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Organophosphorus Pesticide Chlorpyrifos and Its Metabolites Alter the Expression of Biomarker Genes of Differentiation in D3 Mouse Embryonic Stem Cells in a Comparable Way to Other Model Neurodevelopmental Toxicants

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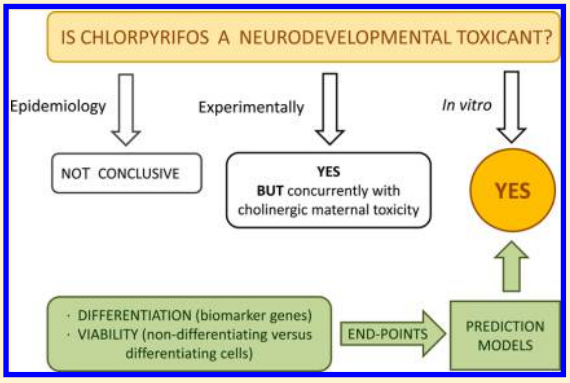
Supporting Information

ABSTRACT: There are discrepancies about whether chlorpyrifos is able to induce neurodevelopmental toxicity or not. We previously reported alterations in the pattern of expression of biomarker genes of differentiation in D3 mouse embryonic stem cells caused by chlorpyrifos and its metabolites chlorpyrifos-oxon and 3,5,6-trichloro-2-pyridinol. Now, we reanalyze these data comparing the effects on these genes with those caused in the same genes by retinoic acid, valproic acid, and penicillin-G (model compounds considered as strong, weak, and non-neurodevelopmental toxicants, respectively). We also compare the effects of chlorpyrifos and its metabolites on the cell viability of D3 cells and 3T3 mouse fibroblasts with the effects caused in the same cells by the three model compounds. We conclude that chlorpyrifos and its metabolites act, regarding these end-points, as the weak neurodevelopmental toxicant valproic acid, and consequently, a principle of caution should be applied avoiding occupational exposures in pregnant women. A second independent experiment run with different cellular batches coming from the same clone obtained the same result as the first one.



INTRODUCTION

Social concerns about the effect of environmental contaminants on neurodevelopment are growing. Chlorpyrifos (CPF) (*O*,*O*-diethyl *O*-(3,5,6-trichloro-2-pyridinyl) phosphorothioate) is an organophosphorus compound widely used as a plant protection product. The studies conducted following official guidelines have proved that CPF might be a neurodevelopmental toxicant but only at doses that display high maternal toxicity for cholinergic syndrome.[1] However, other *in vivo* studies have reported that alterations in development might occur at doses below those needed to cause maternal toxicity,[2] whereas epidemiological studies are not conclusive.[3] We previously reported that CPF and its main metabolites, chlorpyrifos-oxon (CPO) (*O*,*O*-diethyl *O*-(3,5,6-trichloro-2-pyridinyl) phosphate) and 3,5,6-trichloro-2-pyridinol (TClP), are able to alter the gene expression profile of D3 cells in differentiation at concentrations that cause inhibition of acetylcholinesterase compatible with no, or mild, maternal cholinergic symptoms.[4]

The pluripotency of embryonic stem cells suggests that they might be good biological models for testing developmental toxicity by recoding alterations in their differentiation. The embryonic stem cell test (EST) is a validated *in vitro* methodology whose prediction potential is based on alterations in both the differentiation of D3 mouse embryonic stem cells recorded by morphological observations (the beat of spontaneously generated cardiomyocytes (see a beating cardiomyocyte generated following EST protocol at http://www.youtube.com/watch?v=-gPxGM606Ms)) and alterations in cell viability in these D3 and in 3T3 mouse fibroblasts.[5] With these three end points, and using an empirical set of discriminant equations, it is possible to classify chemicals into three categories: strong, weak, and nonembryotoxicants.[5] A neural EST was developed using D3 cells under differentiation forced with certain trophic factors that yielded embryoid bodies with preponderance of neuroectodermal lineage instead of beating cardiomyocytes.[6] The linear discriminant functions of a regular EST cannot be used with a neural EST because the end points are the transcriptomics and morphological measures of differentiated neuron-like cells. A recent meta-analysis has suggested that a combination of a regular EST, and a neural EST improves performance for the detection of embryotoxicants with any of the individual assays.[7]

In a previous study, we demonstrated that the expression of biomarker genes of differentiation can be used, in conjunction with the discriminant equations of a regular EST, to predict the

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embryotoxicity of chemicals. Specifically, by using a predefined set of six biomarker genes, we were able to correctly predict the embryotoxicity of the strong embryotoxicant used as antineoplastic 5-fluorouracil, of the weak embryotoxicant used as antiepileptic drug 5,5-diphenylhydantoin, and of the nonembryotoxicant sweetener saccharin.[8] This procedure conferred one main advantage over a regular EST; the use of a more quantitative end point for monitoring the differentiation of D3 cells (gene expression instead of the individual score of beating cardiomyocytes); but there were also other advantages, such as the greater technical simplicity derived from the use of only monolayer cultures to avoid individual handling and further scoring of differentiated embryoid bodies. In addition, the total length of the assay was reduced to 5 days instead of the 10 days of the regular EST.[5,8] This approach of determining embryotoxicity potency of chemicals on the basis of EST discrimination equations using as end-point alterations on the viability of D3 and 3T3 cells and gene expression was also successfully used previously by several authors.[9−11]

In this work, we use the lineal discriminant functions of EST substituting the end-point based on beating the cardiomyocyte by alterations in the gene expression of biomarkers of differentiation, and together with alterations in viability of D3 and 3T3 cells, we found that CPF, CPO, and TClP behave as the weak neurodevelopmental toxicant valproic acid (VA), with clear differences from the behavior of the strong neurodevelopmental toxicant retinoic acid and the nonembryotoxicant penicillin G.

MATERIALS AND METHODS

Chemicals. Model neurodevelopmental toxicants retinoic acid (RA) and VA and non-neurodevelopmental toxicant penicillin-G (PG) were selected from among those used in the EST validation study for their neurotoxic properties. They were purchased with purity higher than 98% from Sigma Chemicals (RA) and Fluka (VA and PG). Materials for cell culture or molecular biology management were obtained from Sigma-Aldrich Spain, Roche, or local suppliers and were of analytical grade.

