Toxicity Assessment upon Thermal Treatments of Chlorpyrifos and 3,5,6-Trichloro-2-Pyridinol (a Potential Precursor of the Pyridine Analogue of 2,3,7,8-T4CDD) Using the Early Developmental Stage Medaka Embryos (*Oryzias latipes*)

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ABSTRACT

Chlorpyrifos is one of the major pesticides used today which has a chlorinated aromatic moiety of a pyridine analogue of 2,4,5-trichlorophenol and can be a potential direct precursor of another pyridine analogue of 2,3,7,8-T4CDD (2,3,7,8-tetrachlorodibenzo-*p*-dioxin). In the recent study, PCDF compounds have been detected in chlorpyrifos samples, however, the presence of this analogue of 2,3,7,8-T4CDD has not been assessed yet to our knowledge. Here, we analyzed the ecotoxicities of this dioxin-like compound in the early developmental stage embryos (*Oryzias latipes*). Chlorpyrifos and its analogue, 3,5,6-trichloro-2-pyridinol, were thermally treated in closed glass ampoules at 300, 340, and 380°C for 10 minutes and exposed to medaka embryos in early developmental stage for 48 hours, to assess the response of the 20 biomarker gene expressions. The thermally treated 3,5,6-trichloro-2-pyridinol enhanced the expression of CYP1A1 (Cytochrome P450 1A1) and AhRR (aryl hydrocarbon receptor repressor) as 2,3,7,8-T4CDD and PCBs, suggesting that the potentially ecotoxic compounds like 2,3,7,8-T4CDD is newly produced during the thermal treatment of 3,5,6-trichloro-2-pyridinol. The expression profiles of the 20 biomarker genes in the embryos exposed to 2,3,7,8-T4CDD and 3,5,6-trichloro-2-pyridinol were almost the same, strongly suggesting that the thermally treated 3,5,6-trichloro-2-pyridinol sample contained 2,3,7,8-T4CDD-like ecotoxic compounds.

Keywords: chlorpyrifos, medaka embryos, pyridinol, thermal treatment

INTRODUCTION

Pesticide production, use and disposal have contributed significantly to polychlorinated dibenzo-*p*-dioxins (PCDD) and polychlorinated dibenzofuran (PCDF) emissions in the past (Masunaga *et al.*, 2001; Weber *et al.*, 2008). In a recent monitoring of currently used pesticides in Australia in all assessed formulations, PCDD and PCDF were detected at high concentrations in some pesticide formulations (Holt *et al.*, 2010). From a historic perspective, the two pesticides with the highest dioxin production and release were 2,4,5-trichlorophenoxy acetic acid (2,4,5-T) and pentachlorophenol (PCP) (Hagenmaier and Berchtold, 1986; Weber *et al.*, 2008). The production and use of 2,4,5-T and 2,4,5-trichlorophenol, which can be a precursor of 2,3,7,8-T4CDD, resulted in large contamination during the Vietnam War (366 kg TEQ (toxicity equivalency quantity) release from Agent Orange spray) and had a long series of dioxin contamination in the factories with the last accident in Seveso where about 30 kg TEQ have been released (Mocarelli 2001). Today, 2,4,5-T is not available in the market and the use of 2,4,5-trichlorophenol is restricted to the synthesis of the still available

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hexachlorophene, one of the major pesticides used today. Another major pesticide used today, chlorpyrifos, has a chlorinated aromatic moiety of a pyridine analogue, 2,4,5-trichlorophenol (3,5,6-trichloro-2-pyridinol; pyridinol), making this pesticide to be a potential direct precursor of the other pyridine analogue, 2,3,7,8-T4CDD. In the recent study, PCDFs have been detected in chlorpyrifos samples and the presence of chlorpyrifos has been assessed with GC/MS (gas chromatograph/mass spectrometry) analysis by Sakiyama *et al.* (2011). Waste incineration facilities in Japan today are advised to incinerate the wastes at 850°C or higher, so that the pyrolysates of thermally treated pyridinol or chlorpyrifos would be completely decomposed and detoxified. However, inside the waste incinerator significant variation in temperature can occur and we can estimate that dioxins and dioxin-like compounds (e.g. the pyridine analogue of 2,3,7,8-T4CDD) might be formed in the regions at low temperature (e.g. 300 – 380°C) in the waste incinerator or in the case of illegal incineration (e.g. “open burning”, “NOYAKI”). In this study, we analyzed the ecotoxicities of these dioxin-like compounds in the early developmental stage embryos of medaka (*Oryzias latipes*). Chlorpyrifos and pyridinol were thermally treated to form their pyrolysates at moderate temperatures and their precursor potentials were addressed. Medaka embryos in early developmental stage were exposed to these pyrolysates for 48 hours and the ecotoxicogenomics response were assessed by the changes in expressions of the 20 biomarker genes specifically chosen for the evaluation of the ecotoxicities of environmental water.

