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# Cytotoxicity comparison of 35 developmental neurotoxicants in human induced pluripotent stem cells (iPSC), iPSC-derived neural progenitor cells, and transformed cell lines

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ABSTRACT

The Organization for Economic Co-operation and Development (OECD) test guideline 426 for developmental neurotoxicity (DNT) of industrial/environmental chemicals depends primarily on animal experimentation. This requirement raises various critical issues, such as high cost, long duration, the sacrifice of large numbers of animals, and interspecies differences. This study demonstrates an alternative protocol that is simple, quick, less expensive, and standardized to evaluate DNT of many chemicals using human induced pluripotent stem cells (iPSC) and their differentiation to neural progenitor cells (NPC). Initially, concentration-dependent cytotoxicity of 35 DNT chemicals, including industrial materials, insecticides, and clinical drugs, were compared among iPSC, NPC, and two transformed cells, Cos-7 and HepG2, using tetrazolium dye (MTS)-reducing colorimetric and ATP luciferase assays, and $IC_{50}$ values were calculated. Next, inhibitory effects of the 14 representative chemicals (mainly insecticides) on iPSC differentiation to NPC were evaluated by measuring altered expression of neural differentiation and undifferentiation marker genes. Results show that both iPSC and NPC were much more sensitive to most DNT chemicals than the transformed cells, and 14 chemicals induced differential patterns of marker gene expression, highlighting the validity and utility of the protocol for evaluation and classification of DNT chemicals and preclinical DNT tests for safety assessment.

## 1. Introduction

Epidemiological studies suggest close association between embryonic/postnatal exposure to some industrial chemicals and the onset of neurobehavioral disorders, including learning disabilities, attention deficit hyperactivity disorder (ADHD), autism, and the other cognitive abnormalities, in millions of children worldwide (Landrigan et al., 2012; Grandjean and Landrigan, 2014; Ross et al., 2015). These chemicals include insecticides/fungicides, industrial solvents, catalysts/plasticizers, clinical drugs, and research reagents (Pei et al., 2016; Harrill et al., 2018). Some of these chemicals have already been banned. The central nervous system in the fetal and neonatal periods is especially vulnerable to such chemicals, perhaps because the blood-brain barrier is not yet complete (Tohyama, 2016) when critical processes of temporal/regional neural development are ongoing (Rice and Barone Jr., 2000). Neurobehavioral disorders affect ~10% of all newborns/children, and prevalence of ADHD in the US young (3–17 years) population increased from 7.2% (in 2007) to 8.5% (in 2011) (Bloom et al., 2009; Bloom et al., 2012). Genetic factors play substantial roles–perhaps 30%–40% of all neurobehavioral disorders are due to genetics, but non-genetic environmental factors, including chemical exposure, are also involved (Grandjean and Landrigan, 2014).

To date, DNT behavioral/neurological test methodologies depend heavily on experimental animals, mainly rats (TG426; Developmental Neurotoxicity Study). Significant limitations with animal experimentation under this guideline are high cost, long duration, the sacrifice of large numbers of animals, interspecies differences, and lack of skilled laboratory animal technicians in the face of increasing demands (Schmidt, 2009; Tsuji and Crofton, 2012; Tohyama, 2016; Taylor, 2018). The Organization for Economic Co-operation and Development

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(OECD) has begun discussion on a DNT *in vitro* guidance document for protection of developing brains from chemicals that cause DNT (Fritsche et al., 2018b; Sachana et al., 2019). The basic concept is that the complex procedure of brain development can be disassembled into several neurodevelopmental endpoints which can be represented by a combination of different alternative assays (Fritsche et al., 2018a). The discovery of induced pluripotent stem cells (iPSC) and their differentiation to various cell lineages provides an opportunity for application to DNT evaluation (Pei et al., 2016; Ryan et al., 2016; Bal-Price et al., 2018b; Barenys and Fritsche, 2018; Fritsche et al., 2018a). iPSC are not tumor cells but proliferate infinitely and they can differentiate to neural cell lineages.

We assume that DNT in early stage of neural differentiation consists of two components: cytotoxicity to neural cells and differentiation alteration activity on neural stem/progenitor cells; therefore, we initially compared concentration-dependent cytotoxic effects of DNT chemicals selected by National Toxicity Program among iPSC, neural progenitor cells (NPC), and two transformed cell lines. Subsequently, we examined the impacts of 14 representative DNT chemicals on iPSC differentiation to NPC. These results support the utility of iPSC/NPC to supplement animal experimentation for the evaluation of DNT in safety assessment.

## 2. Materials and methods

### 2.1. Chemicals

The 35 DNT chemicals and a negative control, acetaminophen, analyzed in this study are listed in Table 1 with brief notations. All reagents were analytical grade and purchased from Sigma-Aldrich (Merck, Darmstadt, Germany), Wako/Fujifilm (Osaka, Japan), Nacalai Tesque (Kyoto, Japan), Tokyo Chemical Industry (Tokyo, Japan), Santa Cruz (Dallas, TX, USA), and Abcam (Cambridge, UK).

### 2.2. Cells and cell culture

Human iPSC line 253G1, established by retroviral transduction of *OCT4*, *SOX2*, and *KLF4* to adult human dermal fibroblasts (Nakagawa et al., 2008), was obtained from the Riken BRC Cell Bank (Ibaraki, Japan) at passage 27 (Lot no. 022). The cells were acclimatized to feeder-free culture conditions using human embryonic stem cell-qualified Matrigel (BD Biosciences, San Jose, CA, USA) and TeSR-E8 medium (Stemcell Technologies, Vancouver, BC, Canada) at 37 °C in a 5% $CO_2$/95% air incubator. iPSC colonies were dissociated into single cells using Accumax (Innovative Cell Technologies, San Diego, CA, USA) for passage and cultured in TeSR-E8 medium supplemented with Y-27632 (ROCK inhibitor, 10 μM, Wako). Total passage numbers for all iPSC used in this study did not exceed 50.

The dual SMAD inhibition protocol (Chambers et al., 2009) was used for the induction of neural lineages with some modification (Yamada et al., 2017; Yamada et al., 2018a; Yamada et al., 2018b). Briefly, iPSC colonies were dissociated into single cells using Accumax, and cells were seeded into 12-well tissue culture dishes at a density of $6.66 \times 10^4$ cells/cm$^2$ in TeSR-E8 medium on Matrigel-coated plates and become nearly confluent within two days. Then, medium was changed to knockout serum replacement medium consisting of 82% KnockOut DMEM (Gibco/Thermo Fisher Scientific), 15% KnockOut Serum Replacement (Gibco), 1% MEM Non-Essential Amino Acids Solution (NEAA, Gibco), 1% GlutaMAX Supplement (Gibco), 1% penicillin/streptomycin (P/S, Nacalai Tesque), and 3.5 ppm 2-mercaptoethanol (Wako) supplemented with SB431542 (TGF-β inhibitor, 10 μM, Wako) and LDN193189 (Bone Morphogenetic Protein [BMP] inhibitor, 1 μM, Wako). Cells were maintained for four days with medium renewal after two days. From the fourth day on, the medium was stepwise replaced with $N_2$ medium consisting of 95.06% Neurobasal Medium, 1% $N_2$ Supplement, 2% B-27 Supplement (*minus* vitamin A), 0.97% GlutaMAX Supplement (all from Gibco), and 0.97% P/S *plus* 1 μM LDN193189. Replacement steps were 25:75 on the fourth day, 50:50 on the sixth, and 75:25 on the eight (the first value in each ratio represents the $N_2$ medium). After 10 days, established NPCs were dissociated into single cells using Accumax and maintained in neural maintenance medium (NMM) consisting of 47.628% Neurobasal Medium (Gibco), 47.628% DMEM/F-12 medium (Gibco), 20 ng/ml FGF-Basic (R&D Systems, Minneapolis, MN, USA), 20 ng/ml human recombinant EGF (R&D Systems), 1% N-2 Supplement, 2% B-27 Supplement (*minus* vitamin A), 0.486% NEAA, 0.486% GlutaMAX Supplement, 3.4 ppm 2-mercaptoethanol, and 0.972% P/S. NPC were frozen at passage 2 in NMM supplemented with 20 ng/ml FGF-Basic, 20 ng/ml human recombinant EGF, 5 μM Y27932, and 10% DMSO until use. The NPC stocks were thawed, and passages 4–10 were used for the repetitive experiments that gave reproducible results. Giemsa staining of iPSC and NPC at passages 7 and 10 was used for chromosome counting as described previously (Sugawara et al., 2006).

Cos-7 and HepG2 cells were also obtained from the Riken BRC Cell Bank (RCB0539 and RCB1648, respectively). They were maintained in DMEM (Wako), supplemented with 10% fetal bovine serum (French origin; Biowest, Nuaillé, France) and 1% P/S.

### 2.3. Immunocytochemistry

iPSC were cultured on Matrigel-coated glass coverslips and fixed in 10% formaldehyde in phosphate-buffered saline (PBS, pH 7.4) for 1 h at room temperature. Fixed cells were incubated with blocking buffer (10% normal donkey serum [Millipore/Merck] and 0.1% [*v*/*w*] Triton X-100 in PBS) for 1 h. After washing twice with 0.1% [*v*/*w*] Triton X-100 in PBS (PBST), cells were incubated with anti-PAX6 mouse monoclonal antibody (1:100; Santa Cruz, sc-81,649) or anti-OCT3/4 mouse monoclonal antibody (1:100; Santa Cruz, sc-5279) in blocking buffer for 1 h. After washing twice with PBST, cells were incubated with Alexa Fluor 488-labeled donkey anti-mouse IgG (H + L) antibody (1:500; Molecular Probes/Thermo Fisher Scientific, A-21202) in blocking buffer for 1 h. Finally, coverslips were mounted with SlowFade Gold Antifade Mountant with DAPI (Invitrogen/Thermo Fisher Scientific) and examined using a BZ-X810 fluorescent microscope (Keyence, Osaka, Japan) equipped with a CFI Plan Apo λ 20× objective lens (Nikon, Tokyo, Japan).

### 2.4. Cell viability assays

Mitochondrial MTS (5-[3-(carboxymethoxy)phenyl]-3-(4,5-dimethyl-2-thiazolyl)-2-(4-sulfophenyl)-2H-tetrazolium inner salt)-reducing activity and cellular ATP levels were used as two indicators of cell viability. The cells were seeded into 96-well tissue culture dishes at $1.5 \times 10^3$ cells/well ($4.69 \times 10^3$ cells/cm$^2$) for Cos-7 and $2.0 \times 10^3$ cells/well ($6.25 \times 10^3$ cells/cm$^2$) for HepG2, iPSC, and NPC, and cultured overnight at 37 °C in a 5% $CO_2$ incubator for cell adhesion. Cells were then incubated in culture medium with each chemical for 48 h. The MTS-reducing activity was evaluated by measuring the absorbance at 490 nm (and 700 nm for reference) using a CellTiter 96 AQueous One Solution Cell Proliferation Assay kit (Promega, Madison, WI, USA) and an Epoch 2 Microplate Spectrophotometer (BioTek/Agilent, Winooski, VT, USA). Cellular ATP levels were measured using a Cell ATP Assay reagent (TOYO B-Net, Tokyo, Japan) and a LMax II 384 Microplate Luminometer (Molecular Devices, San Jose, CA, USA). $IC_{50}$ values were calculated using a Prism 5 software (GraphPad, San Diego, CA, USA).

### 2.5. Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

The total RNA was isolated with a ReliaPrep RNA Miniprep System (Promega). One μg of RNA was reverse-transcribed (RT) to first-strand cDNA using a ReverTra Ace qPCR RT kit (Toyobo, Tokyo, Japan), and

