Aryl- and alkyl-phosphorus-containing flame retardants induced mitochondrial impairment and cell death in Chinese hamster ovary (CHO-k1) cells[☆]

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ABSTRACT

Phosphorus-containing flame retardants (PFRs) are increasingly in demand worldwide as replacements for brominated flame retardants (BFRs), but insufficient available toxicological information on PFRs makes assessing their health risks challenging. Mitochondria are important targets of various environmental pollutants, and mitochondrial dysfunction may lead to many common diseases. In the present study, mitochondria impairment-related endpoints were measured by a high content screening (HCS) assay for 11 selected non-halogen PFRs in Chinese hamster ovary (CHO-k1) cells. A cluster analysis was used to categorize these PFRs into three groups according to their structural characteristics and results from the HCS assay. Two groups, containing long-chain alkyl-PFRs and all aryl-PFRs, were found to cause mitochondrial impairment but showed different mechanisms of toxicity. Due to the high correlation between cell death and mitochondrial impairment, two PFRs with different structures, trihexyl phosphate (THP) and cresyl diphenyl phosphate (CDP), were selected and compared with chlorpyrifos (CPF) to elucidate their mechanism of inducing cell death. THP (an alkyl-PFR) was found to utilize a similar pathway as CPF to induce apoptosis. However, cell death induced by CDP (an aryl-PFR) was different from classical necrosis based on experiments to discriminate among the different modes of cell death. These results confirm that mitochondria might be important targets for some PFRs and that differently structured PFRs could function via distinct mechanisms of toxicity.

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1. Introduction

Due to the increasingly rigid restrictions on the production and usage of brominated flame retardants (BFRs) by the European Union (EU) and other countries, the production and application of phosphorus-containing flame retardants (PFRs), which are regarded as suitable alternatives for BFRs, have gradually increased

(Betts, 2008; Cox and Efthymiou, 2003). However, as with BFRs, most PFRs are additives and are not chemically bonded to the end-use products, which may allow them to easily diffuse into various environmental media (Rodriguez et al., 2006; van der Veen and de Boer, 2012). Additionally, some PFRs with high bio-concentration factor values have the potential to bio-accumulate in organisms (Hou et al., 2016; van der Veen and de Boer, 2012). Indeed, high concentrations of PFRs have been detected in various environmental and biotic samples in recent decades (Reemtsma et al., 2006; van der Veen and de Boer, 2012). Therefore, PFRs represent a family of chemicals that have become an emerging environmental issue. Increasingly, research has revealed that PFRs, especially chlorinated-PFRs, have the potential to be carcinogenic and

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reprotoxic (EU, 2014). Consequently, three chlorinated PFRs are now restricted by the EU Toy Safety Directive 2014/79/EU. Conversely, non-halogen PFRs are used as flame retardants in various products, and there is an increasing demand for these non-halogen PFRs in the world market (Rakotomalala et al., 2010; Takigawa et al., 2001; van der Veen and de Boer, 2012; Zhou et al., 2008). Previous studies have shown that exposure to non-halogen PFRs has the potential to cause neurological effects, endocrine disruptions, cardiac disorders, carcinogenic changes, and oxidative stress in different organisms (Du et al., 2015; EPA, 2007; Hausherr et al., 2014; Jin et al., 2015; Kluwe et al., 1985; Meeker and Stapleton, 2010; Yuan et al., 2016). Unfortunately, we still lack sufficient safety or toxicological information for these non-halogen PFRs that are currently in use, even though significant numbers of people are exposed to them.

As a type of emerging environmental pollutant, PFRs are structurally similar to organophosphate pesticides (OPs). Although PFRs, like OPs, can induce significant neurotoxicity *in vitro* and *in vivo*, a growing body of research demonstrates that PFRs have low potency compared to OPs with respect to inhibiting acetylcholinesterase (AChE) (Dishaw et al., 2011; Moser et al., 2015; Yuan et al., 2016). Meanwhile, over the last decade, OPs have been shown to exhibit multiple potential non-cholinergic mechanisms, accounting for some of the adverse consequences of OPs exposure (Terry, 2012). Recently, there is substantial evidence has suggested that mitochondria are one of the important non-cholinesterase targets of OPs (dos Santos et al., 2016; Karami-Mohajeri and Abdollahi, 2013; Yamada et al., 2017). In fact, some PFRs were found to acutely decrease the mitochondrial membrane potential ($\Delta\psi$) in HepG2 cells (Attene-Ramos et al., 2015; NCBI, 2012). Therefore, we hypothesize that the induction of mitochondrial dysfunction by PFRs is a potential mechanism behind the adverse consequences of PFRs. A recent study has also revealed that PFRs have the potential to induce oxidative stress, DNA damage, neurotoxicity, and cardiotoxicity (Dishaw et al., 2011; Du et al., 2015; Jin et al., 2015; Yuan et al., 2016). Most of these PFRs-induced toxic effects are more or less associated with the mitochondrial dysfunction. The impairment of mitochondria may further result in some common diseases, including cancer, diabetes, and neurodegenerative and cardiovascular diseases (Ferrari, 1996; Lin and Beal, 2006; Shaughnessy et al., 2014; Whitaker et al., 2016). Hence, there is a need for more detailed work to fully investigate the mitochondrial impairment induced by PFRs.