MATERIALS AND METHODS

Thermal treatment

Chlorpyrifos and 3,5,6-trichloro-2-pyridinol (pyridinol) were purchased from Wako Pure Chemicals (chemical standard grade, Japan). All pyrolysis experiments were carried out in sealed brown glass ampoules; 0.2 mg of chlorpyrifos or 0.8 mg of pyridinol were thermally treated in closed brown glass ampoules at 300, 340, and 380°C for 10 minutes in a GC-oven (Agilent, USA). After cooling down to room temperature, the ampoules were opened carefully. Then, the pyrolysate products were extracted with toluene (pesticide grade; Wako Pure Chemicals) and the toluene was evaporated to the volume of 1.5 mL under gentle nitrogen stream. One hundred and fifty µL of the extracts dissolved in toluene was placed in a vial to be dried up and the pyrolysates were redissolved in DMSO 60 µL. 3,3'-dichlorobiphenyl (PCB11), 3,3',4,4',5-pentachlorobiphenyl (PCB126) and 2,3,7,8-T4CDD were purchased from Wellington Laboratories (Ontario, Canada) in a nonane solution, evaporated, and then redissolved in DMSO at a concentration of 5 µg/L.

Medaka embryos and exposure to waterborne chlorpyrifos

Fertilized eggs and embryos were obtained from natural mating of adult orange-red strain medaka fish originally derived from the colony kept in The University of Tokyo and maintained at $26 \pm 0.5^\circ\text{C}$ under a 14 hours-light and 10 hours-dark cycle. Newly fertilized eggs (200 – 300 embryos) were placed in a 100 mL beaker with distilled water and incubated for 24 hours at $26 \pm 0.5^\circ\text{C}$ to develop. It is noteworthy that in this study the medaka embryos at 25 hours post-fertilization (hpf) were exposed to chlorpyrifos and pyridinol in the early neurula stage (Stage17) to eliminate abnormally developing or dead embryos within the first 24 hours of development. We settled the live embryos

(Stage17) into wells of microtiter plates filled with 200 μ L of waterborne vehicle (DMSO) with 0.32 mg/L of chlorpyrifos, 1.3 mg/L of pyridinol and 1 μ g/L of dioxins (PCB11, PCB126 and 2,3,7,8-T4CDD). The final concentration of the vehicle (DMSO) was to be 0.1% (v/v). In these studies, 3 days post-hatch was considered to represent survival, and we confirmed that the distilled water and DMSO in the range of 0 – 0.2% (v/v) did not induce any abnormalities in the early developmental stage of medaka embryos.

Total RNA isolation and real-time PCR experiments

Total RNA was isolated from the exposed embryos by ISOGEN (Nippon Gene, Japan) and then purified with RNeasy Mini Kit (QIAGEN, USA). The quality and quantity of isolated total RNA were verified by agarose gel electrophoresis and a spectrophotometer (ND-1000, Nano Drop Tech., USA). Total RNA (400 – 1,000 ng/ μ L) with an OD260/OD280 ratio over 2.0 were obtained from 30 – 50 embryos and used. Complementary DNAs (cDNAs) were synthesized from 1 μ g of total RNA using Rever TraAce- α TM (Toyobo, Japan) and oligo(dT)20 primers (Takara, Japan). The relative amounts of the transcripts were quantified using real-time PCR which was performed using SYBR[®] Premix Ex TaqTM in a Smart Cycler[®] II System (Takara, Japan) and specific primers, according to the manufacturer's instructions. For each sample, gene expression was analyzed in triplicate with the following protocol: 95°C for 10 s, followed by 45 cycles of 95°C for 5 s and 60°C for 20 s. At the completion of each PCR run, the obtained PCR products were subjected to melting curve analysis to ensure that only a single product was amplified. At least three technical replicates of each RNA sample were conducted. Expression data were quantified based on threshold cycle (Ct) values. For each gene, the Ct values for each sample were averaged and normalized to the mean Ct of EF-1 α (elongation factor 1-alpha), which is expressed ubiquitously in tissues and widely used as gene expression control. Relative mRNA expression for each gene was calculated as the fold change compared with the solvent control, exposed to DMSO (0.1% (v/v)) for 48 h, according to equations (1) and (2) (Livak and Schmittgen, 2001; Hanno *et al.*, 2010) :

