

Selective Elimination of Human Pluripotent Stem Cells by a Marine Natural Product Derivative

Ting-Fang Kuo,^{§,‡,⊥} Di Mao,^{§,‡,⊥} Nao Hirata,^{§,‡} Bilon Khambu,^{§,‡} Yasuhisa Kimura,[†] Eihachiro Kawase,^{||} Hiroki Shimogawa,[‡] Makoto Ojika,[#] Norio Nakatsuji,^{§,||} Kazumitsu Ueda,^{§,†} and Motonari Uesugi^{*,§,‡}

[§]Institute for Integrated Cell-Material Sciences (WPI-iCeMS), Kyoto University, Kyoto 606-8501, Japan

[‡]Institute for Chemical Research, Kyoto University, Uji, Kyoto 611-0011, Japan

[†]Division of Applied Life Sciences, Graduate School of Agriculture, Kyoto University, Kyoto 606-8502, Japan

^{||}Institute for Frontier Medical Sciences, Kyoto University, Kyoto 606-8507, Japan

[#]Graduate School of Bioagricultural Sciences, Nagoya University, Chikusa-ku, Nagoya 464-8601, Japan

Supporting Information

ABSTRACT: One of the current obstacles to stem cell therapy is the tumorigenic potential of residual undifferentiated stem cells. The present study reports rediscovery of a synthetic derivative of okadaic acid, a marine polyether toxin, as a reagent that selectively induces the death of human pluripotent stem cells. Cell-based screening of 333 cytotoxic compounds identified methyl 27-deoxy-27-oxookadaate (molecule 1) as a substrate of two ATP-binding cassette (ABC) transporters, ABCB1 (MDR1) and ABCG2 (BCRP), whose expression is repressed in human embryonic stem cells and induced pluripotent stem cells. The results demonstrate that selective elimination of human pluripotent stem cells can be achieved by designing cytotoxic small molecules with appropriate ABC-transporter selectivity.

Stem cell therapy using human pluripotent stem cells, including embryonic stem cells (hESCs) and induced pluripotent stem cells (hiPSCs), has the potential to treat a wide variety of diseases. One of the current problems of such therapy is the tumorigenic risk of residual undifferentiated cells.^{1,2} Several strategies for removing undifferentiated cells in therapeutic transplants have been reported, including cell sorting and precipitation using an antibody against a stem cell surface antigen,^{3,4} and selective killing of pluripotent stem cells by a cytotoxic antibody.⁵ Approaches using small molecules to eliminate pluripotent stem cells have also been examined. In one method, an inhibitor of stearoyl-CoA desaturase (SCD1) eliminates hPSCs by blocking the biosynthesis of oleic acid.⁶ In a second method, a glucose-depleted culture medium containing abundant lactate enables selective survival of cardiomyocytes and eliminates non-cardiomyocytes, including undifferentiated stem cells.⁷ A third method uses chemical inhibitors of survivin, one of the antiapoptotic factors overexpressed in pluripotent stem cells.⁸ The discovery of new mechanisms that could be combined with such elimination methods would ensure selective and rapid removal of residual undifferentiated cells.

By screening a chemical library, we previously discovered Kyoto Probe 1 (KP-1), a fluorescent chemical probe that

distinguishes between human pluripotent stem cells and differentiated cells.⁹ The selectivity results primarily from a distinct expression pattern of ATP-binding cassette (ABC) transporters in human pluripotent stem cells and from the unique transporter selectivity of KP-1. Expression of ABCB1 (MDR1) and ABCG2 (BCRP), both of which cause the efflux of KP-1, is repressed in human pluripotent stem cells. However, ABCC1 (MRP1), which is not involved in KP-1 efflux, is expressed in human pluripotent stem cells (Figure 1A). The

