Identification of CYPs involved in biotransformation of Ugilec 141 isomers using rat and human hepatic microsomes.

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Introduction

Ugilec 141®, an industrial mixture of 69 tetrachlorobenzyltoluene (TCBT) isomers, was introduced as a replacement of polychlorinated biphenyls (PCBs) on the European market in the early 1980s. The use of PCBs was prohibited because of their persistency in the environment and toxicological implications of these hydrophobic compounds. Due to structural similarities and similarities in physicochemical properties [1] with PCBs the use of Ugilec 141® was prohibited in 1994 by the European Union as well.

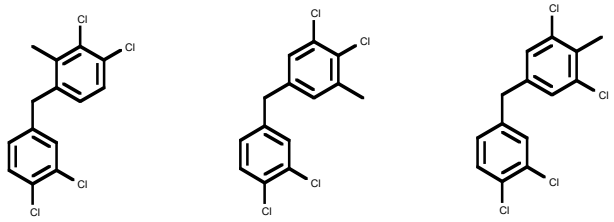

Figure 1 chemical structure of TCBT 87,88, 94 respectively

Isomers 87, 88 and 94 as shown in Fig.1 have characteristics that suggest to interact with the Ah-receptor and as a consequence dioxin-like toxicity could be expected.[2]. These three isomers are metabolised by cytochrome P450 (CYP). However, which CYPs are responsible for the metabolism are still unknown. In order to extrapolate metabolic rates measured in rat liver to human liver we wanted to identify these enzymes. As TCBTs are structurally related to PCBs we studied the metabolic rates of a few PCBs as well.
The experimental methods used in this study included in vitro studies using rat hepatic microsomes to determine elimination rate constants for TCBT, correlation analysis, and chemical inhibition studies. For the correlation analysis we exposed rats to selective inducers to increase the activity of various CYP sub-families such as CYP1A, CYP2B, CYP3A, CYP4A in the liver and determined for each inducer type the elimination rate constants (*k*) of the three TCBTs and selected PCBs. In order to further establish the correlation between specific enzyme activity and *k* we applied specific CYP inhibitors.

Material and Methods

Animals

Five male and five female CPB:uWU rats (300-400 g) were intraperitoneally injected for 4 subsequent days with five selective inducers of cytochrome P450 (Table 1):

Table 1 Overview of the treatment protocol and the main inducible CYP

Inducer	number of rats per sexe	dose (mg/kg/d) 4 days	vehicle	CYP
none (C)	3		none	control
β-naphtoflavone (B)	1	40	corn oil	1A1/1A2
Phenobarbital (P)	2	75	saline	2B1/2B2
Isosafrole (I)	1	230	corn oil	1A2
Clofibric acid (CL)	1	300	corn oil	4A
Dexamethasone (D)	1	75	corn oil	3A

Three male and three female rats received no treatment. These rats served as control animals. The livers were isolated 24 hours after the last dose and perfused with saline until all blood was removed and stored at -80°C till further use.

Isolation of microsomes

About 3 to 4 gram liver was used for the isolation of microsomes as described by Rutten et al. 1987[3]. The protein content was determined according to Bradford[4]. The calibration curve was made using bovine serum albumin as a standard.

Characterisation of the microsomes

Table 2 shows the assays that were performed to determine the enzyme activities in order to characterise the microsomes.

Table 2 enzyme assays performed in order to characterise the microsomes

assay	indicator for specific CYP
regio selective Testosterone hydroxylation	2A1, A2,2B1,2B2,2C11,3A2,3A1
Ethoxy-resorufine-o-deethylase (EROD)	1A1, 1A2
Methoxy-resorufin-o-demethylase (MROD)	1A2
Pentoxyresorufin-o-depentylase (PROD)	2B1/2

Testosterone hydroxylation was performed as described by Wortelboer et al. (1990) [5]. EROD, PROD and MROD were performed as described by Burke et al. 1985 [6].

Biotransformation rates of TCBT isomers

Rat hepatic microsomes from the selectively induced and control rats were incubated (1 mg/ml) at 37°C with TCBT 87, 88, 94 (260 nM) in a total volume of 1 ml consisting of 0.05 M KH_2PO_4/Na_2HPO_4 phosphate buffer (pH=7.4), 1mM EDTA. Ugilec isomers were dissolved in

acetone (1.6 % final concentration). The biotransformation reaction was started by adding 50 µl of the NADPH-regenerating system (final concentration:1 mM NADP, 1 U Glucose-6-phosphate dehydrogenase, 3 mM $MgCl_2$, and 5 mM glucose-6-phosphate). The assay was performed in triplicate. At four time points between 0 and 30 minutes 200 µl of the incubation mix was added to 1 ml ice cold methanol and 800 µl buffer solution to stop the biotransformation of the substrate. Since PCB 153 is hardly metabolised by rat hepatic microsomes [7] it was used as standard (260 nM) to check the mass balance.

Extraction

The parent compound was extracted once by 4 ml n-hexane. Recovery of the extraction ranged between 90 and 100%. The organic phase was collected and evaporated at room temperature under air. The dried samples were then dissolved in 100 µl hexane.