In addition to their role in cellular energy metabolism, mitochondria are now considered central players in cell death (Ott et al., 2007). Previous work has discerned the mechanism of mitochondria-mediated apoptosis (Jiang and Wang, 2004). However, the study of cell death was recently revitalized by the understanding that necrosis can occur in a highly regulated and genetically controlled manner (Berghe et al., 2014). Numerous examples of this process are emerging, and some of them, including parthanatos (David et al., 2009) and ferroptosis (Dixon et al., 2012), depend greatly on mitochondrial participation. Different types of programmed cell death (PCD) are initiated by specific stimuli and recruit diverse core regulators to execute cell death, which results in cells exhibiting distinct morphological and biochemical features during these processes (Xie et al., 2016). The morphological features of the cytoskeleton and mitochondria have proven to be valuable parameters for characterizing compound-induced cell death using cluster analysis (Wolpaw et al., 2011). Biochemical features, including $\Delta\psi$, ATP level, ROS production, intracellular calcium concentration and activation of caspases, could provide further information to distinguish some types of PCD (David et al.,

2009; Dixon et al., 2012; Reed and Pellicchia, 2012; Xie et al., 2016). OPs such as chlorpyrifos (CPF) may induce cell death. Indeed, previous work has found that mitochondria-induced apoptosis was observed in CPF-treated cells (Lee et al., 2012). However, some *in vitro* studies suggested that the cell death induced by TOCP seemed to be distinct from apoptosis (Carlson et al., 2000). Additionally, there is more than one mechanism that could explain the toxicity of organophosphorus compounds via mitochondrial impairment (Karami-Mohajeri and Abdollahi, 2013). Therefore, the mechanisms of cell death induced by different PFRs need to be discerned.

High content screening (HCS) has been shown to be a powerful approach to assess mitochondrial impairment induced by compounds (Attene-Ramos et al., 2015; Tolosa et al., 2015; Wolpaw et al., 2011). HCS integrated with image analysis algorithms and informatics tools is able to collect quantitative data from complex biological systems. These data, containing multiple cellular parameters calculated from HCS, such as morphology and texture, have proven to be useful for characterizing cellular or organellar microstructures in recent studies (Sailem et al., 2015). Consequently, HCS has been widely used with cluster analysis in chemical biology screens to identify compounds that induce specific phenotypes, or in unbiased, general cytological profiling of compounds based on an integrated set of morphological alterations to support structure-activity relationships of chemicals (Adams et al., 2006; Tanaka et al., 2005; Wolpaw et al., 2011).

The main purpose of this study was to examine the mitochondrial impairment of the 11 selected non-halogen PFRs and identify the different possible mechanisms leading to cell death. Therefore, an HCS assay was designed and used to quantitatively analyse multi-parametric features of cellular changes in cytotoxicity, mitochondrial membrane potential and the morphologies or textures of nuclei, mitochondria and cells after PFR exposure. A cluster analysis was also used to analyse data and discern the relationships between mitochondrial toxicity and the structures of these PFRs. Chinese hamster ovary (CHO-k1) cells were chosen because these cells are traditionally used for studying mitochondrial toxicity and function (Ferrer et al., 2009; Stone and Vance, 2000). THP (an alkyl-PFR) and CDP (an aryl-PFR), which had high-level inducible results, were selected from two different groups, and the mechanisms of cells death induced by these compounds were further investigated.

2. Materials and methods

2.1. Chemicals

Trimethyl phosphate (TMP, 98+%), tripropyl phosphate (TPP, 99%), tri-*n*-butyl phosphate (TNBP, 99%), tris(2-ethylhexyl) phosphate (TEHP, 97%), N-acetyl-L-cysteine (NAC, 99+%), dimethyl sulfoxide (DMSO), Pluronic® F-127, HEPES, Hoechst 33,342, propidium iodide (PI), and 2',7'-dichlorofluorescein diacetate (H₂DCF-DA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). TOCP (99+%), tri-*m*-cresyl phosphate (TMCP, 97+%), tri-*p*-cresyl phosphate (TPCP, 99%), cresyl diphenyl phosphate (CDP, 97.1%), triphenyl phosphate (TPHP, 99+%), triethyl phosphate (TEP, 99+%), trihexyl phosphate (THP, 95+%), chlorpyrifos (CPF, 95+%), and carbonyl cyanide meta-chlorophenylhydrazone (CCCP, 98%) were acquired from J&K Chemical (Shanghai, China). Tetramethylrhodamine ethyl ester (TMRE) and Fluo-8® AM were obtained from AAT Bioquest (Sunnyvale, CA, USA). SYTOX® Green and Mito Tracker Deep® Red FM were acquired from Thermo Fisher Scientific (Waltham, MA, USA). All other chemicals were of analytical purity and were purchased from Sinopharm Chemical Reagent Co., Ltd (Shanghai, China).

2.2. Cell culture and exposure

CHO-k1 cells were obtained from the Cell Culture Center, Institute of Basic Medical Sciences of the Chinese Academy of Medical Sciences and School of Basic Medicine of Peking Union Medical College, Beijing, China. Cells were cultured in Dulbecco's modified Eagle medium, Nutrient Mixture F-12 (DMEM/F-12 medium in a ratio of 1:1, Hyclone, Shanghai, China) with 10% defined supplemented foetal bovine serum (PAN Biotech Ltd, Aidenbach, Germany), 1% penicillin-streptomycin (10,000 U/ml, Hyclone, Logan, UT, USA), and 0.1% amphotericin B (2.5 mg/mL, Amresco, Solon, OH, USA). The cells were maintained at 37 °C in a 5% CO₂ atmosphere in a CO₂ incubator (Panasonic, Ehime-ken, Japan), and the medium was refreshed every three or four days during sub-culturing (Westerink et al., 2011). Each experimental condition was assayed in triplicate wells. The stock solutions of the compounds were first prepared and serially diluted to different concentrations in DMSO. Then, all compounds were diluted in culture medium or buffer, and the final DMSO concentration in the medium was 0.5% (v/v). During all experiments, a control culture was treated with an equivalent amount of DMSO.

