# EFFECT OF CHLORINATED DIBENZO-P-DIOXINS ON DRUG-METABOLIZING ENZYMES IN THE RAT LIVER

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## INTRODUCTION

Polychlorinated dibenzo-*p*-dioxins (CDDs) and dibenzofurans are highly toxic chemicals which are formed as by-products of some commercial processes and during the incineration of industrial waste[1,2]. The most toxic isomer, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (2,3,7,8-TCDD) produces carcinogenesis, teratogenicity, reproductive toxicity and immunotoxicity in experiment animals[3-5].

In the rat and other laboratory animals, 2,3,7,8-TCDD induces a limited number of cytochrome P-450 (P450) forms such as the CYP1A subfamily, similar to those inducible by the class of agents typified by β-naphthoflavone (BNF) or 3-methylcholanthrene (MC)[6,7]. These observations suggested that 2,3,7,8-TCDD could serve as a specific pharmacological tool in assessing the importance of CYP1A-mediated toxification and detoxification in the liver. Also, Phase II drug-metabolizing enzymes such as DT-diaphorase, glutathione *S*-transferase (GST) and UDP-glucuronyltransferase (UGT) in the liver of experimental animals are induced by 2,3,7,8-TCDD[8-10]. Thus, measurement of drug-metabolizing enzymes induced by environmental pollutants is an integral part of toxicological research. However, very little has been reported about dioxin compounds except for 2,3,7,8-TCDD.

In this study, to toxicologically assess dioxin compounds, the effects of CDDs on drug-metabolizing enzyme activities in the rat liver were investigated *in vitro* and *in vivo*.

## MATERIALS AND METHODS

### Materials.

Dibenzo-*p*-dioxin (DD), 1-monochlolodibenzo-*p*-dioxin (1-MCDD), 2-MCDD, 2,3-dichlorodibenzo-*p*-dioxin (2,3-DCDD), 1,2,4-trichlorodibenzo-*p*-dioxin (1,2,4-TrCDD) and 1,2,3,4-TCDD were obtained from GL Science Inc. (Tokyo, Japan). All the CDDs were >99% pure as determined by gas chromatography-mass spectrometric analysis. 7-Ethoxycoumarin was purchased from Aldrich Chemical Co. (Milwaukee, WI, U.S.A.). Resorufin, 7-ethoxyresorufin and 7-pentoxyresorufin were purchased from Sigma Chemical Co. (St. Louis. MO, U.S.A.). 7-Methoxyresorufin was from Molecular Probes, Inc. (Junction City, OR, U.S.A.). 2,6-Dichlorophenolindophenol was from E. Merck (Darmstadt, F.R.G.). Other chemicals of reagent grade were obtained from Wako Pure Chemical Ind., Ltd. (Osaka, Japan). Rabbit anti-CYP1A1 immunoglobulin G was prepared by the method described elsewhere[11].

### Enzyme preparation.

Male Wistar rats (100-180 g) were obtained from Nippon Bio-Supp. Center (Tokyo, Japan). The animals were given MC, phenobarbital (PB) and 1,2,4-TrCDD i.p. at doses of 20, 80 and 28.8 mg/kg/day respectively for 3 days, then sacrificed 24 hr after the last injection of each compound. The liver was removed from each rat, perfused with ice-cold physiological saline and homogenized with 3 volumes of 25 mM Tris-HCl (pH 7.6) containing 0.25 M sucrose, 25 mM KCl and 5 mM $MgCl_2$ using a glass-teflon homogenizer. The homogenates were centrifuged at 9,000 x g for 20 min and the supernatant were further centrifuged at 105,000 x g for 60 min. The supernatants were used as the cytosolic fraction. The microsomal pellets were suspended in 25 mM Tris-HCl (pH 7.6) containing 0.15 M KCl, were

centrifuged again at 105,000 x g for 60 min and resuspended in the same buffer. All preparations were stored at -80 °C until use.

**Enzyme assays.**

7-Ethoxycoumarin *O*-deethylase (ECOD) activity was measured by the fluorometric determination of 7-hydroxycoumarin[12]. 7-Ethoxyresorufin *O*-deethylase (EROD) activity was determined fluorometrically from the amount of resorufin produced[13]. The activities of 7-methoxyresorufin *O*-demethylase (MROD) and 7-pentoxyresorufin *O*-depentylase (PROD) were determined by the same procedure used to determine EROD activity, using substrate concentrations of 5 and 10 $\mu$M, respectively. The *N*-Demethylase activities of aminopyrine (AMND), and erythromycin (EMND) were determined by measuring the formaldehyde concentration according to the method of Nash[14]. Aniline 4-hydroxylase (AN4H) was measured by the formation of 4-aminophenol[15]. UGT activity toward 4-nitrophenol (4NP) was determined according to the method of Isselbacher *et al.*[16]. The level of 7-hydroxycoumarin (7HC) glucuronidation was measured fluorometrically at 460 nm with excitation at 390 nm, by the disappearance of substrate[12]. Cytosolic DT-diaphorase was determined spectrometrically, by measuring the decrease in absorbance of 2,6-dichlorophenolindophenol at 600 nm at 25°C by the method of Ernster[17]. GST activities were determined using 1-chloro-2,4-dinitrobenzene (CDNB) and 1,2-dichloro-4-nitrobenzene (DCNB) as substrates[18]. The content of P450 was measured spectrophotometrically as describedby[19]. Protein was determined by method of the Lowry *et al.*[20].