**Table 1**
35 neurotoxicants and developmental neurotoxicants analyzed in this study.

| ID | Chemicals/Abbreviation | CAS | Mol. formula | MW | Stock | Application or origin | Known target |
|---|---|---|---|---|---|---|---|
| A | 1-Methyl-4-phenylpyridinium iodide/MPP + iodide | 36913–39-0 | $C_{12}H_{12}IN$ | 297.13 | 1 M DMSO | Toxic metabolite of MPTP | ETC complex I (Dopaminergic neuron) |
| B | 2-Methoxyethanol | 109–86-4 | $C_3H_8O_2$ | 76.09 | (1 M media) | Solvent/additive | |
| C | 3,3′-Iminodipropionitrile/IDPN | 111–94-4 | $C_6H_9N_3$ | 123.16 | (1 M media) | Synthetic material/ Research reagent | Neurofilament proteins |
| D | 5-Fluorouracil | 51–21-8 | $C_4H_3FN_2O_2$ | 130.08 | 1 M DMSO | Anti-cancer drug | Thymidylate synthase |
| E | 6-Hydroxydopamine hydrochloride/6-OHDA | 8094-15-7 | $C_8H_{12}ClNO_3$ | 205.64 | 0.5 M DMSO | Research reagent | (Dopaminergic/noradrenergic neurons) |
| F | 6-Propyl-2-thiouracil | 51–52-5 | $C_7H_{10}N_2OS$ | 170.23 | 2 M DMSO | Thyrostatic agent | Thyroid peroxidase/ Iodothyronine deiodinase |
| G | Manganese (II) acetate | 638–38-0 | $C_4H_6MnO_4$ | 173.03 | (1 M media) | Catalyst/Fertilizer | (Induction of Parkinson's disease) |
| H | Acrylamide | 79–06-1 | $C_3H_5NO$ | 71.08 | (1 M media) | Industrial material/ Byproduct | DNA (Group 2A carcinogen *via* glycidamide) |
| I | Aldicarb | 116–06-3 | $C_7H_{14}N_2O_2S$ | 190.26 | 1 M DMSO | Carbamate insecticide | Acetylcholinesterase |
| J | Bis(tributyltin) oxide | 56–35-9 | $C_{24}H_{54}OSn_2$ | 596.11 | 1 mM DMSO | Antifoulant/Biocide | (Endocrine disruptor) |
| K | Bisphenol A | 80–05-7 | $C_{15}H_{16}O_2$ | 228.29 | 1 M DMSO | Resin material | (Endocrine disruptor) |
| L | Captan | 133–06-2 | $C_9H_8Cl_3NO_2S$ | 300.59 | 0.1 M DMSO | Phthalimide fungicide | Thiol and amino groups of enzymes |
| M | Carbaryl | 63–25-2 | $C_{12}H_{11}NO_2$ | 201.22 | 1 M DMSO | Carbamate insecticide | Acetylcholinesterase |
| N | Chlorpyrifos | 2921–88-2 | $C_9H_{11}Cl_3NO_3PS$ | 350.59 | 1 M DMSO | Organophosphate insecticide | Acetylcholinesterase |
| O | Colchicine | 64–86-8 | $C_{22}H_{25}NO_6$ | 399.44 | 1 M DMSO | Anti-gout/FMF drug | Tubulin |
| P | Deltamethrin | 52918–63-5 | $C_{22}H_{19}Br_2NO_3$ | 505.20 | 0.5 M DMSO | Pyrethroid insecticide | Voltage-gated sodium channel |
| Q | Di(2-ethylhexyl) phthalate/DEHP | 117–81-7 | $C_{24}H_{38}O_4$ | 390.56 | 0.2 M DMSO | Plasticizer | Androgen receptor |
| R | Dichlorodiphenyltrichloroethane/DDT | 50–29-3 | $C_{14}H_9Cl_5$ | 354.49 | 0.5 M DMSO | Organochlorine insecticide | Voltage-gated sodium channel |
| S | Dieldrin | 60–57-1 | $C_{12}H_8Cl_6O$ | 380.91 | 0.5 M DMSO | Organochlorine insecticide | GABA-gated chloride channel |
| T | Diethylstilbestrol | 56–53-1 | $C_{18}H_{20}O_2$ | 268.35 | 0.5 M DMSO | Synthetic nonsteroidal estrogen | Estrogen receptor |
| U | Heptachlor | 76–44-8 | $C_{10}H_5Cl_7$ | 373.32 | 0.5 M DMSO | Organochlorine insecticide | GABA-gated chloride channel |
| V | Hexachlorophene | 70–30-4 | $C_{13}H_6Cl_6O_2$ | 406.90 | 1 M DMSO | Organochlorine disinfectant | (Protein denaturation) |
| W | Hydroxyurea | 127–07-1 | $CH_4N_2O_2$ | 76.05 | (1 M media) | Anti-cancer drug | Ribonucleoside diphosphate reductase |
| X | Lindane | 58–89-9 | $C_6H_6Cl_6$ | 290.83 | 1 M DMSO | Organochlorine insecticide | GABA-gated chloride channel |
| Y | Methyl Hg (II) chloride | 115–09-3 | $CH_3ClHg$ | 251.08 | (0.5 M media) | Catalyst/Fungicide/ Bacterial product | (Induction of CNS disorder) |
| Z | *n*-Hexane | 110–54-3 | $C_6H_{14}$ | 86.18 | 0.2 M DMSO | Solvent | |
| A' | Permethrin | 52645–53-1 | $C_{21}H_{20}Cl_2O_3$ | 391.29 | 1 M DMSO | Pyrethroid insecticide | Voltage-gated sodium channel |
| B′ | Phenobarbital, Na salt | 57–30-7 | $C_{12}H_{11}N_2NaO_3$ | 254.22 | (0.05 M media) | Anti-epileptic drug | $GABA_A$ receptor |
| C′ | Rotenone | 83–79-4 | $C_{23}H_{22}O_6$ | 394.42 | 0.2 M DMSO | Insecticide/Piscicide | ETC complex I |
| D' | Tebuconazole | 107534–96-3 | $C_{16}H_{22}ClN_3O$ | 307.82 | 1 M DMSO | Triazole fungicide | (Inhibition of sterol C14-demethylation) |
| E' | Tetraethylthiuram disulfide | 97–77-8 | $C_{10}H_{20}N_2S_4$ | 296.54 | 1 M DMSO | Anti-alcoholism drug | Acetaldehyde dehydrogenase |
| F′ | Thalidomide | 50–35-1 | $C_{13}H_{10}N_2O_4$ | 258.23 | 0.5 M DMSO | Anti-cancer (multiple myeloma) drug | |
| G' | Toluene | 108–88-3 | $C_7H_8$ | 92.14 | 1 M DMSO | Solvent | |
| H′ | Valinomycin Valinomycin | 2001-95-8 | $C_{54}H_{90}N_6O_{18}$ | 1111.32 | 0.1 M DMSO | Depsipeptide antibiotic | Potassium ionophore |
| I′ | Valproic acid, Na salt | 1069-66-5 | $C_8H_{15}NaO_2$ | 166.19 | 1 M DDW | Anti-convulsive drug | Histone deacetylase (HDAC) |
| (Negative control) | | | | | | | |
| J' | Acetaminophen | 103–90-2 | $C_8H_9NO_2$ | 151.17 | (0.1 M media) | Anti-fever/pain/headaches | Cyclooxygenase-2 (COX-2) |

MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (=a contaminant of illicitly synthesized meperidine analog MPPP [1-methyl-4-phenyl-propionoxypiperidine]); ETC, electron transport chain; FMF, familial Mediterranean fever; CNS, central nervous system.

5 ng of cDNA from each sample was amplified *via* qPCR using KOD SYBR qPCR Mix (Toyobo), primer sets (Supplementary Table S1), and the Bio-Rad CFX Connect Real-Time PCR Detection System. mRNA levels were quantified using a comparative CT method with glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*, a housekeeping gene) levels for normalization.

### 2.6. Statistical analyses

Data were expressed as mean ± standard error (S.E.) (*n*: numbers of independent experiments with triplicate well samples). Statistical comparison was performed by one-way ANOVA with Tukey's multiple comparison test for $IC_{50}$ values and an unpaired two-tailed Student's *t*-test in RT-qPCR experiments using Prism 5. All *p*-values less than 0.05 denote a significant difference.

## 3. Results

### 3.1. iPSC differentiation to NPC

Using the modified dual SMAD inhibition protocol (*e.g.*, LDN193189 was used instead of Noggin as a BMP inhibitor [Chambers et al., 2009; Yamada et al., 2017]) (Fig. 1A), human iPSC were successfully differentiated to NPC, as confirmed by mRNA induction of neural differentiation markers, *PAX6*, *MAP2*, and *OTX2*, and mRNA repression of stem cell (undifferentiation) markers, *OCT3/4* and *NANOG*. The mRNA level induction in *MAP2* and *OTX2* preceded that in *PAX6*, although all reached plateaus at day 8; the magnitude of *PAX6* induction was most prominent (×1728 *versus* ×169 [*MAP2*] and ×26 [*OTX2*] at day 10; Fig. 1B *left*). Repression of *NANOG* preceded repression of *OCT3/4*, but both finally approached a comparable level at day 10 (1.23% and 1.73% of day 0, respectively) (Fig. 1B *right*). Immunocytochemistry

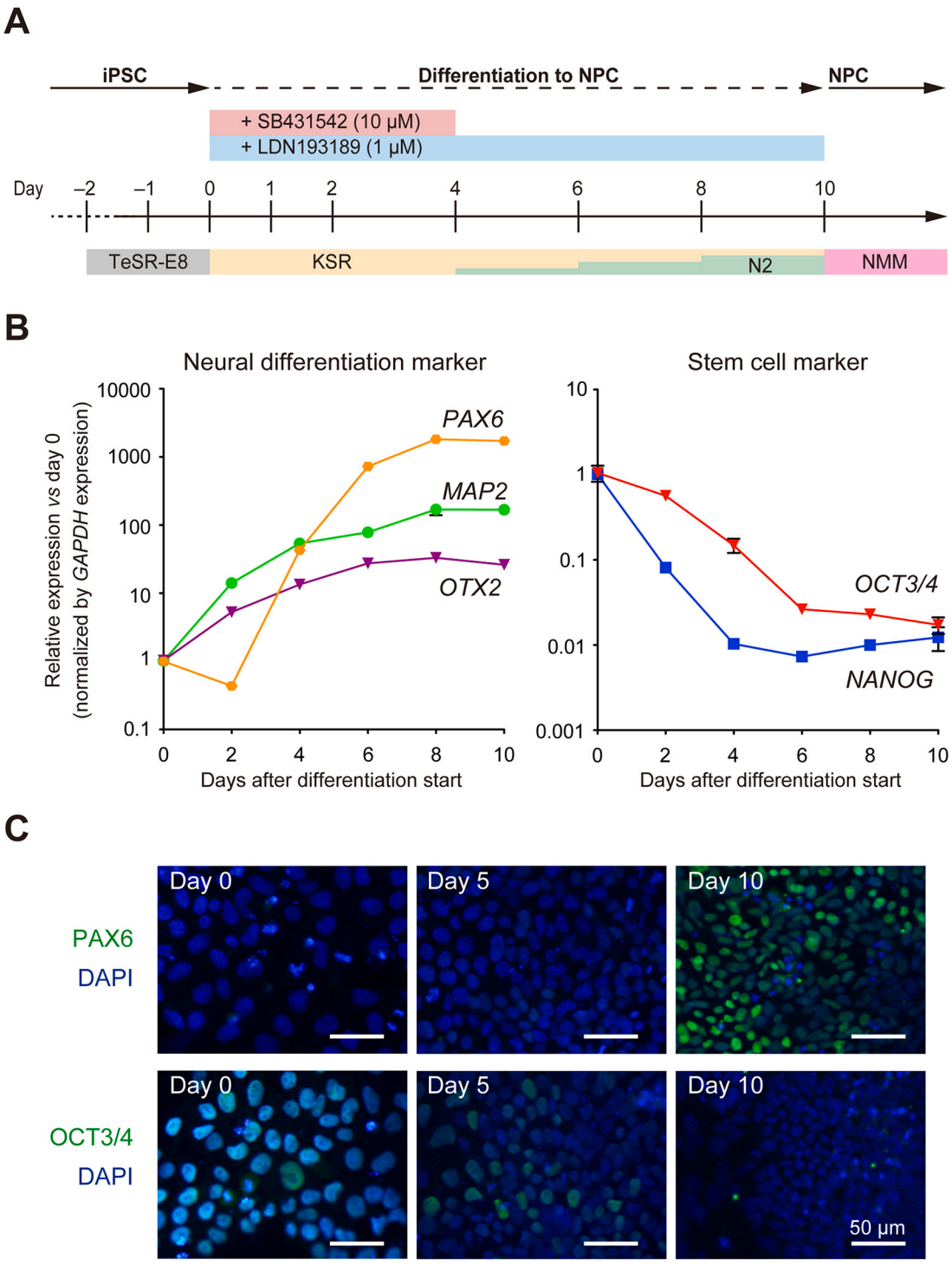



**Fig. 1.** iPSC differentiation to NPC. (A) Differentiation overview by the dual SMAD inhibition protocol. (B) mRNA induction of neural differentiation markers (*PAX6*, *MAP2*, and *OTX2*; *left*) and repression of stem cell (undifferentiation) markers (*OCT3/4* and *NANOG*; *right*) that is normalized by a housekeeping *GAPDH* gene. Representative data from three independent experiments are shown and mean ± standard error (S.E.) of triplicate samples are presented. S.E. bars are not shown when they are smaller than the size of the data points. (C) Immunohistochemistry of PAX6 (*green*; upper three panels) and OCT3/4 (*green*; lower three panels) with DAPI (blue; all six panels) in iPSC (day 0), the differentiation waypoint (to NPC, day 5), and NPC (day 10). Bars are 50 μm.

showed PAX6 protein upregulation and OCT3/4 protein downregulation during differentiation to NPC (Fig. 1C). NPC were thereafter maintained and passaged ten times in NMM, and *PAX6/MAP2* upregulation and *OCT3/4/NANOG* downregulation were maintained. In contrast, *OTX2* upregulation was cancelled–*OTX2* was instead downregulated–during the maintenance period in NMM (Supplementary Fig. S1A). NPC at passages 4–10 were used for ATP(/MTS) assays that produced similar $IC_{50}$ values (Supplementary Fig. S1B, for example). Total chromosomal numbers of original iPSC and NPC at passages 7 and 10 were 46 as evidenced by Giemsa staining (Supplementary Fig. S2).

### 3.2. Cytotoxicity of 35 DNT chemicals on Cos-7, HepG2, iPSC, and NPC

Thirty-five DNT chemicals and a negative control, acetaminophen,

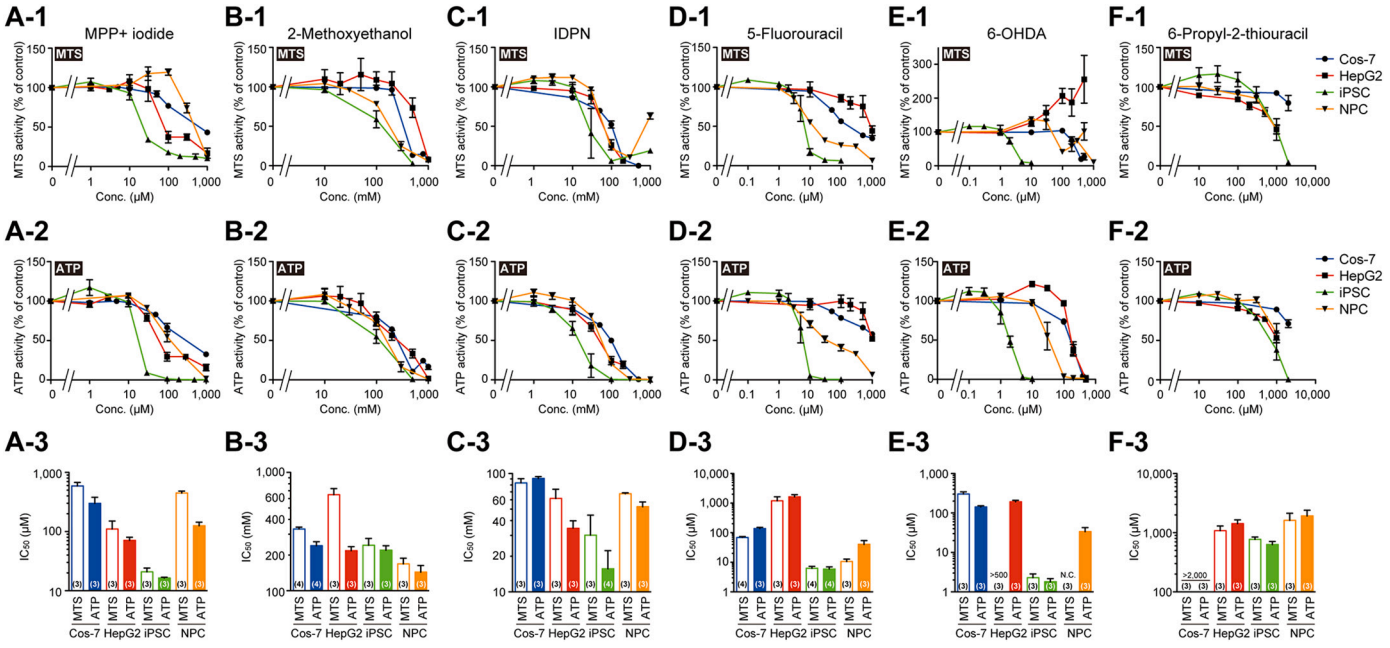



**Fig. 2.** Concentration-dependent inhibition of cell survival activity ("–1," MTS assay; "–2," ATP assay) in four cell types (Cos-7, HepG2, iPSC, and NPC) by six DNT chemicals (A, MPP+ iodide; B, 2-methoxyethanol; C, IDPN; D, 5-fluorouracil; E, 6-OHDA; and F, 6-propyl-2-thiouracil) and calculated $IC_{50}$ values ("–3"). Data are mean ± S.E. of 3–4 (presented in parentheses in "–3") independent experiments that have four sample replicates (wells). N.C., not calculated.