2.3. Cell viability assay

Cell viability was measured by a Cell Counting Kit-8 (CCK-8) Cytotoxicity/Viability Assay (Beyotime Biotechnology, Haimen, China) according to the manufacturer's instructions. CHO-k1 cells were seeded at a density of 3000 cells/well in 96-well microtiter plates (Corning, Tewksbury, MA, USA). After 24 h of pre-culture, cells were exposed for 24 h to 10 concentrations of PFRs. These compound-treated cells were washed with Tyrode's buffer (TB: 145 mM NaCl, 5 mM KCl, 10 mM glucose, 1.5 mM CaCl₂, 1 mM MgCl₂ and 10 mM HEPES; pH adjusted to 7.4 with NaOH). Next, cells in each well were incubated with 100 µl of TB containing 10 µl of CCK-8 solution for 2 h at 37 °C in a humidified 5% CO₂ atmosphere. Finally, the absorbance of each well at 450 nm was measured by a microplate reader (M200, Tecan, Männedorf Switzerland). Cell viability was expressed as a percentage of the control without treatment. Finally, dose-response curves were fitted based on the results of the CCK-8 assay to determine the LC₅₀ using the four-parameter log-logistic model of the R statistical package drc (Ritz and Streibig, 2005).

2.4. Multi-parameter high content screening assay and data analysis

CHO-k1 cells were seeded at a density of 6000 cells/well in 96-well microtiter plates (Corning). After 24 h of pre-culture, cells were exposed for 3 h to 11 PFR at five concentrations (1, 10, 50, 100, and 500 µM). Then, the cells were washed twice with TB. To detect changes in cytotoxicity and $\Delta\Psi$ and to gain insight into subcellular structure after exposing cells to these compounds, CHO-k1 cells were incubated with a fluorophore dye cocktail (diluted in TB) containing Hoechst 33,342 (stains nuclei, 2.7 µM), SYTOX[®] Green (stains dead cells, 20 nM), TMRE (changes in $\Delta\Psi$, 50 nM), and Mito Tracker Deep[®] Red FM (stains mitochondria, 200 nM) for 45 min at room temperature (Bova et al., 2005; Joshi and Bakowska, 2011; Tolosa et al., 2015). An Operetta[™] High Content Screening instrument with Harmony[™] software version 3.5.2 (PerkinElmer, Waltham, MA, USA) and a 40 × NA objective were used for imaging (4 fields per well). Then, the collected images were analysed using the image analysis modules of the Harmony[™] software to obtain multiple quantitative features of the cells according to the different fluorescence signals. Because the exposure time in this assay was reduced to 3 h, the cytotoxicity of 11 PFRs was re-assessed to ensure

that each compound at the concentration selected for obtaining cellular features exhibited suitable cytotoxicity (lower than 50%) for a cluster analysis. Cytotoxicity in the HCS assay was assessed using SYTOX[®] Green to stain dead cells or by counting the cell number in every well. SYTOX[®] Green is an excellent green fluorescent nuclear and chromosome counterstain that cannot permeate live cells, making it a useful indicator of dead cells within a population to determine cytotoxicity or viability (Bova et al., 2005). The loss of cells was also a sensitive index reflecting the cytotoxicity of compounds in the HCS assay (Ye et al., 2014). Cytotoxicity was accounted for and calculated as follows:

$$Cytoto_{\text{sytoxgreen}}(\%) = 100 \times \frac{N_{\text{dead}}}{N_{\text{control}}}$$

$$Cytoto_{\text{count}}(\%) = 100 \times \left(1 - \frac{N_{\text{dead}}}{N_{\text{control}}}\right)$$

$Cytoto_{\text{sytoxgreen}}$ means the cytotoxicity determined by using SYTOX[®] Green to stain dead cells, N_{dead} = number of cells which were stained with SYTOX[®] Green in selected compound-treated wells, N_{control} = the number of cells in DMSO-treated wells.

$Cytoto_{\text{count}}$ means the cytotoxicity determined by counting cell number in every well to reflect loss of cells after compound exposure. N_{treat} = number of cells in compound-treated wells, N_{control} = the number of cells in DMSO-treated wells.

In each well, 100 cells were randomly selected under the Hoechst 33,342 channel. These cells were used to obtain multiple parameters to describe nuclear mass, morphology and texture (Hoechst 33,342 channel); cell membrane integrity and cell viability (SYTOX[®] Green channel); mitochondrial mass, texture, and cell morphology (Mito Tracker Deep[®] Red FM channel); and changes in $\Delta\Psi$ (TMRE channel). Unlike in previous studies (Bova et al., 2005; Tolosa et al., 2015), the image analysis used here could quantitatively describe the compound-induced specific phenotypes in the nuclei, mitochondria, and cells. Parameters such as texture have proven to be useful for characterizing cellular or organellar microstructures in recent research (Sailem et al., 2015). More detailed annotations of all parameters are provided in the supplementary material (Appendix A. Supplementary data, Table 1). Finally, cellular features of the cells treated with the 11 PFR cells were selected for cluster analysis at the highest treatment concentration with cytotoxicity lower than 50%. A hierarchical cluster analysis was applied to cluster the 11 PFRs according to the multiple parameters obtained from the HCS assay using the hclust function of the R statistical package (R Development Core Team, 2010).

2.5. Real-time measurement of mitochondrial membrane potential in live cells

To better characterize the kinetics of the PFR-induced reductions in $\Delta\Psi$, $\Delta\Psi$ values were monitored in real time with live CHO-k1 cells. The procedure was modified according to the methods of Bova et al. (2005). In brief, CHO-k1 cells were seeded at a density of 6000 cells/well in 96-well microtiter plates (Corning) to grow for 24 h. Cells were stained with Hoechst 33,342 and TMRE as described above. TMRE is a non-fixable cationic dye that is actively pumped into and accumulates within energized mitochondria. When mitochondria become de-energized, the dye will rapidly wash out of the cell, resulting in a decrease in fluorescence intensity indicative of a loss of $\Delta\Psi$ (Bova et al., 2005). THP (100, 75, and 10 µM), CDP (500, 100, and 10 µM), and CPF (200, 100, and 10 µM) were added to cells simultaneously, and the fluorescent images were instantly recorded in live cells in real time for nearly 2 h using

Table 1
OPFRs selected for the study of mitochondrial impairment.