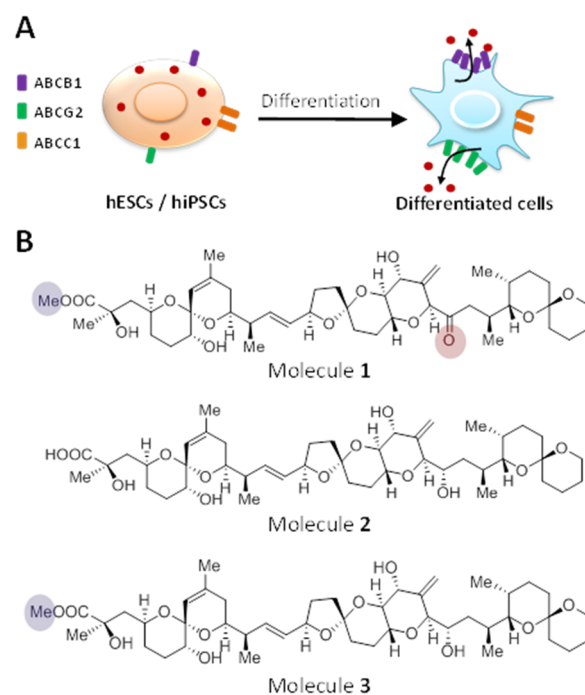


Figure 1. (A) Distinct expression pattern of ATP-binding cassette (ABC) transporters in human pluripotent stem cells and differentiated cells. (B) Chemical structures of methyl 27-deoxy-27-oxookadaate (molecule 1), okadaic acid (molecule 2), and methyl okadaate (molecule 3).

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distinct expression pattern of ABC transporters in human pluripotent stem cells could provide the basis for a new small molecule-based approach for selective elimination of those cells. In theory, selective death of human pluripotent stem cells should be achieved by cytotoxic molecules with ABC transporter selectivity similar to that of KP-1.

An in-house chemical library of cytotoxic small molecules (333 natural product derivatives and anticancer drugs) was screened for their effects on the viability of four cell lines: KB3-1 cells, which express no detectable levels of ABC transporters, and KB3-1-derived cells that overexpress ABCB1,¹⁰ ABCB1,¹¹ or ABCG2⁹ (KB3-1/ABCB1, KB3-1/ABCC1, and KB3-1/ABCG2). The screening details are described in Table S1. We selected compounds that had stronger cytotoxic effects on wild-type KB3-1 and KB3-1/ABCC1 cells than on KB3-1/ABCB1 and KB3-1/ABCG2 cells. Molecule 1 (methyl 27-deoxy-27-oxookadaate), a synthetic derivative of a well-known marine natural product, okadaic acid, exhibited the clearest and most desirable profile.

Molecule 1 was originally synthesized as a derivative of okadaic acid (molecule 2) by Yamada and co-workers at Nagoya University about 25 years ago, but was never described in the literature. We resynthesized molecule 1 and confirmed its structure and activity. The chemical structure and synthetic route of molecule 1 are shown in Figure 1B and Scheme S1, respectively. We then studied the structure–activity relationships of eight okadaic acid derivatives previously synthesized (Table S2).^{12,13} Okadaic acid (molecule 2) itself did not act as a substrate for any of the transporters, and exhibited potent cytotoxicity to all of the cells tested. Esterification of the carboxyl group (e.g., molecule 3) enhanced transport by ABCB1, consistent with the reported preference of ABCB1 for hydrophobic molecules.^{14–16} Among the okadaic acid derivatives we tested, molecule 1 was the only derivative that exhibited clear efflux both by ABCB1 and ABCG2, although the structural modifications in molecule 1 compromised overall cytotoxicity, compared to okadaic acid. The reason why the simple oxidation of a hydroxyl group to a keto group makes molecule 1 a suitable substrate of ABCG2 is unknown.

To confirm that the ABC transporters were responsible for the observed cell resistance, we examined the effects of selective inhibitors for ABCB1 and ABCG2, on IC₅₀ values (Table 1A and Table S3). Treatment with selective inhibitors of ABCB1, cyclosporine A (CsA) and its non-immunosuppressive derivative PSC833,¹⁷ greatly reduced the resistance of KB3-1/ABCB1 cells to molecule 3, whereas Ko143, a potent non-toxic inhibitor of ABCG2,¹⁸ had no detectable effect on its activity to KB3-1/ABCG2 cells. On the other hand, the cytotoxicity of molecule 1 in KB3-1/ABCB1 and KB3-1/ABCG2 cells was restored by the ABCB1 and ABCG2 inhibitors, respectively, confirming that molecule 1 is a substrate of both ABCB1 and ABCG2. Neither inhibitor had detectable effects on the activity of molecule 2.