were administered to four cell types (Cos-7, HepG2, iPSC, and NPC) at varying concentrations (Table 1). After two days, cell survival was examined with two common assays, mitochondrial MTS reduction activity and cellular ATP level, and $IC_{50}$ values were calculated (Fig. 2, Supplementary Fig. S3, and Table 2). The highest concentrations of DNT chemicals were individually set so that the chemical did not precipitate in medium. Most, but not all, DNT chemicals displayed concentration-dependent inhibition by MTS reduction and cellular ATP activity (Fig. 2 and Supplementary Fig. S3). Some DNT chemicals, including aldicarb [I], *n*-hexane [Z], thalidomide [F′], and toluene [G'], were not cytotoxic when administered at their highest concentration. Generally, similar $IC_{50}$ values were obtained from two cellular assays (Supplementary Fig. S4), demonstrating the accuracy and validity of the assays. The $IC_{50}$ values for most DNT chemicals for NPC, and especially iPSC, were > 1–2 orders of magnitude less than $IC_{50}$ of Cos-7 and HepG2 cells (Fig. 3). Further, the differences in $IC_{50}$ values of most DNT chemicals, based on ATP assays, were more apparent between Cos-7 and HepG2 (Fig. 4A) than between iPSC and NPC (Fig. 4B). The $IC_{50}$ values of most DNT chemicals were one order of magnitude higher in NPC than in iPSC (Fig. 4B). NPC passage numbers did not affect the concentration-dependent inhibition and thus $IC_{50}$ values (Supplementary Fig. S1B, for examples in ATP assays).

### 3.3. Differential inhibition of iPSC differentiation to NPC by DNT chemicals

To consider the application of the differentiation system for evaluation/classification of DNT chemicals, effects of 14 representative DNT chemicals (mainly insecticides) on day 0–4 differentiation from iPSC to NPC were investigated (Fig. 5A). Specifically, total RNA was isolated from cells at day 4, and expression of differentiation *vs* undifferentiation marker genes was examined by RT-qPCR (Fig. 5B and Supplementary Fig. S5). DNT chemicals induced different patterns of gene expression (Table 3). For example, aldicarb, a carbamate insecticide used worldwide and is a known environmental toxicant, which did not show cytotoxic activity on iPSC (Supplementary Fig. S3, I-1–3), downregulated *PAX6* expression but upregulated *MAP2* expression without significantly altering *OTX2*, *OCT3/4*, and *NANOG* expression (Fig. 5B *top column panels*). Another carbamate insecticide, carbaryl, also downregulated *PAX6* and upregulated *MAP2* but downregulated *OTX2* and *OCT3/4* at its highest concentration (100 μM; Fig. 5B *third column panels*). Among the four organochlorine insecticides (DDT, dieldrin, heptachlor, and lindane), the registrations of which have expired in most developed countries (Aktar et al., 2009), *PAX6* expression was suppressed by all four chemicals, but *OTX2* expression was only suppressed by dieldrin and lindane, and *MAP2* expression was upregulated by dieldrin, heptachlor, and lindane, (Supplementary Fig. S5 *top, second, and fourth column panels*) but not DDT (Fig. 5B *bottom column panels*). The organochlorine disinfectant, hexachlorophene, and insecticide/piscicide, rotenone, displayed typical inhibition patterns that repress induction of three neural differentiation markers while maintaining *OTX2* and *OCT3/4* expression (Supplementary Fig. S5 *third and sixth column panels*). In contrast, the two pyrethroid insecticides (deltamethrin and permethrin) that have been developed as safer replacements of organochlorine insecticides due to relatively low mammalian toxicity and rapid environmental biodegradation (Jayaraj et al., 2016) did not modify expressions of marker genes to such an extent (Fig. 5B *fifth column panels* and Supplementary Fig. S5 *fifth column panels*). Captan and colchicine showed limited ability to affect marker gene expression (Fig. 5B *second and fourth column panels*), whereas valproic acid exerted significant effects except for *PAX6* (Supplementary Fig. S5 *bottom column panels*). Constant yields of total RNA from each well and viable cell appearances in microscopic analyses suggest that such expressional alteration did occur at concentrations below cytotoxic ranges (data not shown).

## 4. Discussion

This study modeled *in vitro* the impacts of DNT chemicals on early stages of neural differentiation among various key neurodevelopmental processes (Bal-Price et al., 2018b; Fritsche et al., 2018a), by examining the cytotoxicity activity to iPSC/NPC as one measurement endpoint and alteration of gene expression of neural differentiation marker genes during neural differentiation as a second endpoint. A previous study investigated the cytotoxic effects of 80 drugs and environmental chemicals provided by the National Toxicology Program (US Department of Health and Human Services). Toxicity was evaluated in human iPSC

**Table 2**
The $IC_{50}$ values of 35 (developmental) neurotoxicants and a negative control acetaminophen in four cell types calculated from MTS and ATP assays.

| | | | Cos-7 (a) | | HepG2 (b) | | iPSC (c) | | NPC (d) | |
|---|---|---|---|---|---|---|---|---|---|---|
| ID | Chemicals | Unit | MTS | ATP (*) | MTS | ATP (*) | MTS | ATP (*) | MTS | ATP (*) |
| A | MPP+ iodide | μM | 593 ± 83$^{b,c}$ (3) | 299 ± 81$^{b}$ (3) | 111 ± 40$^{a,d}$ (3) | 71.0 ± 9.3$^{a}$ (3) | 20.9 ± 3.4$^{a,d}$ (3) | 16.5 ± 0.4$^{a}$ (3) | 450 ± 35$^{b,c}$ (3) | 126 ± 18* (3) |
| B | 2-Methoxyethanol | mM | 333 ± 12$^{b}$ (4) | 240 ± 19$^{d,*}$ (4) | 648 ± 82$^{a,c,d}$ (3) | 218 ± 18* (3) | 243 ± 33$^{b}$ (3) | 220 ± 20 (3) | 169 ± 20$^{b}$ (3) | 144 ± 20$^{a}$ (3) |
| C | IDPN | mM | 83.5 ± 7.1$^{c}$ (3) | 91.1 ± 3.1$^{b,c,d}$ (3) | 61.6 ± 12.0 (3) | 34.5 ± 5.4$^{a}$ (3) | 30.2 ± 14.4$^{a}$ (3) | 15.7 ± 6.6$^{a,d}$ (4) | 67.7 ± 1.1 (3) | 52.7 ± 4.9$^{a,c,*}$ (3) |
| D | 5-Fluorouracil | μM | 69.4 ± 5.1$^{b}$ (4) | 138 ± 15$^{b,*}$ (3) | 1199 ± 452$^{a,c,d}$ (3) | 1649 ± 302$^{a,c,d}$ (3) | 6.32 ± 1.03$^{b}$ (4) | 5.92 ± 1.06$^{b}$ (4) | 10.8 ± 2.0$^{b}$ (3) | 41.1 ± 13.5$^{b}$ (3) |
| E | 6-OHDA | μM | 304 ± 41$^{c}$ (3) | 142 ± 12$^{b,c,d,*}$ (3) | N.C. (3) | 195 ± 15$^{a,c,d}$ (3) | 2.29 ± 0.56$^{a}$ (3) | 1.83 ± 0.35$^{a,b}$ (3) | N.C. (3) | 34.0 ± 8.7$^{a,b}$ (3) |
| F | 6-Propyl-2-thiouracil | μM | > 2000 (3) | > 2000 (3) | 1087 ± 214 (3) | 1431 ± 219 (3) | 766 ± 76 (3) | 631 ± 84 (3) | 1621 ± 512 (3) | 1926 ± 489 (3) |
| G | Manganese (II) acetate | μM | N.C. (3) | 68.3 ± 15.6$^{b}$ (3) | N.C. (3) | 688 ± 204$^{a}$ (3) | N.C. (3) | N.C. (3) | N.C. (3) | 408 ± 13 (3) |
| H | Acrylamide | mM | 2.37 ± 0.28$^{c,d}$ (5) | 2.93 ± 0.45$^{c,d}$ (3) | 2.20 ± 0.34$^{c,d}$ (3) | 2.34 ± 0.51$^{c,d}$ (3) | 0.458 ± 0.086$^{a,b}$ (3) | 0.274 ± 0.039$^{a,b}$ (3) | 0.753 ± 0.041$^{a,b}$ (3) | 0.635 ± 0.055$^{a,b}$ (3) |
| I | Aldicarb | μM | > 1000 (3) | > 1000 (3) | > 1000 (3) | > 1000 (3) | > 1000 (3) | > 1000 (3) | > 1000 (3) | > 1000 (3) |
| J | Bis(tributyltin) oxide | μM | > 1 (3) | > 1 (3) | 0.944 ± 0.204$^{c}$ (4) | 0.512 ± 0.043$^{c}$ (3) | 0.220 ± 0.093$^{b}$ (4) | 0.220 ± 0.070$^{b}$ (4) | 0.596 ± 0.100 (3) | 0.410 ± 0.082 (3) |
| K | Bisphenol A | μM | 126 ± 6$^{b,c}$ (3) | 111 ± 14$^{c}$ (3) | 242 ± 22$^{a,c}$ (4) | 184 ± 30$^{c,d}$ (4) | 19.8 ± 3.1$^{a,b,d}$ (4) | 15.2 ± 1.5$^{a,b}$ (3) | 190 ± 13$^{c}$ (3) | 65.9 ± 7.2$^{b,*}$ (3) |
| L | Captan | μM | > 100 (3) | > 100 (3) | > 100 (3) | > 100 (3) | 0.454 ± 0.085$^{d}$ (3) | 0.497 ± 0.130$^{d}$ (3) | 6.72 ± 1.73$^{c}$ (3) | 6.44 ± 1.35$^{c}$ (3) |
| M | Carbaryl | μM | > 500 (3) | > 500 (3) | > 500 (4) | > 500 (4) | 20.7 ± 7.8 (4) | 22.4 ± 6.4 (4) | 24.3 ± 10.0 (3) | 20.2 ± 4.3 (3) |
| N | Chlorpyrifos | μM | > 1000 (4) | > 1000 (4) | > 1000 (4) | > 1000 (3) | 86.1 ± 34.9 (3) | 19.9 ± 2.3$^{d}$ (3) | 217 ± 57 (3) | 136 ± 38$^{c}$ (3) |
| O | Colchicine | nM | 32.1 ± 6.9 (3) | 26.1 ± 5.5$^{b}$ (3) | 38.2 ± 13.5$^{c,d}$ (3) | 75.9 ± 21.2$^{a,c,d}$ (3) | 5.73 ± 0.67$^{b}$ (4) | 5.32 ± 0.54$^{b}$ (4) | 2.26 ± 0.68$^{b}$ (3) | 3.29 ± 0.84$^{b}$ (3) |
| P | Deltamethrin | μM | > 200 (3) | > 200 (3) | > 200 (4) | > 200 (4) | 7.34 ± 0.33 (4) | 3.42 ± 0.50$^{d,*}$ (4) | > 200 (3) | 83.7 ± 19.4$^{c}$ (3) |
| Q | DEHP | μM | > 200 (3) | > 200 (3) | > 200 (3) | > 200 (3) | > 200 (4) | 75.1 ± 31.1 (5) | 38.8 ± 11.5 (4) | 15.7 ± 7.1 (4) |
| R | DDT | μM | > 500 (3) | > 500 (3) | > 500 (3) | > 500 (3) | 3.83 ± 0.26$^{d}$ (3) | 2.33 ± 0.37$^{d,*}$ (4) | 16.6 ± 1.5$^{c}$ (3) | 11.4 ± 2.9$^{c}$ (3) |
| S | Dieldrin | μM | > 500 (3) | 55.7 ± 13.6$^{c,d}$ (3) | > 500 (4) | > 500 (3) | 5.64 ± 0.90$^{a}$ (4) | 3.17 ± 0.47$^{d}$ (4) | 13.3 ± 1.5$^{a}$ (4) | 9.22 ± 0.95$^{c}$ (4) |
| T | Diethylstilbestrol | μM | 47.2 ± 6.8$^{c,d}$ (4) | 56.8 ± 4.2$^{c,d}$ (3) | N.C. (4) | N.C. (4) | 2.24 ± 0.8$^{a,d}$ (6) | 2.09 ± 0.91$^{a,d}$ (6) | 24.9 ± 1.8$^{a,c}$ (3) | 18.8 ± 1.6$^{a,c}$ (3) |
| U | Heptachlor | μM | > 200 (3) | > 200 (3) | > 200 (3) | > 200 (3) | 4.60 ± 0.76$^{d}$ (4) | 4.33 ± 0.33$^{d}$ (4) | 16.8 ± 0.3$^{c}$ (3) | 14.5 ± 1.3$^{c}$ (3) |
| V | Hexachlorophene | μM | 24.9 ± 3.5$^{c,d}$ (4) | 35.0 ± 4.3$^{b,c,d}$ (3) | 18.0 ± 2.3$^{c,d}$ (3) | 16.9 ± 1.9$^{a,c,d}$ (3) | 0.0851 ± 0.0164$^{a,b}$ (5) | 0.0911 ± 0.0222$^{a,b}$ (5) | 5.30 ± 0.59$^{a,b}$ (3) | 5.19 ± 0.33$^{a,b}$ (3) |
| W | Hydroxyurea | μM | N.C. (3) | > 10,000 (3) | N.C. (3) | > 10,000 (3) | 193 ± 24 (3) | 111 ± 5$^{d,*}$ (3) | N.C. (3) | 414 ± 61$^{c}$ (3) |
| X | Lindane | μM | > 200 (3) | > 200 (3) | > 200 (3) | > 200 (3) | 33.3 ± 7.4$^{d}$ (3) | 15.0 ± 1.3$^{d}$ (3) | 150 ± 17$^{c}$ (3) | 124 ± 14$^{c}$ (3) |
| Y | Methyl Hg (II) chloride | μM | 1.58 ± 0.38$^{c,d}$ (4) | 1.48 ± 0.52$^{c}$ (4) | 1.16 ± 0.39 (3) | 0.865 ± 0.251 (3) | 0.0835 ± 0.0304$^{a}$ (4) | 0.0774 ± 0.0266$^{a}$ (4) | 0.190 ± 0.021$^{a}$ (3) | 0.152 ± 0.027 (3) |
| Z | *n*-Hexane | μM | > 200 (3) | > 200 (3) | > 200 (3) | > 200 (3) | > 200 (3) | > 200 (3) | > 200 (3) | > 200 (3) |
| A' | Permethrin | μM | > 500 (3) | > 500 (3) | > 1000 (3) | > 1000 (3) | 311 ± 105$^{d}$ (3) | 11.1 ± 0.9$^{d,*}$ (3) | 825 ± 67$^{c}$ (3) | 300 ± 43$^{c,*}$ (3) |
| B′ | Phenobarbital, Na salt | mM | 5.48 ± 0.16$^{b,c,d}$ (3) | 4.75 ± 0.09$^{b,c,d,*}$ (3) | 2.89 ± 0.75$^{a}$ (3) | 1.49 ± 0.38$^{a}$ (3) | 1.37 ± 0.25$^{a}$ (3) | 0.984 ± 0.175$^{a,d}$ (3) | 3.03 ± 0.61$^{a}$ (3) | 2.25 ± 0.27$^{a,c}$ (3) |
| C′ | Rotenone | μM | 3.35 ± 1.00 (3) | 5.28 ± 2.13 (3) | 6.05 ± 1.57$^{c,d}$ (3) | 4.49 ± 0.98 (3) | 0.0162 ± 0.054$^{b}$ (3) | 0.00823 ± 0.0023 (3) | 0.0787 ± 0.0071$^{b}$ (3) | 0.0598 ± 0.0071 (3) |
| D' | Tebuconazole | μM | > 1000 (4) | > 1000 (4) | 16.8 ± 8.2$^{d}$ (4) | 5.00 ± 3.16$^{c,d}$ (4) | 45.1 ± 7.0 (3) | 28.8 ± 4.9$^{b}$ (3) | 57.9 ± 11.3$^{b}$ (3) | 44.0 ± 3.2$^{b}$ (3) |
| E' | Tetraethylthiuram disulfide | μM | 0.388 ± 0.108$^{c,d}$ (3) | 0.363 ± 0.073$^{c}$ (3) | > 100 (4) | > 100 (4) | 2.45 ± 0.15$^{a}$ (3) | 1.99 ± 0.33$^{a}$ (3) | N.C. (3) | N.C. (3) |
| F′ | Thalidomide | μM | > 500 (3) | > 500 (3) | > 500 (3) | > 500 (3) | > 500 (4) | > 500 (4) | > 500 (3) | > 500 (3) |
| G' | Toluene | μM | > 1000 (3) | > 1000 (3) | > 1000 (3) | > 1000 (3) | > 1000 (3) | > 1000 (3) | > 1000 (3) | > 1000 (3) |
| H′ | Valinomycin | nM | 55.2 ± 19.6$^{c}$ (3) | 58.7 ± 17.2$^{b,c,d}$ (3) | 16.7 ± 5.1 (5) | 9.96 ± 4.62$^{a}$ (5) | 5.68 ± 1.24$^{a}$ (3) | 5.94 ± 1.91$^{a}$ (3) | 28.6 ± 9.5 (5) | 6.89 ± 2.23$^{a}$ (5) |
| I′ | Valproic acid, Na salt | μM | 2314 ± 203$^{c}$ (3) | 2342 ± 257$^{b,c}$ (3) | 1395 ± 670$^{d}$ (3) | 393 ± 102$^{a,d}$ (4) | 186 ± 17$^{a,d}$ (4) | 319 ± 122$^{a,d}$ (4) | 3594 ± 340$^{b,c}$ (3) | 2763 ± 385$^{b,c}$ (3) |
| (Negative control) | | | | | | | | | | |
| J' | Acetaminophen | mM | 3.22 ± 0.68$^{c}$ (3) | 4.63 ± 0.32$^{c,d}$ (5) | > 20 (3) | > 20 (3) | 0.919 ± 0.025$^{a,d}$ (4) | 0.918 ± 0.051$^{a,d}$ (4) | 2.94 ± 0.45$^{c}$ (3) | 2.49 ± 0.23$^{a,c}$ (3) |