Name	Abbr.	CAS	Structure		Log K _{ow}
Tri-o-cresyl phosphate	TOCP	78-30-8	R1 = R2 = R3 =		6.34
Tri-m-cresyl phosphate	TMCP	563-04-2	R1 = R2 = R3 =		6.34
Tri-p-cresyl phosphate	TPCP	78-32-0	R1 = R2 = R3 =		6.34
Cresyl diphenyl phosphate	CDP	26,444-49-5	R1 = R2 =		5.25
			R3 =
Triphenyl phosphate	TPHP	115-86-6	R1 = R2 = R3 =		4.70
Trimethyl phosphate	TMP	512-56-1	R1 = R2 = R3 =		−0.60
Triethyl phosphate	TEP	78-40-0	R1 = R2 = R3 =		0.87
Tripropyl phosphate	TPP	513-08-6	R1 = R2 = R3 =		2.35
Tri-n-butyl phosphate	TNBP	126-73-8	R1 = R2 = R3 =		3.82
Trihexyl phosphate	THP	2528-39-4	R1 = R2 = R3 =		6.76
Tris (2-ethylhexyl) phosphate	TEHP	78-42-2	R1 = R2 = R3 =		9.49

Log K_{ow} have been estimated using EPI Suite software with KOWWIN v1.67 models.

the Operetta™ High Content Screening instrument. CCCP (100 μM), which potentially uncouples oxidative phosphorylation in mitochondria, was added to cells and used as a positive control.

2.6. Biochemical features to discriminate among different cell death modes

The appearance of phosphatidylserine (PS) residues (normally hidden within the plasma membrane) on the surface of the cell is an early event in apoptosis. Annexin-V has a strong, Ca²⁺-dependent affinity for PS and therefore can be used as a probe for detecting early apoptosis (Darzynkiewicz et al., 1997). The analysis of Annexin-V binding was performed with an Annexin-V FITC Apoptosis Detection Kit (Dojindo, Tokyo, Japan) according to the manufacturer's protocol. In brief, CHO-k1 cells were seeded at a density of 60,000 cells/well in 12-well cell culture plates (Corning). After 24 h of pre-culture, cells were treated with THP (75, 100 μM), CDP (200, 500 μM), and CPF (100, 200 μM) for 3 h. Treated cells were harvested and stained with Hoechst 33,342 (2.7 μM), PI (2.2 μM) and FITC-labelled Annexin-V for 20 min. The stained cells were deposited onto slides, and then these slides were mounted and observed under the 20 × NA objective of an Operetta™ High Content Screening instrument with the Harmony™ software.

Image analysis was used to determine cell area under the Hoechst 33,342 channel and to calculate the labelled Annexin-V and PI intensity in each cell area.

The induction of caspases and changes in the level and intracellular concentration of ATP are different in different types of PCD (Xie et al., 2016). Therefore, three bioassays were further conducted to determine these biochemical features in CHO-k1 cells. All these assays were performed at the same cell density and using the same concentration of each compound. In brief, cells were seeded at a density of 6000 cells/well in 96-well microtiter plates (Corning). After 24 h of pre-culture, cells were exposed for 3 h to five concentrations of THP (1, 10, 50, 75, 100 μM), CDP (1, 10, 100, 200, 500 μM), and CPF (1, 10, 50, 100, 200 μM). Then, various methods were used to determine these biochemical features.

Apoptosis is a highly regulated mechanism of cell death, which converges on caspase activation (Jiang and Wang, 2004). The induction of caspase-3/7 and 9 activities by THP, CDP, and CPF was determined using the Promega (Madison, WI, USA) Caspase-Glo® 3/7 Assay Kit and Caspase-Glo® 3/7 Assay Kit and was measured according to the manufacturer's protocol. In brief, Caspase-Glo® reagent was added to compound-treated cells, and cells were incubated for 45 min at room temperature. Luminescence intensity was detected using a microplate reader (M200). The luminescence

intensity was normalized to the protein concentration in each well.

Some types of PCD, including apoptosis and necroptosis, could elevate intracellular calcium concentrations (Nomura et al., 2014; Rasola and Bernardi, 2011). To determine changes in cytosolic free calcium concentrations after exposing cells to the compounds, cells were washed twice with TB and stained with 4 μ M Fluo-8 AM (diluted in TB) containing Pluronic® F-127 (0.02%, W/V) by incubation for 30 min at 37 °C in a dark and humidified 5% CO₂ atmosphere. Then, this dye solution was replaced with TB containing 2 mM probenecid. Each well in the 96-well microtiter plate was observed using the 20 $\times$ NA objective of the Operetta™ High Content Screening instrument with Harmony™ software. Image analysis was used to determine the cell area according to Hoechst 33,342 and to calculate the mean Fluo-8 intensity in each cell area.

Whether cells activate apoptosis or succumb to necrosis highly depends on the residual intracellular ATP level (Rasola and Bernardi, 2011). In this study, the cellular ATP level was assessed by the PhosphoWorks™ Luminometric ATP Assay Kit (AAT Bioquest) according to the manufacturer's protocol. In brief, the ATP assay solution was added into compound-treated cells, and cells were incubated for 20 min at room temperature. Luminescence intensity was detected using a microplate reader (M200). The value of the luminescence intensity in each well was normalized to the protein concentration, which was measured using the Enhanced BCA Protein Assay Kit (Beyotime Biotechnology).

2.7. Measurement of intracellular ROS

ROS production has been implicated in various forms of cell death (Reed and Pellicchia, 2012). To monitor intracellular ROS production, fluorescent DCF, which is converted from non-fluorescent H₂DCF by ROS, was used to measure ROS production. The procedure for detecting intracellular ROS was modified according to the method of Wang and Joseph (1999). In brief, CHO-k1 cells were seeded at a density of 6000 cells/well in 96-well microtiter plates (Corning) to grow for 24 h. First, cells were washed twice with TB and loaded with 5 μ M H₂DCF (diluted in TB) by incubation for 45 min at 37 °C in a dark and humidified 5% CO₂ atmosphere. The cells were then washed again and exposed for 3 h to five concentrations of THP (1, 10, 50, 75, 100 μ M), CDP (1, 10, 100, 200, 500 μ M), and CPF (1, 10, 50, 100, 200 μ M) in a dark and humidified 5% CO₂ atmosphere. Finally, the induction of intracellular ROS production in cells was measured directly using a microplate reader (M200). The fluorescence intensity in each well was normalized to the protein concentration, which was measured using the Enhanced BCA Protein Assay Kit (Beyotime Biotechnology).