To confirm that molecule 1 is a direct substrate for ABCB1, we carried out *in vitro* biochemical assays, in which ATPase activity of purified, reconstituted ABCB1 was monitored in the presence of various concentrations of molecules 1–3 (Figure S1).¹⁹ The amounts of ADP produced were measured using HPLC. Verapamil, a known substrate of ABCB1, drove the ATPase activity of ABCB1.²⁰ Molecules 1 and 3 activated ATPase activity to comparable levels, while molecule 2 failed to do so, consistent with the selectivity of their cytotoxicity to the

Table 1. IC₅₀ Values of (A) Molecules 1–3 in Model Cells and (B) Molecule 1 in hiPSCs, Human Primary Cells, and Cancer Cells, with or without the Addition of CsA, PSC833, or Ko143^a

μM			
	Molecule 1	Molecule 2	Molecule 3
KB3-1	1.24	0.021	0.185
ABCB1	25.76	0.075	> 50
ABCB1+ CsA	5.02	0.078	2.21
ABCB1+PSC833	1.02	0.030	0.386
ABCC1	1.34	0.102	0.238
ABCG2	6.58	0.04	0.216
ABCG2+ Ko143	2.812	0.03	0.209

μM			
	1	1 + CsA	1 + Ko143
hiPS	0.78 ± 0.064	0.70 ± 0.073	0.81 ± 0.064
SNL feeder cells	13.8 ± 0.29	2.94 ± 0.2	9.28 ± 0.51
Adrenal microvascular	5.0 ± 0.18	0.77 ± 0.02	4.95 ± 0.19
Astrocyte	2.1 ± 0.08	1.3 ± 0.01	1.2 ± 0.05
Brain microvascular	5.3 ± 0.2	1.2 ± 0.03	3.9 ± 0.02
Prostate epithelial	6.8 ± 0.31	2.1 ± 0.11	5.8 ± 0.13
Hepatocyte	5.9 ± 0.09	1.23 ± 0.02	3.8 ± 0.11
Bronchial epithelial	6.2 ± 0.11	2.1 ± 0.07	4.78 ± 0.15
HeLa	1.23 ± 0.02	1.11 ± 0.02	1.31 ± 0.02
HepG2	14.67 ± 0.25	2.08 ± 0.08	10.96 ± 0.5

^aPart A shows the IC₅₀ values of molecules 1, 2, and 3 in KB3-1-derived cell lines (*n* = 12). The level of resistance is indicated in blue. Cyclosporine A (CsA; 10 μM) and PSC833 (1 μM) are selective inhibitors of ABCB1, and Ko143 (10 μM) is a selective inhibitor of ABCG2. Panel B shows the IC₅₀ values of molecule 1 in hiPSCs, human somatic primary cells, and human cancer cells (*n* = 8). The level of cytotoxic potency is indicated in red. It is evident that CsA and Ko143 restored activity of molecule 1 in resistant cells.

model cells. Collectively, the results indicated that further analysis should focus on molecule 1.

KP-1 has been shown to selectively stain human pluripotent stem cells and neuronal cells, which have low expression of ABC transporters, including ABCB1 and ABCG2.⁹ As molecule 1 has a selectivity profile similar to that of KP-1, molecule 1 was expected to induce cell death selectively in KP-1-positive cells. Therefore, we compared the IC₅₀ values of molecule 1 in hiPSCs, human somatic primary cells, and human cancer cells (Table 1B). Molecule 1 was highly toxic to hiPSCs, with an IC₅₀ value of 0.78 μM (Figure S2), even lower than the IC₅₀ value of astrocytes (2.1 μM, Table 1B), a neuronal cell lineage known to express low levels of ABC transporters.²¹ In contrast, cells primarily involved in secretory functions (adrenal gland), metabolic functions (liver), barrier functions (bronchia), and reproductive organs (prostate) tend to express higher levels of ABC transporters²¹ and exhibited greater resistance to molecule 1 (IC₅₀ > 5 μM). Addition of CsA or Ko143 increased cytotoxicity of molecule 1 in these resistant somatic cells. HepG2 cells, a well-differentiated human hepatocellular carcinoma cell line that expresses high levels of ABC transporters,²² had IC₅₀ values as high as 14.7 μM. In contrast, HeLa cells, a human cervical carcinoma cell line that displays low levels of ABC transporters,²² exhibited IC₅₀ values as low as that of hiPSCs (1.2 μM). SNL cells, a mouse embryonic