Cell Cultures. The cell culture conditions of the D3 mouse embryonic stem cells (undifferentiated and under spontaneous differentiation) and of the 3T3 mouse fibroblasts cells were those which have been previously described.[4] The cellular media for culturing D3 cells in undifferentiated conditions contained Dulbecco's modified Eagle's medium supplemented with 15% heat-inactivated fetal calf serum, 1% nonessential amino acids, 50 units of penicillin/mL, 100 μg of streptomycin/mL, 0.1 mM β-mercaptoethanol, and 1000 units of leukemia inhibition factor (LIF)/mL. The spontaneous differentiation of D3 cells was triggered removing LIF from the media. The medium for 3T3 fibroblast culture contained Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 50 units of penicillin/mL, and 100 μg streptomycin/mL. For both cell lines, culture environmental conditions included 37 °C and an atmosphere of 5% CO_2 with 95% humidity. D3 and 3T3 cells were cultured in 96-well plates for the viability experiments (initial cellular density seeding 2×10^5 D3 cells/well and 2×10^4 3T3 cells/well), while D3 cells were cultured in P100 plates (initial cellular density seeding 2×10^6 cells/plate) for the gene expression experiments.

Cell Exposures. The D3 cells under spontaneous differentiation (in the absence of LIF) and 3T3 cells were exposed for 3 days to different concentrations of RA, VA, PG, CPF, CPO, and TClP, which were freshly added to the cell culture media immediately prior to starting the experiment. CPO was renewed daily due to the reported degradation.[4]

Cell Viability Tests. The D3 cells under spontaneous differentiation and 3T3 cells were exposed during 3 days to different concentrations of model embryotoxic chemicals (RA, VA, and PG) and CPF and its metabolites CPO and TClP. Afterward, the viability of the resulting cultures was assessed with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (used in the EST validation study) according to Estevan and co-workers.[4] For that, cells were exposed during 3 h to 200 μL of 1 mg MTT/mL solution. At the end of this period, the MTT solution was removed, cultures washed with phosphate buffer saline, and cells lysed with 200 μL of dimethyl sulfoxide added to each well with the aim to dissolve the reduced formazan product formed by mitochondrial dehydrogenases. Formazan was finally quantified by recording absorbance at 540 nm and by correcting for background absorbance at 690 nm. Control cultures (nonexposed to chemicals) were taken as 100% of viability, and the absorbance of other cultures was referred to these controls. Two independent experiments for each chemical with 12 biological replicates of each experimental condition were run in each set of experiments. The data addressing the effects of CPF, CPO, and TClP on cell viability were taken in the first set of experiments from Estevan and co-workers,[4] where experiments were run in similar conditions; while the data for RA, VA, and PG of the first set of experiments were explicitly obtained for this work. A second set of experiments were also run in our laboratory using the same cellular batches for all tested chemicals.

Quantification of Gene Expression. The D3 cells under spontaneous differentiation were exposed to chemicals, and afterward, RNA was extracted, retrotranscribed, and quantified using the StepOnePlus Real-Time PCR System (Applied Biosystems) equipment as described in detail by Estevan and co-workers.[4]

RNA Purification. After exposure, trypsinized cells were pelleted by centrifugation and 1 mL of Tripure isolation solution (guanidinium thiocyanate) was added with further shaking for 10 s. Once the mixture was homogeneous, it was kept for 5 min at room temperature to produce the cellular lysate, and afterward 200 μL of chloroform was added until a pink color appeared. Then, after another 10 min of incubation at room temperature, centrifugation (12,000*g*, 15 min, 4 °C) was applied to obtain RNA in the colorless phase. This phase was collected and transferred to an Eppendorf tube for RNA precipitation by adding 500 μL of isopropanol at −20 °C and waiting for at least 3 h. After RNA precipitation, isopropanol was removed and the pellet washed with 1 mL of ethanol (75%, v/v) at −20 °C. The pellet was dried for 3 min at room temperature with further homogenization in 25 μL of 0.1% (w/v) diethylpyrocarbonate (DEPC) in water. The tube containing RNA was incubated at 55−60 °C for 15 min and stored at −80 °C until use. RNA was quantified, and its purity was determined according to the 260/280 nm optical density ratio.

RNA Retrotranscription. The extracted RNA was reverse-transcribed using the Expand Reverse Transcriptase kit and oligo-dT primers (Roche) according to the supplier's indications. The reaction mixture (1 μg of RNA and 10 μM polydT in a final volume of 10.5 μL of 0.1% (w/v) DEPC in water) was heated for 10 min at 65 °C, and afterward, the following components were added to the mixture: 1 μL (50 units) of RNA expand reverse transcriptase, 2 μL of 100 mM DTT, 4 μL of buffer for expand reverse transcriptase, 2 μL of 10 mM dNTPs, and 0.5 μL of RNAase inhibitor (Roche). The resulting mixture was further heated at 37 °C for 60 min with a final step of 5 min at 93 °C.

Gene Expression Quantification. The patatin-like phospholipase domain containing six (*Pnpla6*) gene expressions was monitored by the specific Taqman kit, while α-fetoprotein (*Afp*), fetal liver kinase 1 (*Flk1*), nestin (*Nes*), neurofilament medium polypeptide (*Nefm*), and β-actin gene expression was quantified with the Power SYBR Green kit (both supplied by Applied Biosystems). β-Actin was used as the housekeeping control gene in each sample. Table S1 (Supporting Information) displays the primer sequences and annealing temperatures used in the quantification of the gene expression by SYBR green.

For the power SYBR green analysis, each sample contained 0.9 μL of forward primer plus 0.9 μL of reverse primer (both containing 25 pmol DNA/μL), 6.2 μL of 0.1% (w/v) DEPC in water, plus 2.0 μL of cDNA sample (0.1 ng/mL), and 10 μL of master mix. The cDNA amplification consisted in an initial step of 10 min at 95 °C, followed

by 40 cycles of 15 s at 95 °C, 60 s at the respective annealing temperature (see Table S1, Supporting Information), 15 s at 95 °C, and a final melting step of 60 s at 60 °C.

