Dependence of Estrogenic Activity on the Shape of the 4-Alkyl Substituent in Simple Phenols

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Received September 6, 1999; accepted October 27, 1999

The ability of certain chemicals to mimic the effects of natural steroid hormones and their potential to disrupt the delicate balance of the endocrine system in animals has attracted much interest in recent years. Alkylphenolic chemicals have been reported to be weakly estrogenic. Estrogen receptor (ER) binding is primarily the result of interaction of the receptor with both a phenolic residue, and a hydrophobic pharmacophore. We have prepared and screened various phenols having a bulky 4-alkyl group, which may interact hydrophobically with the receptor, for estrogenic activity by using a previously described reporter gene assay employing COS-1 cells transfected with rat ERα-expression plasmid and an appropriate reporter plasmid. Some of the tested compounds, such as 4-(1-adamantyl)phenol and 4-cycloalkylphenols, exhibited much more potent activity than the typical estrogenic alkylphenol, 4-*tert*-octylphenol.

Key words estrogen; hydrophobic interaction; structure–activity relationship; reporter gene assay

The steroid hormone estrogen influences the growth, differentiation, and functioning of many target tissues. Estrogens play an important role in the female and male reproductive systems, and also in bone maintenance. The first step in the appearance of these activities is mediated by the binding of hormonal ligands to the estrogen receptors α and β (ERα and β).[1,2] The ER undergoes a conformational change, allowing the receptor to dimerize. The dimer binds with high affinity to chromatin to modulate the transcription of target genes.[3]

There is currently much debate over the health risks associated with the estrogenic activity of compounds that are either present in the environment or used industrially.[4] High binding affinity for ER and the appearance of substantial estrogenic activity require a phenolic ring, an appropriate hydrophobic group adjacent to the phenolic ring, and another hydroxyl group located at a suitable position on the molecule, as in 17β-estradiol (**1**). However, estrogenic activity has been found in a large range of structural prototypes, including non-steroidal compounds isolated from plants, such as flavonoids, genistein and coumesterol.[5] One of the reasons for the estrogenic activity of such a wide variety of organic chemicals appears to be that ER binding is primarily the result of interaction between the receptor and a phenolic residue.

Recently, we have reported potent estrogenic agonists bearing dicarba-*closo*-dodecaborane as a hydrophobic pharmacophore.[6] Compound **2**, which satisfies the above structural requirements for an estrogen, exhibited potent transcriptional activity for ERα in the concentration range of 10^{-10} M; its potency is at least 10-fold greater than that of 17β-estradiol (**1**). We also found that compound **3**, which lacks the alcoholic hydroxyl group, exhibited potent activity comparable to that of **1**. Furthermore, 4-(1-adamantyl)phenol (**5**), in which the substituent resembles the carborane cage in molecular size and shape, also showed moderate activity. It is important to clarify the structure–activity relations of phenolic compounds in order to evaluate the health risks of these chemicals. In this paper, we describe the dependence of estrogenic activity on the shape of the alkyl substituent in simple phenols having a bulky alkyl group at the 4-position, which may be present in the environment or used industrially.

MATERIALS AND METHODS

Materials 4-(1,1,3,3-Tetramethylbutan-1-yl)phenol (4-*tert*-octylphenol, **4**) and 4-(1-adamantyl)phenol (**5**) were purchased from Sigma-Aldrich Co., Ltd. 4-(1-Diamantyl)phenol (**6**) was prepared by the method previously reported.[7] The compounds having a secondary alkyl group at the 4-position of phenol (**7**, **9**, **12**, **13**, **14**) were prepared from 4-iodoanisole. Palladium-catalyzed coupling of 4-iodoanisole and the corresponding 5-, 6-, 7-, 8- or 12-membered cyclic alkenes followed by catalytic hydrogenation afforded 4-cyclic alkylanisoles. The 4-cyclic alkylanisoles were demethylated with BBr_3 to give 4-cyclic alkylphenols (**7**, **9**, **12**, **13**, **14**). The compounds having a tertiary alkyl group at the 4-position of phenol (**8**, **10**, **11**, **15**) were prepared by Friedel–Crafts alkylation of phenol with 1-methylcyclopentanol, 1-methylcyclohexanol, 1-ethylcyclohexanol or 3-ethylpentan-3-ol catalyzed by sulfuric acid.