2.8. Using NAC to inhibit intracellular ROS production

To investigate the role of ROS in the induction of cell death, NAC was used as a ROS scavenger to inhibit intracellular ROS production and reduce the effectiveness of ROS-mediated cell death (Sun, 2010). A stock solution of NAC (100 mM) was prepared in PBS. The final concentration of NAC as the ROS scavenger was 2 mM in the cell culture medium. Cytotoxicity changes after NAC treatment and co-exposure to the tested compounds (THP, CDP and CPF) for 3 h were measured using a CCK-8 cytotoxicity assay. Changes in the induction of intracellular caspase 3/7 by the tested compounds after NAC treatment were also measured using the method described above.

2.9. Statistical analysis

Each measurement was performed in single cells, and the values

for the same treatment (e.g., triplicate wells) were averaged and then normalized to the average value of the solvent-treated cells. Each experimental condition for each method was repeated at least three times in three independent cell preparations. The statistical data analysis was performed using an ANOVA followed by Bonferroni's test from the agricolae package (version 1.2–4). A *p* value below 0.05 was considered statistically significant. Most of the presented figures were produced using the ggplot2 package (Wickham, 2016).

3. Results

3.1. Cytotoxic effects of PFRs in CHO-k1 cells

In this study, 11 selected PFRs were first analysed with respect to their potency to inhibit cell viability in CHO-k1 cells. A brief summary of the information on all the tested PFRs is shown in Table 1. All tested PFRs except for TMP, TEP, and TEHP significantly inhibited cell viability in a dose-dependent manner (Fig. 1), and their half-maximal inhibiting concentrations (LC₅₀) are presented in the legend of Fig. 1. All aryl-PFRs exhibited similar cytotoxic effects in CHO-k1 cells, and their LC₅₀ values ranged from 74 to 92 μ M. THP, an alkyl-PFR, had the lowest LC₅₀ (58 μ M) of all the PFRs, but TMP and TEP, both of which have a short alkyl chain, could not inhibit cell viability at their highest concentration. The LogKow values of the PFRs seemed to be related to their LC₅₀ values, especially for the alkyl-PFRs. A counter-example is TEHP, which had the highest LogKow value but relatively low cytotoxicity.

3.2. A multi-parametric assessment of PFR-induced toxicity was conducted regarding the effects on nuclei, mitochondria, and cells

Fig. 2 shows the $\Delta\Psi$ values of three types of alkyl-PFRs (TNBP,

Fig. 1. The dose-response curves illustrated cytotoxic effects of PFRs on CHO-k1 cells and fitted with log-logistic model. The CHO-k1 cells were exposed to a range of concentrations of (a) aryl-OPFRs and (b) alkyl-OPFRs for 24 h, and cell viability was measured by CCK-8 method. The values presented are the mean of at least three independent experiments. The LC₅₀ of each compound is shown in the legend.

Fig. 2. Effects of test compounds on mitochondrial membrane potential. The CHO-k1 cells were exposed to a range of concentrations of (a) aryl-OPFRs and (b) alkyl-OPFRs for 3 h, and mitochondrial membrane potential was detected by staining with TMRE. The values presented are the mean $\pm$ SEM of at least three independent experiments. A significant difference among the groups of $p < 0.05$ (ANOVA) is indicated by differences in the letters.

THP, and TEHP), and all aryl-PFRs were found to significantly decrease $\Delta\psi$ in a dose-dependent manner. Additionally, all these compounds had relatively high LogKow values, as shown in Table 1. However, TMP, TEP, and TPP, which have lower LogKow values (<2.5), lacked the ability to decrease $\Delta\psi$ in CHO-k1 cells. This result indicated that the ability of selected PFRs to decrease $\Delta\psi$ might be associated with their hydrophobicity and could affect their cytotoxicity. Fig. 3 indicates that all aryl-PFRs and TEHP can increase the mitochondrial mass in a dose-dependent manner, but TNBP at a high concentration decreased the mitochondrial mass. There was no significant difference from the negative control group in cellular mitochondrial mass between cells treated with TMP, TEP, TPP, or THP ($p > 0.05$).

Cellular features for clustering were obtained at the concentration where the cytotoxicity of each compound was lower than 50% (Table 2). These concentrations were: 500 μM for TMCP, TOCP, TPCP, CDP, TMP, TEP, TPP, TNBP, and TEHP, and 100 μM for THP. The results of the hierarchical cluster analysis showed that the 11 selected compounds can be divided into 3 groups (Fig. 4). The first group contained TMP, TEP, and TPP, which have relatively short straight-chain alkyl groups. The control group (only DMSO-treated) also clustered with this group, which indicates that these 3 PFRs

Fig. 3. Effects of test compounds on mitochondrial mass. The CHO-k1 cells were exposed to a range of concentrations of (a) aryl-OPFRs and (b) alkyl-OPFRs for 3 h, and mitochondrial mass was detected by staining with Mito Tracker Deep® Red FM. The values presented are the mean $\pm$ SEM of at least three independent experiments. A significant difference among the groups of $p < 0.05$ (ANOVA) is indicated by differences in the letters.

Table 2

Cytotoxicity of compounds for clustering at selected concentration.

