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Uterotropic and Enzyme Induction Effects of 2,2',5-Trichlorobiphenyl

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There is a growing concern regarding the potential risk from exposure to endocrine-disrupting chemicals in wildlife and humans (Colborn et al. 1993). Polychlorinated biphenyls (PCBs) are among those ubiquitously persistent chemicals possessing endocrine-disrupting effects. For example, estrogenic activity has been ascribed to some lower-chlorinated Aroclor mixtures and *ortho*-chlorinated chlorobiphenyl (CB) congeners (Bitman and Cecil 1970; Ecobichon and Mackenzie 1974; Jansen et al. 1993; Li et al. 1994; Soontornchat et al. 1994). Furthermore, some putative PCB metabolites were found to have relatively high estrogen receptor binding affinities *in vitro* and to stimulate uterine weight increases *in vivo* (Korach et al. 1988; Jansen et al. 1993). Most of the environmentally abundant CB congeners have one or more *ortho* chlorines in the biphenyl ring. Because *ortho*-chlorinated CB congeners do not avidly bind to the aryl hydrocarbon (*Ah*) receptor, this clearly suggests that the estrogenicity of PCBs is not related to potency as *Ah* receptor agonists. It is important to investigate the estrogenicity of those *ortho*-chlorinated CB congeners in order to more accurately predict the hazards from PCB residues in the environment.

The di-*ortho* 2,2',4,4',5,5'-hexachlorobiphenyl (CB 153) is estrogenic in 22-day-old prepubertal rats at doses between 20 and 100 mg/kg, but at total doses greater than 50 mg/kg, estrogenic activity is attenuated (Li et al. 1994). Near doses of 120 mg/kg, serum residues are lower than at 100 mg/kg (Li et al. 1994), presumably due to more rapid metabolism by induced P450 2B1 (Kaminsky et al. 1981; Sipes and Schnellmann 1987); nevertheless, P450 2B1 activity, as measured by pentoxyresorufin O-dependentylase (PROD), is also attenuated. Even though CB 153 is recalcitrant to oxidative metabolism, induced P450 2B1 catalyzes significant formation of hydroxylated metabolites in dogs and rats (Sipes and

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Schnellmann 1987). The hydroxylated metabolites are presumably more reactive with the estrogen receptor (Korach et al. 1988; Jansen et al. 1993), but induction of phase 2 enzymes at the higher doses could sequester the metabolites resulting in the lower serum residues of parent compound and attenuated estrogenic activity observed at higher doses. It was of interest to determine if a di-*ortho* CB congener which is more readily metabolized and an ineffective PROD inducer exhibited similar nonlinear dose:response relationships for estrogenicity and serum residues as observed for CB 153. CB 18 (2,2',5-trichlorobiphenyl) is very rapidly metabolized to more polar products by sheep liver microsomes (Hansen et al. 1977), rats (Saghir et al. 1994) and house flies (Saghir and Hansen 1992). The detection of greater amounts of mono-hydroxylated metabolites from slower metabolized congeners than from CB 18 (Borlakoglu and Wilkins 1993) reflects further secondary metabolism in these extended (up to 1 hour) microsomal incubations. Such secondary metabolism should be relatively constant up to very high doses *in vivo*, since CB 18 should be a very poor P450 2B1 inducer. Even though CB 18 commonly occurs in environmental samples (Hornbuckle et al. 1993), there is little information regarding its biological effects. In this study, we used immature Sprague-Dawley female rats as an animal model to examine the uterotropic response, thyrotoxic effect and enzyme induction potency of CB 18.