The *Pnpla6* expression was recorded with 10.0 μL of the Taqman master mix, 1.0 μL of the Taqman assay mix, 2.0 μL of sample, and 7.0 μL of 0.1% (w/v) DEPC in water. According to supplier indications, the program of temperature was an initial step at 50 °C for 2 min, plus 10 min at 95 °C, followed by 40 cycles of 15 s at 95 °C and 60 s at 60 °C.

In all cases, relative changes in gene expression were estimated based on the expression of the same gene in the control plate (not exposed to chemicals). Quantification was performed using $2^{-\Delta\Delta Ct}$ calculations according to Schmittgen and Livak.[12] This method uses the expression of β-actin (gene not involved in development) in each experimental condition for normalizing the expression of the analyzed genes regarding cellular proliferation that might be affected due to cytotoxicity caused by the assessed chemicals.

The data on the effects of CPF, CPO, and TClP on gene expression were taken in the first set of experiments from Estevan and co-workers,[4] where experiments were run in similar conditions, while the data for RA, VA, and PG for the first set of experiments were explicitly obtained for this work. A second set of experiments were also run in our laboratory using the same cellular batches for all tested chemicals. Each determination was always done using three biological replicates.

Classification of the Neurodevelopmental Potential of the Test Compounds. The neurodevelopmental potential (strong, weak, and non-neurodevelopmental) of the assessed chemicals was assigned according to EST criteria and by introducing certain changes into the prediction models. We maintained the alterations in the viability of D3 and 3T3 cells as end points using IC50D3 and IC503T3 as the concentrations of the assessed chemical that reduces cell viability by 50% after 3 days of exposure (instead of 10 days, as considered in a regular EST) in D3 and 3T3 cells, respectively. Alterations in the generation of the beating cardiomyocytes used in a regular EST to monitor alterations in cell differentiation were substituted by the ECD parameter, defined as the concentration of the studied chemical causing either a 50% reduction in gene expression or a 200% increase in gene expression, after 3 days of exposure of D3 cells to the tested chemical. Table 1 presents the classification functions and criteria employed in each case. All of the genes employed in this study yielded a classification (strong, weak, or non-neurodevelopmental) for each tested chemical. We considered as the final assay classification the category with the highest percentage of individual gene predictions. We applied this procedure to RA, VA, and PG to validate our approach. Afterward, we reanalyzed our previously published data[4] for CPF, CPO, and TClP by the same procedure. The consistency in the prediction of our assay was further confirmed in a second set of experiments where all chemicals were assayed in the same set of experiments, and therefore, the RNA used for determining gene expression was coming from the same cellular batch.

Table 1. Prediction Models and Classification Criteria Employed for Assessing the Neurodevelopmental Toxicity of Chemicals[a]

strong embryotoxic if	weak embryotoxic if	nonembryotoxic if
III > I	II > I	I > II
and	and	and
III > II	II > III	I > III
I = 5.916 log(IC503T3) + 3.500 log (IC50D3) − 5.307 [(IC503T3-ECD)/IC503T3] −15.27		
II = 3.651 log (IC503T3) + 2.394 log (IC50D3) − 2.033 [(IC503T3-ECD)/IC503T3] − 6.85		
III = −0.125 log (IC503T3) − 1.917 log (IC50D3) + 1.500 [(IC503T3- ECD)/IC50 3T3] − 2.67		

[a]ECD was defined as the effective concentration of the tested chemical that either decreases the expression of the biomarker gene by 50% or increases the expression of the biomarker gene by 200% in spontaneously differentiated D3 cells after 3 days of exposure. IC50D3 was defined as the concentration that reduces the viability of D3 cells under spontaneous differentiation by 50% after 3 days of exposure. IC503T3 was defined as the concentration that reduces the viability of 3T3 cells by 50% after 3 days of exposure. IC50D3, IC503T3, and ECD must necessarily be expressed in μg/mL. These linear discriminant functions and classification criteria were all equally considered in a classic EST,[28] but an exposure of 3 days was contemplated instead of 10 days, and ID50 (concentration of the tested compound that brings a 50% decrease in the differentiation of undifferentiated D3 cells into beating cardiomyocytes) was changed by ECD.

RESULTS

Cytotoxicity. *Set of Experiments 1.* In the set of experiments 1, we used the data about alterations in viability caused by CPF, CPO, and TClP previously published by Estevan and co-workers,[4] and we ran experiments for model embryotoxic chemicals RA, VA, and PG. The temporal differences between both groups of experiments were a little bigger than one year.

Cytotoxicity exerted by the strong neurodevelopmental toxicant RA over D3 was several orders of magnitude higher than the cytotoxicity exerted over 3T3 cells (Table 2). In other cases (weak neurodevelopmental toxicant VA, CPF, and TClP), the cytotoxicity of the chemicals was higher (between 2.4 and 11 times) for D3 cells than for the nondifferentiating 3T3 cells (Table 2). CPO was the only case where the cytotoxicity for differentiating and nondifferentiating cells was comparable. Finally, the negative control (non-neurodevelopmental toxicant) PG was 4 times more cytotoxic for 3T3 cells than for D3 cells (Table 2).

Set of Experiments 2. We ran a second set of experiments where all chemicals (CPF and its metabolites) and the three embryotoxic model chemicals were simultaneously assayed with the same D3 and 3T3 cellular batches. The second set of experiments yielded comparable results to those found in the first set (Table 2). The differences of cytotoxicity between 3T3 and D3 cells for VA, CPF, and TClP ranged between 1.4 and 4.8 times (Table 2). The main difference between both sets of experiments was found for PG because in the second set, the sensitivity of both cellular lines to the antibiotic was in the same order of magnitude. Nevertheless, the IC50 for 3T3 and D3 cells was always (for both sets of experiments) in the order of mg/mL, and therefore, stem cells and fibroblasts were always quite resistant to the cytotoxicity induced by this compound used as a model of a nonembryotoxicant chemical.