fibroblast cell line often used as feeder cells for hESC and hiPSC culture, were resistant to molecule 1, and addition of CsA or Ko143 increased cytotoxicity of molecule 1 in these cells. Thus, the selectivity profile of molecule 1 paralleled KP-1 labeling.

To determine the ability of molecule 1 to distinguish hiPSCs from differentiated cells, we first added molecule 1 to hiPSCs cultured on SNL feeder cells. As expected, molecule 1 ($5 \mu\text{M}$) selectively eliminated hiPSC colonies, leaving the feeder cells on the plates (Figure 2A, left panel). A 50-fold lower concentration of okadaic acid (molecule 2) killed both the

hiPSCs and feeder cells. We next added molecule 1 ($5 \mu\text{M}$) to partially differentiated hiPSCs, prepared by treating hiPSCs with $5 \mu\text{M}$ of all-trans retinoic acid (RA) for 72 h. These experiments were carried out under both feeder cell (Figure 2A, right panel) and feeder cell-free (Figure 2B) conditions. Unlike hiPSCs, which form colonies, the partially differentiated cells were flat and spread out on the culture plates. While $0.1 \mu\text{M}$ molecule 2 killed hiPSCs, feeder cells, and differentiated cells, $5 \mu\text{M}$ molecule 1 appeared to selectively eliminate hiPSCs, leaving the differentiated cells. We obtained similar results using hESCs, in place of hiPSCs (Figure S3).

To confirm the selectivity of molecule 1, we used fluorescence staining with a stem cell marker, SSEA-4, and a differentiation marker, SSEA-1. Partially differentiated hiPSCs displayed a $\sim 1:1$ distribution of SSEA-4-positive cells and SSEA-1-positive cells. Treatment of the partially differentiated sample with DMSO alone for 48 h had no effect on the distribution of these cells (Figure 2C, upper panels). In contrast, treatment with molecule 1 for 48 h selectively eliminated the SSEA-4-positive cells (Figure 2C, lower panels). Fluorescence-activated cell sorting (FACS) analysis showed that molecule 1 removed SSEA-4-positive (Figure 2D) and SSEA-1-negative cells (Figure 2E), confirming the ability of molecule 1 to selectively eliminate undifferentiated cells from the mixture.

Okadaic acid (molecule 2) exerts its cytotoxic activity by inhibiting the protein serine/threonine phosphatases, PP1 and PP2A.^{23,24} To examine whether molecule 1 also targets PP1 and PP2A, we carried out *in vitro* biochemical assays, using recombinant PP1 and PP2 proteins. Okadaic acid (molecule 2) showed potent inhibition: IC_{50} was 1 nM for PP2A and 2.5 nM for PP1 (Figure S4A). Molecules 1 and 3 also inhibited PP1 and PP2A in a dose-dependent manner (Figure S4BC), but less potently: $\text{IC}_{50} \approx 1 \mu\text{M}$ for PP2A and $\text{IC}_{50} \approx 5 \mu\text{M}$ for PP1, consistent with their cytotoxicity. Thus, it is likely that molecule 1 exerts its cytotoxicity at least in part by inhibiting protein phosphatases.