The IC50 values calculated from Fig. 2 and supplementary Fig. S1-1–5 are shown as mean ± S.E. (*n* as experimental replicates of 4 [well] samples/treatment).
Significant differences were observed *versus* the respective Cos-7(a), HepG2(b), iPSC(c), NPC(d), and MTS(*) data at $P < 0.05$ by the Student *t*-test.
N.C.: not calculated mostly because of irregular inhibition patterns.

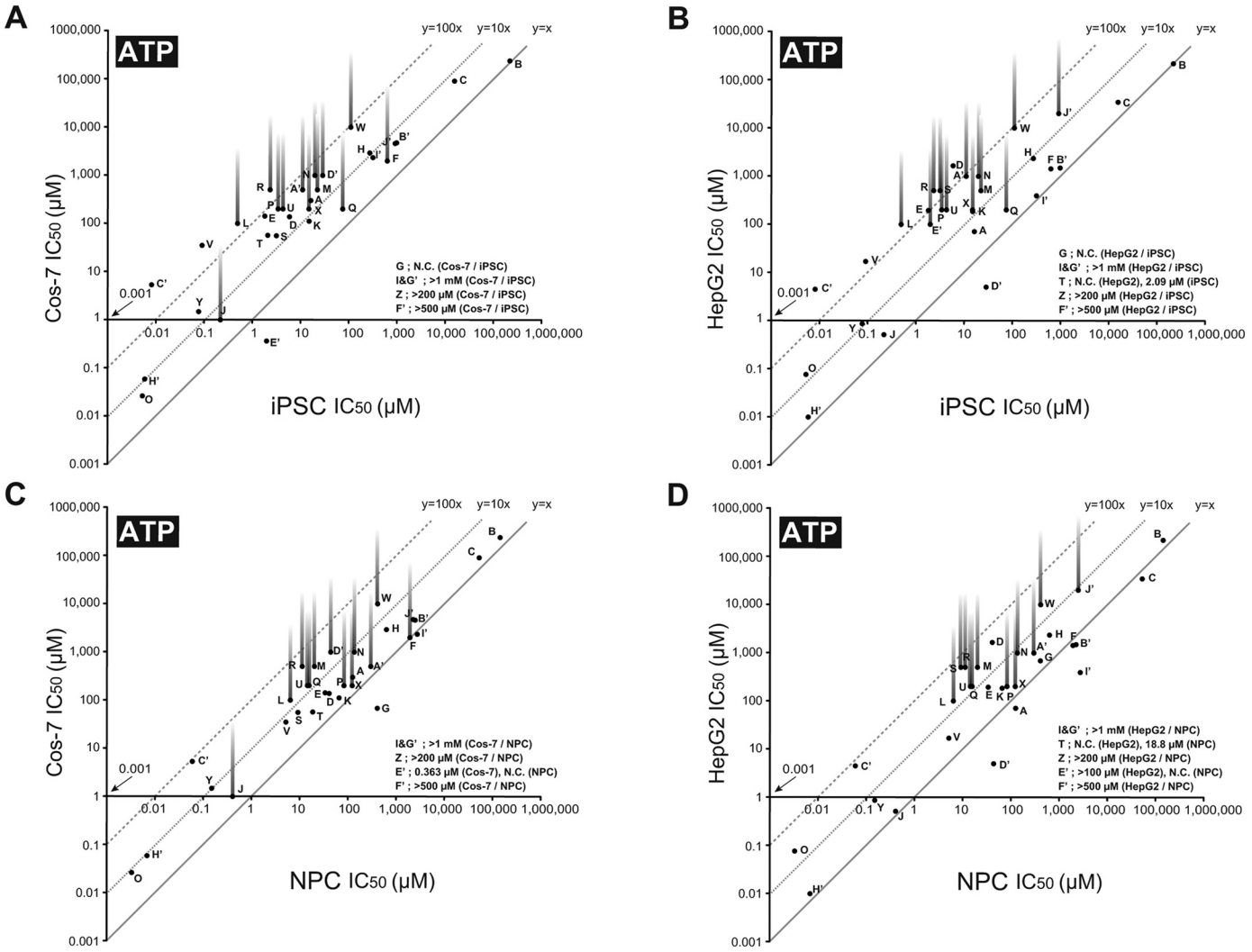



**Fig. 3.** Comparisons of $IC_{50}$ values of 35 DNT chemicals and acetaminophen in ATP assays between (A) iPSC and Cos-7 cells, (B) iPSC and HepG2 cells, (C) NPC and Cos-7 cells, and (D) NPC and HepG2 cells. Tail portions of the comets represent $IC_{50}$ values higher than the dots. The three lines indicate 1× (solid), 10× (dotted), and 100× (bar dotted) divergence in magnitude.

(BC1 cell line originated from newborn cord blood mononuclear cells [Chou et al., 2011]), human iPSC-derived neural stem (= progenitor) cells, neurons, and astrocytes, using only two concentrations (10 and 100 μM) by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay (Pei et al., 2016). Except for this study, information on cytotoxicity or inhibition of neural differentiation by those chemicals on human iPSC(/NPC) is lacking in the published literature. All the 35 DNT chemicals tested in this study (Table 1) were included in their 80-chemical list, and they demonstrated cytotoxicity of 14 DNT chemicals (MPP+ iodide, 2-methoxyethanol, 6-propyl-2-thiouracil, acrylamide, aldicarb, captan, carbaryl, DEHP, DDT, dieldrin, deltamethrin, heptachlor, rotenone, and valinomycin) on iPSC and those *plus* manganese (II) acetate, bisphenol A, colchicine, and *n*-hexane (total 18 DNT chemicals) on neural stem cells at 100 μM concentration (Pei et al., 2016). All other chemicals were considered nontoxic to iPSC or neural stem cells (Pei et al., 2016); however, our present study demonstrated that IDPN, 5-fluorouracil, 6-OHDA, bis(tributyltin)oxide, chlorpyrifos, DEHP, diethylstilbestrol, hexachlorophene, hydroxyurea, lindane, methyl Hg (II) chloride, permethrin, phenobarbital, tubuconazole, and tetraethylthiuram disulfide (but not thalidomide/toluene) display concentration-dependent inhibition of MTS or ATP activity in iPSC, even at concentrations lower than 100 μM (Fig. 2 and Supplementary Fig. S3). The discrepancies in sensitivity could be attributable to differences in human iPSC lines (XCL1 deriving from < 1-month-old male CD34-positive cord blood cells *vs* 253G1 from 36-year-old female dermal fibroblasts in this study), assay methods (MTT *vs* MTS), cell densities ($3.2 \times 10^4/cm^2$ *vs* $6.25 \times 10^3/cm^2$ at experiment start), or culture duration (1 day *vs* 2 days).

We utilized two independent assays, MTS and ATP. The former measures mitochondrial enzyme activity in living cells and the latter quantifies ATP content in cells living just before assay. Our results obtained by these two different cellular assays assessing mitochondrial function indirectly were almost identical (Supplementary Fig. S4); however, monophasic inhibition curves were not observed in some cases. 6-OHDA (Fig. 2, E-1) and hydroxyurea (Supplementary Fig. S3, W-1) upregulated the activity in MTS assays of HepG2 cells *via* unknown mechanisms. In such cases, our independent ATP assay was found to be beneficial as a backup (Fig. 2, E-2, and Supplementary Fig. S3, W-2). The ATP luciferase assay could also be influenced by unknown factors/mechanisms as shown in biphasic effects of manganese (II) acetate on iPSC (Supplementary Fig. S3, G-2). One possibility for the reversed effects and biphasic effects is the reduction potential or the pH-altering effect of the chemicals independent of biological activity. When $IC_{50}$ values of 200 μM were used as activity cutoffs in either MTS or ATP assays on iPSC, 25 out of 35 DNT chemicals (71.4%) were identified as DNT-positives while the remaining 10 chemicals,

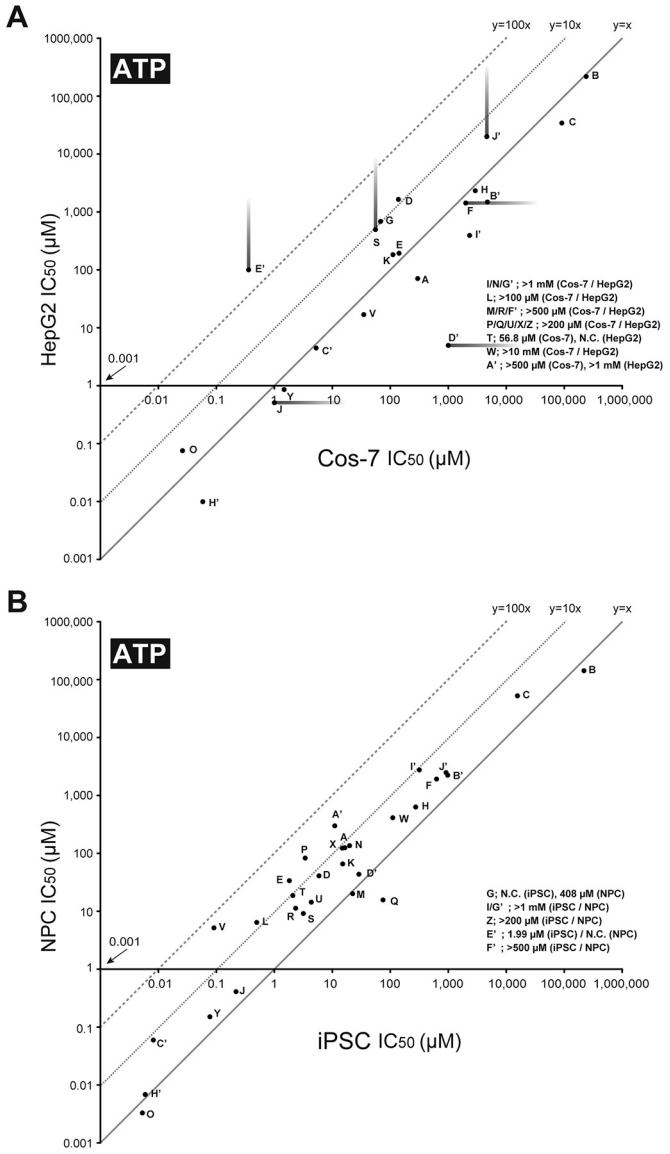



**Fig. 4.** Comparisons of $IC_{50}$ values of 35 DNT chemicals and acetaminophen in ATP assays between (A) Cos-7 and HepG2 cells and (B) iPSC and NPC cells. Tail portions of the comets represent $IC_{50}$ values higher than the dots. The three lines indicate 1× (solid), 10× (dotted), and 100× (bar dotted) divergence in magnitude.

including 2-methoxyethanol, 3,3′-iminodipropionitrile (IDPN), 6-propyl-2-thiouracil, manganese (II) acetate, acrylamide, aldicarb, *n*-hexane, phenobarbital, Na salt, thalidomide, and toluene, (*plus* control acetaminophen) as DNT-negatives (Table 2). These results suggest the substantial potentials of these assays for the large-scale DNT chemical screening.