Compound	Concentration (μM)	Cyto _{count} (%)	Cyto _{sytoxgreen} (%)
TOCP	500	40	6
TMCP	500	34	3
TPCP	500	35	19
CDP	500	26	4
TPHP	500	23	0
TMP	500	7	0
TEP	500	8	0
TPP	500	–1	0
TnBP	500	23	17
THP	100	30	9
TEHP	500	41	43

Cyto_{count} the cytotoxicity determined by counting cell number in every well to reflect loss of cells after compound treating, Cyto_{sytoxgreen} the cytotoxicity determined by using SYTOX® Green to stain dead cells.

have little effect on cells and mitochondria. Although PFRs containing aryl or long alkyl substituent groups have a similar effect on $\Delta\psi$ (Fig. 2), the cluster analysis successfully divided these PFRs into two groups according to their structure. All 5 aryl-PFRs, including TOCP, TMCP, TPCP, CDP, and TPHP, clustered into the second group. The third group contained THP, TEHP, and TNBP, which have long alkyl substituent groups. The distinct multiple parameters that

Fig. 4. Cluster analysis of cellular features from the multi-parameter HCS assay. Cellular features of each compounds in CHO-k1 cells obtained from HCS assay were compared with those of vehicle-treated (DMSO) cells in heat map. The hierarchical cluster analysis was applied to cluster 11 OPFRs according the multi-parameters. The values presented are the mean of at least three independent experiments. The more detail annotations of all parameters were provided in the supplementary material ([Appendix A. Supplementary data, Table 1](#)).

Fig. 5. Representative images of multi-parameter HCS assay for cluster analysis. Vehicle-treated cells (control), exposed to 500 μM of TMP, 100 μM of THP, and 500 μM of CDP in CHO-k1 cells, are shown. Fluorescence of SYTOX® Green (green) or TMRE (yellow) or Mito Tracker Deep® Red FM (red) is exemplified. Nuclei were identified by Hoechst 333,342 (blue) in all the images for determining the position of the cells and detecting changes in nuclei mass, morphology and texture. Scales bar = 20 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

describe the effects of the two classes of PFRs on cells are: 1) the area of the nuclei, 2) the texture of the nuclei, 3) the morphology of

cell, and 4) the mitochondrial mass. The fluorescence images of different treated cells further confirmed these results ([Fig. 5](#)). The

long alkyl-PFRs led to smaller and more condensed nuclei than the aryl-PFRs. Apoptotic bodies were also observed in THP-treated cells. Most PFRs could cause mitochondrial impairment and had significant cytotoxicity in the HCS assay; however, the cell death induced by aryl-PFRs and alkyl-PFRs showed distinct features. According to the results, THP (alkyl-PFRs) and CDP (aryl-PFRs) with high-level inducible results were selected as the representative molecules for aryl- and alkyl-PFRs to conduct further research into discerning the possible mechanisms of mitochondrial impairment and cell death, respectively. An organophosphorus pesticide, CPF, was also assessed in our study, and its effects were compared with THP and CDP.

3.3. Monitoring the kinetics of THP, CDP, and CPF-induced decreases in mitochondrial membrane potential

The reductions of $\Delta\Psi$ in response to short-term exposure to THP and CDP were obvious, which suggests that both compounds induced a fairly fast decrease in $\Delta\Psi$. Therefore, real time monitoring of THP-, CDP-, and CPF-induced changes to $\Delta\Psi$ in CHO-k1 cells was conducted to obtain the kinetics of the compound-induced reductions in $\Delta\Psi$. THP (100 μM), CDP (500 μM) and CPF (200 μM) also resulted in relatively rapid and pronounced reductions in $\Delta\Psi$, which indicated that mitochondria are one of the first targets influenced by these compounds (Fig. 6).

3.4. Biochemical features of cell death induced by THP, CDP, and CPF

Cell death was first measured by detecting PS expression and membrane permeability. When cells were exposed to the compounds for 3 h, THP, CDP and CPF significantly increased the proportions of cells in Q2 (early apoptosis), Q3 (cell fragments from necrosis) and Q4 (necrosis or late apoptosis) (Fig. 7a). THP and CPF at high concentrations could also significantly elevate intracellular calcium concentrations and decrease ATP levels (Fig. 7b and c). However, when cells were treated with a high concentration of CDP (500 μM), there was no difference for either index between CDP-treated and control cells (Fig. 7b and c). As shown in Fig. 8a and b, exposure to THP and CPF for 3 h induced caspase-9 and caspase 3/7 in a concentration-dependent manner. As with intracellular calcium concentrations and ATP levels, CDP treatment could also not produce caspase 9 or caspase 3/7, even at high concentrations (Fig. 8a and b).

3.5. Involvement of reactive oxygen species in THP, CDP, and CPF-induced cell death

Fig. 7d indicates that THP, CDP, and CPF all produced a concentration-dependent increase in DCF fluorescence intensity relative to the control. This result indicated that ROS were produced in treated cells. All compounds exhibited significant cytotoxicity in the CCK-8 assay, but combined exposure to an individual compound and 2 mM NAC inhibited the cytotoxicity of the test compounds to a certain degree at different concentrations (Fig. 9). Additionally, 2 mM NAC used as an ROS scavenger could also inhibit the induction of caspase 3/7 that was elicited by THP (Fig. 8c).