MATERIALS AND METHODS

Corn oil was purchased at a local grocery. All solvents were analytical grade (Burdick and Jackson, Muskegon, MI). All chemicals and reagents used in enzyme assays were obtained from Sigma Chemical Co. (St. Louis, MO). Radioimmunoassay kits (Coat-A-Count TT4) for determining serum total T4 (TT4) concentration were purchased from Diagnostic Products Corp. (Los Angeles, CA). Alumina (80-200 mesh) was from Fisher Scientific (Springfield, NJ), and AX-21 charcoal was from Anderson (Adrian, MI). CB 18 was provided from previously synthesized stock (Hansen 1979) and was repurified before use by column chromatography (3 g of 2% deactivated alumina mixed with 1.5% AX-21 activated charcoal; elution with hexane). The concentration of CB 18 in the corn oil solution was confirmed with a Hewlett Packard 5790 gas liquid chromatograph (GLC) with electron capture detection on a 60m DB-5 capillary column (Li et al. 1994).

Sprague-Dawley breeder rats were obtained from SASCO (Lincoln, NE). Pups were culled to 10 animals per litter on the day of birth (day 0) and were weaned at 20 days of age. Female pups were injected ip with CB 18 dissolved in 0.1 mL corn oil (8, 16 and 64 mg/kg/day) or corn oil alone between 1:00 and 2:00 p.m. on day 20 and day 21. Estradiol (0.02

mg/kg/day) was used as a positive control. Rats were decapitated between 9:00 and 11:00 a.m. on day 22 and their uteri were excised, trimmed of fat and connective tissue, and weighed. The uterotropic effects were determined by comparing (uterine wet wt in mg) / (body wt in g) ratios to the ratios found in control animals. Blood was collected immediately after decapitation and allowed to clot. Serum was separated by centrifugation and stored at -20°C until analyzed for TT4 by RIA and CB 18 residues by GLC.

Liver microsomes were prepared by standard centrifugation procedures and stored in liquid nitrogen or at -80°C until assayed. Ethoxyresorufin O-deethylase (EROD) and PROD were determined by a modification of the method of Pohl and Fouts (1980). In brief, the reaction mixture contained 5 mM $MgCl_2$, 1 mg bovine serum albumin, microsomal suspension (200 to 500 µg of microsomal protein for EROD and 400 to 800 µg for PROD), 2.5 µM 7-ethoxyresorufin or 10 µM 7-pentoxyresorufin in 0.05 M Tris-HCl (pH 7.5) and a NADPH-generating system (0.8 mg $NADP^+$, 1.5 mg glucose-6-phosphate, 1 unit glucose-6-phosphate dehydrogenase). The reaction was initiated by adding the NADPH-generating system and stopped by the addition of 2 mL of methanol. The reaction was carried out 4 min for EROD and 10 min for PROD at 37°C. The formation of resorufin was determined by measuring sample fluorescence relative to a known amount of resorufin with excitation at 550 nm and emission at 585 nm in a Perkin-Elmer 203 Fluorescence Spectrophotometer. Microsomal protein was determined by the method of Lowry (1951) using bovine serum albumin as a standard. All samples were run in duplicate.

The serum concentrations of CB 18 in animals following treatment were analyzed by GLC as above. In brief, serum (100 µL) was extracted by adding 1.0 mL of acetone followed by 1.0 mL of hexane; after thorough mixing, enough sodium sulfate was added in small amounts to adsorb all the water. Then 0.2 mL of the CB 18 treated serum extract was added to 0.8 mL of hexane containing 50 ng/mL mirex as an internal standard. The extraction efficiency was 107.4% ± 5.0% (mean ± SE). Extract (1 µL) was injected onto the column and the oven temperature was programmed for 175°C to 266°C at 3°C per min. The temperature of the injection port and electron capture detector were 250°C and 325°C, respectively. Hydrogen was used as carrier gas and argon/methane was used for make-up gas. Quantification was accomplished by comparing to known amounts of CB 18 standards.

The data were first analyzed by F test to determine the homogeneity of variance between treated and control groups. If variances were homogeneous, Student's t test was used to compare the difference between

treated and control groups. The Mann-Whitney U test was used when variances were not homogeneous. Differences were considered significant when $P \leq 0.05$.

