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Short Communication

ESTROGENICITY OF SELECTED BIPHENYLS EVALUATED USING A RECOMBINANT YEAST ASSAY

T. WAYNE SCHULTZ,*†‡ DANIEL H. KRAUT,† GARY S. SAYLER,‡ and ALICE C. LAYTON‡
†College of Veterinary Medicine ‡Center for Environmental Biotechnology,
University of Tennessee, P.O. Box 1071, Knoxville, Tennessee 37901-1071, USA

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Abstract—The estrogenic activity of biphenyl and 4-hydroxylated derivatives with varied levels of chloro- and/or hydroxyl substitution was measured in a *Saccharomyces cerevisiae*-based *lac*-Z (β-galactosidase) reporter assay. β-Galactosidase activity was compared with competitive binding to soluble mouse uterine estrogen receptor protein. The comparison of relative potency for biphenyls hydroxylated on one ring and chlorinated on the other ring ($n = 5$) revealed excellent correlation between the two systems ($r^2 = 0.995$). However, estrogenicities of biphenyls hydroxylated and chlorinated on the same ring were not in agreement. Although weak ligand binding was demonstrated for these compounds, β-galactosidase activity was not observed. Rather, these compounds were shown to be cytotoxic to yeast. The results of this study further support the hypothesis that both an unhindered phenolic ring and molecular symmetry are structural features associated with estrogenicity.

Keywords—Estrogen activity Biphenyls Structure–activity relationship *Saccharomyces cerevisiae*

INTRODUCTION

The role of industrial organic chemicals in the alteration of estrogen function has been the object of recent studies. Many of these investigations have been in vitro in design [1]. Because of structural similarity with the polarized phenolic A-ring of 17-β-estradiol, several classes of hydroxy-substituted aromatic hydrocarbons have been linked to estrogenicity [2]. Hydroxylated biphenyls were found as metabolites of the parent halogenated biphenyls [3]. In the mouse uterine estrogen receptor system, the binding affinity of a series of hydroxylated biphenyls, including chloro-derivatives, was quantified by Korach et al. [4].

Paraphrasing the review of Ing and O'Malley [5], estrogenicity is mediated by binding to specific intracellular proteins known as receptors. This binding causes a conformational change in the receptor enabling the estrogen–estrogen receptor complex to bind to specific sites on DNA. Once bound to DNA, the complex alters expression of estrogen-responsiveness genes. Steroidal estrogens exert their effects through this change in gene expression.

A chemical can alter this receptor-mediated process by a number of mechanisms. The chemical can vary the level of endogenous estrogen at a particular site by altering its synthesis, metabolism, distribution, or clearance. Alternatively, the chemical may modify tissue responsiveness to estrogen by changing receptor levels or by acting through a secondary pathway to affect receptor function. Finally, a chemical may interact directly with the estrogen receptor to either mimic or block estrogenicity.

As noted by Zacharewski [6], a number of in vitro estrogenic activity assays have been developed. These include competitive ligand-binding assays such as the one described by Korach et al. [7] and yeast-based assays such as the one described by Routledge and Sumpter [8].

Chemicals that interact directly with the receptor have the potential to produce estrogenic effects at extremely low concentrations. However, binding to an estrogen receptor is not sufficient to determine if a compound is estrogenic. Ligand-binding assays also cannot distinguish between receptor agonists and antagonists. High concentrations of competitor ligand may result in noncompetitive displacement. Moreover, ligand-binding assays cannot evaluate the acute toxic potential of the compound in question.

Although yeasts do not contain steroid or nuclear receptors, they do possess proteins homologous to those in mammalian cells for activated transcription. This allows for the identification of chemicals that induce estrogen receptor-mediated transcriptional activity. Advantages of using yeasts to study estrogenicity are several fold. Yeasts are easy to manipulate and stable transformants are attained quickly. Yeasts provide the opportunity to process large numbers of samples quickly and inexpensively (a necessity for structure–activity work). Moreover, yeasts provide a means to concomitantly evaluate acute toxicity. In vitro estrogenicity testing with recombinant yeasts commonly employ *Saccharomyces cerevisiae* strains transfected with the estrogen receptor cDNA and *lac*-Z reporter gene that encodes for the β-glactosidase enzyme [8–11].

The aim of this study was to evaluate the estrogenicity of selected biphenyls using a recombinant yeast-based *lac*-Z reporter assay and compare activity with that reported from competitive ligand-binding studies.

MATERIALS AND METHODS

The recombinant *S. cerevisiae* assay employed here was previously described by Routledge and Sumpter [8]. Briefly, this strain has the constitutively expressed gene for the human estrogen receptor integrated into the yeast genome. On a separate expression plasmid, the estrogen-responsive sequences

* To whom correspondence may be addressed (tschultz@utkux.utcc.utk.edu).