Neurodevelopmental Toxicity of Model Chemicals. Our aim was to test the capability of CPF, CPO, and TClP to alter the differentiation of D3 cells in a way that might suggest the disruption of neurodevelopment. For that, we initially selected a battery of 5 different genes with the potential to act as biomarker for this purpose. Table 3 shows the selected genes together with their physiological role on differentiation. *Afp* and *Flk1* were included because they were shown to be good biomarkers of exposure to embryotoxicants such as 5-fluorouracil and 5,5-diphenylhydantoin.[8−13] *Nes* is a neuronal progenitor marker typically used in immunostaining of neural tissues[14] also with good capability to differentiate between strong, weak, and nonembryotoxicants.[8] *Pnpla6* is a gene strongly involved in neural differentiation, as was recently shown when the silencing of this gene caused disturbance in several pathways related to the formation of respiratory, neural, and vascular tubes,[15] and also previously shown to be a biomarker of exposure to embryotoxicants.[8] Finally, we used

Table 2. Effect of Chemicals on the Cell Viability of D3 and 3T3 Cultures[a]

	chemical	run (date)	units	IC50D3	IC503T3
embryotoxic model chemicals	RA	set 1 (July 2013)	μM	0.053	97
			μg/mL	0.016	29
		set 2 (June 2014)	μM	0.043	80
			μg/mL	0.013	24
	VA	set 1 (July 2013)	μM	867	9910
			μg/mL	125	1430
		set 2 (June 2014)	μM	1680	8110
			μg/mL	242	1170
	PG	set 1 (July 2013)	μM	47800	12000
			μg/mL	16000	4000
		set 2 (June 2014)	μM	11400	14700
			μg/mL	3800	4900
chlorpyrifos and metabolites	CPF	set 1 (2012)	μM	140	340
			μg/mL	49	119
		set 2 (June 2014)	μM	117	211
			μg/mL	41	74
	CPO	set 1 (2012)	μM	200	200
			μg/mL	67	67
		set 2 (June 2014)	μM	212	200
			μg/mL	71	67
	TClP	set 1 (2012)	μM	191	620
			μg/mL	38	123
		set 2 (June 2014)	μM	407	578
			μg/mL	81	115

[a]An MTT test was used to determine the cell viability of the D3 and 3T3 cultures of the cells previously exposed to several concentrations of each chemical for 3 days. In each case, IC50D3 and IC503T3 were estimated as the compound concentration capable of reducing cellular viability by 50%. Two different sets of experiments were run on the dates indicated in each case. Each set consisted in all cases of two independent experiments performed, each one with 12 biological replicates within the same experiment. The mean of each of these two independent experiments is displayed. The data for CPF, CPO, and TClP of set 1 were taken form Estevan and co-workers[4] in experiments performed in our laboratory under comparable conditions. IC50D3 for PG in set 1 was estimated by extrapolation because it was higher than the highest concentration employed.

Table 3. Genes Employed in the Work as Biomarkers of Differentiation[a]

gene symbol	NCBI ID	name	role
Nes	18008	nestin	intermediate filament protein
Pnpla6	50767	patatin-like phospholipase domain containing 6	neural and vascular development
Flk1	16542	fetal liver kinase 1	receptor for vascular endothelial growth factor
Afp	11576	alpha fetoprotein	major plasma protein produced by the yolk sac and the liver during fetal development
Nefm	18040	neurofilament, medium polypeptide	maintenance of axoskeleton

[a]The NCBI ID is always stated for *Mus musculus*.

Nefm as a neuroectodermal marker, as was also previously done to study the neurodevelopmental toxicity of several chemicals, such as methyl mercury.[16] The capability of all these genes to differentiate among strong, weak. and non-neurodevelopmental chemicals was initially tested using RA, VA, and PG as model compounds.

We determined the concentrations of the strong neurodevelopmental toxicant RA, of the weak neurodevelopmental toxicant VA, and of the non-neurodevelopmental toxicant PG, which brought either a 200% overexpression or a 50% underexpression in the genes previously reported as biomarkers of differentiation and that altered their expression after exposure to CPF and its main metabolites.[4] RA, VA, and PG were included in the EST validation study.[5] We performed two different sets of experiments with a temporal difference of about 1 year. The results are displayed in Table 4 and Figures S1 and S2 (Supporting Information). The differences in the ECDs reported for both sets of experiments were more qualitative (overexpression versus under-expression) than quantitative because the ECD records for all three model chemicals were always in the same order of magnitude (Table 4). RA was able to alter D3 differentiation at lower concentrations than VA and PG, ECD being in the order of nanomolar and subnanomolar. We were not able to estimate ECD for the *Pnpla6* gene because at the highest RA tested concentration, gene expression did not reach either 50% or 200%. Therefore, we concluded that the ECD must be higher than 10 μM. VA displayed ECD records from tens of micromolar to units of millimolar (Table 4). PG, despite its consideration as a non-neurodevelopmental toxicant, produced a 50% or 200% alteration in the expression of biomarker genes at concentrations ranging between 160 μM and more than 1.8 mM (Table 4).

When the prediction models displayed in Table 1 were applied using the ECD records displayed in Table 4 and IC50D3 and IC503T3 records displayed in Table 2, all tested genes offered predictions of strong, weak and non- neurodevelopmental toxicants for RA, VA, and PG, respectively in both sets of experiments (Table 5). Thus, the *in vitro* prediction was consistent in all cases with *in vivo* outcomes for all three model chemicals. The only doubtful case was *Pnpla6* with RA, where we predicted an ECD higher than 30 ng/mL. In this scenario and analyzing the prediction model using the IC503T3 and IC50D3 reported in Table 2, we found that the prediction of strong neurodevelopmental toxicant is maintained in the range between 0 and 160 ng/mL (for the set of experiments 1) and between 0 and 70 μg/mL (for the set of experiments 2). However, the exposure to RA concentrations higher than 160 ng/mL is physiologically odd since this concentration is 6.2 times higher than the IC503T3 and 10000 times higher than IC50D3 and therefore would be noncompatible with the survival of both cell lines. In the same way, in the second set of experiments, the cutoff point of 70 μg/mL also is 2.9 times higher than the IC503T3 and 5400 times higher than the IC50D3 and therefore leads to the same conclusion as in the set of experiment 1. Consequently, the prediction of the toxicity induced by RA using *Pnpla6* must be strong neurodevelopmental toxicity. Similarly, the mathematical analysis of the prediction models on the basis of the IC503T3 and IC50D3 for both sets of experiments showed that for ECD records higher than 600 μM the only possible prediction for PG is nonembryotoxicant (Table 5).