Transfection and Luciferase Assays The estrogen receptor expression plasmid, pCI-rERα, was constructed by inserting the rat uterus estrogen receptor cDNA (pUCER6, which was obtained from the Health Science Research Resources Bank, Osaka, Japan) into the expression vector pCI-neo (Promega). The reporter plasmid, ERE×5-pGL-TK, was constructed by introducing five copies of the estrogen response element (ERE), caAGGTCAcccTGACCTcc, and the herpes simplex virus thymidine kinase promoter into the *Nhe*I-*Hind*III sites of the pGL3-Basic luciferase reporter vector (Promega). The COS-1 cells were obtained from

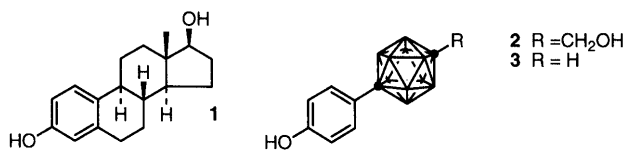

Fig. 1. Structures of 17β-Estradiol and Potent Estrogenic Agonists Bearing Carborane as a Hydrophobic Pharmacophore

In icosahedral cage structure, closed circles (●) represent carbon atoms and other vertices represent BH units.

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Japanese Cancer Research Resources Bank (JCRB) and were maintained in Phenol Red free Dulbecco's modified essential medium (DMEM), supplemented with 5% charcoal-stripped fetal bovine serum (FBS). For reporter gene assay, COS-1 cells were seeded in 24-well tissue culture plates at 6×10^4 cells per well. The cells were cultured at 37 °C in 5% CO_2 overnight and allowed to attach to the plates. Then, the medium was removed and transfection was performed with the cationic lipid transfection reagent Tfx-20 (Promega). Liposomes were formed by incubating 100 ng of pCI-rERα, 50 ng of ERE×5-pGL-TK, and 100 ng of the reference plasmid pCMVβ (Clontech) with 0.75 μl of Tfx-20 in phenol red-free DMEM (final 200 μl) for 10—15 min at room temperature. These DNA/Tfx-20 mixtures were added to the cells and the culture plates were returned to the CO_2 incubator. After 2 h, 800 μl of DMEM was supplemented with charcoal-stripped FBS (final 5%). After an additional 2 h, 5 μl of ethanol solution of ligands was added. After 18 h of incubation, the cells were harvested, and the luciferase assay was performed with the Luciferase Assay System (Promega). The luciferase activities were normalized to β-galactosidase activities. Assay was done in triplicate, under each condition.

RESULTS AND DISCUSSION

We have screened phenols having a bulky alkyl group at the 4-position (Fig. 2, **5**—**15**) for estrogenic activity by using a previously described reporter gene assay employing COS-1 cells transfected with rat ERα-expression plasmid and an appropriate reporter plasmid.[6] As a reference standard, we used 4-*tert*-octylphenol (**4**), a typical estrogenic chemical[8,9] and well-known component of the detergent Triton X-100. 4-*tert*-Octylphenol (**4**) at 1×10^{-7} M induced the expression of luciferase. The potency was approximately 1000 times weaker than that of **1**. Figure 3 summarizes the transcriptional activation by the tested compounds. 4-(1-Adamantyl)phenol (**5**) and 4-(1-diamantyl)phenol (**6**) exhibited more potent activity than **4**. On the other hand, a compound having a small alkyl group, 4-cyclopentylphenol (**7**) showed almost no activity below 1×10^{-7} M concentration. However, substitution of a methyl group at the 1′-position (4-(1-methylcyclopentan-1-yl)phenol (**8**)) or enlargement of the ring (4-cyclohexylphenol (**9**)) afforded compounds that produced a weak response at the concentration of 1×10^{-7} M. The response increased with each additional carbon, up to a maximum of 8 carbon atoms (4-cyclooctylphenol (**13**)). The activity seems to decrease when the carbon number exceeds 8—10 because 4-cyclododecanylphenol (**14**) was somewhat less potent than **13**.