4. Discussion

Mitochondria play a central role in maintaining cell and organ function and are involved in several key pathways when cells respond to environmental stressors (Meyer et al., 2013). Recently, increasing evidence has supported that mitochondrial dysfunction and altered organelle regulation are related to many common diseases, including cancers, neurodegenerative diseases, and

Fig. 6. Monitoring the kinetics of THP, CDP, and CPF-induced drop of $\Delta\Psi$ in real time with live cells. The CHO-k1 cells were loaded with TMRE, firstly. Then, THP (a), CDP (b), and CPF (c), as well as DMSO were applied to the cells and their images were collected as fast as possible for almost 2 h. A drop in $\Delta\Psi$ was calculated according the TMRE channel. The values presented are the mean of at least three independent experiments.

diabetes (Shaughnessy et al., 2014; Whitaker et al., 2016). A much smaller but growing body of literature also indicates that numerous environmental pollutants can target mitochondria and cause some specific diseases (Meyer et al., 2013). PFRs are structurally similar to OPs. Some evidence has indicated that most PFRs have a lower ability to influence the function of AChE than OPs do (Moser et al., 2015; Yuan et al., 2016), but PFRs, like OPs, can cause significant mitochondrial impairment (Attene-Ramos et al., 2015; Lee et al.,

Fig. 7. Biochemical features of cell death induced by THP, CDP, and CPF. Cell apoptosis and necrosis were quantitative analysed by staining with Hoechst 33,342, PI and FITC labelled Annexin-V after 3 h exposures of compounds (a). Q1, Q2, Q3 and Q4 showed in legend represent live cells, early apoptosis cells, cell fragments from necrosis, and necrosis or late apoptosis, respectively. The values presented are the mean $\pm$ SEM of at least three independent experiments. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ (ANOVA) as compared to control cells. Effects of test compounds on ATP level (b), intracellular calcium concentration (c), and intracellular ROS production (d) after the 3-h treatment were measured. The values presented are the mean $\pm$ SEM of at least three independent experiments. A significant difference among the groups of $p < 0.05$ (ANOVA) is indicated by differences in the letters.

2012; NCBI, 2012). Therefore, mitochondria may be a potential target for some PFRs. Although PFRs have been increasingly used to cater to the rigid demands of flame retardants, there is a lack of sufficient toxicity and health data available for PFRs, which can easily release into the surrounding environments. In this study, we hypothesized that mitochondrial dysfunction induction by non-halogen PFRs could be a potential mechanism to explain the adverse consequences of these compounds.

Two classes (containing aryl or alkyl substituent groups) of non-halogen PFRs were selected as our research subject. The LC_{50} of the tested PFRs was assessed, and the LC_{50} of all PFRs except TEHP correlated with the LogKow values. In concordance, it has been shown that aryl-PFRs with high LogKow values cause acute toxicity in zebrafish embryos (Du et al., 2015). Although TEHP had the highest LogKow value (9.49) of all the PFRs tested, the cytotoxicity of TEHP was relatively low. TEHP has a low acute toxicity for mammals (NCBI, 2016a), and the cytotoxicity of this compound is inactive or inconclusive in most qHTS cytotoxicity assays from Tox21 (NCBI, 2016b). Based on Richard Horobin's QSAR decision rules (Horobin et al., 2007), it is suspected that the extremely high LogKow values of TEHP result in this compound having difficulty penetrating cell membranes.

The outcome of the multi-parameter HCS assay indicated that all PFRs except TMP, TEP, and TPP caused significant damage to mitochondria as indicated by a reduction in $\Delta\Psi$ and changes in mitochondrial mass (Fig. 4). The PFRs that reduced $\Delta\Psi$ shared high

LogKow values (>3.8). Traditionally, lipophilic cations are regarded as the most common structural type of compounds that accumulates in the mitochondrial matrix (Wallace and Starkov, 2003). However, electrically neutral esters with high LogKow values can be converted to weak acids by cellular esterases, and then these lipophilic acids are suspected to be retained in mitochondria (Horobin et al., 2007). Because PFRs are one type of ester, the hydrophobicity of PFRs was believed to help them target and undermine the function of mitochondria. Similar effects were also observed in mitochondrial membrane potential assays of Tox21 (Attene-Ramos et al., 2015), in which compounds with longer side chains have been shown to be more potent than those with shorter side chains. In general our results indicated that the hydrophobicity of PFRs can influence the ability of these compounds to induce mitochondrial impairment. Additionally, because previous studies have suggested mitochondrial impairment, such as disrupting $\Delta\Psi$, can lead to opening of the mitochondrial permeability transition pore (PTP) and eventually to cell death (Tirmenstein et al., 2002), mitochondrial-mediated cytotoxic effects (Nakagawa and Moldeus, 1998) are also suspected to exist for some PFRs.

The cluster analysis results revealed that the toxicity of the aryl-PFRs and long-chain alkyl-PFRs seemed to depend on the different mechanisms used. Increasing research has shown the power of HCS to probe the toxicity mechanisms of compounds (Hirsch et al., 2017; Tolosa et al., 2015; Wolpaw et al., 2011). In this study, the cluster analysis successfully distinguished aryl-PFRs and long chain

Fig. 8. The induction of caspase activities by THP, CDP, and CPF. CHO-k1 cells were exposed to a range of concentration of PFRs for 3 h, and caspase 3/7 (a) and 9 (b) activity were measured. The values presented are the mean $\pm$ SEM of at least three independent experiments. A significant difference among the groups of $p < 0.05$ (ANOVA) is indicated by differences in the letters. NAC were used as ROS scavenger to inhibit Caspase 3/7 activity (c). The values presented are the mean $\pm$ SEM of at least three independent experiments. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ (ANOVA) as compared to cells without NAC treatment.

alkyl-PFRs based on the quantitative cellular or organellar features obtained from HCS. These features of the nucleus, mitochondria and cells were good indicators for discerning the mechanism of cell death (Bova et al., 2005; Tolosa et al., 2015; Wolpaw et al., 2011). Fig. 5 further indicates long chain alkyl-PFR-treated cells undergoing apoptosis showed characteristic morphological features. Although dead cells were also found

Fig. 9. Effects of THP, CDP and CPF on cytotoxicity by inhibit intracellular ROS production by using NAC. CHO-k1 cells were co-exposing 2 mM NAC and a range of concentration of THP (a), CDP (b), and CPF (c) for 3 h. Cytotoxicity was measured by using CCK-8 cytotoxicity assay. The values presented are the mean $\pm$ SEM of at least three independent experiments. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ (ANOVA) as compared to cells without NAC treatment.

among aryl-PFR-treated cells, those dead cells are mainly due to a loss of cell membrane integrity without obvious apoptotic characteristics (Fig. 5). Therefore, according to the results of the multi-parameter HCS assay, clarification of the cell death mechanisms induced by representative alkyl- and aryl- PFRs is needed.