Neurodevelopmental Toxicity of CPF and Its Metabolites. Having validated our model, we reanalyzed our previously published results where we reported alterations in the gene expression profile of several biomarkers of differentiation after exposures in situations similar to those used in this work.[4] We found that 100% of genes predicted weak neurodevelopmental toxicity for CPF and TClP (Table 5). Eighty percent of genes predicted weak neurodevelopmental

Table 4. Alterations in the Expressions of Biomarker Genes after Exposure to Chemicals[a]

	chemical	gene	ECD set 1 (μM (μg/mL))	ECD set 2 (μM (μg/mL))
embryotoxic model chemicals	RA	*Afp*	↓0.060 (↓0.018)	↑0.00027 (↑0.00008)
		Flk1	↓0.070 (↓0.021)	↑ 0.00060 (↑0.00018)
		Nes	↑0.067 (↑0.020)	↑ 0.025 (↑0.0075)
		Pnpla6	higher than 0.010 (higher than 0.030)	higher than 0.010 (higher than 0.030)
		Nefm	↑0.013 (↑0.004)	↑0.033 (↑0.010)
	VA	*Afp*	↑83 (↑12)	↑300 (↑43)
		Flk1	↑1420 (↑205)	↑130 (↑18)
		Nes	↑350 (↑50)	↑1300 (↑187)
		Pnpla6	↓1730 (↓250)	↑700 (↑100)
		Nefm	↑250 (↑36)	higher than 2100 (higher than 300)
	PG	*Afp*	↑160 (↑53)	↓310 (↓105)
		Flk1	↑250 (↑82)	higher than 1800 (higher than 600)
		Nes	↓300 (↓100)	higher than 1800 (higher than 600)
		Pnpla6	↓210 (↓69)	↓ 600 (↓200)
		Nefm	↓160 (↓53)	higher than 1800 (higher than 600)
chlorpyrifos and metabolites	CPF	*Afp*	↑20 (↑6.9)	↑14 (↑4.9)
		Flk1	↑24 (↑8.4)	↑19 (↑6.6)
		Nes	↓94 (↓33)	↓48 (↓17)
		Pnpla6	↑3.2 (↑1.1)	↑150 (↑51)
		Nefm	↑18 (↑6.4)	↓110 (↓37)
	CPO	*Afp*	↑120 (↑40)	↓60 (↓20)
		Flk1	↑19 (↑6.3)	↓42 (↓14)
		Nes	↑760 (↑255)	↓66 (↓22)
		Pnpla6	↑66 (↑22)	higher than 370 (higher than 125)
		Nefm	↑140 (↑48)	↓90 (↓30)
	TClP	*Afp*	↓705 (↓140)	↑1.8 (↑0.35)
		Flk1	↑1.7 (↑0.33)	↑39 (↑7.7)
		Nes	↑18 (↑3.5)	↓260 (↓51)
		Pnpla6	↓106 (↓21)	↓130 (↓25)
		Nefm	↑4.8 (↑0.96)	↑360 (↑7.1)

[a]The concentration of each chemical able to either reduce the expression to 50% of control or to increase the expression to 200% of control (ECD) is displayed. Cells were exposed to different concentrations of each chemical present in the culture media during 3 days. Afterward, the RNA of cells was extracted, retrotranscripted, and the expression of each gene quantified as described in Material and Methods. The ECD records for embryotoxic model chemicals were graphically deduced from Figures S1 and S2 (Supporting Information). The ECD record for CPF and metabolites in sets of experiment 1 was deduced from Estevan and co-workers[4] in experiments performed in our laboratory in comparable conditions. The ECD record for CPF and metabolites in sets of experiment 2 was graphically deduced from Figure S3 (Supporting Information). Data are expressed in micromolar units with the equivalence in μg/mL between parentheses. The symbol ↑ represents gene over-expression, while ↓ represents gene under-expression. Set 1 for embryotoxic model chemicals was performed in July 2013. Set 2 was totally performed in May−June 2014, and therefore, embryotoxic model chemicals and CPF and metabolites were simultaneously assayed with the same cellular batch. Each set of experiments consisted of three biological plates for each of the experimental conditions. When the highest tested concentration did not reach either 50% or 200% of expression, we indicated that ECD must be higher than this concentration without indication of whether the effect is over or under-expression.

toxicity for CPO; thus, this was the category assigned to this chemical (Table 5).

We performed a second set of experiments (Table 4 and Figure S3, Supporting Information) where model chemicals and CPF and its metabolites were simultaneously assayed, and therefore, the comparisons of the predictions between both groups of chemicals were done using the same cellular batches. The predictions for CPF, CPO, and TClP were again as weak embryotoxicants (Table 5), as was previously reported for the set of experiment 1 run in experiments temporally spaced by more than 1 year.

DISCUSSION

We demonstrated that a reduced set of biomarker genes is able to correctly discriminate among the power of the strong neurodevelopmental toxicant RA and the power of the weak neurodevelopmental toxicant VA, and also to discriminate these two chemicals from the non-neurodevelopmental toxicant PG. When we applied this same procedure to CPF, CPO, and TClP, it predicted weak neurodevelopmental toxicity for all of them (Table 5).

The approach displayed in this work (use of the prediction model of classic EST on the basis of gene expression of biomarkers of differentiation) for identification of developmental toxicants was previously applied with success. In this way, with a small set of chemicals, we previously published that it is possible to enhance EST performance using the expression of the gene biomarkers of differentiation as end points for monitoring D3 cell differentiation.[8] We used four of the five genes employed in this work, where we added *Nefm* as a specific biomarker of neurodifferentiation, on the basis of previous studies that consider the expression of mRNA of neurofilaments as a relevant tools to identify developmental neurotoxicants using *in vitro* approaches.[17] Others also used this approach, including (1) Seiler and co-workers,[9] who correctly classified the embryotoxicity of 5-fluorouracil, RA, and PG on the basis of the expression of myosin heavy chain and α-actinin genes; (2) Festag and co-workers,[10] who were able to