Table 1. Comparative activities of selected biphenyls

Compound	Binding ratio[a]	Binding potency[b]	β-Galactosidase activity[c]
17-β-Estradiol	1	2.25×10^{-10} [d]	1.26×10^{-10}
2′,4′,6′-Trichloro-4-biphenylol	42	9.45×10^{-9}	5.08×10^{-9}
2′,3′,4′,5′-Tetrachloro-4-biphenylol	95	2.14×10^{-8}	1.26×10^{-8}
2′,5′-Dichloro-4-biphenylol	506	1.14×10^{-7}	1.27×10^{-7}
4′Chloro-4-biphenylol	3,900	7.78×10^{-7}	1.05×10^{-6}
4,4′-Biphenol	>5,000	—	5.42×10^{-6}
4-Hydroxybiphenyl	>5,000	—	5.90×10^{-5}
3,4′,5-Trichloro-4-biphenylol	1,000	2.25×10^{-7}	Nonactive
2-Chloro-4-biphenylol	2,500	5.63×10^{-7}	Nonactive
3,3′,5,5′-Tetrachloro-4-4′biphenyldiol	5,000	1.13×10^{-6}	Nonactive
Biphenyl	—	—	Nonactive

[a] [4].
[b] Determined in M from the binding ratio and the reported 50% binding potency for 17-β-estradiol.
[c] The 50% estrogenic concentration (EC50) value in M.
[d] [11].

control the expression of the *lac*-Z reporter gene. Natural or environmental estrogens interact with the bound receptor on the hybrid promoter to activate transcription of the genes, resulting in the production of β-galactosidase. β-Galactosidase activity is measured colorimetrically using the chromogenic substrate chlorophenol red-β-D-galactopyranoside (CPRG). In the medium, β-galactosidase transforms the initially yellow chromogen into a red product that is measured by absorbance at 540 nm. Therefore, this simple, yet elegant, molecular toxicology assay allows measurement of β-galactosidase activity as a surrogate for estrogenicity. In addition, absorbance at 650 nm was used to estimate cell density and mortality.

The test procedure was performed as described by Routledge and Sumpter [8]. The 0.08-nM detection limit in the *lac*-Z reporter gene yeast system is higher than for MCF-7 cell proliferation or foci formation assays [6]. However, its 1,000-fold responsiveness, short duration, and low cost more than offset its sensitivity.

Serial dilutions of test chemicals were combined with 200 μl of medium containing yeast and CPRG per well in 96-well microtiter plates and incubated at 32 ± 1°C. Plates were agitated daily and following 3 d of incubation, absorbance at both 540 and 610 nm was recorded. All chemicals were evaluated in triplicate with a minimum of two replicates. Each replicate included the positive control 17-β-estradiol. The chemicals evaluated here are listed in Table 1. They include biphenyl and 4-hydroxylated derivatives with varied levels of chloro- and/or hydroxyl substitution. All the chloro-derivatives are known metabolites of polychlorinated biphenyls [3]. Chemicals were purchased from Aldrich Chemical Company (Milwaukee, WI, USA) or ULTRAScientific (Kingstown, RI, USA). Chemicals were not repurified prior to use. Stock solutions of each were prepared in ethanol. The 50% estrogenic concentration (EC50) and 50% lethal concentration (LC50) were determined by probit analysis using Statistical Analysis System (SAS) software [12] with the absorbency normalized as percentage of control as the dependent variable the toxicant concentration in mg/L as the independent variable.

For comparative purposes, ligand-binding data were taken from the literature [4]. These data quantify in vitro competitive binding to soluble mouse uterine estrogen receptor protein [7].

The correlation relationship was examined using logs of the estrogen-receptor binding and β-galactosidase activity in mM. These data were modeled using least squares regression (general linear model procedure of SAS [12]). The coefficient of determination (r^2 value), root of the mean square for error (s value), Fisher statistic (F value), and the probability greater than the F value ($Pr > F$) were reported.

RESULTS

The estrogen-receptor binding data and β-galactosidase activity are listed in Table 1. Three structural characteristics affecting estrogenicity are demonstrated by the data in Table 1. The comparison of estrogenicity between biphenyl and 4-hydroxybiphenyl showed that a polar moiety (i.e., hydroxyl group) is required for β-galactosidase activity. A comparison of estrogenic activity between the 2′,4′,6′-trichloro- and the 3,4′,5,-trichloro-derivatives as well as the 4′-chloro- and 2-chloro-derivatives revealed estrogenicity to be inhibited by chloro-substitution on the phenolic ring. In addition, the comparison of estrogenic potency of the symmetrical 2′,4′,6′-trichloro-4-biphenylol and the nonsymmetrical 2′,3′,4′,5′-tetrachloro- or 2′,5′-dichloro-derivatives indicated the importance of molecular symmetry to estrogenicity.

