Caffeine, Acetanilide, and Methoxyresorufin Metabolism by Rat and Human CYP1A2 SUPERSOMES and their Inhibition by 2,3,7,8,-Tetrachlorodibenzo-*p*-dioxin (TCDD)

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Introduction

Cytochrome P450 1A2 (CYP1A2) is an inducible hepatic protein which binds dioxins and produces a dose-dependent hepatic sequestration. Dioxins bind to the Ah receptor (AhR), which regulates a variety of effects including the expression of CYP1A2. There is a direct concordance between the ability to bind AhR and the toxic potency of each chemical. Polyhalogenated aromatic hydrocarbons (PHAHs) have been classified as dioxin-like or non-dioxin-like based on their ability to bind the aryl-hydrocarbon receptor (AhR), induce toxicity, and bioaccumulate. 90-day subchronic mouse studies have shown dose-dependent increases in liver/fat concentrations of several dioxin-like chemicals, supporting the presence of an inducible hepatic binding protein[1]. Our laboratory demonstrated the importance of CYP1A2 as the specific hepatic binding protein responsible for hepatic sequestration of TCDD and dioxin-like compounds by exposing CYP1A2 knockout mice and parental strains to TCDD, 4-PeCDF (a dioxin-like PHAH), and PCB 153 (a nondioxin-like PCB)[2]. The ability of the liver to sequester TCDD and 4-PeCDF was significantly decreased in the knockouts resulting in increased concentrations of these chemicals in extrahepatic tissues, while the distribution of PCB 153 was unaltered in the knockout mice. These studies demonstrate the influence of CYP1A2 on the distribution of dioxins in rodents.

Human data demonstrating hepatic sequestration is limited. Although the data is inconclusive, several human studies have failed to show a dose-dependent increase in CYP1A2 activity following high levels of exposure to dioxin[3]. However, there is some evidence for human hepatic sequestration based on analysis of liver and adipose tissue levels in exposed people[5]. In current risk assessments, it is assumed that the disposition of these chemicals in humans is similar to rodents. This *in vitro* study investigates this assumption by comparing the metabolism of three prototype CYP1A2 substrates in rat and human CYP1A2 SUPERSOMES, as well as the ability of dioxin and dioxin-like chemicals to inhibit these reactions.

Materials and Methods

Microsomes: Rat and human CYP1A2 + P450 reductase SUPERSOMES were obtained from GenTest Corporation (Woburn, MA). Microsomes were kept at –80°C until use, then rapidly thawed at 37°C and stored on ice. A 1:10 dilution of both human or rat microsomes was prepared daily using .05M (pH 8) TRIS buffer.

Chemicals: All PHAHs (Table 1) from AccuStandard Inc. (New Haven, CT) were 99% or greater purity and dissolved in DMSO and stored at 4°C. β-Nicotinamide adenenine dinucleotide phosphate (β-NADPH), methoxyresorufin, and resorufin were purchased from Sigma Chemical Co. (St. Louis, MO) and prepared daily.

MROD assay: Methoxyresorufin O-deethylase (MROD), a specific marker for CYP1A2 enzymatic activity, was used to detect the presence of inhibition by a series of dioxin-like chemicals. CYP1A2 metabolizes methoxyresorufin to resorufin, which produces fluorescence. Resorufin production was recorded using a Spectromax Gemini SX plate reader. Each well contained 50 µl microsome solution, 110 µl of .05M TRIS buffer, 5 µl chemical, 50 µl methoxyresorufin solution, and 25 µl NADPH

Acetanilide 4-hydroxylase (ACOH) assay: SUPERSOMES were incubated for 20 minutes at 37° C in a buffer containing 50mM Tris, 0.3mM MgCl2, 0.6 mM NADPH, and 1mg bovine serum albumin/ml. The reaction as initiated by the addition of 20 µl 20 nM acetanilide in acetone. The reaction was stopped and 4-hydroxyacetanilide was extracted by the addition of 2.5 ml ethyl acetate containing 0.1 ug 3-hydroxyacetanilide. Recovery of 4-hydroxyacetanilide was estimated based on the recovery of 3-hydroxyacetanilide. Reverse phase HPLC was used to separate and quantitate the hydroxlated acetanilide derivatives using a UV detector and ODS column (4.6 x 25 cm).