Table 5. Assigning the Neurodevelopmental Potential to the Assessed Chemicals[a]

		set of experiments 1						set of experiments 2				
		in vivo		functions			*in vitro*	functions			*in vitro*	
		outcome	gene	I	II	III	outcome	I	II	III	outcome	
embryotoxic model chemicals	RA	strong	*Afp*	−18	−8	2	strong	**strong** (100% as strong)	−19	8	2	strong	**strong** (100% as strong)
			Flk1	−18	−8	2	strong		−19	−8	2	strong	
			Nes	−18	−8	2	strong		−19	−8	2	strong	
			Pnpla6	> −18	> −8	<2	strong		> −12	> −6	<0	strong	
			Nefm	−18	−8	2	strong		−19	−8	2	strong	
	VA	weak	*Afp*	5	8	−6	weak	**weak** (100% as weak)	6	8	−6	weak	**weak** (100% as weak)
			Flk1	5	8	−6	weak		6	8	−6	weak	
			Nes	5	8	−6	weak		7	8	−6	weak	
			Pnpla6	5	8	−6	weak		7	9	−7	weak	
			Nefm	5	8	−6	weak		>6	>8	<−6	weak	
	PG	Non-	*Afp*	16	14	−10	nonembryotoxicant	**nonembryotoxicant** (100% as nonembryotoxicant)	14	13	−9	nonembryotoxicant	**nonembryotoxicant** (100% as nonembryotoxicant)
			Flk1	16	14	−10	nonembryotoxicant		>14	>13	<−9	nonembryotoxicant	
			Nes	16	14	−10	nonembryotoxicant		>14	>13	<−9	nonembryotoxicant	
			Pnpla6	16	14	−10	nonembryotoxicant		14	13	−9	nonembryotoxicant	
			Nefm	16	14	−10	nonembryotoxicant		>14	>13	<−9	nonembryotoxicant	
chlorpyrifos and metabolites	CPF	Unclear	*Afp*	−2	3	−5	weak	**weak** (100% as weak)	−4	2	−5	weak	**weak** (100% as weak)
			Flk1	−2	3	−5	weak		−3	2	−5	weak	
			Nes	−1	3	−5	weak		−3	2	−5	weak	
			Pnpla6	−2	3	−5	weak		0	3	−6	weak	
			Nefm	−2	3	−5	weak		−1	3	−5	weak	
	CPO	Unclear	*Afp*	−0.2	3	−6	weak	**weak** (80% as weak)	−2	3	−5	weak	**weak** (100% as weak)
			Flk1	−3	2	−5	weak		−2	3	−5	weak	
			Nes	17	10	−11	nonembryotoxicant		−2	3	−5	weak	
			Pnpla6	−2	3	−5	weak		>7	>6	<−8	unclear	
			Nefm	0.4	4	−6	weak		−1	3	−6	weak	
	TClP	Unclear	*Afp*	3	5	−6	weak	**weak** (100% as weak)	−2	3	−5	weak	**weak** (100% as weak)
			Flk1	−3	3	−5	weak		−1	3	−5	weak	
			Nes	−3	3	−5	weak		1	4	−6	weak	
			Pnpla6	−3	3	−5	weak		−1	4	−5	weak	
			Nefm	−3	3	−5	weak		−1	3	−5	weak	

[a]We used the prediction model of a classic EST, which was modified as described in Table 1. The IC_{50} and ECD records were taken from Tables 2 and 4.

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differentiate among the embryotoxicity of 2 strong, 2 weak, and 2 nonembryotoxicants using the expression of platelet-endothelial cell adhesion molecule-1 and vascular endothelial cadherin genes; and (3) Suzuki and co-workers,[11] who used the expression of heart and neural crest derivatives expressed transcript 1 and cardiomyopathy associated 1 for differentiating the embryotoxicity of a set of model compounds containing 24 different chemicals. In addition, it has been recently demonstrated that the strategy of using gene expression as biomarkers of differentiation for ranking developmental toxicity on the basis of *in vitro* EST is feasible.[18] Thus, all these studies support the methodology employed in the current work for assigning weak neurodevelopmental toxicity to CPF, CPO, and TClP.

We performed two sets of independent experiments temporally separated by more than one year. The reproducibility of the end-points addressing viability was quite good (Table 2). However, we found significant qualitative and relatively important quantitative differences in the genomic end-points (ECD) obtained in the two sets of experiments (Table 4). We do not have a clear explanation for the differences in the ECDs. We believe that these might be attributed to different batches of undifferentiated D3 cells coming from the same clone. D3 cells can be maintained in an undifferentiated state in the presence of LIF, but even in this scenario, passages temporally separated (more than 1 year in our case) might start differentiation not exactly at the same point. We might also consider that the differentiation of D3 cells is spontaneous, and it is not directed or forced toward specific lineages by trophic factors. Consequently, small differences in the composition of the media (i.e., different Dulbecco's modified Eagle's medium and fetal calf serum batches) might also determine the spontaneous differentiation address and consequently the extension in the expression of the different genomic pathways. However, it is important to highlight that, despite these differences, the predictivity of the system was not affected and that both experiments yielded correct proposals of classification for the model embryotoxic chemicals and the same classification for CPF and its metabolites too (Table 5). The consistency and reliability of the prediction is based on the use of a set of different genes and not on a single end-point, as in differentiation to beating cardiomyocytes in the classical EST. Other authors already showed that a reduced subset of genes (five in our case) is sufficient to effectively separate teratogens from nonteratogens.[19]

Other *in vitro* studies have proposed neurodevelopmental toxicity for CPF and CPO. In this way, CPF promoted the differentiation of PC12 cells into the dopamine[20] and serotonin[21] phenotypes. CPF and CPO also have proven capability to inhibit the outgrowth of axon-like neurites of differentiating mouse N2a neuroblastoma[22,23] and rat C6 glioma[24] cells through reduction of the expression of different cytoskeletal proteins as neurofilament heavy chain, microtubule-associated protein, and tubulin. Nevertheless, all these effects are reported using differentiating adult cells under neurodifferentiation, while we used in this work embryonic stem cells, which might be a more appropriate model for testing toxicity in earlier stages of development. Our data are also supported by Buzanska and co-workers[25] and by Visan and co-workers,[26] who also reported neurodevelopmental toxicity *in vitro* using a human neural stem cell line derived from umbilical cord blood and D3 cells forced to neuroectodermal differentiation, respectively.