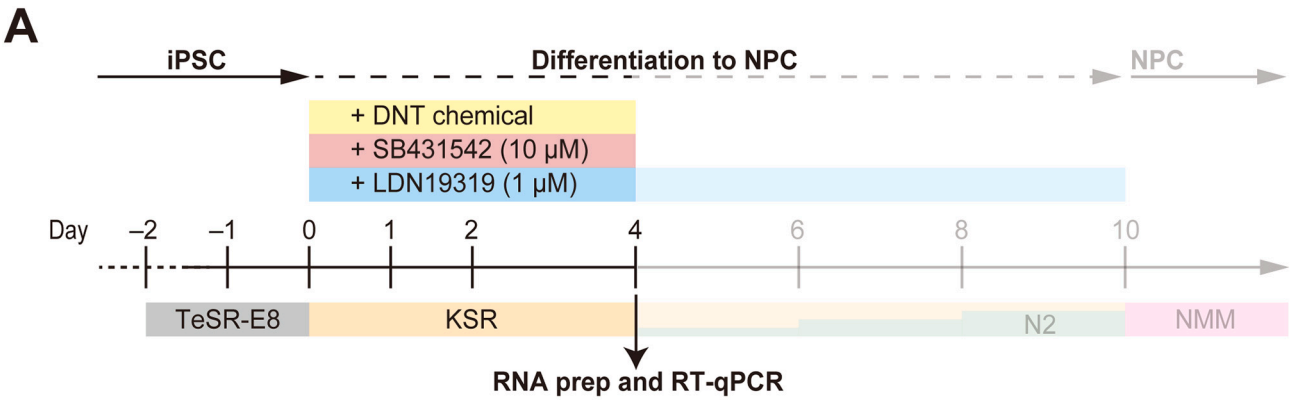
The iPSC and NPC were more sensitive to almost all DNT chemicals tested than in two popular transformed cell lines, Cos-7 (a fibroblast-like cell line obtained by immortalizing CV-1 African green monkey kidney cells with SV40 large T antigen) and HepG2 (a hepatocellular carcinoma cell line derived from a 15-year-old hepatoma patient; also referred as hepatocellular blastoma [Lopez-Terrada et al., 2009]) (Fig. 3). In new drug development, nonclinical safety tests using mammalian cells and experimental animals are mandatory. Notably, no rules/guidelines for selecting cell types for the safety examinations exist, and human (or other mammalian) transformed cells such as HepG2 and HEK293 are most often used because they, unlike normal human cells, proliferate eternally. Human iPSC (and their derivatives such as NPC) are proliferative and have a much closer resemblance to normal human cells with normal chromosomal numbers (*e.g.*, 253G1 cells [46XX; http://cellbank.brc.riken.jp/cell_bank/CellInfo/?cellNo=HPS0002&lang=En]; HepG2 [51–53; http://cellbank.brc.riken.jp/cell_bank/CellInfo/?cellNo=RCB1648&lang=En]; HEK293 [63–71; http://cellbank.brc.riken.jp/cell_bank/CellInfo/?cellNo=RCB1637&lang=En]). Therefore, human iPSC might be better suited as a standard cell type for drug safety tests, especially for clinical evaluation of DNT chemicals, such as 5-fluorouracil, colchicine, hydroxyurea, phenobarbital, tetraethylthiuram disulfide, thalidomide, and valproic acid (Table 1).

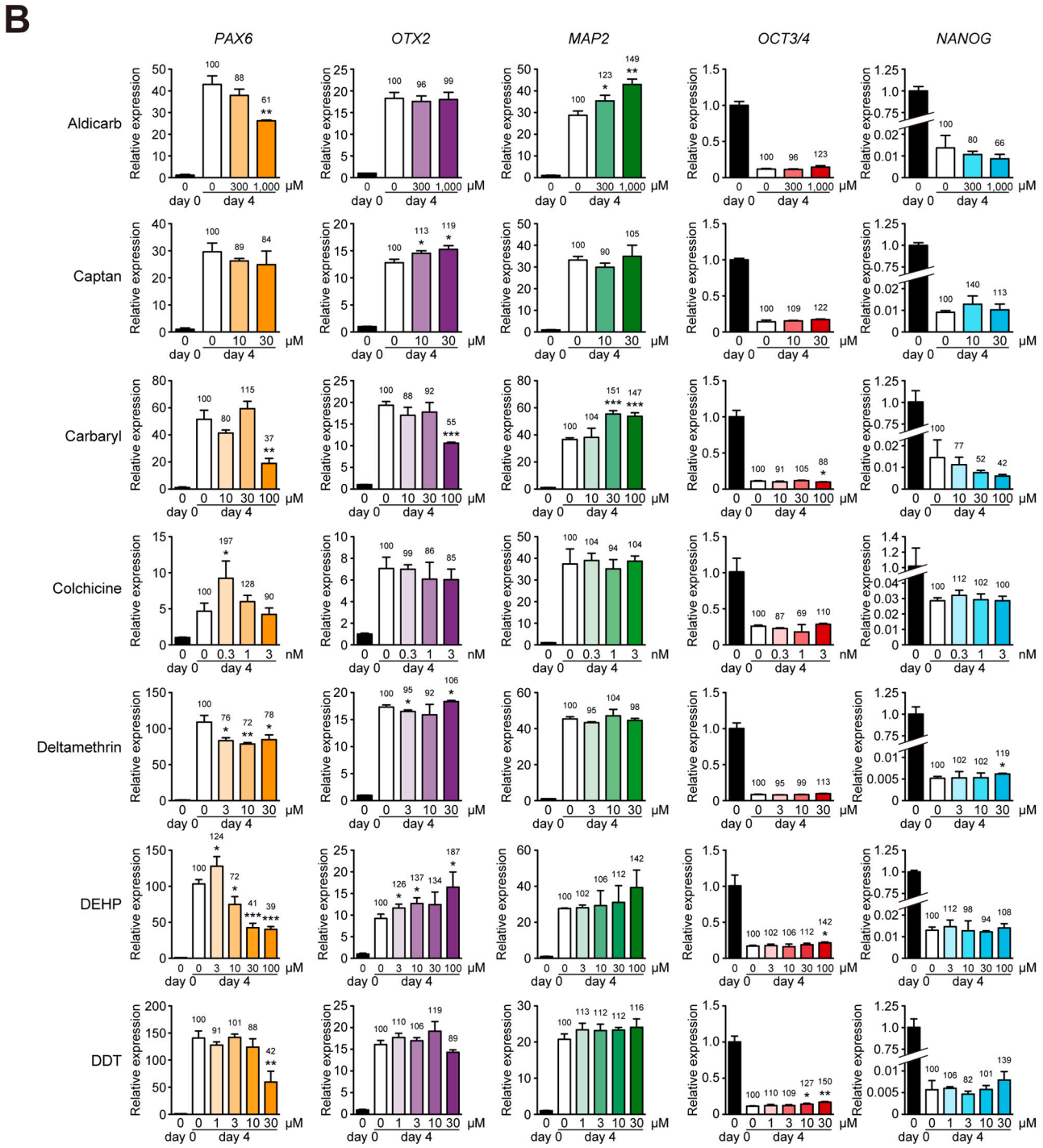
The use of differentiation of pluripotent stem cells into neural precursor cells for DNT evaluation is classified as a "UKN1" test in a recent OECD/European Food Safety Authority (EFSA) testing battery (Bal-Price et al., 2018a). This study has become the first to evaluate the impacts of DNT chemicals on iPSC differentiation to NPC by examining the altered expression of neural differentiation as well as undifferentiation (stem cell) marker genes. For neural differentiation, *PAX6*, *OTX2*, and *MAP2* were selected because the magnitude of mRNA induction was greater than other markers such as *Nestin* and *HOXB4* (*Homeobox B4*) (data not shown). Induction of PAX6, a key transcription factor that regulates multiple downstream genes and balances proliferation/differentiation of neural stem/progenitor cells (Kikkawa et al., 2019), was most prominent (Fig. 1B). We previously reported that all three DNT chemicals, tributyltin (Yamada et al., 2018a), chlorpyrifos (Yamada et al., 2017), and 5-fluorouracil (Yamada et al., 2018b), inhibit *PAX6* induction in the process of iPSC differentiation to NPC, and herein add 10 DNT chemicals (aldicarb, carbaryl, deltamethrin, DEHP, DDT, dieldrin, heptachlor, hexachlorophene, lindane, and rotenone) to our list (Table 3). In contrast, *PAX6* was somewhat upregulated by 0.3 μM colchicine (197%), 3 μM DOP (124%), and 1 μM hexachlorophene (129%) by unknown mechanisms (Fig. 5B and Supplementary Fig. S5). *PAX6* is considered one of the autism spectrum disorder (ASD)-susceptible loci, and therefore, PAX6 regulation by those chemicals could be implicated in ASD pathogenesis (Yamamoto et al., 2014).

The homeodomain-containing transcriptional factor OTX2 also plays an important role in brain development (Acampora et al., 2000; Boncinelli and Morgan, 2001; Maheu and Ressler, 2017). It is essential for early specification of the anterior neural plate, and Otx2-deficient mice were embryonically lethal, around embryonic day 9, due to lack of the rostral neuroectoderm that forms the forebrain, midbrain, and rostral hindbrain (Acampora et al., 1995; Matsuo et al., 1995). Because *OTX2* upregulation is not maintained in NMM (Supplementary Fig. S1A), its temporal expression regulated by OTX4–OTX2 axis may occur when cells exit from ground state pluripotency (Yang et al., 2014). Several *OTX2* mutations have been found in patients with brain malformations and neurological disorders (Beby and Lamonerie, 2013), and polymorphisms in the *OTX2* gene are considered risk factors for bipolar disorders (Sabunciyan et al., 2007). We observed that *OTX2* expression is also bidirectionally regulated by 14 DNT chemicals during iPSC differentiation to NPC–captan and DEHP upregulated *OTX2* induction, while carbaryl, dieldrin, hexachlorophene, lindane, rotenone, and valproic acid suppressed it (Fig. 5B and Supplementary Fig. S3).

MAP2 belongs to the MAP2/Tau family of microtubule-associated proteins (MAPs) and regulates the interaction of microtubule and F-actin that is critical for neuromorphogenic processes, such as neurite initiation (Nunez and Fischer, 1997). MAP2 anomalies are implicated in the onset of mood disorders (*e.g.*, depression and bipolar disorders) and schizophrenia (Marchisella et al., 2016). Aldicarb, carbaryl, dieldrin, heptachlor, lindane, and valproic acid induced *MAP2* expression, while hexachlorophene and rotenone suppressed it (Fig. 5B and Supplementary Fig. S3).

