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Estrogenicity of Biphenylols: Activity in the Yeast Gene Activation Assay

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Polychlorinated biphenyls (PCBs) are man-made contaminants, which have been identified in a variety of ecosystems (McFarland and Clarke 1989). Commercial formulations of PCBs are mixtures of biphenyl compounds differing in the degree and location of chlorination (Larsen et al. 1992). The biochemical and toxicological responses elicited by individual PCB congeners are diverse and complex and affect several systems including the estrogen and thyroid hormone systems (Brouwer et al. 1999). Due to structural similarity with the polarized phenolic A-ring of 17-ß-estradiol, hydroxy-substituted PCBs have been linked to estrogenicity (Korach et al. 1988; McKinney and Waller 1994; Gierthy et al. 1997; Schultz et al. 1998; Kramer and Giesy 1999). Various methods have been developed to study these effects and to screen potential endocrine disrupters (Zacharewski 1997). The recombinant yeast estrogenic system (YES) is particularly useful for modeling estrogenic structure-activity relationships (Schultz and Seward 2000).

Although PCBs are considered extremely persistent compounds, many PCB congeners are metabolized *in vivo* to more polar derivatives (Sundstrom et al. 1976; Sipes and Schnellmann 1987). In mammals, hydroxylated biphenyls are found as metabolites of the parent halogenated biphenyls (Kato et al. 1980; Bergman et al. 1994). PCBs containing chlorines in the *meta-* and *para-* positions are less susceptible to biotransformation than those lacking chlorines in those positions (Borlakoglu and Wilkins 1993). Metabolic transformation of PCBs is mainly by formation of arene oxides or by direct insertion of a hydroxy-group (Koga et al. 1992).

The Glaxo Welcome-derived *Saccharomyces cerevisiae* strain is a molecular toxicology assay that measures ß-galactosidase activity; as a proxy for estrogenicity this allows for the identification of chemicals that induce estrogen receptor-mediated transcriptional activity (Routledge and Sumpter 1996). This yeast estrogen assay, here after referred to as the YES assay, has a low cost, a 0.08 nM detection limit and a 10,000-fold responsiveness. The strain constitutively-expresses the gene for the alpha human estrogen receptor integrated into the yeast genome. On a separate expression plasmid, the estrogen-responsive sequences control the expression of the *lac*-Z reporter gene. Natural or xenoestrogens interact with the receptor to activate transcription. The net result is the production

of ß-galactosidase. The ß-galactosidase activity is measured colorimetrically as in the medium; ß-galactosidase transforms the initially yellow chromogen into a red product that is measured by absorbance at 540 nm.

Using this YES assay, Schultz et al. (1998) reported estrogenicity for selected hydroxylated PCBs following 3-days of exposure. However, recent work in this laboratory with selected phenols, oxyphenols, hydroxybenzoates, and phytoestrogens (Schultz et al. 2000) revealed that some compounds, while not exhibiting any activity following 3 days of exposure, demonstrated ß-galactosidase activity after 5 days of exposure. These findings raised the issue of false negatives for congeners previously reported as not active in this gene activation assay (Schultz et al. 1998). Therefore, the nine biphenylols previously examined by Schultz et al. (1998) were re-assayed after 5 days of exposure and the data set was expanded to include 13 additional hydroxylated biphenyls. The aims of this study were to evaluate the estrogenicity of these selected biphenyls and probe the results for structure-activity relationships.

METHODS AND MATERIALS

The YES assays were performed following the protocol of Schultz et al. (2000). Briefly, all biphenylols were evaluated in triplicate with a minimum of two replicates. Each replicate included the positive control 17-ß-estradiol and ethanol as the negative control. Biphenylol stock solutions were prepared fresh in ethanol for each replicate. Each compound was serially diluted in ethanol with 10 µL of each concentration being added to a well of a 96-well plastic microtiter plate and allowed to dry. After drying, 200 µL of medium containing yeast and 0.1 mg/mL CPRG were added to each well. Plates were incubated at 30 ± 1°C and agitated daily. Following 3, 5 and 7 days of incubation, absorbencies at 540 nm and 620 nm were recorded. The EC_{50} (the concentration eliciting an activity equal to 50% of the positive control 17-ß-estradiol) was determined for each compound and each time point by Probit Analysis of Statistical Analysis System (SAS) software (SAS Inc. 1989). The dependent variable was the absorbency normalized as percentage of control. The independent variable was the toxicant concentration in g/L or moles/L. The EC_{50} values are reported in molar units.

