Biotransformation of Coplanar PCBs, PCDDs, and PCDFs and Specific Cytochrome P450 Isozyme Activities in Harbour Seal (*Phoca vitulina*): Selective Inhibition of *In Vitro* Metabolism in Hepatic Microsomes

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Introduction

Marine mammals are high in the food web, and bioconcentrate and bioaccumulate substantial concentrations of persistent organochlorines (OCs) such as polychlorinated biphenyls (PCBs) [1]. Of the 12 cytochrome P450 (CYP) families known to exist in mammals, three (CYP1A, CYP2B and CYP3A) of the 22 subfamilies are of primary importance for the metabolism of anthropogenic compounds. PCB biotransformation can determine the toxicokinetics, and ultimately the PCB congener pattern and levels in a given species. CYP isozyme profiles, whether constituent or OC-induced, determine the metabolic capacity towards PCBs, and is species-dependent. In captive Dutch harbour seals, animals fed a diet of OC-contaminated Baltic herring had very low levels of PCDDs, PCDFs and dioxin-like non-*ortho*- and mono-*ortho*-PCBs, relative to seals fed relatively uncontaminated North Sea herring [2]. Greater OC exposure and greater elimination efficiency of dioxin-like compounds appeared to be correlated despite a higher daily OC intake for the seal group fed Baltic herring.

In marine mammals, pharmacokinetic models for PCB biotransformation and methyl sulfonyl-PCB metabolite formation and clearance, catalytic activity (e.g., EROD) and immunochemical studies have illustrated the inter-species differences in biotransformation capacity [1,3-5]. However, the precise CYP isoform specificity toward PCB congener metabolism has only been demonstrated for a few species of laboratory animals, and not for free-ranging marine mammals [5]. A recently developed *in vitro* metabolism bioassay using viable hepatic microsomes has shown the biotransformation of PCB-77 and chlorobornane (toxaphene) congeners by white-beaked dolphin, harbour seal and eider duck [6,7]. Nevertheless, the identity of specific CYP isozymes participating in OC metabolism remains unclear. Unlike humans and rats [8], studies using compounds known to inhibit the activity of specific microsomal CYP isozymes or subfamilies in a competitive or a mechanism-based

manner, have not been forthcoming for wild mammalian species. Inhibition of OC-substrate metabolism *in vitro* has been limited to ketoconazole inhibition of the metabolism of selected toxaphene congeners in harbour seal [9]. Using the *in vitro* metabolism bioassay and CYP-specific inhibitor compounds, we investigated the role of CYP1A-, CYP2B- and CYP3A-type isozymes in the metabolism of dioxin-like PCBs in harbour seal. Further, we evaluated the importance of phenyl ring co-planarity in dioxin-like PCBs, to CYP1A-/CYP3A-mediated biotransformation, by comparison to easily metabolized PCDD/F congeners.

Materials and Methods

The liver microsomes were from a free-ranging female harbour seal (*Phoca vitulina*) of ca. 4 years of age, stranded at Camperduin (Dutch Coast, October 1996). After the isolation of microsomes [9], the pre-screening for EROD activity (ca. 500 pmol/mg. prot./min) revealed a suitably high biotransformation potential necessary for the *in vitro* metabolism assay. Three acetone solutions of, #1) PCB-15, -26, -31, -44, -52, -77, -101, -105, -118, -126, -153 and -156, 5 to 13 ng/μl, #2) PCB-26, -31, -44, -52, -101, -105, -118, -126, -153 and -156, 2 to 3 ng/μl, and #3) PCB-15, -77 and -153, 2,7-DiCDD, and 1,2,3,4,8-PnCDF, 4 to 11 ng/μl, were used in the *in vitro* bioasssays without and with (mixture #1 and #3) inhibitor compound solutions (n = 3 or 4 replicates) of variable concentration. The degree of metabolism was measured by the decrease of the parent compounds relative to the internal standard, the highly recalcitrant PCB-153. The PCB congeners were representative of the PCB metabolism structural classes known for marine mammals, and dominant congeners persisting in North Sea and Baltic seals. The clean-up of unmetabolized PCBs and their GC/ECD detection has been described [6,9]. Reference assays were run in the absence of the NADPH reaction initiator to determine the percent recovery of the PCBs (> 75% recovery minimum). Control assays (with NADPH and without inhibitors) represented the maximum PCB depletion. The CYP-specific inhibitor compounds ellipticine (CYP1A1; 0.03, 0.06, 0.1, 0.5, 5.0, 10 and 20 μM) and ketoconazole (CYP3A; 0.01, 0.1, 0.5, 1.0, 5.0, 10 and 20 μM) were added to separate assays. The inhibitor concentrations listed are those in the final assay mixture.

