Interactive Effect of EROD Induction in the Japanese Medaka (*Oryzias latipes*) Treated with Mixtures of Dioxin Congeners

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Introduction

Polychlorinated dibenzo-p-dioxins (PCDDs) and polychlorinated dibenzofurans (PCDFs) are widespread environmental contaminants. These compounds are usually present in the environment as complex mixtures. Levels and the patterns of different congeners in environmental metrics vary and depend upon the source(s) of the contamination. To evaluate the human or ecological risk associated with the exposure to a dioxin mixture, a dioxin toxic equivalent (TE) concept has been proposed[1,2] and used by different agencies.

The TE concept assumes that the toxicity of all dioxin and furan isomers are simply additive; however, some studies have reported not-additive results. The combination of 1,3,6,8- and 2,4,6,8-TCDF(tetrachloro-dibenzofuran) antagonized the enzyme induction by TCDD(tetrachlorodibenzo-*p*-dioxin) in rat hepatoma H4IIE cells[3]; however, 1,3,6,8-TCDF was found to be synergistic to TCDD in rainbow trout larvae mortality[4]. The objective of this study was to examine the biochemical responses, measured by the EROD(7-ethoxyresorufin *O*-deethylation) assay, elicited by exposing the Japanese medaka (*Oryzias latipes*) larvae to mixtures of different dioxin congeners were additive.

Similar to adult fish, medaka in early life stages also exhibits cytochrome P-4501A (CYP1A) activity, which is a sensitive indicator for dioxin exposure. The EROD reaction is widely used because of the high specificity for the P-4501A-type enzyme, and its simplicity.

Material and Methods

Groups of fifty to sixty larvae were exposed to one of the four dioxin congeners, 2,3,7,8-TCDD, 2,3,7,8-TCDF, 1,2,3,7,8-pentachlro-dibenzo-*p*-dioxin(PeCDD), and 1,2,3,4,7,8-hexachloro-dibenzo-*p*-dioxin(HeCDD), or their binary mixture for 72 hours. The concentrations used in the binary mixture group were approximately equal to 25% of the concentration (EC_{25}) at the maximal EROD induction as determined in the previous studies

(unpublished data). Larvae were sacrificed after 72 hours and the whole microsomal fraction was prepared according to Prince[5]. The protein concentration was determined using the commercialized BioRad® protein assay. The observed induction by the mixture was compared with the expected induction, which was the summation of activities induced by both congeners.

Results and Discussion

EROD induction in larvae, by a single congener or a binary mixture of both congeners at the concentrations as treated singularly, was determined. The expected induced activity was assumed to be the sum of the mean activities for the three replicated groups treated with the single congener in each binary mixture.

The result from the TCDD and TCDF combination is shown in Figure 1a. Larvae treated with TCDD at 0.35 ng/l had an induced activity of 91.8 pmole of resorufin/min/mg of protein, while larvae exposed to 1 ng/l of TCDF exhibited an EROD activity of 70 pmole/min/mg. When another group of larvae were exposed to a mixture containing 0.35 ng/l of TCDD and 1 ng/l of TCDF, the EROD activity of larvae was only induced to 105 pmole/min/mg (35.1% less than the expected induction). The expected induced activity, which was 161.5 pmole/min/mg, was not statistically different from the observed activity.

For the TCDD and PeCDD mixture (Figure 1b), the observed mean activity induced by the mixture was less than the expected induction (a 44.6% decrease), but was not significantly different from the expected value. Figure 1c demonstrated that the observed activity induced by the TCDD/HeCDD mixture was less than the total activity evoked by both congeners treated singularly. This led to a significant difference ($p<0.01$) between the expected and the observed values, indicating an antagonistic effect.

When larvae were exposed to a TCDF and PeCDD mixture (Figure 1d), the mean induced activity was lower than the activity induced by 2 ng/l of TCDF only, and was significantly less than the expected induction ($p<0.01$). The results of the combination of HeCDD with either PeCDD (Figure 1e) or TCDF (Figure 1f) showed that the EROD induction by both mixtures were additive due to no statistical differences between the observed and the expected activities (24.4% and 33.3% decreases for the two combinations, respectively).

Some of our results of antagonism between two different dioxin congeners were in agreement with other studies using other biological systems. Bannister *et al.*[6] reported MCDF (6-methyl-1,3,8-trichlorodibenzofuran) as a TCDD antagonist on mice. By using 3 different cell lines, Merchant *et al.* [7] also demonstrated that co-treatment of TCDD plus different concentrations of MCDF or α-naphthoflavone (ANF), a weak Ah-receptor agonist, resulted in a concentration-dependent decrease in EROD activity and TCDD-induced CYP1A1 mRNA levels. ANF and BNF (β-naphthoflavone), both bind to the Ah-receptor, also exhibited fully or partially antagonistic effects on murine splenocyte EROD activity[8]. For two of the combinations, TCDD/HeCDD and TCDF/PeCDD, there was statistically significant reduction in EROD activity. For other combinations (TCDD/PeCDD, PeCDD/HeCDD, TCDF/HeCDD, and TCDD/TCDF), the observed inductions were lower than the expected responses, although

they were not significantly different. This lack of statistical significance may be due to the large variations between replicates and to the number of replicates used (three for each group) in each test. However, there was no instance where the observed value was greater than the expected induction.

In this study, one group of larvae was treated with a higher concentration of either TCDD (1.4 ng/l) or TCDF (16 ng/l) in each EROD mixture study (except for TCDD/PeCDD and TCDD/HeCDD combinations). This treatment was expected to induce the maximal enzyme activity. For the HeCDD/TCDF and HeCDD/PeCDD combinations, the expected enzyme activities induced by either mixture did not exceed the induction by 16 ng/l of TCDF. For the TCDF/PeCDD combination, the expected induction was at about the same level as that induction by 16 ng/l of TCDF. Thus, the lack of additive effects for these combinations was not due to exceeding the maximal induction being achieved which may result in a decreased activity.

One possible mechanism for the EROD or AHH antagonism was suggested to be competition for cytosolic Ah-receptor binding sites by low affinity ligands, such as MCDF or ANF [6,7,8]. Astroff and Safe[9] also determined the Ah-receptor binding affinities of 6-substituted compounds used in their study, and suggested that these analogues competitively displaced TCDD from the Ah-receptor resulting in antagonism in CYP1A1 induction. Similar studies have not been carried out in fish, but this is a plausible explanation for the results presented above.

There is little information available concerning the combined effects of TCDD mixtures on fish. This type of information would be useful to risk assessors. The TE approach has been used in the risk assessment associated with the dioxin mixture exposures. This approach assumes that the toxic effects are additive. Based on our findings, the TE approach may not overestimate the risk of mixtures. This is based on the medaka's EROD assay in which the responses were either additive or less than additive.

References

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Figure 1.EROD activity in the medaka larvae treated with binary mixtures of dioxins. (a) TCDD+TCDF, t=2.65; (b) PeCDD+TCDD, t=2.58; (c) HeCDD+TCDD, t=4.9, $p<0.01$; (d)TCDF+PeCDD, t=6.2, $p<0.01$; (e) HeCDD+PeCDD, t=2.0; (f) TCDF+HeCDD, t=2.76. EX:expected induction.