$$\text{Relative mRNA expression} = 2^{-\Delta\Delta C_t} \quad (1)$$

$$\Delta\Delta C_t = [C_{t(\text{target samples})} - C_{t(\text{EF-1}\alpha)}]_{\text{exposed 48 h}} - [C_{t(\text{solvent control})} - C_{t(\text{EF-1}\alpha)}]_{\text{exposed 48 h}} \quad (2)$$

Medaka-customized microarray experiments.

Medaka-customized microarray (Agilent, USA) contains 36,398 medaka gene probes. Following hybridization at 65°C for 17 hours, 10% Triton X-100 was added to the array to make a final concentration of 0.005% and washed with Wash Buffer1 and Wash Buffer2. Fluorescence signals were scanned using Agilent DNA Microarray Scanner. All microarray data were quantified using Feature Extraction Software (Agilent, USA).

Statistical analysis

Statistical significance was determined by the *t*-test. The results were presented as fold difference between the chemicals treated groups and the control groups.

RESULTS AND DISCUSSION

We classified the biomarker genes into four groups, using a medaka-customized microarray: Group1: *CYP1A1*, *UDPGT* (uridine diphosphate glucuronyl transferase), *AhR1b-1*, *AhR2a*, *AhRR*, *ER-β* (estrogen receptor-beta) – detoxication metabolism genes; Group2: *CACHD1* (Cache Domain containing 1), *RAR-α* (retinoic acid receptor-alpha), *ER-α*, *VEGF-R* (vascular endothelial growth factor receptor) – endocrine effect/reproduction genes; Group3: *AGXT* (alanine-glyoxylate aminotransferase), *MTF1* (metal-regulatory transcription factor 1), *Tropomyosin*, *HSP90* (heat shock protein 90) – cell proliferation genes; and Group4: *TBP* (TATA-box binding protein), *TNF-R* (tumor necrosis factor receptor), *CDC37* (cell division control protein 37), *HSP70*, *MT* (metallothionein), ependymin1 – immunity effect/nervous system genes, considering the physiological functions and induction by chemicals. In particular, Group1 genes were highly inducible by dioxins and dioxin-like compounds exposure. Induced expressions of the biomarker genes mRNA are shown in Table 1. The shaded boxes represent the relative mRNA expression which showed up-regulated (> 2-fold) or down-regulated (< 0.5-fold) and significant difference at $p < 0.001$ (***) from the corresponding control value.

Group1 genes

The functions of the Group1 genes were detoxication and metabolism. *CYP1A1* and *AhRR* genes are well known to be highly inducible by dioxin and dioxin-like compounds exposure. Both genes are regulated through the aryl hydrocarbon receptor (AhR)-mediated pathway, which is ubiquitously functional in both mammalian tissues and medaka (*Oryzias latipes*) embryos. Although pyridinol blank did not induce the *CYP1A1* expression, thermal treated pyridinol with higher temperature at 300 – 380°C induced higher expression of *CYP1A1* in medaka embryos (Fig. 1). In contrast, chlorpyrifos didn't enhance *CYP1A1* expression with thermal treatment. The relative expression of *CYP1A1* was highly related to the WHO-TEF (World Health Organization – Toxic Equivalency Factor) for mammals (Hanno *et al.*, 2010) and 380°C thermally treated pyridinol induced *CYP1A1* expression as dioxins or dioxin-like PCBs did.