Results and Discussion

Relative to reference assays, two congeners (PCB-15 (4,4′-DiCB) and PCB-77 (3,3′,4,4′-TeCB)) in mixture #1 underwent significant metabolic depletion (two-tailed t-test, $P < 0.01$) of ca. 85% and 30%, respectively. Murk *et al.* [6] observed a similar degree of metabolic depletion for PCB-77 with Dutch harbour seal microsomes having a ca. 5-fold greater level of EROD activity. It may be that a maximal rate of CYP1A-type metabolism *in vitro* of dioxin-like PCBs is reached at moderately low level of CYP1A-type activity in harbour seal. In 3-MC induced rats, hepatic microsomal metabolism also showed a high elimination rate constant for PCB-15, whereas the PCB-126 rate constant was almost negligible [10]. A marginal, but significant ($P < 0.05$) metabolism occurred for PCB-26, -31, and -52 (ca. 15-20%), and to an even lesser extent for non-*ortho* PCB-126 (<10%), but was difficult to reproduce in subsequent assays. PCB-15 and -77 were therefore used in the inhibition studies. Relative to mixture #1, a marginally higher but significant ($P < 0.05$) metabolic depletion was observed for PCB-26, and -31 (ca. 28%) from mixture #2, suggesting that these congeners

were better able to compete for CYP enzyme active sites in the absence of the monopolizing PCB-15 and -77 congeners. Significant metabolic depletion of mono-*ortho* PCB-105 and -118 from Clophen A40 has been observed using seal microsomes with a 5-fold higher EROD activity level. Pharmacokinetic models for PCB metabolism in captive harbour seal relative to their herring diet has also shown a decrease in the group III PCB-105 and -118 which is indicative of a high CYP1A-type biotransformation capacity. A slow rate of metabolism *in vitro* may have occurred for some congeners, and thus the congener depletion was below the sensitivity of the assay. Seal microsomes with greater CYP activity are required to study the CYP induction-response effect on the metabolism of other PCB structural classes. The absence of significant depletion *in vitro* may have also been the result of the non-physiological nature of the *in vitro* microsomal assay, which includes high NADPH and O_2 concentrations and a differing pH.

The competitive CYP inhibitor ellipticine has been shown to be a selective inhibitor of CYP1A1 activity in humans, but also of CYP1A2 activity in rodents [8]. Significant concentration-dependent inhibition (ANOVA for multiple comparison with posthoc SNK test, $P < 0.01$) of PCB-15 and -77 metabolism commenced coincidentally at ca. 0.25 µM, with ca. 80 % maximal inhibition at ca. 1.0 µM and ca. 20 %, at ca. 0.5 µM, respectively. The CYP3A-type ketoconazole inhibited PCB-15 and -77 metabolism starting at 0.5 µM and 1.0 µM, respectively, and maximized at ca. 90 % and 40%, respectively at ca. 10 µM. Assuming a similar specificity of the inhibitor compounds to CYP isoforms in harbour seal as for humans and rats, both CYP1A- and CYP3A-type enzymes appeared to be responsible for dioxin-like PCB metabolism in harbour seal. The CYP2B1 inhibitor chloramphenicol had no effect on PCB-15 or -77 metabolism for treatment concentrations up to 10 µM.