The chemicals assayed included a series of 22 biphenylols (Table 1). Each was purchased from ULTRAScientific (Kingstown, RI, USA), Aldrich Chemical Company (Milwaukee, WI, USA) or Lancaster Synthesis Inc. (Windham, NH, USA). All had purities greater that 97% and none were repurified prior to use.

RESULTS AND DISCUSSION

The positive control, 17-ß-estradiol, had an EC_{50} value for estrogenic activity of 3.91×10^{-11} M. The negative control, ethanol exhibited no activity. Seventeen of the 22 hydroxylated biphenyls tested exhibited gene expression as indicated by ß-galactosidase activity (Table 1). For those derivatives exhibiting activity, potency (EC_{50}) varied from 5×10^{-5} to 1×10^{-9} M. Several structural characteristics affecting estrogenicity are demonstrated by these data.

Table 1. Estrogenic activity of hydroxylated biphenyls in the standard 96-well microtitre plate YES assay.

Compound	EC_{50} of Estrogenic Activity (M)[a]
1. 2-biphenylol	1.84×10^{-5}
2. 3-biphenylol	9.18×10^{-6}
3. 4-biphenylol	1.15×10^{-6}
4. 2,2'-biphenyldiol	Not active
5. 2,3-biphenyldiol	1.68×10^{-5}
6. 2,5-biphenyldiol	Not active
7. 3,4-biphenyldiol	4.20×10^{-6}
8. 3,3'-biphenyldiol	8.4×10^{-6}
9. 4,4'-biphenyldiol	2.63×10^{-7}
10. 2,3',4,5',6'-biphenylpentaol	Not active
11. 2-chloro-4-biphenylol	3.82×10^{-6}
12. 3-chloro-4-biphenylol	3.82×10^{-6}
13. 4'-chloro-4-biphenylol	5.98×10^{-8}
14. 2',5'-dichloro-2-biphenylol	5.23×10^{-5}
15. 2',5'-dichloro-3-biphenylol	2.04×10^{-7}
16. 2',5'-dichloro-4-biphenylol	3.00×10^{-8}
17. 2',4',6'-trichloro-4-biphenylol	1.29×10^{-9}
18. 2,2',5'-trichloro-4-biphenylol	1.78×10^{-7}
19. 3,4',5-trichloro-4-biphenylol	Not active
20. 2',3',4',5'-tetrachloro-3-biphenylol	1.58×10^{-7}
21. 2',3',4',5-tetrachloro-4-biphenylol	6.30×10^{-9}
22. 3,3',5,5'-teterachloro-4,4'biphenylol	Not active

[a]Estrogenic activity was measured by colormetric detection of ß-galactosidase activity after the yeast reporter strain was exposed to the compound for 5 days.

A comparison of galactosidase activity for compounds 1 - 3 and 11 - 13 reveals that gene expression is typically increased by an order of magnitude where there is an *ortho-* to *meta-* to *para*-hydroxyl substitution. Comparing the activity between compounds 15 and 16 further substantiates this relationship. Comparisons of estrogenicity between the 2',4',6'-trichloro-4-biphenylol, 2,2'5'-trichloro-4-biphenylol, and 3,4',5,-trichloro-4-biphenylol (compounds 17 –19) reveal that

expression is impaired by chloro-substitution on the phenolic ring. The latter is supported by data for compounds 11 and 12 compared to compound 13. As a rule, an increase in the number of chlorines on the non-phenolic ring increases activity (Schultz et al. 1998). The increased activity is due to an increase in electron withdrawing capacity, which results in higher pK_a values and better hydrogen-bond donor capacity (Ebner and Braselton 1985) rather than an increase in hydrophobicity. Moreover, examination of the data for compounds 19 and 22 reveals that bracketing the hydroxyl group with chlorines (i.e., 3,5-chloro and 4-hydroxyl substituted) inhibits estrogenic gene activation probably by steric hindrance of the hydroxyl group.