Stem cell transcription factors *OCT3/4* and *NANOG* were used as undifferentiation markers; the former is a Yamanaka factor (Takahashi et al., 2007), and NANOG is a superior marker for pluripotency used for




*(caption on next page)*

**Fig. 5.** Impacts of seven DNT chemicals (aldicarb, captan, carbaryl, colchicine, deltamethrin, DEHP, and DDT) on iPSC differentiation to NPC revealed by altered expression of differentiation/undifferentiation marker genes. (A) Experimental overview for the RT-qPCR analyses. (B) The effects of seven DNT chemicals on mRNA expression of differentiation (*PAX6*, *MAP2*, and *OTX2*) and undifferentiation (*OCT3/4* and *NANOG*) markers normalized by *GAPDH*. The expression in iPSC cells was set at one on the *y*-axis and the numbers above the bars indicate the percentages of expression assuming that day 4 cells without the DNT chemical (unfilled bars) are 100. Data are mean ± S.E. ($n=3$). Differences *versus* expression in day 4 cells without the DNT chemical (unfilled bars) were significant at $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

**Table 3**
Impacts of the representative (developmental) neurotoxicants on the expression of neural differentiation and undifferentiation marker genes during neural differentiation of iPSC.

| ID | Chemicals | *PAX6* | *OTX2* | *MAP2* | *OCT3/4* | *NANOG* |
|---|---|---|---|---|---|---|
| I | Aldicarb | ↓↓ | – | ↑↑ | – | – |
| L | Captan | – | ↑ | – | – | – |
| M | Carbaryl | ↓↓↓ | ↓↓ | ↑↑ | ↓ | – |
| O | Colchicine | ↑↑↑ | – | – | – | – |
| P | Deltamethrin | ↓ | ↑(↓) | – | – | ↑ |
| Q | DEHP | ↓↓↓ | ↑↑↑ | – | ↑↑ | – |
| R | DDT | ↓↓↓ | – | – | ↑↑ | – |
| S | Dieldrin | ↓↓↓ | ↓↓ | ↑↑↑ | (↓↓) | ↑↑↑ |
| U | Heptachlor | ↓↓↓ | – | ↑↑ | ↑↑↑ | ↑↑↑ |
| V | Hexachlorophene | ↓↓↓ | ↓↓↓ | ↓↓↓ | ↑↑ | ↑↑↑ |
| X | Lindane | ↓↓ | ↓ | ↑ | – | – |
| A' | Permethrin | – | – | – | – | ↓↓ |
| C′ | Rotenone | ↓↓↓ | ↓↓↓ | ↓↓↓ | ↑↑↑ | ↑↑↑ |
| I′ | Valproic acid, Na salt | – | ↓↓↓ | ↑↑↑ | ↑↑↑ | ↑↑↑ |

During neural differentiation, differentiation marker genes (*PAX6*, *OTX2*, and *MAP2*) are upregulated while undifferentiation marker genes (*OCT3/4* and *NANOG*) are downregulated. The impact of each neurotoxicant was indicated as follows when significant differences were observed *versus* control: ↑, 1–20% up; ↑↑, 21–50% up; ↑↑↑, > 50% up; ↓,1–20% down; ↓↓, 21–50% down; ↓↓↓, > 50% down.

establishing "the 2nd generation" NANOG iPSC (Okita et al., 2007). Their expression was significantly suppressed during differentiation to NPC (Fig. 1B). Compared to the differentiation markers, the impacts of 14 DNT chemicals on undifferentiation markers were much smaller (Fig. 5B and Supplementary Fig. S3).

Indeed, the 14 DNT chemicals displayed differential profiles for the regulation of such marker genes, suggesting their differential impacts on neural development in humans. Low-level childhood exposures to deltamethrin may negatively affect neurocognitive (learning and social) development by six years of age (Viel et al., 2015). Plasma level increases in DEHP (and bisphenol A) might be associated with a Pervasive Developmental Disorder-Not Otherwise Specified (PDD-NOS) (Kondolot et al., 2016). Such phenotypic differences could be fundamentally attributable to altered regulation of neural differentiation (Fig. 5). The majority of epidemiological studies do not strongly implicate any particular pesticide as causally related to adverse neurodevelopmental impacts in infants and children (Burns et al., 2013); however, dose-related correlations between maternal exposures to chlorpyrifos (and other organophosphates) and small head circumference at birth and neurobehavioral deficits, along with correlations between serum concentrations of DDT or its metabolite dichlorodiphenyldichloroethylene (DDE) and neurodevelopmental performance, are reported (Grandjean and Landrigan, 2014).

Also, many "not negligible" concerns and evidence from animal experiments are observed. Oral administration of a single dose of the carbamate, aldicarb (0.35 mg/ kg body weight), markedly inhibited acetylcholinesterase activity (up to 30%–40% of the basal) in the brains of postnatal day 17 (PND17) rats (and in adult rats at higher doses), thereby inducing tremors and gait ataxia (Moser, 1999), while another carbamate, carbaryl (5.0 mg/kg body weight), inhibited acetylcholinesterase activity only slightly (88% of the basal at maximum) in the brains of PND10 mice but induced persistent adult behavior alteration and cognitive impairment (Lee et al., 2015). In this study, the assessment of aldicarb as a DNT chemical was only realized by a combination of different alternative *in vitro* assays on iPSC.

## 5. Conclusion

We evaluated the cytotoxicity of the 35 DNT chemicals on iPSC, NPC, Cos-7, and HepG2 cells, and found that iPSC/NPC are more vulnerable to the majority of these chemicals than the two transformed cell lines. Further, we observed that 14 DNT chemicals differentially affected iPSC differentiation to NPC. The CAS registry now includes over 100 million chemicals, and more than 74,000 compounds are in commercial use (Schmidt, 2009); however, only 12 chemicals have been identified as human DNT chemicals (Grandjean and Landrigan, 2014; Oulhote et al., 2016; Fritsche et al., 2018b). Current screening protocols cannot keep pace with the backlog of untested chemicals (Fritsche et al., 2018b). This study provides a useful screening test that precedes animal experimentation. The test displays many characteristics recommended for proper evaluation of DNT (Crofton et al., 2011). Our methods 1) incorporate two endpoints that model key events of neurodevelopment; 2) correctly and accurately measure the intended endpoints with MTS/ATP and RT-PCR assays; 3) are supported by both positive (responsive) and negative (non-responsive) training/testing sets of chemicals (as found in Figs. 2 and 5, Supplementary Figs. S3 and S5); and 4) can feasibly screen large numbers of chemicals (*e.g.*, 35 chemicals in this study), although their specificity and sensitivity need to be scrutinized in the future experiments. Also, our methods might be more easily reproducible in many labs and performed on large numbers of chemicals in less time than recently described methods using human iPSC-derived 3D-neurospheres (Kobolak et al., 2020). Selection of the other differentiation/undifferentiation marker genes may enable more versatile DNT evaluation.

## Declaration of Competing Interest

The authors have no conflict of interest to declare.

## Acknowledgments

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.tiv.2020.104999.

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# Supplementary Data

## Cytotoxicity comparison of 35 developmental neurotoxicants in human induced pluripotent stem cells (iPSC), iPSC-derived neural progenitor cells, and transformed cell lines

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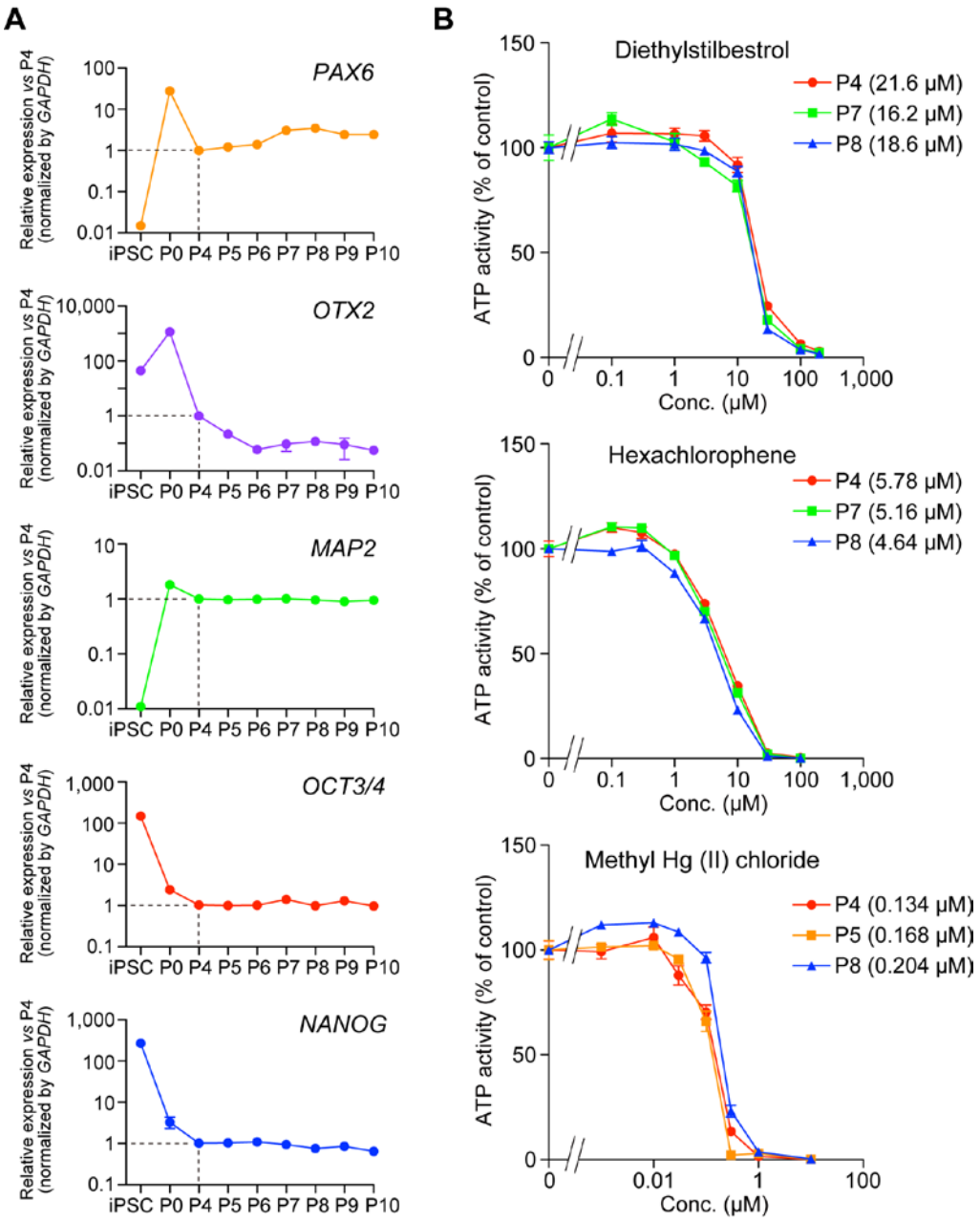
## Contents

**Supplementary Table S1.**

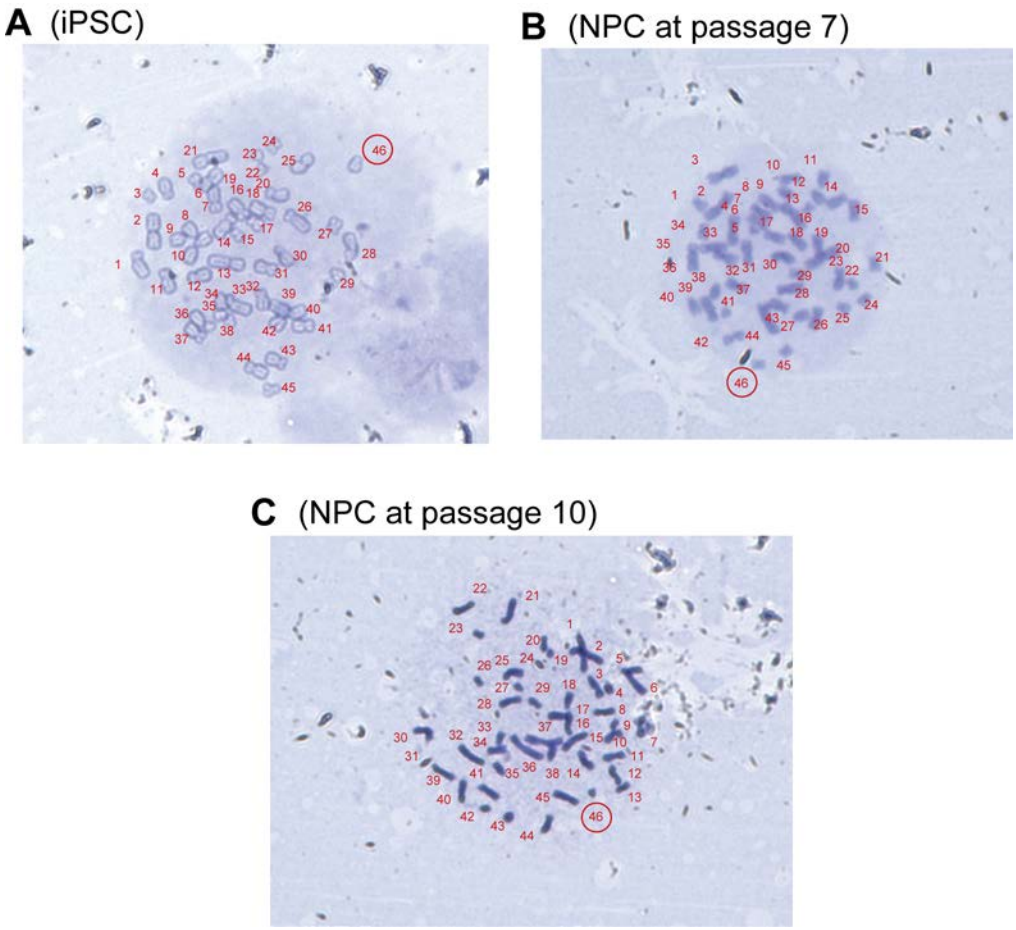
Sequences of primer sets for RT-qPCR.

| Gene | Primer sequence | Size |
|---|---|---|
| **Neural differentiation marker genes** | | |
| *PAX6* | 5′-ATGTGTGAGTAAAATTCTGGGCA-3′(Forward) | 103 bp |
| | 5′-GCTTACAACTTCTGGAGTCGCTA-3′(Reverse) | |
| *MAP2* | 5′-TCTCCCAAGACCTTCCTCCA-3′(Forward) | 144 bp |
| | 5′-CTCTTCCCTGCTCTGCGAAT-3′(Reverse) | |
| *OTX2* | 5′-ACAAGTGGCCAATTCACTCC-3′(Forward) | 122 bp |
| | 5′-GAGGTGGACAAGGGATCTGA-3′(Reverse) | |
| **Stem cell marker genes** | | |
| *OCT3/4* (*POU5F1*) | 5′-GGGTGGAGGAAGCTGACAAC-3′(Forward) | 114 bp |
| | 5′-GGTTGCCTCTCACTCGGTTC-3′(Reverse) | |
| *NANOG* | 5′-GATGCCTCACACGGAGACTG-3′(Forward) | 170 bp |
| | 5′-TCTTGACCGGGACCTTGTCT-3′(Reverse) | |
| **Housekeeping gene (as a control)** | | |
| *GAPDH* | 5′-GCCATCAATGACCCCTTCAT-3′(Forward) | 112 bp |
| | 5′-TGACAAGCTTCCCGTTCTCA-3′(Reverse) | |