RESULTS AND DISCUSSION

CB 18 caused essentially the same uterine wet wt increase over an 8-fold range in doses (Table 1). CB 153 did not change the relative uterus wt at either 16 or 118 mg/kg, but the maximum increase (163% control at 50 mg/kg) was greater than for CB 18 (Li et al. 1994). The lowest effective total dose for CB 18 must be lower than 16 mg/kg, 120-140% of the control value appears to be the maximum effect and there does not appear to be antagonism at the highest dose.

Table 1. Uterotropic effect of CB 18 administered on day 20 and 21 to immature Sprague-Dawley female rats.

Treatment	Total Dose (mg/kg)	n	% (UWW mg)/(g BW)[a]	% Control
Control		5	40.5 ± 1.8	100
17ß-Estradiol	0.04	4	128.0 ± 2.4**	316
CB 18	16	5	52.8 ± 3.9**	130
	64	5	49.0 ± 2.7**	121
	128	4	55.4 ± 5.8**	137

[a] % Uterine wet wt mg/g body wt expressed as Mean ± SE.
** Significantly different from control; $P \leq 0.05$.

Neither CB 153 (Li et al. 1994) nor CB 18 (Table 2) induced EROD activity significantly, confirming that they are poor agonists for the *Ah* receptor. The major differences between CBs 18 and 153 are rates of metabolism and potency as inducers of their own metabolism. CB 18 is a very weak PROD inducer, increasing activity 2.5-fold at 128 mg/kg (Table 2). CB 153 increased PROD activity nearly 9-fold at a slightly lower dose (Li et al. 1994). Neither CB 18 (Table 3) nor CB 153 (Li et al. 1994) decreased serum TT4 significantly.

It is possible that CB 18 produces a biphasic response similar to CB 153 if the maximum effective dose occurs in a relatively narrow window which

Table 2. Liver microsomal enzyme activities in immature female rats administered CB 18.

Treatment	Total Dose (mg/kg)	n	Microsomal enzyme activities[a] EROD[b]	PROD[b]
Control		5	145.9 ± 21.0	2.9 ± 0.7
17ß-Estradiol	0.04	4	157.0 ± 20.0	2.2 ± 0.2
CB 18	16	5	155.4 ± 27.3	4.5 ± 1.0
	64	5	188.2 ± 52.5	3.1 ± 0.4
	128	4	251.1 ± 75.8	7.5 ± 1.1**

[a] Mean ± SE.
[b] Activity expressed as pmol resorufin formed/min/mg protein.
** Significantly different from control; P≤0.05.

Table 3. Serum TT4 and CB 18 concentrations in immature female rats treated with CB 18.

Treatment	Total Dose (mg/kg)	n	TT4[a] (ug/dL)	CB 18[a] (ppb)
Control		5	3.2 ± 0.5	ND[b]
17ß-Estradiol	0.04	4	2.9 ± 0.3	ND
CB 18	16	5	2.6 ± 0.2	35.6 ± 8.7
	64	5	2.5 ± 0.2	72.9 ± 3.3
	128	4	2.8 ± 0.3	131.3 ± 13.3

[a] Mean ± SE.
[b] Not Determine.

was missed by the larger dosing increments. Nevertheless, the more expected (suggested sigmoid) increase in serum residues with dose (Table 3) argues against the same dose:response shape as with CB 153. As expected, the serum residues of the rapidly metabolized CB 18 were about 100-fold lower than those of CB 153 at comparable doses. At much higher (but less environmentally relevant) doses, CB 18 may show a similar nonlinear dose:response relationship. More importantly, the more likely event of co-exposure to CB 18 and active PROD inducers may alter the

dose: response relationships entirely. If the PROD inducer is also estrogenic, the estrogenicity of CB 18 might merely be additive or the PROD induction may synergize CB 18 actions since the 4'-hydroxy metabolite binds actively to the rat uterine estrogen receptor (unpublished results). Once individual actions of dissimilar di-ortho congeners are described and determined over a range of doses, interactions of this type can be quantified and this will greatly facilitate accurate hazard assessment for environmental PCB residues.

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