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In Vitro Estrogenic Actions in Rat and Human Cells of Hydroxylated Derivatives of D16726 (Zindoxifene), an Agent with Known Antimammary Cancer Activity *in Vivo*[1]

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ABSTRACT

A series of 2-phenyl-1-ethyl-3-methylindoles with or without a hydroxyl group in the para position of the phenyl ring and the 5 or 6 position of the indole nucleus were compared with 17β-estradiol in the stimulation of (*a*) prolactin production in rat pituitary cells in primary culture, (*b*) progesterone receptor synthesis in MCF-7 cells, and (*c*) proliferation of MCF-7 cells. All compounds were less active than estradiol but all derivatives including D15414, the hydroxylated metabolite of D16726 (zindoxifene, a known antitumor agent against mammary cancer) were fully estrogenic.

Hydroxyl groups at the para position of the phenyl ring and 6 position of the indole nucleus conferred the highest estrogen potency [ED_{50} (drug concentration producing 50% of maximum activity) in all assays around 10^{-10} M]. Moving or eliminating the hydroxyl on the indole ring markedly reduced the estrogen potency; however, an even more dramatic reduction in estrogenic activity was produced by removing the hydroxyl of the phenyl ring.

INTRODUCTION

The first clue that direct antagonism of estrogen action was possible was discovered from the finding that dimethylstilbesterol could prevent the full vaginal cornification produced by estradiol alone (1, 2). A similar result can be obtained with 3,3′,5,5′-tetra-methyl-α,β-diethylstilbesterol (3). However, neither of these compounds is an antiestrogen when administered systemically (1, 3).

Since these early reports, a series of nonsteroidal antiestrogens have been developed. These have consistantly been triphenylethylene type structures containing an alkylaminoethoxy or glycerol side chain (4). The structural importance of this side chain for the antiestrogenic action of these agents has been demonstrated (5). Of the antiestrogens so far developed, nafoxidine (6–8), enclomiphene (9, 10), tamoxifen (11, 12), and trioxifene (13, 14) have been tested in phase I and II breast cancer clinical trials. The objective response rate of each antiestrogen was similar (~30%) but only tamoxifen has a low incidence of side effects (15, 16) and is at present the only antiestrogen available for the treatment of breast cancer. The success of antiestrogen therapy has prompted a search for new and more potent estrogen antagonists for clinical applications.

Systematic structural variation of the estrogens diethylstilbestrol and hexestrol has recently led to the development of a new group of compounds based on the 2-phenylindole skeleton (17). While some of these agents have been demonstrated to be full estrogen agonists, one (D16726 or zindoxifene, see Fig. 1) has been reported to be an impeded estrogen with high affinity for the estrogen receptor (18). Mammary tumor inhibitory activity has been reported for D16726 *in vivo* and its hydroxy derivative D15414, (compound I, Fig. 1) *in vitro* (17, 19). This is of particular interest if the tumor inhibitory activity of D16726 is by estrogen antagonism as this compound does not have a structural equivalent of the alkylaminoethoxy side chain.

We now report the activity of a series of 2-phenylindole derivatives, including D15414, in the stimulation of prolactin production in rat pituitary cells in primary culture and the stimulation of progesterone receptor synthesis and growth of MCF-7 cells.

MATERIALS AND METHODS

The derivatives of 2-phenylindole, synthesized as described previously (18), were a gift from Dr. E. Von Angerer, University of Regensburg, Federal Republic of Germany. 17β-Estradiol was purchased from Sigma Chemical Company (St. Louis, MO).

MCF-7 Cells. Stock MCF-7 cells were maintained in MEM[3] (GIBCO, Grand Island, NY) with phenol red, containing nonessential amino acids, 10 mM HEPES, 6 ng/ml insulin, 100 units/ml penicillin, 100 μg/ml streptomycin, and 5% calf serum (GIBCO) that was charcoal stripped. Experiments were performed in phenol red-free MEM (Sigma) made as described for phenol red-containing media.