**Western immunoblotting.**

Rat liver microsomal protein were separated electrophoretically on a sodium dodecyl sulfate-polyacrylamide gel using 7.5% polyacrylamide[21]. Thereafter, the gel was transferred to nitocellulose membranes according to Towbin *et al.*[22], then probed with rabbit anti-rat CYP1A1 immunoglobulin G, which recognizes both CYP1A1 and CYP1A2. Bound antibody was visualized using 4-chloro-1-naphthol.

## RESULTS AND DISCUSSION

**Effect of CDDs on ECOD activity *in vitro*.**

It has been reported that substrate specificity of ECOD exists for CYP1A1, CYP2B1 and CYP2B2[23]. In this study, ECOD activities were increased 21.9 and 5.8-fold over the control in MC- and PB-pretreated rats, respectively. When rat liver microsomes were incubated with 7-ethoxycoumarin in the presence of DD, 1-MCDD, 2-MCDD, 2,3-DCDD, 1,2,4-TrCDD and 1,2,3,4-TCDD, there were no remarkable effects on ECOD activity in untreated rat liver microsomes. On the other hand, ECOD activities in the liver microsomes of rats treated with MC and PB, were inhibited from 18 to 70% and from 31 to 91% of the control activity at a concentration of 100 $\mu$M DD or CDDs, respectively. In MC-treated rat liver microsomes, 1,2,4-TrCDD remarkably inhibited the ECOD activity to 18% of the control activity. This ratio of inhibition was similar to that of the positive control, BNF.

To obtain further information about the type of ECOD inhibition by DD and CDDs, Lineweaver-Burk plots were constructed for each compound. All compounds competitively inhibited ECOD activities in all microsomes, although at different rates. The positive controls BNF, isosafrole, PB, and dexamethasone also competitively inhibited ECOD activities in MC- or PB-pretreated rat liver microsomes. The summarized data from all inhibition kinetics are given in Table 1. In liver microsomes from MC-pretreated rats, the Ki for 1,2,4-TrCDD was the lowest, which was the same as that of BNF. On the other hand, in PB-pretreated rat liver microsomes, the Ki values for 2,3-DCDD, 1,2,4-TrCDD and 1,2,3,4-TCDD were higher than those of DD, 1-MCDD and 2-MCDD. The ratio of $PB_{Ki}/MC_{Ki}$ for 1,2,4-TrCDD was the highest among the tested DD and CDDs. Therefore, it is considered that 1,2,4-TrCDD induces CYP1A1 as well as MC, BNF or 2,3,7,8-TCDD.

**Effect of 1,2,4-TrCDD on P450-mediated monooxygenase activities.**

Table 2 summarizes the effects of 1,2,4-TrCDD ,administration upon different P450-mediated monooxygenase activities in rat liver microsomes. EROD and MROD activities were induced 32.9-and 5.7-fold, respectively by 1,2 4-TrCDD. Also, ECOD and PROD activities were increased 4.2- and 2.6-fold over those of control rats, respectively. On the other hand, the relative induction of AN4H and EMND activities by 1,2,4-TrCDD were less than 2-fold. 1,2,4-TrCDD did not seem to effect AMND activity.

Furthermore, the results of this study showed that 1,2,4-TrCDD increased the CYP1A1 and CYP1A2 levels in liver microsomes, as shown by Western immunoblotting using rabbit anti-CYP1A1

immunoblobulin G. Especially, 1,2,4-TrCDD caused a remarkable induction of CYP1A2 protein; the band was more intense than that of CYP1A1. Mason *et al.* have reported detailed *in vivo* structure-activity relationships for PCDDs and there was an excellent linear correlation between the *in vivo* results ($ED_{50}$ values for aryl hydrocarbon hydroxylase/EROD induction, thymic atrophy and body weight loss) and their corresponding *in vitro* aryl hydrocarbon hydroxylase or EROD induction potencies in rat hepatoma H-4-IIE cells[24]. In the paper, the $ED_{50}$ value of 2,3,7,8-TCDD for *in vivo* EROD induction was 0.003 μmol/kg, and the relative potency of 1,2,4-TrCDD/2,3,7,8-TCDD for *in vivo* was about 0.0001. Therefore, base upon the $ED_{50}$ value of 1,2,4-TrCDD for *in vivo* EROD induction, dose used in this study (0.1 mmol/kg x 3) is high, the ability is reasonable.