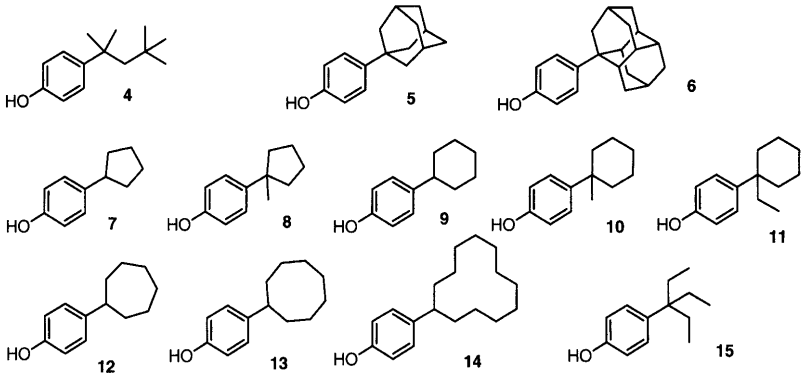

Fig. 2. Structures of Phenols Having a Bulky Alkyl Substituent at the 4-Position

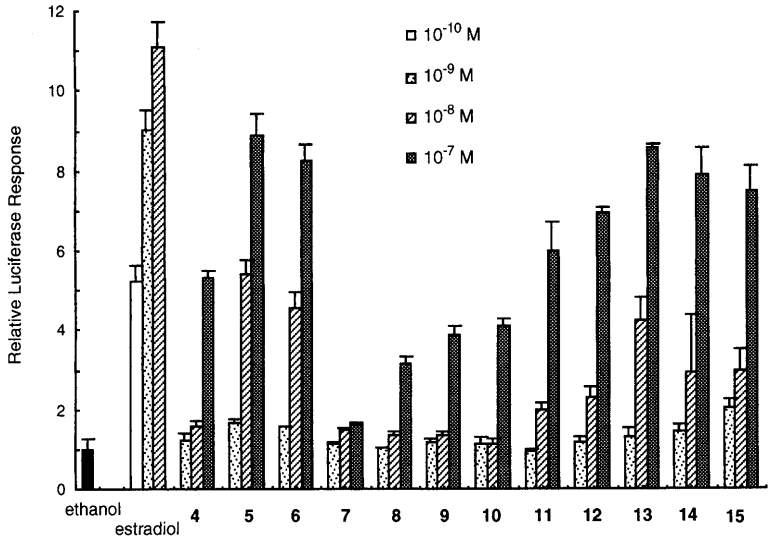

Fig. 3. Transcriptional Activation by the Test Compounds

COS-1 cells were transfected with ERE×5-pGL-TK and pCI-rERα and incubated with the test compounds at the indicated concentrations (10^{-9}—10^{-7} M). Results are shown as means±S.D. for triplicate transfections.

4-(3-Ethylpentan-3-yl)phenol (**15**) showed a comparable activity to **13**.

The effects of altering the chain length and of branching on the estrogenic potency of commercially available 4-substituted alkylphenols have been examined using an estrogen-inducible expression system in yeast.[9] The report indicated that 4-*tert*-octylphenol (**4**) had the greatest activity among the tested compounds and suggested that branching at the 1′-carbon may be an important feature for estrogenic activity.[9] Recent studies on the three-dimensional structure of the complex formed by 17β-estradiol and the human estrogen receptor-α ligand binding domain (hERαLBD) have revealed the structural requirements for the appearance of estrogenic activity.[10] 17β-Estradiol (**1**) is oriented in the hERα ligand-binding pocket by two types of contacts: hydrogen bonding at both ends and hydrophobic van der Waals contacts along the body of the skeleton. We have discussed the remarkable activity of 4-(1,12-dicarba-*closo*-dodecaboranyl)phenols (**2** and **3**) and suggested that the carborane cage works as a hydrophobic group for binding to the hydrophobic cavity of ER, and the hydrophobic van der Waals contacts along the spherical carborane cage produce a stronger interaction than that in the case of 17β-estradiol.[6] In the present experiment, phenols having medium-ring cyclic alkyl group at the 4-position exhibited much more potent activity than **4**. The relatively high activity of **5**, **6**, **13**—**15** also suggests that hydrophobic contact along a spherical shape produces a stronger interaction than that in the case of other alkylphenols.

In summary, we found that simple phenols having a bulky alkyl group at the 4-position exhibited much more potent activity than the typical estrogenic chemical, 4-*tert*-octylphenol. Although the potency of these compounds is moderate (approximately 200—300 times weaker than 17β-estradiol), the present finding should be helpful for the discovery of so-far-unidentified estrogenic chemicals and for predicting possible risk to humans.

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