MCF-7 cells were washed and plated (24-well plate; Falcon, Cockeysville, MD) at either 25,000 or 15,000 cells/well in 1 ml of phenol red-free media. Media on the cells plated at the higher density was changed after 48 h and the compounds added in ethanol (10 μl/well). These cells were cultured for a further 48 h before the progesterone receptor number was determined. Media on the cells plated at the lower density was changed after 24 and 72 h before media containing the compounds was added. This media was changed every day for 5 days before the DNA content was determined.

Progesterone Receptor Assay. Progesterone receptor content was determined in attached MCF-7 cells by whole cell uptake of [^{3}H]R5020 (specific activity, 86.9 ci/mmol; New England Nuclear, Boston, MA) in MEM (phenol red free). Total binding was estimated by incubating cells with 2 nM [^{3}H]R5020 alone and nonspecific binding, in separate wells, by the addition of 200-fold excess unlabeled R5020. After incubating with 0.5 ml of the [^{3}H]R5020 for 45 min at 37°C the cells in the wells were washed five times with 0.5 ml of HBSS (GIBCO) containing 25 mM HEPES. Wells designated for protein estimations were similarly washed. The radiolabeled cells were extracted with 1 ml of ethanol which was subsequently transferred to 10 ml of ACS scintillation liquid (Amersham, Arlington Heights, IL) and the radioactivity determined. Specific binding was calculated as total binding minus nonspecific binding. Estimates of each were made in duplicate. Cells in the protein wells were sonicated in 1 ml of HBSS diluted 1:9 with water and samples (100 μl) taken for protein determination. Protein was measured using the commercially available BioRad assay (BioRad Laboratories, Richmond, CA) and an IgG protein standard.

DNA Assay. Cells were sonicated in 1 ml of HBSS diluted 1:9 with water and DNA measured on samples fluorimetrically using Hoechst 33258 (Calbiochem-Behring, La Jolla, CA) according to the method of Labarca and Paigen (20).

Prolactin Assay in Primary Cultures of Pituitary Cells. Immature (18–21 days old) female rats of the Sprague-Dawley strain were obtained

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[3] The abbreviations used are: MEM, minimum essential medium; ED_{50}, drug concentration producing 50% of maximum activity; HBSS, Hanks' balanced salt solution; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid.

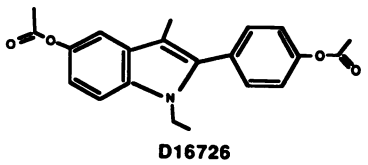
from the Holtzman Company (Madison, WI). The procedure for the maintenance of primary pituitary cell cultures and analysis of prolactin synthesis have been described in detail (21).

Results were calculated as percentage of prolactin synthesis relative to total protein synthesis. Values were plotted as percentage of prolactin synthesis to be consistent with our previous data (21, 22).

RESULTS

Prolactin Synthesis in Primary Cultures of Pituitary Cells. All the 2-phenylindole compounds examined (see Fig. 1) produced stimulation of prolactin production approaching the maximum levels produced by 17β-estradiol (see Fig. 2). The most active 2-phenylindole derivative, compound II (ED_{50}, 9×10^{-11} M), had hydroxyl groups in the para position of the phenyl ring and the 6 position of the indole nucleus and was about 1/30 as potent as estradiol (ED_{50}, 3×10^{-12} M). Moving the hydroxyl on the indole nucleus from position 6 to position 5 reduced the potency a further 30-fold (ED_{50} of compound I, 2.8×10^{-9} M). Removal of the hydroxyl on the indole nucleus (compound III) decreased the estrogenic activity to about 1/5000 of estradiol and demonstrated the structural requirement of this hydroxyl for estrogenic potency. However more important than hydroxyl substitutions on the indole ring was the hydroxyl on the phenyl ring. Removal of this para hydroxyl caused a dramatic reduction in estrogenic activity regardless of the hydroxyl groups on the indole nucleus (ED_{50} of compound IV, 3.5×10^{-7} M; compound V, 6.4×10^{-7} M).