The induced expression of *AhRR* mRNA is shown in Fig. 2. In the adult killfish, *AhRR* mRNA is widely expressed in tissues and is induced by both 2,3,7,8-T4CDD or dioxin-like PCBs (Hahn *et al.*, 2009). *AhRR* might play significant roles in modulating the activities of *AhRs* and mediating the induction of *CYP1A1* expression in fish as in mammals. In this study, *AhRR* expression was enhanced 2.0 – 2.5 fold higher than the control upon exposure to 380°C thermally treated pyridinol, which was very similar when exposed to PCB-126 (1 µg/L) and 2,3,7,8-T4CDD (1 µg/L). Four *AhR* genes (*AhR1b-1*, *AhR1b-2*, *AhR2a* and *AhR2b*) were identified in the medaka genome and *AhR2a* is most strongly induced by 2,3,7,8-T4CDD, which suggests that *AhR2a* plays an important role as dioxins receptors in mediating the induction of *CYP1A1* and the ecotoxicities of dioxins in medaka embryos (Hanno *et al.*, 2010).

In contrast, *AhR1b-1* was only slightly induced by 2,3,7,8-T4CDD and endogenous expression increased during normal development in unexposed embryos, suggesting that *AhR1b-1* possesses physiological functions distinct from the dioxin response in medaka (Hanno *et al.*, 2010). Induction of *AhR2a* mRNA expression is shown in Fig. 3.

Table 1 - The relative mRNA expression of the biomarker genes in medaka embryos.

No.	Group	gene	PCB11	PCB126	2,3,7,8-T4CDD	Pri_BL	Pri_300	Pri_340	Pri_380	Chl_300	Chl_340	Chl_380	p
1	1	CYP1A1	1.47	34.9	327	0.901	3.38	6.58	312	0.749	1.39	0.529	**
2		UDPGT	0.922	0.611	1.06	1.18	2.23	0.918	2.01	1.44	2.22	1.39	*
3		AhR1b-1	1.10	0.803	1.19	0.629	1.01	0.464	0.694	0.903	1.11	0.685	**
4		AhR2a	1.29	1.72	2.86	0.833	1.14	0.942	1.50	2.62	1.38	0.874	*
5		AhR	1.38	2.84	2.99	0.584	0.866	0.823	2.24	0.913	0.936	0.508	***
6		ER-β	1.71	1.12	1.52	0.706	1.52	0.773	1.13	1.19	1.35	0.798	*
7	2	CACD1	1.61	1.08	0.940	1.73	2.21	1.82	2.27	2.66	1.90	1.08	NS
8		RAR-α	1.74	2.82	1.25	0.821	1.15	1.23	1.40	1.53	1.65	0.940	NS
9		ER-α	0.914	0.858	1.00	0.979	1.08	1.29	1.57	1.35	1.10	0.796	**
10		VEGF-R	1.16	0.939	1.68	0.833	0.884	0.844	0.994	0.717	0.792	0.688	**
11		AGXT	2.00	1.19	1.87	0.912	1.36	1.14	1.31	1.77	1.67	1.35	**
12	3	MTF1	1.55	0.990	1.54	0.974	1.35	1.06	1.28	1.20	1.16	0.948	NS
13		Tropomyosin	1.67	1.29	1.33	1.45	2.41	1.29	1.92	1.50	1.71	0.631	**
14		HSP90	1.04	0.703	0.822	0.696	0.650	0.546	0.643	0.659	0.551	0.274	***
15		TBP	1.25	0.707	1.24	0.671	1.01	0.637	0.835	0.920	0.877	0.719	**
16	4	TNF-R	1.51	0.933	1.46	0.999	1.47	1.02	1.31	1.36	1.46	1.20	*
17		CDC37	1.61	1.83	1.26	1.01	1.38	0.986	1.39	1.32	1.23	1.17	*
18		HSP70	0.903	0.951	1.04	0.373	0.480	0.380	0.469	0.626	0.431	0.258	***
19		MT	0.615	0.575	0.638	0.170	0.306	0.150	0.432	0.227	0.423	0.248	***
20		Ependymin1	1.77	1.52	1.94	0.933	1.31	0.734	1.28	1.11	1.16	0.859	*

Medaka embryos were exposed to dioxins and the pyrolysates of chlorpyrifos and 3,5,6-trichloro-2-pyridinol for 48 hours (n = 3). Statistical comparisons between the solvent control (DMSO 0.1%) and the thermal treatment groups were performed using the student's *t*-test. Pri_BL; pyridinol-blank (non-thermal treatment), Pri; pyridinol, Chl; chlorpyrifos, _300; pyrolysates under 300°C treatment. *p < 0.05, **p < 0.01, ***p < 0.001, NS; not significant (p > 0.05).

