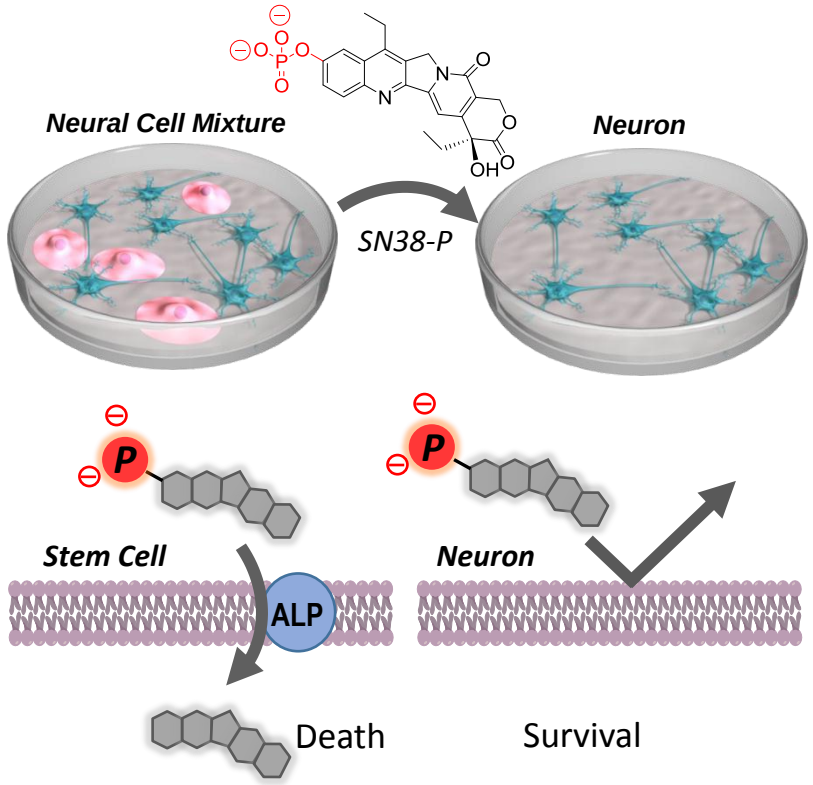

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