Relative to the reference assays, the percent metabolism of PCB-15 (93%), 2,7-Cl_2-CDD (76%), 1,2,3,4,8-Cl_5-CDF (61%), and to a lesser extent PCB-77 (26%) from mixture #3 were significant ($P < 0.01$, Figure 1). To test whether the flexibility of the phenyl ring coplanarity of non-*ortho* PCB-15 and -77 permitted binding to the active sites of both CYP1A- and CYP3A-type isoforms, the metabolic inhibition by maximally inhibitive concentrations of ketoconazole and ellipticine was compared with the easily metabolized and rigidly planar 2,7-Cl_2-CDD and 1,2,3,4,8-Cl_5-CDF (Figure 1). Complete metabolic inhibition occurred for all four substrates with ellipticine, whereas the inhibitive effect of ketoconazole was incomplete and variable. Inhibition of 80 and 85% of the metabolism occurred for PCB-15 and 2,7-Cl_2-CDD, respectively, and 44 and 48% of the metabolism for PCB-77 and 1,2,3,4,8-Cl_5-CDF, respectively. These results suggested that for CYP3A-type biotransformation mediation *in vitro*, dioxin/furan and dioxin-like PCB metabolism may be contingent on the degree of chlorination, and not on an obligatory low energy barrier to phenyl ring rotation. It was not possible to be conclusive regarding the inhibitor compound specificities for the CYP1A- and CYP3A-type isoforms in harbour seal. Complementary studies on the effects of the inhibitors on CYP-specific catalytic activities should be investigated using the same harbour seal microsomes. CYP-mediated hydroxylations of 17β-estradiol and testosterone at different carbons atoms are more or less specific to some CYP1A-, CYP3A-, and CYP2B-type isozymes. In summary, it was possible to partially define the type of CYP isozyme activity responsible for coplanar PCB, PCDD, and PCDF metabolism *in vitro* in harbour seal. This may extend to PCB metabolism pharmacokinetics in harbour seal *in vivo*, and perhaps to pinniped and marine mammals in general.

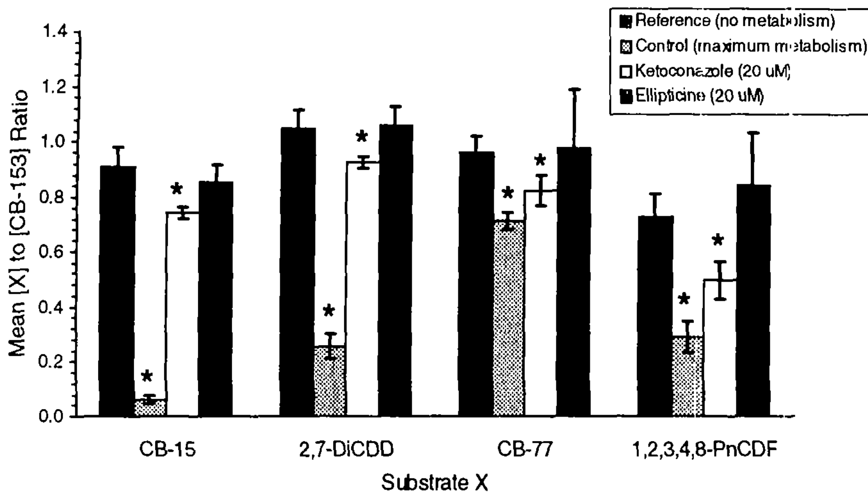

Figure 1. The inhibitive effects of ellipticine and ketoconazole on PCB, PCDD and PCDF metabolism *in vitro* in harbour seal microsomes. The significance of the difference (* $P < 0.01$) of controls and inhibitor-treated assays from the references is shown (two-tailed *t*-test).

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