A comparison of expression for the symmetrical 2',4',6'-trichloro-4-biphenylol (compound 17) and the non-symmetrical 2',3',4',5'-tetrachloro-4-biphenylol (compound 21) or 2',5'-dichloro-4-biphenylol (compound 16) indicates the importance of molecular symmetry in estrogenic gene expression and further indicates the importance of pK_a rather than hydrophobicity in explaining relative gene expression for this set of molecules.

The comparison of estrogenic potency among the non-chlorinated derivatives reveals that the 2,3-dihydroxyl-derivative exhibited activity equivalent to 2-biphenylol. Similarly, the 3,3'-biphenyldiol and to a lesser extent 3,4-biphenyldiol exhibit gene expression equivalent to 3-biphenylol. In contrast, 4,4'-biphenyldiol is an order of magnitude more potent that 4-biphenylol. This difference may reflect the molecular symmetry of the former compound. One diol, compound 6, is not active. Based on the structure-activity relationships noted one would expect this compound to have an activity in the 10^{-5} range. The reason for this discrepancy is that the LC_{50} value for this compound is 3×10^{-5}. Thus, this derivative is eliciting an acute toxic response at the concentration estimated to reduce ß-galactosidase activity by 50% (i.e., acute toxicity is inhibiting gene activation).

While cellular uptake is critical to reaching the molecular site of action (i.e., the cytosolic receptor protein), this is rarely the rate-limiting step in gene expression. However, gene expression assays are sensitive to subtle toxic effects that can effect cell growth, cellular energetics, and protein synthesis. These factors may explain the time course for expression of estrogenicity.

Environmental estrogen disrupters can be grouped into one of four categories: direct-acting agonists, direct-acting antagonists, indirect-acting agonists, and indirect-acting antagonists (Cooper and Kavlock 1997). For direct-acting compounds, competitive binding is a required condition and can be measured using either ligand-binding or gene expression assays. However, binding to an estrogen receptor is not sufficient to determine if a compound is estrogenic. Ligand-binding assays cannot distinguish between receptor agonists and antagonists, regardless of whether the "parent" compound is active, or as in the case of PCBs, where metabolic activation is necessary (Kramer et al. 1997).

Estrogenicity of PCBs is attributed to the formation of hydroxylated metabolites (Korach et al. 1988). Hydroxylated PCBs that interact directly with the receptor

produce estrogenic effects at very low concentrations in rainbow trout hepatocytes (Andersson et al. 1996, 1999; Blom et al. 1998). Hydroxylated PCBs have been found in plasma of humans (Bergman et al. 1994). Typically, hydroxylated PCBs are readily excreted (Bergman et al. 1994). However, hydroxylated congeners that contain the hydroxyl-moiety in the *para*-position and chlorine atoms in the adjacent positions such as 3,5-chloro and 4-hydroxyl substituted are retained due to binding to plasma proteins (Bergman et al. 1994). The YES assay shows a strong correlation between estrogen receptor-binding and gene activation measured by ß-galactosidase activity in the alpha human estrogen-receptor containing recombinant yeast assay for biphenyls hydroxylated on one ring and chlorinated on the other ring (Schultz et al. 1998). Previously, the compound 2-chloro-4-biphenylol was not active following 3-days of exposure in the YES assay (Schultz et al. 1998). However, in this study weak estrogenic activity was measured after 5-days of exposure. This result is now in agreement with the statement that *para*-hydroxylated biphenyls chlorinated in the *ortho*-positions on the same ring are weak binders to the estrogen receptor (Korach et al. 1988) but often exhibit anti-estrogenic activity (Kramer et al. 1997). Therefore, all hydroxylated PCBs tested are thought to bind to the estrogen receptor in the YES except for those sterically hindered (i.e, halogenated in both positions next to the *para*-hydroxyl group).

In summary, the estrogenic activity of biphenyl and derivatives with varied levels of chloro- and /or hydroxyl substitution was measured in recombinant yeast estrogen system, i.e., a *S. cerevisiae*-based *lac*-Z (ß-galactosidase) reporter assay. The analysis of galactosidase activity confirms that a polar moiety on the biphenyl ring is required for estrogenic activity. Estrogenic activity is increased typically by an order of magnitude in going from an *ortho*- to *meta*- to *para*-hydroxyl substitution. Estrogenic activity is impaired by chloro-substitution on the phenolic ring, with 3,5-substitution on the *para*-phenolic ring inhibiting galactosidase activity. Molecular symmetry enhances estrogenic activity.

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