A mathematical analysis of the prediction models reveals that, with the IC50D3 and IC503T3 records displayed in Table 2, the assigned classification for CPF, CPO, and TClP will be always weak neurodevelopmental toxicants for ECD lower than 186, 113, and 196 μg/mL, respectively, in the first set of experiments and 128, 112, and 170 μg/mL, respectively, in the second set of experiments. The same analysis also allows one to conclude that it is mathematically impossible to reach the category of strong neurodevelopmental toxicant. In the hypothetical situation where ECD overcomes the above stated cutoff points, the category would move from weak to nondevelopmental toxicant. However, these concentrations are between 1.6 and 3.8 times higher than the respective IC50D3 and consequently not compatible with survival of the embryonic differentiating cell.

CPF, CPO, and TClP were classified as weak neurodevelopmental toxicants according to the procedure developed in this work (Table 5). *In vivo* and epidemiological reports about the capability of CPF to induce neurodevelopmental toxicity are contradictory.[2,3] We previously reported that CPO and TClP (the main metabolites of CPF) were able to alter the expression of gene biomarkers of differentiation after exposure, which, *in vivo*, may be expected to cause severe cholinergic maternal toxicity.[4] Nevertheless, the required concentrations of the CPF were not that high, and it was possible to make out exposure scenarios (those with low CPF bioactivation, i.e., after inhalatory or dermal exposures) where alterations of the gene expression profile were significant, with low or no acetylcholinesterase inhibition, which causes poor or no maternal cholinergic effects.[4] These uncertainties about the *in vivo* neurodevelopmental hazards of CPF in animals and humans and the possibility of CPF exerting effects on neurodevelopment only after exposures causing very low or no maternal toxicity are consistent with the weak embryotoxicant definition employed to classify chemicals according to EST, given that these weak embryotoxicants were defined as those chemicals capable of inducing embryotoxicity with low or mild maternal toxicity.[27] In conclusion, the intrinsic neurodevelopmental toxicity of CPF would be seen under exposures at which toxic activation to the highly acute anticholinesterase inhibitor oxon-metabolite (CPO) is limited. This could explain why the results reported in different epidemiological and experimental studies are contradictory and suggest that occupational exposures should be avoided with pregnant women as a precaution.

■ ASSOCIATED CONTENT

Supporting Information

Primer sequences and annealing temperatures used in the quantitative RT-PCR experiments; effect of RA, VA, and PG on the expression of the biomarker genes; and effect of CPF, CPO, and TClP on the expression of the biomarker genes. This material is available free of charge via the Internet at http://pubs.acs.org.

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■ ABBREVIATIONS

Afp, α-fetoprotein; CPF, chlorpyrifos (*O*,*O*-diethyl *O*-(3,5,6-trichloro-2-pyridinyl) phosphorothioate); CPO, chlorpyrifos-oxon (*O*,*O*-diethyl *O*-(3,5,6-trichloro-2-pyridinyl) phosphate); DEPC, diethylpyrocarbonate; ECD, effective concentration of the tested chemical that causes either an increase of 200% or a reduction of 50% in the expression of the biomarker gene in spontaneously differentiated cells after 3 days of exposure; EST, embryonic stem cell test; *Flk1*, fetal liver kinase 1; IC503T3, concentration of the tested compound that causes a decrease of 50% of viability in 3T3 cells after 3 days of exposure; IC50D3, concentration of the tested compound that causes a decrease of 50% of viability in D3 cells after 3 days of exposure; LIF, leukemia inhibition factor; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay; *Nefm*, neurofilament medium polypeptide; *Nes*, nestin; PG, penicillin-G; *Pnpla6*, patatin-like phospholipase domain containing six; RA, retinoic acid; TClP, 3,5,6-trichloro-2-pyridinol; VA, valproic acid

■ REFERENCES

(1) Food and Agriculture Organization of the United Nations and World Health Organization. (1999) Chlorpyrifos, in *Pesticide Residues in Food* (Food and Agriculture Organization of the United Nations and World Health Organization, Eds.) available at http://www.inchem.org/documents/jmpr/jmpmono/v99pr03.htm.

(2) Eaton, D. L., Daroff, R. B., Autrup, H., Bridges, J., Buffler, P., Costa, L. G., Coyle, J., McKhann, G., Mobley, W. C., Nadel, L., Neubert, D., Schulte-Hermann, R., and Spencer, P. S. (2008) Review of the toxicology of chlorpyrifos with an emphasis on human exposure and neurodevelopment. *Crit. Rev. Toxicol. 38* (Suppl2), 1−125.

(3) Mink, P. J., Kimmel, C. A., and Li, A. A. (2012) Potential effects of chlorpyrifos on fetal growth outcomes: implications for risk assessment. *J. Toxicol. Environ. Health, Part B 15*, 281−316.

(4) Estevan, C., Vilanova, E., and Sogorb, M. A. (2013) Chlorpyrifos and its metabolites alter gene expression at non-cytotoxic concentrations in D3 mouse embryonic stem cells under in vitro differentiation: considerations for embryotoxic risk assessment. *Toxicol. Lett. 217*, 14−22.

(5) Sogorb, M. A., Pamies, D., de Lapuente, J., Estevan, C., Estévez, J., and Vilanova, E. (2014) An integrated approach for detecting embryotoxicity and developmental toxicity of environmental contaminants using in vitro alternative methods. *Toxicol. Lett.* DOI: 10.1016/j.toxlet.2014.01.037.

(6) Theunissen, P. T., Schulpen, S. H., van Dartel, D. A., Hermsen, S. A., van Schooten, F. J., and Piersma, A. H. (2010) An abbreviated protocol for multilineage neural differentiation of murine embryonic stem cells and its perturbation by methyl mercury. *Reprod. Toxicol. 29*, 383−392.

(7) Theunissen, P. T., Pennings, J. L., van Dartel, D. A., Robinson, J. F., Kleinjans, J. C., and Piersma, A. H. (2013) Complementary detection of embryotoxic properties of substances in the neural and cardiac embryonic stem cell tests. *Toxicol. Sci. 132*, 118−130.