GC-analysis

The samples were analysed on a Carlo Erba Mega 5360 GC-ECD equipped with a 15 m DB5 column (ID 0.32 mm, film thickness 0.25 µm) using split injection with a split ratio of 30 (T inj=250°C, Tcol=250°C). The area of TCBT relative to the area of PCB 153 was determined for each sample. The disappearance of the parent compound (TCBT) was described by first order kinetics. The elimination rate constant was calculated by linear regression analysis after logarithmic transformation of the relative areas.

Correlation analysis

Spearman correlation test was performed between the microsomal enzyme activities and *k* using SPSS for Windows®. The statistically significant correlations indicated the CYPs that play a role in the metabolism of TCBT. These relationships were further substantiated by investigating the effect of a specific inhibitor of the involved enzyme on the elimination rate constant. As *k* for TCBT 87, 88 and 94 correlated with CYP 2B, CYP 3A1 and 2A1/2 we used inhibitors for these enzymes: Orphenadrine as inhibitor for CYP 2B1 and 2B2[8], Troleandomycin for CYP 3A1[9] and Hematoxylin for CYP 2A1 and 3A2[10] . 30µM of the inhibitor in acetone was added tot the incubation mix with TCBT using dexamethansone induced microsomes. The inhibition of *k* for the individual TCBTs was determined and compared with the non-inhibited value.

Results and discussion

Figure 2 shows that the elimination rate constants vary with the type of hepatic microsomes used. Dexamethasone, phenobarbital and isosafrole induced microsomes metabolise the TCBTs more rapidly than β-naphtoflavone, clofibric acid induced microsomes. This indicates that enzymes induced by these inducers play an important role in metabolism. Table 4 shows the Spearman's correlation coefficients for the enzyme activities and *k*. From this table it can be concluded that CYP 2B1, 2B2 and 3A1 as well as 2A1 and 2A2 are involved in the metabolism of TCBT. Preliminary results from the inhibiting studies confirm these conclusions. TCBT 87 does not correlate with CYP 2A1 or 2A2 activity. Some isomeric preference seems to exist between the various CYPs. Studies with human microsomes are still under investigation as well as the studies on the PCBs (e.g. PCB126). At the conference the CYPs involved in metabolism of TCBT and PCB in rats and humans will be compared and discussed.

Table 4 Overview of the statistically significant correlations ($p<0.05$) between *k* and the substrates or the hydroxylated testosterone metabolites. CYPs involved in the formation of metabolites are listed as well

	Metabolites or substrate										
cyp	15α 2A2	16α 2B1,2C 11	2α 2C11	7α 2A1,2A 2	and 2B1, 2B2, 2C	12β	15β 3A1	16β 2B1,2B 2	2β 3A2	6β 1A1, 1A2, 2A2, 2C13,3A1 , 3A2	prod 2B1, 2B2
94				0.64		0.53	0.48	0.75	0.52		0.70
88	0.67	0.47			0.61	0.72	0.69	0.77	0.70	0.69	0.54
87						0.47	0.49	0.70	053		0.49

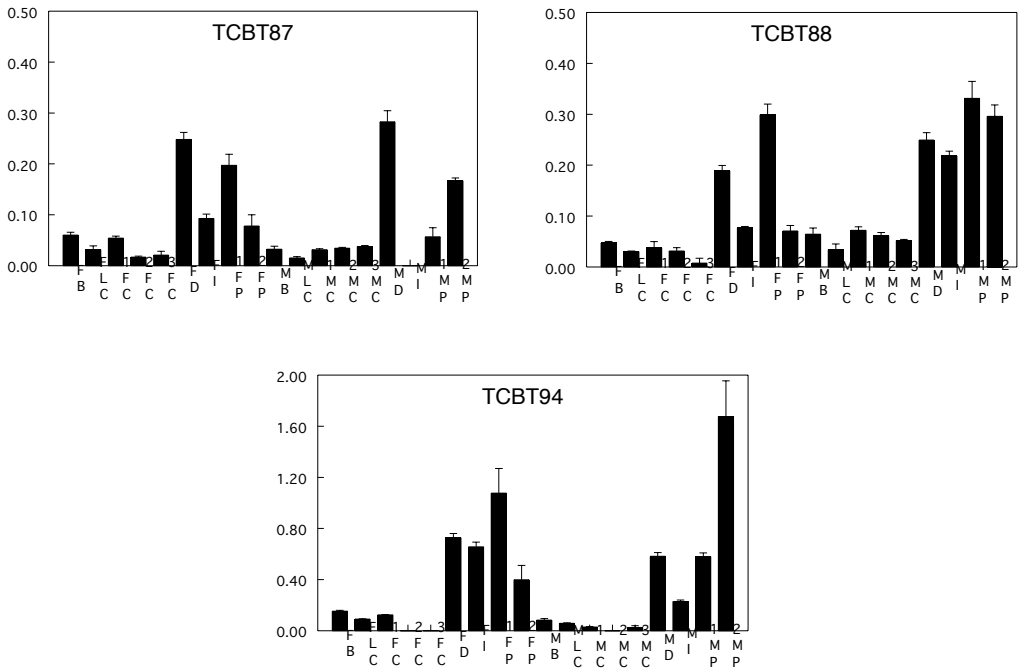

Figure 2 The elimination rate constants for TCBT 87, 88 and 94 (mg prot.$^{-1}$ min.$^{-1}$) for the different microsomes from induced and non-induced rats. The bars represent the average *k* and the standard error of 3 determinations. Table 1 sums up the inducers and their corresponding codes. F= female and M=male.

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