Progesterone Receptor Induction in MCF-7 Cells. The 2-phenylindole derivatives tested in this assay induced increases in progesterone receptor concentration to the maximum levels stimulated by estradiol (see Fig. 3). The order of potency of these compounds to stimulate the progesterone receptor (II>I>III>IV>V) was the same as the order of potency for stimulating prolactin synthesis in the pituitary primary cell culture assay.



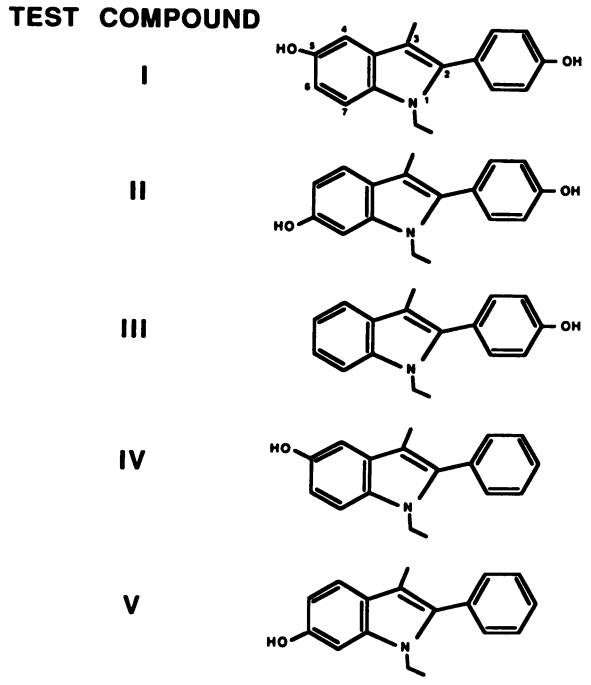


Fig. 1. Structures of the 2-phenylindole derivate D16726 (zindoxifene) and the hydroxylated 2-phenylindole derivatives used in the present study. Compound I (D15414) corresponds to the hydroxylated metabolite of D16726.

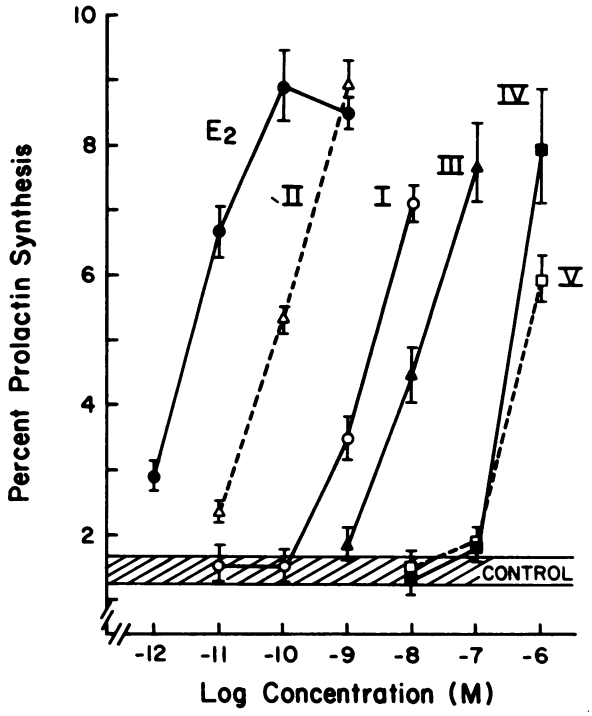


Fig. 2. Comparison of effects of E_2 and hydroxylated-2-phenylindole compounds (structures shown in Fig. 1) on prolactin synthesis of primary cultures of rat pituitary cells. Monodispersed pituitary cells (2×10^5/dish) were cultured for 6 days, as previously described (22), in media containing the indicated concentrations of compounds. Prolactin synthesis, expressed as a percentage of total protein synthesis, was determined as described previously (22). *Values,* means ± SE of three cultures per point.