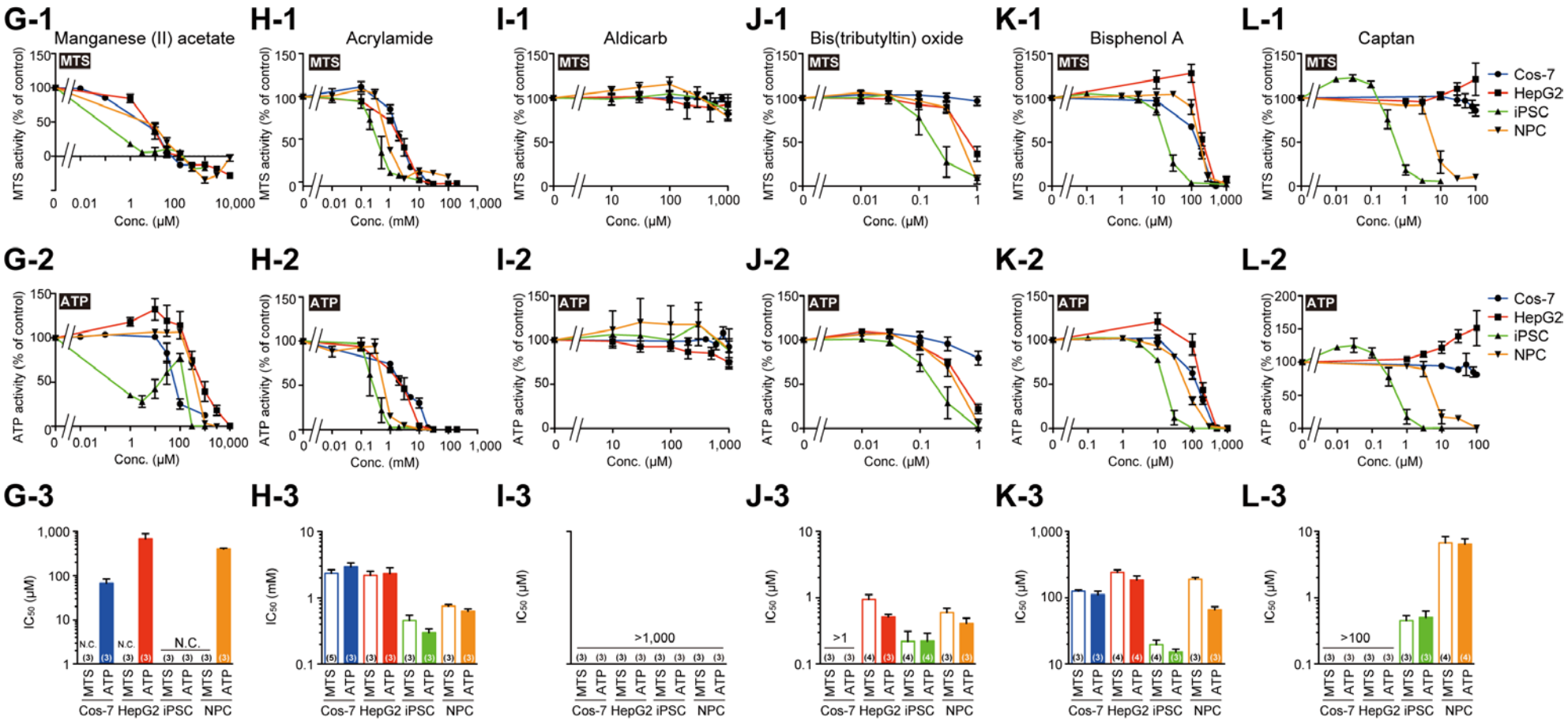
bp: base pair



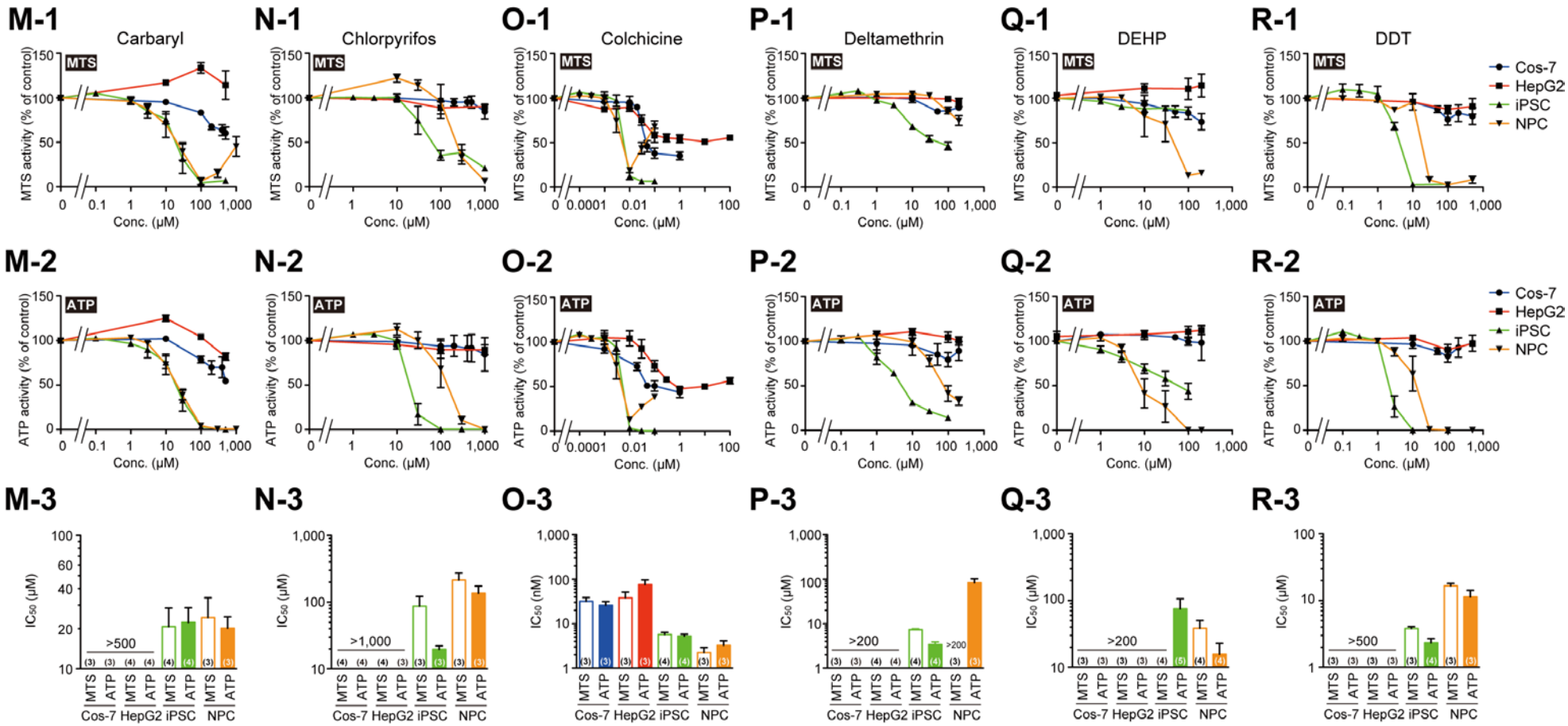

**Supplementary Fig. S1** NPC passage number does not significantly affect $IC_{50}$ values in ATP assays. (A) Expression changes of five neural differentiation/undifferentiation marker genes in iPSC and NPC at passages (P) 0 and 4–10. iPSC and NPC P0 are identical to Day 0 and 10 in Fig. 1B, respectively. Relative expression at P4 (normalized by *GAPDH* expression) is set at 1. (B) Repetitive experiments using NPC at various passage numbers in ATP assay to calculate $IC_{50}$ values from concentration-dependent inhibition by diethylstilbestrol, hexachlorophene, and methyl Hg (II) chloride. Data are mean ± S.E. of triplicate samples and calculated $IC_{50}$ values are shown in parentheses.



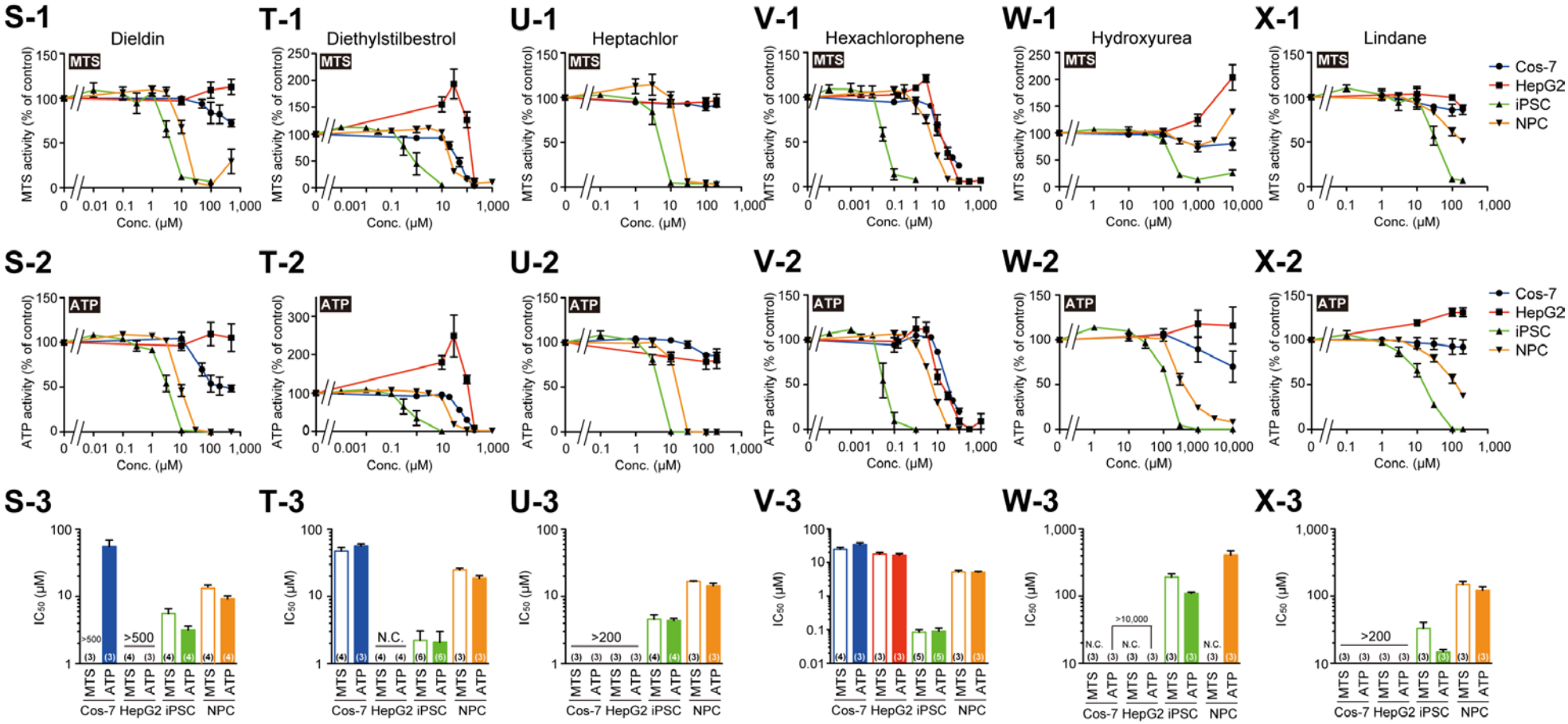

**Supplementary Fig. S2** Giemsa staining for chromosome counting in iPSC before differentiation to NPC (A), and NPC at passage 7 (B) and 10 (C). Most cell spreads indicate the presence of 46 chromosomes.