The relationship of relative activity for biphenyls hydroxylated on one ring and chlorinated on the other ring is reported in the following equation:

$$\log(\text{binding potency}) = 0.89\,(\log \beta\text{-galactosidase activity}) - 0.73;$$

$$n = 5, \quad r^2 = 0.995, \quad s = 0.11, \quad F = 591, \quad Pr > F = 0.0001$$

This equation revealed near perfect correlation between estrogen-receptor binding and β-galactosidase activity with a slope of approximate unity and an intercept approaching zero. However, activities of biphenyls hydroxylated and chlorinated on the same ring were not in agreement. Specifically, ligand-binding was demonstrated for the latter compounds. However, no β-galactosidase activity was observed for these derivatives. Rather, as compared with controls, diminished absorbance at 650 nm showed these compounds to be cytotoxic to yeast.

DISCUSSION

The mean estrogenic activities reported here for 17-β-estradiol and 4-hydroxybiphenyl are consistent with those presented graphically by Routledge and Sumpter [13], 1×10^{-10} and 5×10^{-5}, respectively. These findings provide an interlaboratory validation of the present results.

The excellent correlation between estrogen receptor-binding and β-galactosidase activity for biphenyls hydroxylated on one ring and chlorinated on the other ring reveals that for these compounds, receptor-binding is the critical step in expressing estrogenicity. It is hypothesized that these chemicals interact directly with the estrogen receptor to mimic steroidal estrogen. Biphenyls hydroxylated and chlorinated on the same ring are weak binders to the estrogen receptor and are cytotoxic.

The recombinant yeast assays represent in vitro models with which to screen chemicals for estrogen modulating activity. In such a system, molecular interactions may be evaluated without possible organismal interactions that can cloud the identification of molecular structure or properties that are related to the actual mechanism of action. Recombinant yeast assays allow for reproducible empirical biological data to be obtained in a time- and cost-effective manner. This latter point is critical to the development of data for structure–activity assessment.

The same characteristics that make yeast systems so attractive are their short comings. Namely, they fail to account for the toxicokinetic and toxicodynamic complexity of the whole animal. Specifically, yeasts lack the ability to metabolically alter chemicals. As with other toxicologic endpoints, an array of in vitro assays is often more informative than is a single assay.

Although structure–activity relationships derived from in vitro databases may only qualitatively predict in vivo responses, the fact that recombinant yeast assays allow for time- and cost-effective data acquisition makes them a choice for structure–activity assessments. Moreover, yeast assays can be used as tools to screen compounds and aid in establishing priorities for future in vivo evaluation.

Structure–activity assessment is based on the principle that the behavior and properties of chemicals are derived directly from their molecular structural characteristics. Two conceptual approaches to structure–activity relationships, a pattern recognition approach [2,14] and a correlative approach [15] have been implemented in estrogenicity studies. Pattern recognition approaches such as comparative molecular field analyses attempt to identify common characteristics among structures that elicit similar toxicologic activity. Correlative approaches, on the other hand, relate variation in molecular structure, assessed quantitatively by molecular descriptors within a congeneric series of compounds to variance in toxicologic properties.

Recently, in an effort to elucidate structural features associated with estrogenicity, Routledge and Sumpter [13] examined the activity of a large number of alkylphenols. They noted that a free-ring hydroxyl group was necessary for activity, with estrogenicity being enhanced as the hydroxyl group was transferred from the ortho- to meta- to para- position. Comparisons of activity for para-alkyl derivatives, both normal and branched structures, indicated that estrogenicity required at least a three-carbon alkyl group and in general branched derivatives, especially tertiary ones, were more active than their corresponding normal-structured isomer [13].

In the present study, the symmetrical 2′,4′,6′-trichloro-derivative is more estrogenic than the nonsymmetrical 2′,3′,4′,5′-tetrachloro- or 2′,5′-dichloro-derivatives. This trend is consistent with the fact that maximal activity of alkylphenols was associated with derivatives of similar size and symmetry, namely a single tertiary-branched group consisting of six to eight carbons [13].

The absence of estrogenicity for the 3,4′,5,-trichloro- and 2-chloro-derivatives is consistent with the observations of Routledge and Sumpter [13] who noted that the position of alkyl-substitution on the phenolic ring, irrespective of homolog, reduced or completely abolished activity. The results of this study further support the hypothesis that an unhindered phenolic ring is important to estrogenic potency.

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