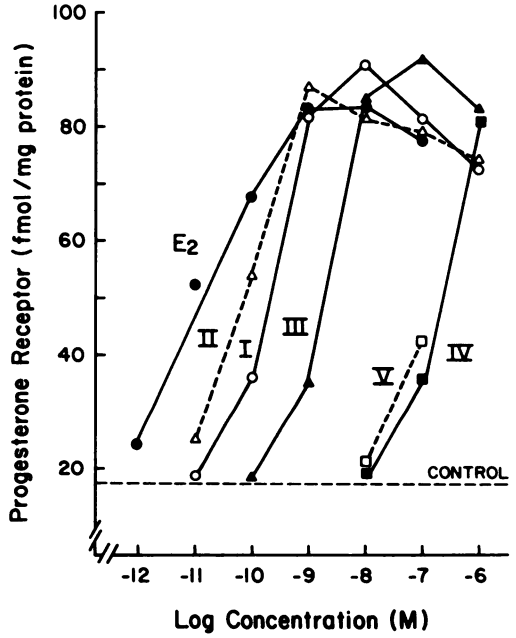


Fig. 3. Comparison of effects of estradiol and hydroxylated 2-phenylindole compounds (structures shown in Fig. 1) on progesterone receptor induction in MCF-7 cells. Cells were cultured for 2 days with compounds at the concentration indicated and then progesterone receptors determined by whole cell uptake of [^{3}H]R5020 (see "Materials and Methods"). *Values,* receptors per mg protein, mean of duplicate estimates.

The ED_{50} value for estradiol in this assay (1.5×10^{-11} M) was within a log concentration of the ED_{50} value for estradiol in the prolactin synthesis assay (3×10^{-12} M). The ED_{50} values for the 2-phenylindole derivatives in the progesterone receptor induction assay were similarly within a log concentration of their ED_{50} concentration for the prolactin synthesis assay.

Stimulation of Growth of MCF-7 Cells. The 2-phenylindole derivatives, compounds I and II, were both found to stimulate MCF-7 cell growth to the same maximum as estradiol (see Fig. 4). The order of potency of these two compounds again was consistent with the potency order determined in the prolactin synthesis assay. However this assay was more sensitive to the effects of estradiol (ED_{50}, 5×10^{-13} M) than either the prolactin synthesis assay or the progesterone receptor induction assay. In contrast similar sensitivity was seen for the 2-phenylindole derivatives in this assay (ED_{50} of compound I, 6×10^{-10} M; compound II, 3×10^{-10} M) as for the progesterone assay (ED_{50} compound I, 8×10^{-10} M; compound II, 1×10^{-10} M).

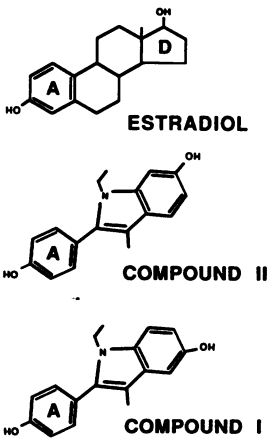


Fig. 5. Structural similarities between E_2 and the two most potent hydroxylated 2-phenylindole compounds. In the three *in vitro* assays used, compound II was more potent than compound I (D15414). The para hydroxyl on the phenyl ring may represent the hydroxyl on the A ring of E_2. The D ring hydroxyl of E_2 may then be represented by the hydroxyl substitution on the indole nucleus. From the estrogenic potencies compound II appears to fit the estrogen receptor more favorably than compound I.

DISCUSSION

Using three different assay systems *in vitro* we have demonstrated that all the 2-phenylindole compounds examined are fully estrogenic. This includes D15414 (compound I) which has been reported to be readily formed from D16726 (zindoxifene, an antitumor agent), by esterase activity *in vivo*, and believed to be the active metabolite (23).

