

# 1-Benzyl-2-phenylindole- and 1,2-Diphenylindole-based Antiestrogens. Estimation of Agonist and Antagonist Activities in Transfection Assays

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In the 2-phenylindole system, the side chain at the nitrogen atom dominates the endocrine profile both in respect to the reduction of estrogenic action and the increase of antiestrogenic potency. In previous papers we reported on 2-phenylindoles with aliphatic side chains and various functional groups [Biberger, C. and von Angerer, E., *J. Steroid Biochem. Molec. Biol.*, 1996, 58, 31-43 and references therein]. In this study, we incorporated one or two phenyl rings into the side chain in order to lower the flexibility of the side chain. The sulfone group which was used as a polar function was linked to various positions of a benzyl or a phenyl group attached to the indole moiety. The relative binding affinities (RBA) ranged from 1.5 to 8.4% of estradiol. Agonist and antagonist activities were estimated in transfection assays using transiently transfected HeLa cells (cotransfected with the HEG0 vector) and stably transfected MCF-7/2a human breast cancer cells. The reporter plasmid contained the ERE from the Vitellogenin 2A gene, a viral tk promoter and the luciferase gene. Many of the new derivatives showed no or only very low estrogenic activity except for the compound 4e which contained two benzyl elements in the side chain. The antiestrogenic potency was very variable when concentrations 100-fold higher than that of estradiol were applied. The compound with the *para*-substituted benzyl fragment (4b) proved to be a pure antagonist in the transfection assays. It antagonized the effect of estradiol (10 nM) with an  $IC_{50}$  value of  $10^{-7}$  M. It also inhibited strongly the growth of estrogen-sensitive human MCF-7 mammary carcinoma cells ( $IC_{50}$ , 3 nM). Its activity was comparable to the one of the corresponding aliphatic 2-phenylindole derivative ZK 164.015. The data from the transcription and proliferation assays suggest that a phenyl ring can be incorporated into the side chain of pure antiestrogens without reducing their potency, provided the aromatic ring is *para*-substituted and a methylene group between the indole nitrogen and the phenyl group can act as hinge. © 1998 Elsevier Science Ltd. All rights reserved.

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## INTRODUCTION

In the last two decades, the antiestrogen tamoxifen has become the most important drug for the endocrine treatment of hormone-dependent breast cancer in postmenopausal women [1]. This development was accompanied by an intensive search for new estrogen antagonists with no or weaker estrogenic side effects

than those observed with tamoxifen [2]. Jean Bowler and Alan Wakeling were the first who succeeded to synthesize an antiestrogen, the estradiol derivative ICI 164,384, which was completely devoid of agonist activity [3]. Replacement of the amide function in the side chain by a sulfinyl group and terminal fluorination led to ICI 182,780 with considerably increased potency [4]. Similar structural elements were also found in another steroid-based antiestrogen, the  $11\beta$ -substituted estradiol derivative RU 58668 [5]. We have converted some heterocyclic structures such as the 2-phenylindole system [6] and 2-phenylbenzothiophene [7] into pure antiestrogens by

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Abbreviations: DMF, dimethyl formamide; ER, estrogen receptor; ERE, estrogen response element; RBA, relative binding affinity.

the introduction of side chains containing sulfone or sulfoxide functions.

Extensive studies of the last years have demonstrated that side chains, characterized by a polar function such as an amide, sulfone or sulfoxide group linked by a polymethylene spacer group to the receptor-binding moiety, determine the endocrine profile in respect to antagonism and potency. The lack of crystallographic data and the inherent flexibility made it difficult to model the steric structure of the side chain and calculate the distance between the functional group and the attachment point in the basic structure. An aliphatic chain consisting of six or more methylene groups can adopt a linear structure, but also has the potential to form a loop that brings both ends close together. A cyclic element such as a phenyl group would make the side chain more rigid and impose a distinct orientation of the functional group on it. One of the first examples for the use of this element was RU 58668 with a phenyl ring directly linked to carbon-11 of estradiol [5].