(8) Romero, A. C., Vilanova, E., and Sogorb, M. A. (2011) Shortening and improving the embryonic stem cell test through the use of gene biomarkers of differentiation. *J. Toxicol.* DOI: 10.1155/2011/286034.

(9) Seiler, A., Visan, A., Buesen, R., Genschow, E., and Spielmann, H. (2004) Improvement of an in vitro stem cell assay for developmental toxicity: the use of molecular endpoints in the embryonic stem cell test. *Reprod. Toxicol. 18*, 231−240.

(10) Festag, M., Viertel, B., Steinberg, P., and Sehner, C. (2007) An in vitro embryotoxicity assay based on the disturbance of the differentiation of murine embryonic stem cells into endothelial cells. II. Testing of compounds. *Toxicol. in Vitro 21*, 1631−1640.

(11) Suzuki, N., Ando, S., Yamashita, N., Horie, N., and Saito, K. (2011) Evaluation of novel high-throughput embryonic stem cell tests with new molecular markers for screening embryotoxic chemicals in vitro. *Toxicol. Sci. 124*, 460−471.

(12) Schmittgen, T. D., and Livak, K. J. (2008) Analyzing real-time PCR data by the comparative C(T) method. *Nat. Protoc. 3*, 1101−1108.

(13) Pamies, D., Vicente-Salar, N., Sogorb, M. A., Roche, E., and Reig, J. A. (2010) Specific effect of 5-fluorouracil on alpha-fetoprotein gene expression during the in vitro mouse embryonic stem cell differentiation. *Int. J. Toxicol. 29*, 297−304.

(14) Pamies, D., Sogorb, M. A., Fabbri, M., Gribaldo, L., Collotta, A., Scelfo, B., Vilanova, E., Harris, G., and Bal-Price, A. (2014) Genomic and phenotypic alterations of the neuronal-like cells derived from human embryonal carcinoma stem cells (NT2) caused by exposure to organophosphorus compounds paraoxon and mipafox. *Int. J. Mol. Sci. 15*, 905−926.

(15) Pamies, D., Vilanova, E., and Sogorb, M. A. (2013) Functional pathways altered after silencing Pnpla6 (the codifying gene of neuropathy target esterase) in mouse embryonic stem cells under differentiation. *In Vitro Cell Dev. Biol. Anim. 50*, 261−273.

(16) Stummann, T. C., Hareng, L., and Bremer, S. (2009) Hazard assessment of methylmercury toxicity to neuronal induction in embryogenesis using human embryonic stem cells. *Toxicology 257*, 117−126.

(17) Hogberg, H. T., Kinsner-Ovaskainen, A., Coecke, S., Hartung, T., and Bal-Price, A. K. (2010) mRNA expression is a relevant tool to identify developmental neurotoxicants using an in vitro approach. *Toxicol. Sci. 113*, 95−115.

(18) Schulpen, S. H., Pennings, J. L., Tonk, E. C., and Piersma, A. H. (2014) A statistical approach towards the derivation of predictive gene sets for potency ranking of chemicals in the mouse embryonic stem cell test. *Toxicol. Lett. 225*, 342−349.

(19) Jergil, M., Forsberg, M., Salter, H., Stockling, K., Gustafson, A. L., Dencker, L., and Stigson, M. (2011) Short-time gene expression response to valproic acid and valproic acid analogs in mouse embryonic stem cells. *Toxicol. Sci. 121*, 328−342.

(20) Slotkin, T., and Seidler, F. (2009) Transcriptional profiles reveal similarities and differences in the effects of developmental neurotoxicants on differentiation into neurotransmitter phenotypes in PC12 cells. *Brain Res. Bull. 78*, 211−225.

(21) Slotkin, T. A., and Seidler, F. J. (2008) Developmental neurotoxicants target neurodifferentiation into the serotonin phenotype: Chlorpyrifos, diazinon, dieldrin and divalent nickel. *Toxicol. Appl. Pharmacol. 233*, 211−219.

(22) Sachana, M., Flaskos, J., Alexaki, E., Glynn, P., and Hargreaves, A. J. (2001) The toxicity of chlorpyrifos towards differentiating mouse N2a neuroblastoma cells. *Toxicol. in Vitro 15*, 369−372.

(23) Flaskos, J., Nikolaidis, E., Harris, W., Sachana, M., and Hargreaves, A. J. (2011) Effects of sub-lethal neurite outgrowth inhibitory concentrations of chlorpyrifos oxon on cytoskeletal proteins and acetylcholinesterase in differentiating N2a cells. *Toxicol. Appl. Pharmacol. 256*, 330−336.

(24) Sachana, M., Flaskos, J., Sidiropoulou, E., Yavari, C. A., and Hargreaves, A. J. (2008) Inhibition of extension outgrowth in differentiating rat C6 glioma cells by chlorpyrifos and chlorpyrifos oxon: effects on microtubule proteins. *Toxicol. in Vitro 22*, 1387−1391.

(25) Buzanska, L., Sypecka, J., Nerini-Molteni, S., Compagnoni, A., Hogberg, H. T., del Torchio, R., Domanska-Janik, K., Zimmer, J., and Coecke, S. (2009) A human stem cell-based model for identifying adverse effects of organic and inorganic chemicals on the developing nervous system. *Stem Cells 27*, 2591−2601.

(26) Visan, A., Hayess, K., Sittner, D., Pohl, E. E., Riebeling, C., Slawik, B., Gulich, K., Oelgeschläger, M., Luch, A., and Seiler, A. E. (2012) Neural differentiation of mouse embryonic stem cells as a tool to assess developmental neurotoxicity in vitro. *Neurotoxicology 33*, 1135−1146.

(27) Brown, N. A. (2002) Selection of test chemicals for the ECVAM international validation study on in vitro embryotoxicity tests. European Centre for the Validation of Alternative Methods. *Altern. Lab. Anim. 30*, 177−198.

(28) Genschow, E., Spielmann, H., Scholz, G., Pohl, I., Seiler, A., Clemann, N., Bremer, S., and Becker, K. (2004) Validation of the embryonic stem cell test in the international ECVAM validation study on three in vitro embryotoxicity tests. *Altern. Lab. Anim. 32*, 209−244.