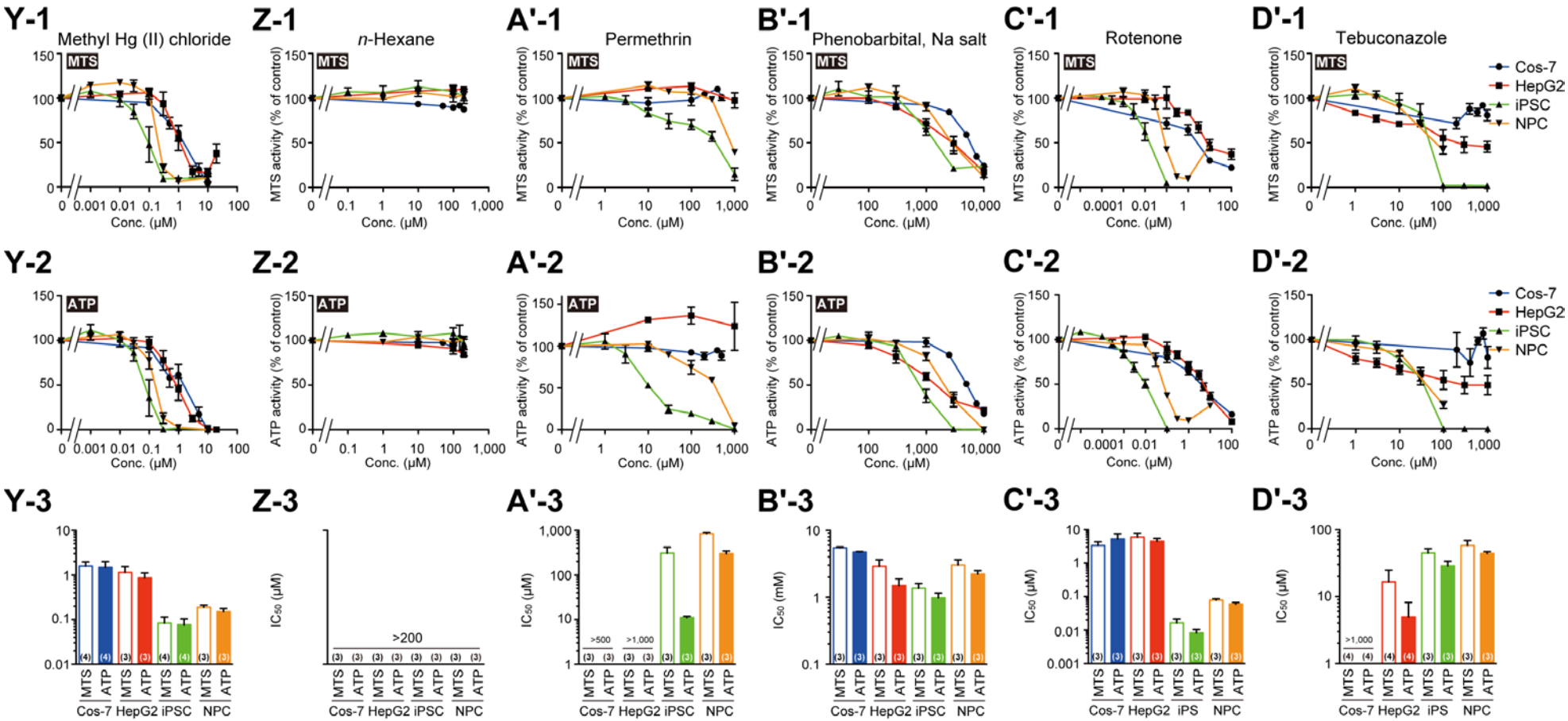

**Supplementary Fig. S3 (continues-1)**



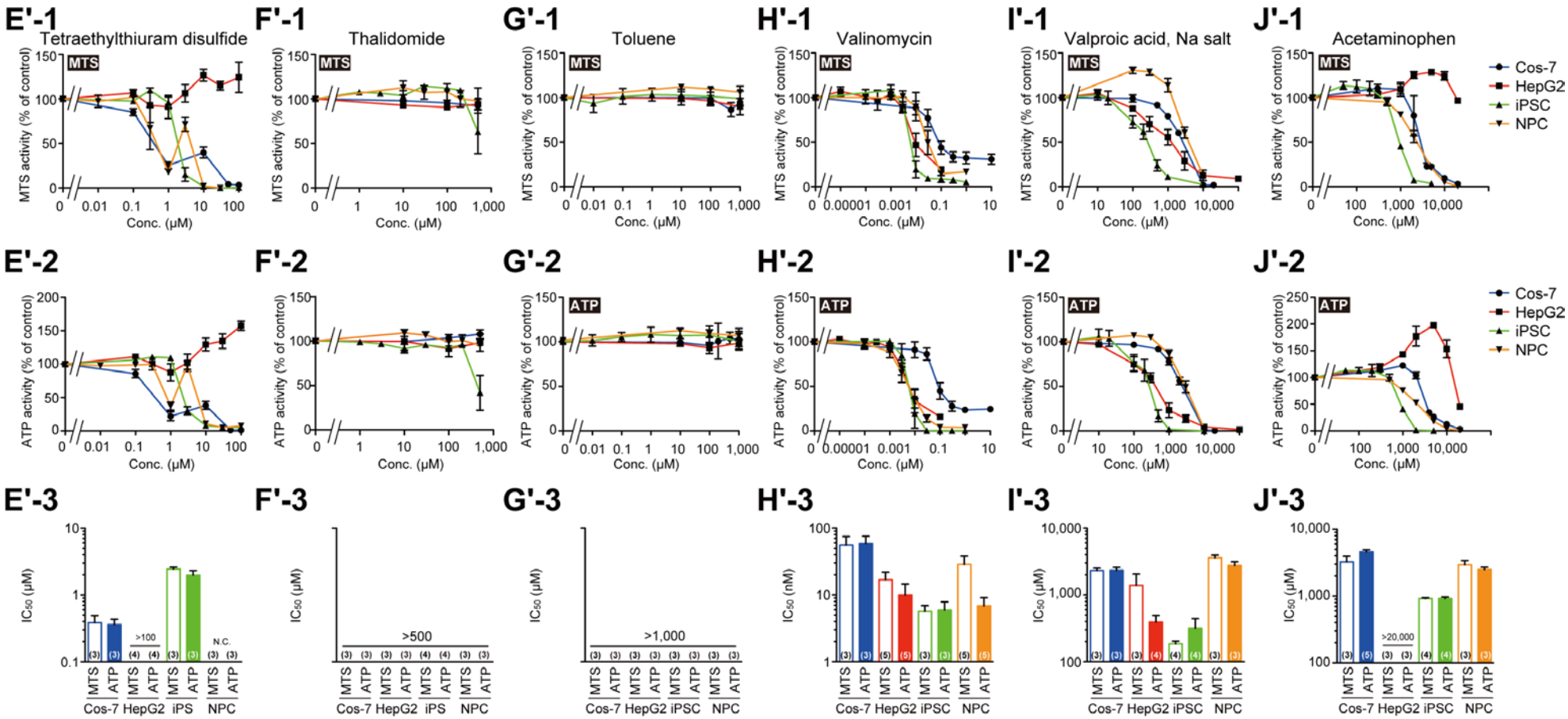

**Supplementary Fig. S3 (continues-2)**



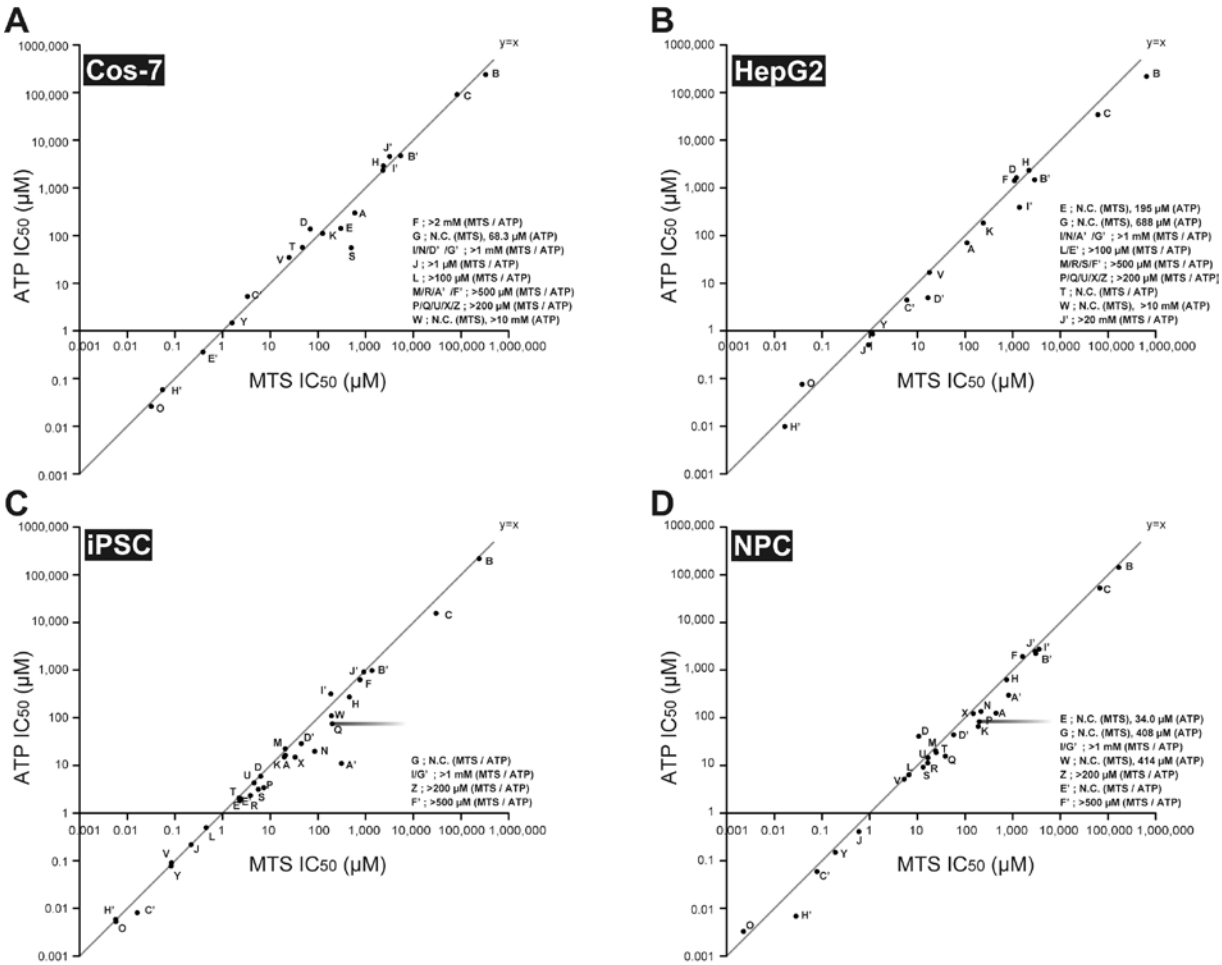

**Supplementary Fig. S3 (continues-3)**



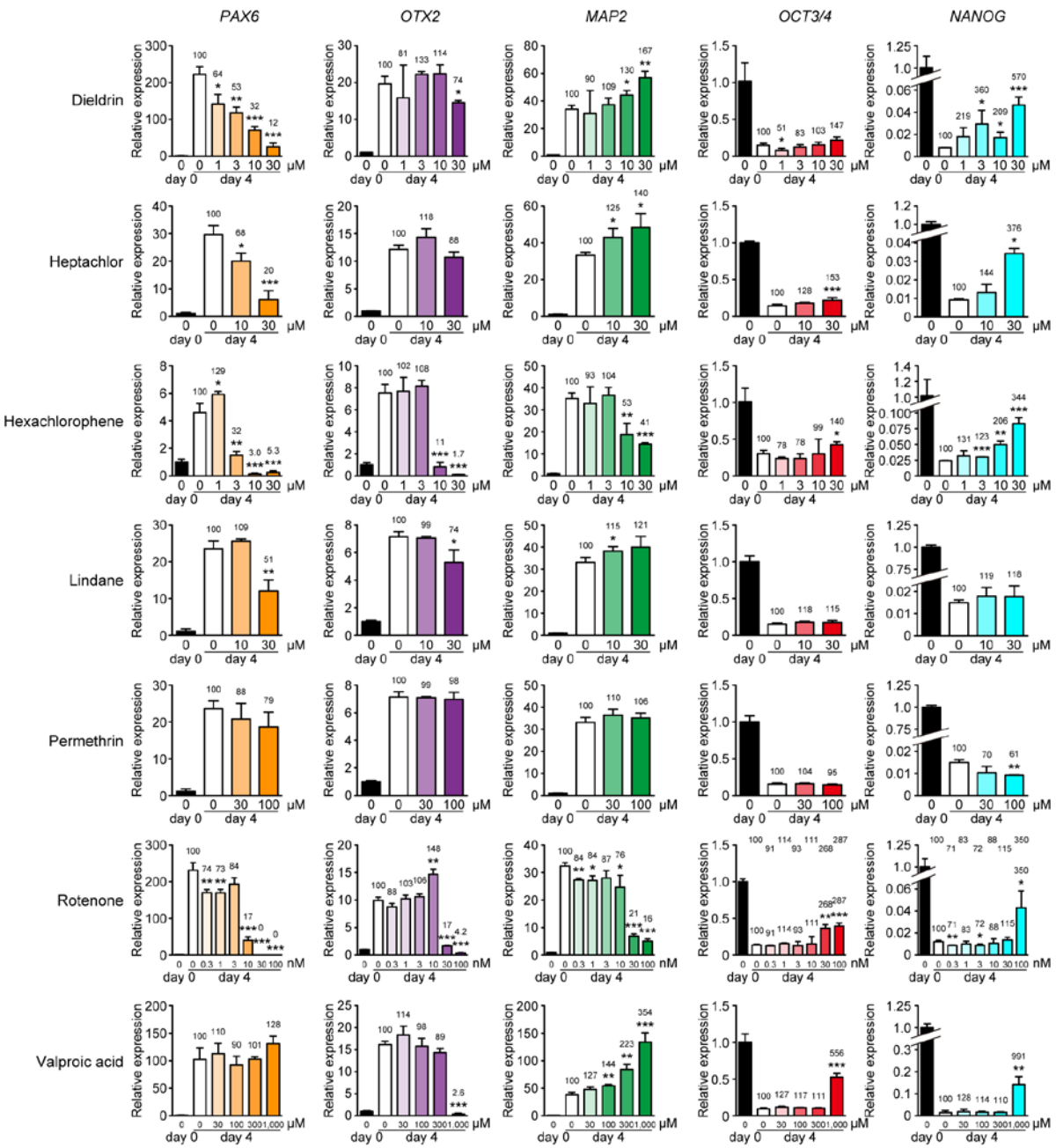

**Supplementary Fig. S3 (continues-4)**




**Supplementary Fig. S3 (ends)** Concentration-dependent inhibition of cell survival activity ("–1," MTS assay; "–2," ATP assay) in four cell types (Cos-7, HepG2, iPSC, and NPC) by 29 DNT chemicals (G, manganese (II) acetate; H, acrylamide; I, aldicarb; J, bis(tributyltin) oxide; K, bisphenol A; L, captan; M, carbaryl; N, chlorpyrifos; O, colchicine; P, deltamethrin; Q, DEHP; R, DDT; S, dieldrin; T, diethylstilbestrol; U, heptachlor; V, hexachlorophene; W, hydroxyurea; X, lindane; Y, methyl Hg (II) chloride; Z, *n*-hexane; A′, permethrin; B′, phenobarbital, Na salt; C′, rotenone; D′, tebuconazole; E′, tetraethylthiuram disulfide; F′, thalidomide; G′, toluene; H′, valinomycin; and I′, valproic acid, Na salt) and acetaminophen (J′, negative control) and calculated $IC_{50}$ values ("–3"). Data are mean ± S.E. of 3–5 (presented in parentheses in "–3") independent experiments that have four sample replicates (wells). N.C., not calculated.




**Supplementary Fig. S4** Comparisons of $IC_{50}$ values of 35 DNT chemicals and acetaminophen calculated from MTS and ATP assays in (A) Cos-7 cells, (B) HepG2 cells, (C) iPSC, and (D) NPC. Tail portions of the comets represent $IC_{50}$ values higher than the dots. Please refer to Table 1 for chemicals ID, A–J′.




**Supplementary Fig. S5** Impacts of seven DNT chemicals (dieldrin, heptachlor, hexachlorophene, lindane, permethrin, rotenone, and valproic acid) on iPSC differentiation to NPC revealed by altered expression of differentiation/undifferentiation marker genes in RT-qPCR analyses. Effects of seven DNT chemicals on the mRNA expression of differentiation (*PAX6*, *MAP2*, and *OTX2*) and undifferentiation (*OCT3/4* and *NANOG*) markers normalized by *GAPDH*. The expression in iPSC cells was set at one on the *y*-axis, and the numbers above the bars indicate the percentages of the expression with that on day 4 cells without the DNT chemical (unfilled bars) as 100. Differences *vs* the expression on day 4 cells without the DNT chemical (unfilled bars) were significant at $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$.