In our studies, we used the 2-phenylindole system as a basic structure because it has proved to be a very versatile molecule for the synthesis of potent antiestrogens [6, 8, 9]. The acidic hydrogen at the nitrogen allows the introduction of a variety of side chains by simple nucleophilic substitution reactions. The phenyl group as the rigid element was linked to the indole nitrogen either by a methylene group or directly. The overall length of the side chain including the functional group was kept constant in order to avoid alterations in lipophilicity. Estrogenic and antiestrogenic activities of the new 1-benzyl-2-phenylindoles and 1,2-diphenylindoles, respectively, were estimated *in vitro* using different transfection models. Estrogen receptor negative HeLa cells cotransfected with the EREwtc luc reporter plasmid and the HEG0 expression vector for the human estrogen receptor has proved to be a very sensitive instrument for the detection of residual agonist activity in antiestrogens and, in this respect, it turned out to be superior to the stably transfected MCF-7/2a cell line [9]. The latter lineage, however, is appropriate for quantifying antiestrogenic potencies of compounds *in vitro* [10]. In addition to these experiments with transfected cells cytostatic activities were determined in hormone sensitive MCF-7 breast cancer cells. Non-specific cytotoxic effects were estimated by experiments with hormone-independent MDA-MB 231 breast cancer cells.

## MATERIALS AND METHODS

### Chemistry

<sup>1</sup>H NMR spectra were obtained on a Bruker AC-250 spectrometer with TMS as internal standard and CDCl<sub>3</sub> as solvent, and are consistent with the

assigned structures. Mass spectra were recorded on a Varian MAT 311A spectrometer. All of the crystalline compounds were analyzed for C, H and N and the analyses were within  $\pm 0.40\%$  of the calculated values. The syntheses of the starting material 5-methoxy-2-(4-methoxyphenyl)-3-methylindole [11] and the reference compounds 5-hydroxy-2-(4-hydroxyphenyl)-3-methyl-1-(6-pyrrolidinohexyl)indole (ZK 119,010) [8] and 5-hydroxy-2-(4-hydroxyphenyl)-3-methyl-1-[10-(pentylsulfonyl)decyl]indole (23, ZK 164,015) [6] have been described previously.

For the synthesis of the new *N*-substituted 2-phenylindole derivatives two different approaches were applied. The 1-benzyl-2-phenylindole-derived compounds 4a-e and the reference compound 22 were prepared by the introduction of the complete side chain into the 2-phenylindole system with both phenolic hydroxy functions protected as tetrahydropyranyl ethers as outlined in Scheme 1. In the 1,2-diphenylindole derivatives 10a and 10b the side chains were successively built up before the methoxy functions were cleaved with lithium iodide in collidine. The phenolic compounds 10a and 10b were converted into the chemically more stable diacetates 11a and 11b. The synthetic details for the preparation of the new compounds used in this study will be published elsewhere. The analytical data for the final products are: 5-hydroxy-2-(4-hydroxyphenyl)-3-methyl-1-[4-[4-(pentylthio)butoxy]benzyl]indole (4a): yellow resin; C<sub>31</sub>H<sub>37</sub>NO<sub>3</sub>S (503.7); <sup>1</sup>H-NMR  $\delta$  0.69–2.11 (m, 13H, -(CH<sub>2</sub>)<sub>2</sub>-, -(CH<sub>2</sub>)<sub>3</sub>CH<sub>3</sub>), 2.19 (s, 3H, Ar-CH<sub>3</sub>), 2.30–2.72 (m, 4H, S(CH<sub>2</sub>-)<sub>2</sub>), 3.75–4.03 (m, 2H, -OCH<sub>2</sub>-), 5.07 (s, 2H, ArN-CH<sub>2</sub>-Ar), 6.54–7.36 (m, 11H, Ar-H). 5-Hydroxy-2-(4-hydroxyphenyl)-3-methyl-1-[4-[4-(pentylsulfonyl)butoxy]benzyl]indole (4b): m.p. (CHCl<sub>3</sub>) 137–138°C; C<sub>21</sub>H<sub>37</sub>NO<sub>5</sub>S (535.7); <sup>1</sup>H-NMR  $\delta$  0.86 (t, <sup>3</sup>J=7 Hz, 3H, -CH<sub>3</sub>), 1.20–1.41 (m, 4H, -(CH<sub>2</sub>)<sub>2</sub>CH<sub>3</sub>), 1.58–1.98 (m, 6H, -(CH<sub>2</sub>)<sub>2</sub>-, -CH<sub>2</sub>-), 2.10 (s, 3H, Ar-CH<sub>3</sub>), 2.99–3.18 (m, 4H, SO<sub>2</sub>(CH<sub>2</sub>-)<sub>2</sub>), 3.82–3.99 (m, 2H, -OCH<sub>2</sub>-), 5.10 (s, 2H, ArN-CH<sub>2</sub>-Ar), 6.58 (dd, <sup>3</sup>J=9 Hz, <sup>4</sup>J=2 Hz, 1H, indole-H<sup>6</sup>), 6.76 (s, 4H, Ar-H), 6.80 (d, <sup>4</sup>J=2 Hz, 1H, indole-H<sup>4</sup>), 6.85, 7.16 (AA'BB', <sup>3</sup>J=9 Hz, 4H, Ar-H), 7.06 (d, <sup>3</sup>J=9 Hz, 1H, indole-H<sup>7</sup>), 8.69 (s, br, 1H, -OH), 9.66 (s, broad, 1H, -OH), FD-MS (MeOH) m/z (%) = 535 (100; M<sup>+</sup>). 5-Hydroxy-2-(4-hydroxyphenyl)-3-methyl-1-[3-[5-(pentylsulfonyl)pentyl]oxy]benzyl]indole (4c): amorphous solid; C<sub>32</sub>H<sub>39</sub>NO<sub>5</sub>S (549.7); <sup>1</sup>H-NMR  $\delta$  0.68–2.29 (m, 15H, -(CH<sub>2</sub>)<sub>3</sub>-, -(CH<sub>2</sub>)<sub>3</sub>CH<sub>3</sub>), 2.15 (s, 3H, Ar-CH<sub>3</sub>), 2.72–3.15 (m, 4H, SO<sub>2</sub>(CH<sub>2</sub>-)<sub>2</sub>), 3.60–3.94 (m, 2H, -OCH<sub>2</sub>-), 5.06 (s, 2H, ArN-CH<sub>2</sub>-Ar), ~5.2 (s, broad, 1H, -OH), 6.12–7.24 (m, 12H, Ar-H, -OH). 5-Hydroxy-2-(4-hydroxyphenyl)-3-methyl-1-[2-[6-(pentylsulfonyl)hexyl]oxy]benzyl]indole (4d): amorphous solid; C<sub>33</sub>H<sub>41</sub>NO<sub>5</sub>S (563.8); <sup>1</sup>H-NMR  $\delta$  0.69–2.33 (m, 17H, -(CH<sub>2</sub>)<sub>4</sub>-, -(CH<sub>2</sub>)<sub>3</sub>CH<sub>3</sub>), 2.22 (s, 3H, Ar-