The expression of *AhR2a* was enhanced by 1.5 fold upon exposure to 380°C thermally treated pyridinol. In contrast, exposure to 300°C thermally treated chlorpyrifos induced *AhR2a* more than 2.5 – 3.0 fold, but suppressed it at higher thermal treatment, whereas *AhR1b-1* was slightly induced by exposure to either chlorpyrifos or pyridinol.

Fig. 1 - CYP1A1 mRNA expression in medaka embryos exposed to pyrolysates of chlorpyrifos and 3,5,6-trichloro-2-pyridinol. The amount of CYP1A1 mRNA was measured by quantitative real-time PCR and normalized to the solvent control (0.1% DMSO) for 48 h exposure, the error bars represents SD (n = 3). BL; Blank (DMSO 0.1%), Pri_BL; pyridinol-blank (non-thermal treatment), Pri; pyridinol, Chl; chlorpyrifos, _300; pyrolysates under 300°C treatment. *p < 0.05, **p < 0.01, ***p < 0.001, NS; not significant (p > 0.05).

Fig. 2 - AhRR mRNA expression in medaka embryos exposed to pyrolysates of chlorpyrifos and 3,5,6-trichloro-2-pyridinol.

Group2 genes

The functions of the Group2 genes were endocrine and reproductive effects. Induction of *CACHD1* (Cache domain-containing protein 1) mRNA expression is shown in Fig. 4. *CACHD1* contains two cache domains and a VWFA (von Willebrand factor A) domain which is known to be involved in homeostasis (Anantharaman and Aravind, 2000). This gene was induced by the exposure to heavy metals or PCBs with low TEF, but was suppressed by PCDDs with high TEF (e.g. 2,3,7,8-T4CDD). The expression of *CACHD1* mRNA was enhanced by pyridinol exposure, but was suppressed by chlorpyrifos exposure at higher thermal treatment. The mRNA expression of *RAR-α* and *ER-α* also showed similar patterns as *CACHD1* at thermal treatment.

Fig. 3 - AhR2a mRNA expression in medaka embryos exposed to pyrolysates of chlorpyrifos and 3,5,6-trichloro-2-pyridinol.

Fig. 4 - CACHD1 mRNA expression in medaka embryos exposed to pyrolysates of chlorpyrifos and 3,5,6-trichloro-2-pyridinol.

Group3 genes

The function of the Group3 genes was cell proliferation. Induction of *HSP90* mRNA expression is shown in Fig. 5. Heat shock proteins (HSPs) are stress-defensive proteins and are highly induced in response to stresses caused by changes in environmental factors such as high temperature, heavy metal exposure, reactive oxygen production, salinity, etc (Beckmann *et al.*, 1990) and protect the structure and function of proteins and cells from damages, maintaining the cellular homeostasis. *HSP90* mRNA expression was suppressed by exposure to both pyridinol and chlorpyrifos.

Group4 genes

The functions of the Group4 genes were immunity effects and nervous system control. Induction of *Metallothionein (MT)* mRNA expression is shown in Fig. 6. MT is a

Fig. 5 - HSP90 mRNA expression in medaka embryos exposed to pyrolysates of chlorpyrifos and 3,5,6-trichloro-2-pyridinol.

Fig. 6 - MT mRNA expression in medaka embryos exposed to pyrolysates of chlorpyrifos and 3,5,6-trichloro-2-pyridinol.

protein that has high cysteine content, low molecular mass (about 7 kDa), and high affinity for metals. It binds metals and regulates the homeostasis of essential trace metals such as copper and zinc, and also takes a part in counteracting the toxic effects of heavy metals such as cadmium, mercury, and silver (Choi *et al.*, 2007). The mRNA expressions of *MT* were suppressed by exposure to both pyridinol and chlorpyrifos. The same expression patterns were shown by *HSP70* and *TBP*.