Caffeine assay: SUPERSOMES were incubated for 5 minutes at 37°C in a buffer (pH 7.4) containing 0.1 M sodium phosphate and caffeine (0.1-64mM) The reaction was initiated by the addition of 50µl 25mg/ml NADPH and incubated for 40 minutes. The reaction was stopped using cold zinc sulphate followed by the addition β-hydroxyethyltheophyline and 25ul of 2M HCl. Caffeine, theophylline (1,3-dimethylxanthine), paraxanthine (1,7-dimethylxanthine), theobromine (3,7-dimethylxanthine), and the internal standard were extracted twice by ethyl acetate/isopropanol. Reverse phase HPLC was used to separate and quantitate the metabolites using a UV detector and ODS colum (4.6 x 25cm). Recovery of caffeine metabolites was based on recovery of β-hydroxyethyltheophyline.

Analysis: The data were initially analyzed using Lineweaver-Burke plots (Prism Software, CA). Assuming competitive inhibition, inhibitor constant (K_I) approximations were calculated using the lowest inhibitor concentration and average K_{Mobs} and K_M values according to the following equation: $K_I = [\text{Inhibitor}] / [(K_{M,obs} - 1)/ K_M]$

Results

Human and rat CYP1A2 SUPERSOMES metabolized all three prototype substrates, however, methoxyresorufin was the most efficient and most sensitive substrate used in this study. K_M and V_{max} values for each substrate are given in Table 1. The metabolism of caffeine by CYP1A2 results in different metabolite profiles between human and rat supersomes, which is consistent with the literature. 1,7-Dimethylxanthine (paraxanthine) is the predominate metabolite from human CYP1A2, while in rats, there is also significant production of theobromine (3,7-dimethylxanthine) and theophylline (1,3-dimethylxanthine).

TCDD inhibited CYP1A2 activity as measured by decreases in metabolism of methoxyresorufin, caffeine, and acetanilide (Figure 1). TCDD, PCDD, TCDF, 4-PeCDF, PCBs 126, 169, 105, 118, and 156 produced a concentration-dependent inhibition of MROD activity in both human and rat CYP1A2 supersomes. Inhibition appeared to be competitive for all chemicals except 4-PeCDF, for which it appeared to me of a mixed type. Approximate K_I values are given in Table 2. PCB 153 did not inhibit CYP1A2 metabolism of methoxyresorufin.

Substrate	KM		Vmax	
	Human	Rat	Human	Rat
MROD	2.5	1.9	1.8	2.8
ACOH	15	50.2	97.7	156
Caffeine	11.7	10.6	0.5	0.086

Table 1. Approximate K_M and V_{max} values. K_M = μM for MROD and mM for Caffeine and ACOH. V_{max} = pmol/min/pg P450.

Chemical	Concentration Range	KI - Human (+ /- SD)	KI - Rat (+/- SD)
2,3,7,8-Tetrachlorodibenzo-*P*-dioxin (TCDD)	.106 - 10.6	0.32 (+/- 0.4)	0.064 (+/- 1.0008)
1,2,3,7,8-Pentachlorodibenzo-*P*-dioxin (PCDD	.430 - 43.0	0.48 (+/- 0.052)	0.055 (+/- 1.016)
2,3,4,7,8-Pentachlorodibenzofuran (4-PeCDF)	.1021 - 10.21	0.054 (+/- 0.002)	0.027 (+/- 0.0004)
2,3,7,8-Tetrachlorodibenzofuran (TCDF)	.1008 - 10.08	0.082 (+/- 0.019)	0.016 (+/- 0.00004)
3,3',4,4',5,5'-Hexachlorobiphenyl (169)	.3902 - 39.02	0.28 (+/- 0.037)	0.077 (+/- 0.0013)
3,3',4,4',5-Pentachlorobiphenyl (126)	.541 - 54.1	0.064 (+/- 0.0002)	0.069 (+/- 0.00059)
2,3,3',4,4',5-Hexachlorobiphenyl (156)	2.205 - 220.5	2.43 (+/- 1.2)	3.31 (+/- 8.32)
2,3',4,4',5-Pentachlorobiphenyl (118)	2.386 -238.6	1.26 (+/- 0.082)	2.45 (+/- 0.54)
2,3,3',4,4'-Pentachlorobiphenyl (105)	2.857 - 285.7	2.72 (+/- 0.85)	4.45 (+/-10.5)
2,2',4,4',5,5'-Hexachlorobiphenyl (153)	2.052 - 220.5	No Inhibition	