The rapid decrease in $\Delta\Psi$ suggests that mitochondria might be the one of the earliest targets affected by exposure to THP, CDP and CPF (Bova et al., 2005). These compounds are suspected to have a direct effect on the electron transport chain complexes or to possibly modulate the PTP (Bova et al., 2005). Additionally, a great deal of evidence supports that mitochondria are one of the primary targets of compound-induced cytotoxicity (Preston et al., 2001; Rasola and Bernardi, 2011). Considering that cell death is highly related to mitochondrial damage, the mechanisms of cell death induced by THP, CDP, and CPF need to be identified. Previous studies have indicated that CPF can induce classical mitochondrial-dependent apoptosis in PC12 cells (Lee et al., 2012). The results of this study confirmed that the cell death induced by THP and CPF in CHO-k1 cells occurs through a similar apoptotic pathway. This apoptotic pathway is triggered by the release of cytochrome c from

the mitochondria, leading to sequential activation of caspase-9 and 3. Caspase-3 finally functions as the ultimate “executioner” of cell death (Stine and Brown, 2015). The mitochondrial permeability transition (MPT), in which the opening of the PTP leads to a dramatic increase in the permeability of the mitochondria, may allow mitochondrial constituents, including cytochrome c, ATP, Ca^{2+} , etc., to leak out from mitochondria (Rasola and Bernardi, 2011). Importantly, ATP levels must be high enough to activate caspases in apoptosis. Otherwise, rapid depletion of ATP would make it impossible for cells to coordinate the apoptotic machinery and force them to turn to necrosis (Rasola and Bernardi, 2011; Richter et al., 1996). THP- and CPF-treated cells in this study successfully induced caspase-9 and 3, and the cells seemed to have enough ATP to support apoptotic signalling. The elevation of intracellular calcium concentrations and ROS levels further supported that the apoptosis induced by THP and CPF was mediated by mitochondria (Rasola et al., 2010; Wang et al., 2008). The reduction of $\Delta\psi$ indicated that the PTP might be persistently open in THP- and CPF-treated cells, such that the mitochondria no longer functioned as “firewalls” to control calcium homeostasis. Therefore, Ca^{2+} resting in the mitochondrial matrix was released, followed by cessation of oxidative phosphorylation and ROS production (Rasola and Bernardi, 2011). Further work should discern whether persistent opening of the PTP results directly from damage to mitochondria by THP and CPF or from Ca^{2+} overload in which mitochondria accumulate excessive Ca^{2+} from either the endoplasmic reticulum or opening of plasma membrane Ca^{2+} channels.

Some aryl-PFRs, such as TOCP, can induce the mitochondria-mediated apoptotic pathway in adult hens (Zou et al., 2013). However, Carlson et al. found that 1 mM TOCP could not only activate caspase-3 but also be caused necrotic morphological changes in SH-SY5Y cells (Carlson et al., 2000). The cell death induced by TOCP seems to depend on an unusual mechanism. A similar conclusion from Karami-Mohajeri and Abdollahi (2013) also indicated that OPs can induce mitochondrial dysfunction and that there is more than one mechanism to explain the toxicity of OPs. In this study, CDP could inhibit cell viability, decrease $\Delta\psi$, and lead to a failure of cell membrane integrity (Figs. 1, 2a, 5, 9b). All these results indicate that 3 h of exposure of CHO-k1 cells to both CDP and THP could induce cell death. However, CDP treatment of cells maintained normal ATP and intracellular calcium levels and could not activate caspase-3 and 9, cause chromatin condensation, or form apoptotic bodies (Fig. 7b and c, 8a, b). As a consequence, the cell death induced by CDP is different from traditional apoptosis (activation of caspase-3) or hydrogen peroxide (H_2O_2)-induced necrosis with MPT (ATP depletion) (Dixon et al., 2012). Thus, CDP-induced cell death might be a different type of necrosis. Necrosis can occur in a highly regulated and genetically controlled manner, and numerous examples of this process are emerging, including pyroptosis, necroptosis, parthanatos, ferroptosis, etc (Vanden et al., 2014). CDP-induced cell death seemed to be highly related to mitochondrial impairment, and this type of cell death did not cause chromatin condensation as in pyroptosis (Bergsbaken et al., 2009) or seem to be initiated through increasing cytoplasmic calcium as in necroptosis (Nomura et al., 2014). Two possibilities for the mode of cell death are parthanatos (David et al., 2009) and ferroptosis (Dixon et al., 2012). Both of these mechanisms cause cell death that is highly related to the mitochondria and not dependent on caspase. ROS can be produced in the processes of parthanatos and ferroptosis (David et al., 2009; Dixon et al., 2012), and in fact, ROS have been implicated in various forms of cell death. As a result, it is possible that apoptosis or another form of cell death could occur in “pure” parthanatos or ferroptosis (Reed and Pellecchia, 2012). Nevertheless, challenging work remains to discern the mechanisms of cell death that CDP or other PFRs induce.

5. Conclusions

In conclusion, mitochondrial impairment in cells has been observed upon treatment with most selected non-halogen PFRs except for those compounds with short alkyl chains. The results from the HCS assay indicated that PFRs with different structures are likely associated with distinct mechanisms of toxicity. THP was found to function via a similar pathway as CPF to induce apoptosis. CDP-induced cell death was distinct from classical necrosis. Further studies should be conducted to elucidate the cell death mechanisms of other PFRs and clarify the relationship between the cell death mechanism and the PFR structures. Finally, the results of the present work give insight into the toxicity of PFRs. The HCS analysis of mitochondrial toxicity has also been proven to be a promising *in vitro* strategy for evaluating the toxicity and identifying the mechanisms of environmental pollutants.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.envpol.2017.07.024>.

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