Toxicity assessment of thermal treatments of chlorpyrifos and pyridinol

The pyrolysate of pyridinol (1.3 mg/L) after 380°C thermal treatment induced *CYP1A1*, *AhR2a*, *AhRR* (Group1) and *CACHD1*, *RAR-α* (Group2), but suppressed *HSP90* (Group3) and *MT* (Group4). 2,3,7,8-T4CDD (1 µg/L) induced *CYP1A1*, *AhR2a*, and *AhRR* (Group1), but suppressed *HSP90* (Group3), *HSP70* and *MT* (Group4).

In this study, we investigated the adverse effects of the pyrolysate of pyridinol (1.3 mg/L) with 380°C thermal treatment on the occurrence of developmental abnormalities in medaka embryos by observation with a microscope. The pyrolysate caused blood circulation deficiencies, including slowed blood flow, blood clots, and hemorrhage, in the caudal vein and arteries the same as 2,3,7,8-T4CDD (1 µg/L). These results suggest that the thermal treatment at 380°C of pyridinol might form the pyridine analogue of 2,3,7,8-T4CDD which has the very similar ecotoxicities to 2,3,7,8-T4CDD in medaka embryos. In contrast, the pyrolysates of chlorpyrifos did not induce *CYP1A1* and *AhRR*, which were well known to be sensitive target genes for dioxins and dioxin-like compounds.

The formation potential of the pyridine analogue of 2,3,7,8-T4CDD was considerably lower for chlorpyrifos compared to pyridinol, indicating that the phosphoric acid ester moiety might considerably reduce the dioxin-precursor potential of the compounds (Fig. 7). Sakiyama *et al.* (2011) confirmed that the yield of the pyridine analogue of 2,3,7,8-T4CDD was considerably higher in the pyrolysate of pyridinol than that of chlorpyrifos by GC/MS analysis. Wheelock *et al.* (2005) studied chlorpyrifos and clarified that chlorpyrifos had little effect on *CYP1A* protein levels in salmon, suggesting OPs (organophosphates) may not be strong regulators of *CYP1A* in fish. The main effect of OPs on *CYP1A* appears to be catalytic suppression (Flammarion *et al.*, 1996; Paolini *et al.*, 1997; Flammarion *et al.*, 1998; Tang *et al.*, 2002). Organophosphate effects on CYPs occur as a result of CYP-mediated metabolism, where OPs, such as chlorpyrifos, undergo desulfuration by CYPs leading to the release

Fig. 7 - Structures of 3,5,6-trichloro-2-pyridinol and chlorpyrifos.

of a free sulfur ion, which binds to the CYP heme and inhibits catalytic activity (Fukuto, 1990; Tang *et al.*, 2002). These results suggest that the pyrolysates of chlorpyrifos would contain the unknown ecotoxic chemicals other than the predicted pyridine analogue of 2,3,7,8-T4CDD. Further assessment is necessary to clarify the potential ecotoxicities of chlorpyrifos and its analogues and the advantages of thermal treatment on these chemicals to reduce their ecotoxicities.

CONCLUSIONS

In this study, we evaluated the toxicities of the pyrolysates formed in thermal treatments of pyridinol and chlorpyrifos at 300, 340, 380°C for 10 minutes, obtaining the following conclusions :

1. The pyridine analogue of 2,3,7,8-T4CDD would be formed in thermal treatment of pyridinol which possesses the very similar ecotoxicities to 2,3,7,8-T4CDD.
2. The formation potential of the pyridine analogue of 2,3,7,8-T4CDD was considerably lower for chlorpyrifos compared to pyridinol, indicating that the phosphoric acid ester moiety might considerably reduce the dioxin-precursor potential.

The pyridine analogue of 2,3,7,8-T4CDD may also be formed and released when chlorpyrifos-impacted crop residues and biomass are burned and when chlorpyrifos-contaminated soil is heated. These findings need further assessment considering that chlorpyrifos is a major pesticide used worldwide and can be involved in fires in pesticide productions and storages and in the combustion of post-harvest residues with associated thermal stress for present dioxin and dioxin-like substance precursors. Further assessment on chlorpyrifos at higher temperature thermal treatment is therefore needed to assess the potentialities of the ecotoxic chemical formation.

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