Table 2. Approximate K_I values for all chemicals tested based on MROD activity (μ M).

Discussion

The ability of human and rat CYP1A2 to bind dioxin and dioxin-like chemicals influences the disposition and body burden of these chemicals. The *in vitro* assays presented in this study using three prototype CYP1A2 substrates demonstrate that both human and rat CYP1A2 enzymatic activity is inhibited by TCDD. Other dioxin-like chemicals such as PCDD, TCDF, 4-PeCDF, and PCBs 126 and 169 also inhibited CYP1A2 activity similar to TCDD. The PCBs 105, 118, and 156 inhibited MROD activity, but to a lesser extent. The inhibition of CYP1A2 activity by these chemicals may explain the lack of induction of CYP1A2 activity in some human cohorts highly exposed to TCDD. This may also explain the discrepancy between maximal binding induction of CYP1A2 activity, protein and mRNA concentrations found in some experimental studies in rodents. Because of the potential for inhibition of CYP1A2 activity by TCDD and other dioxins, studies using CYP1A2 enzymatic activity as a biomarker of exposure in these populations should be view cautiously.

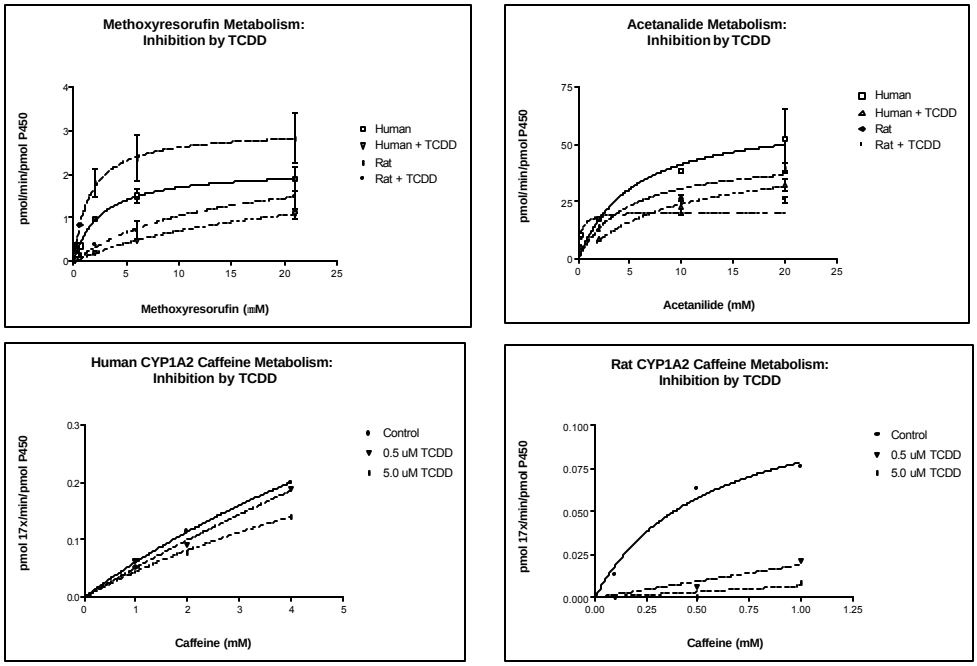

Figure 1. Metabolism of three prototype substrates by human and rat CYP1A2 supersomes and the inhibition of activity by TCDD. TCDD concentrations were 10.6 µm and 7.3 µM for MROD and acetanalide, respectively.

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