Table 1. The inhibition of ECOD activity in rat liver microsomes by 450 inducers, DD and CDDs

| | Ki (μM) | | |
|---|---|---|---|
| | MC | PB | PBKi/MCKi |
| BNF | 5.12 | 693 | 135 |
| Isosafrole | 14.9 | 9.28 | 0.62 |
| PB | NI | 433 | --- |
| Dexamethasone | NI | 46.4 | --- |
| Clofibrate | NI | NI | --- |
| DD | 37.2 | 26.9 | 0.72 |
| 1-MCDD | 17.3 | 24.4 | 1.41 |
| 2-MCDD | 11.6 | 25.9 | 2.23 |
| 2,3-DCDD | 24.7 | 166 | 6.72 |
| 1,2,4-TrCDD | 5.41 | 156 | 28.8 |
| 1,2,3,4-TCDD | 18.0 | 357 | 19.8 |

Ki values were calculated from Lineweaver-Burk plots of inhibition kinetics.
NI, not inhibited.

Table 2. The effect of 1,2,4-TrCDD on 450-mediated monooxygenase activities in rat liver microsomes

| | Control | 1,2,4-TrCDD |
|---|---|---|
| EROD[a] | 49.3 ± 17.1 | 1623 ± 233‡ |
| ECOD[b] | 0.63 ± 0.14 | 2.66 ± 0.26‡ |
| MROD[b] | 0.26 ± 0.07 | 1.49 ± 0.18‡ |
| PROD[a] | 29.4 ± 12.6 | 77.4 ± 15.1† |
| APND[b] | 4.19 ± 0.70 | 4.97 ± 0.53 |
| AN4H[b] | 0.80 ± 0.14 | 1.05 ± 0.04* |
| EMND[b] | 6.21 ± 0.98 | 8.24 ± 0.88* |

Each value represents the mean ± SD of four animals.
a) pmol/min/mg protein.
b) nmol/min/mg protein.
* Significantly different from the control ($P<0.05$).
† Significantly different from the control ($P<0.01$).
‡ Significantly different from the control ($P<0.001$).

**Effect of 1,2,4-TrCDD on Phase II drug-metabolizing enzyme activities.**

DT-diaphorase, GST and UGT are induced by various xenobiotics, such as MC, 2,3,7,8-TCDD, polychlorinated biphenyls and PB[8-10,25-28]. Phase II drug-metabolizing enzyme inducers can be classified into two types of bifunctional inducers (*e. g.*, 2,3,7,8-TCDD, MC, BNF) and monofunctional inducers (*e. g.*, diphenols, thiocarbamates, isothiocyanates)[29,30]. Bifunctional inducers elevate both *Phase II* drug-metabolizing enzymes such as DT-diaphorase, GST and UGT and Phase I drug-metabolizing enzymes such as aryl hydrocarbon hydroxylase, whereas monofunctional inducers

primarily elevate Phase II drug-metabolizing enzymes without significantly affecting those of Phase I. As shown in Table 3, 1,2,4-TrCDD increased the DT-diaphorase, GST and UGT levels in the rat liver, although the relative induction to control activity was not so high compared with those of CYP1A1-mediated monooxygenase activities such as EROD and ECOD. Therefore, we considered that 1,2,4-TrCDD is a bifunctional inducer. However, lower CDDs are not usually considered "dioxin-like", further studies are required to identify the mechanisms of various enzyme inductions.

Table 3. The effect of 1,2,4-TrCDD on Phase II drug-metabolizing enzyme activities in rat liver

| | Control | 1,2,4-TrCDD |
|---|---|---|
| DT-Diaphorase[a)] | 0.12 ± 0.04 | 0.32 ± 0.13* |
| GST | | |
| CDND[a)] | 1.28 ± 0.07 | 1.88 ± 0.13† |
| DCNB[b)] | 37.3 ± 5.6 | 56.6 ± 1.7† |
| UGT | | |
| 4NP[b)] | 44.3 ± 1.0 | 87.5 ± 7.2† |
| 7HC[b)] | 41.7 ± 3.4 | 91.2 ± 4.5† |

Each value represents the mean ± SD of four animals.
a) μmol/min/mg protein.
b) nmol/min/mg protein.
* Significantly different from the control ($P<0.05$).
† Significantly different from the control ($P<0.001$).

In conclusion, these kinetic and immunochemical studies showed that 1,2,4-TrCDD induces the CYP1A subfamily of P450. DT-diaphorase, GST and UGT in the rat liver were also induced by 1,2,4-TrCDD, suggesting that 1,2,4-TrCDD is a bifunctional inducer of drug-metabolizing enzymes. Moreover, measuring both Phase I and II drug-metabolizing enzymes in the liver may be useful for the toxicological assessment of dioxin compounds.

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