In conclusion, the present study offers a simple small molecule-based approach to eliminating undifferentiated pluripotent stem cells from cell mixtures. The selective cytotoxicity of molecule 1 is based on its unique ABC-transporter selectivity. The limitation of this new approach is the inability of molecule 1 to distinguish between pluripotent stem cells and neuronal cells, similarly to KP-1 staining. Despite this limitation, molecule 1 or its analogs might serve as useful tools for both cell biology and stem cell therapy. Combining molecule 1 or its analogues with other reagents might improve the purity of differentiated cell samples for transplantation.

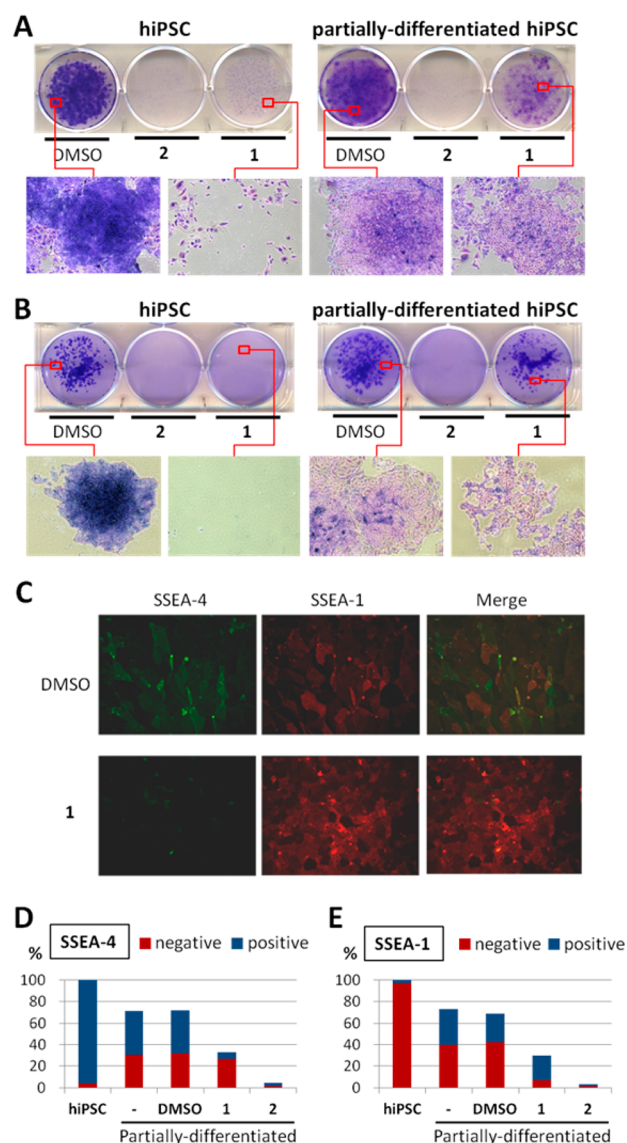


Figure 2. (A,B) Cytotoxic effects of molecule 1 on hiPSCs or partially differentiated hiPSCs. The cells were treated with molecule 2 ($0.1 \mu\text{M}$) or molecule 1 ($5 \mu\text{M}$) for 48 h on feeder cells (A) or under feeder-free conditions (B), $n = 4$. (C) Double staining of partially differentiated hiPSCs by a stem cell marker (SSEA-4) or a differentiation marker (SSEA-1), after treatment with molecule 1 ($5 \mu\text{M}$) or DMSO alone for 48 h under feeder-free conditions. (D,E) FACS analysis of partially differentiated hiPSCs immunostained by SSEA-4 (D) or SSEA-1 (E), after treatment with molecule 2 ($0.1 \mu\text{M}$) or molecule 1 ($5 \mu\text{M}$) for 48 h under feeder-free conditions. Each percentage was normalized to the corresponding viability, $n = 3$.

■ ASSOCIATED CONTENT

📄 Supporting Information

Supplementary data, experimental procedures, and characterization data. This material is available free of charge via the Internet at <http://pubs.acs.org>.

■ AUTHOR INFORMATION

Corresponding Author

uesugi@scl.kyoto-u.ac.jp

Author Contributions

[†]T.-F.K. and D.M. contributed equally to the work.

Notes

The authors declare the following competing financial interest(s): N.N. is a founder and shareholder of ReproCELL, Inc.

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