D15414 (compound I) has previously been reported to markedly decrease [^{3}H]thymide incorporation into MCF-7 cells and the number of MCF-7 cells growing in culture with concentration between 10^{-8}–10^{-5} M (6, 8, 12). This is inconsistent with the findings presented here, where compound I caused maximal stimulation of MCF-7 cell growth at 10^{-8} and 10^{-7} M and maximal PgR induction at 10^{-8} and 10^{-7} M with a slight reduction at 10^{-6} M. The reasons for the different findings are at present unclear, however, phenol red was not present in the media used in our studies and concentrations lower than 10^{-8} M were examined.

Relative binding affinities for four of these 2-phenylindole derivatives with the calf uterus estrogen receptor has been reported (17) and the order of affinities (II>I>III>V) is the same as the order of estrogen potency reported in the present study.

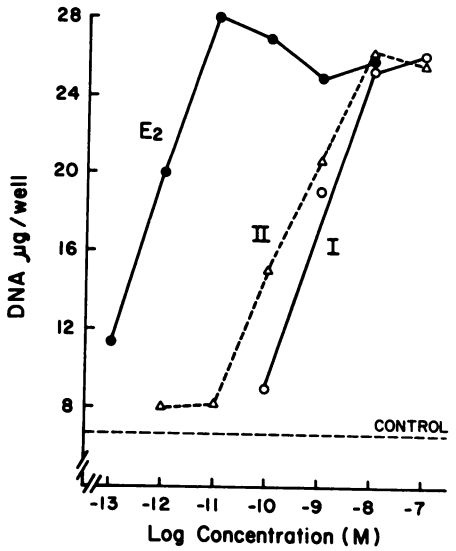


Fig. 4. Comparison of the effects of estradiol and hydroxylated 2-phenylindole compounds (structures shown in Fig. 1) on the proliferation of MCF-7 cells. Cells were cultured for 5 days with compounds at the concentrations indicated before being disrupted by sonication and DNA measured fluorometrically (see "Materials and Methods"). *Values*, mean of four estimates.

The acetylated version of compound II (structural equivalent of D16726 with the acetoxy group at the 6 position of the indole ring) has been reported to be a strong estrogen in the mouse uterine weight test, but nevertheless to have antitumor activity (18). Esterase activity *in vivo* would be expected to deacetylate this agent rapidly to form compound II, as with D15414 formation from D16726 (23). High dose estrogen therapy has been reported to cause regression in a number of hormone dependent tumors (24, 25). From our results with D15414 the antitumor action reported for D16726 (17–19) may also be a consequence of estrogen activity.

The change of estrogen activity observed with systematic alteration of the position of the hydroxyl groups on the 2-phenylindole derivatives indicates a structure activity relationship. Compound II, the most potent estrogen of the 2-phenylindole derivatives, has hydroxyl groups in the para position of the phenyl ring and 6 position of the indole nucleus, positions which can match the hydroxyl groups on the A and D rings of estradiol (see Fig. 5). The phenolic A ring of estradiol has been suggested to play a primary role in estrogen receptor binding while the D ring is involved in controlling activity (26). Compound I is a less potent estrogen than compound II as a consequence of moving the hydroxyl on the indole ring from position 6 to 5. This structural change presumably makes the interaction with the estrogen receptor less favorable for response mediation. Derivatives of 2-phenylindole without hydroxyl groups on the indole or in particular the phenyl ring emphasize the importance of these groups in producing potent estrogenic activity.

The lack of an alkylaminoethoxy side chain or equivalent is inconsistent with what is presently known of structural requirements for antiestrogens (4). The finding in this study that D15414 (compound I) is fully estrogenic is therefore in keeping with current models of drug interaction with the estrogen binding site of the estrogen receptor programming the cell for estrogenic or antiestrogenic activity.

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