CH<sub>3</sub>), 2.75–3.18 (m, 4H, SO<sub>2</sub>(CH<sub>2</sub>)<sub>2</sub>), 3.75–4.13 (m, 2H, -OCH<sub>2</sub>-), 5.07 (s, 2H, ArN-CH<sub>2</sub>-Ar), 5.15 (s, broad, 1H, -OH), 6.32–7.31 (m, 11H, Ar-H), 6.49 (s, broad, 1H, -OH). 5-Hydroxy-2-(4-hydroxyphenyl)-3-methyl-1-[3-[3-(pentylsulfonylmethyl)benzyloxy]benzyl]indole (**4e**): amorphous solid; C<sub>35</sub>H<sub>37</sub>NO<sub>5</sub>S (549.7); <sup>1</sup>H-NMR 0.66–1.98 (m, 9H, -(CH<sub>2</sub>)<sub>3</sub>CH<sub>3</sub>), 2.16 (s, 3H, Ar-CH<sub>3</sub>), 2.60–2.94 (m, 2H, -SO<sub>2</sub>CH<sub>2</sub>-), 4.15 (s, 2H, Ar-CH<sub>2</sub>SO<sub>2</sub>-), 4.86 (s, 2H, Ar-OCH<sub>2</sub>-Ar), 5.04 (s, 2H, ArN-CH<sub>2</sub>-Ar), 6.30–7.44 (m, 16H, Ar-H, -OH). 5-Hydroxy-2-(4-hydroxyphenyl)-3-methyl-1-[4-[4-(pentylsulfonyl)-butoxymethyl]-phenyl]-indole (**10a**): yellow resin; C<sub>31</sub>H<sub>37</sub>NO<sub>5</sub>S (535.7); <sup>1</sup>H-NMR 0.68–2.18 (m, 13H, -(CH<sub>2</sub>)<sub>2</sub>-, -(CH<sub>2</sub>)<sub>3</sub>CH<sub>3</sub>), 2.33 (s, 3H, Ar-CH<sub>3</sub>), 2.74–3.15 (m, 4H, SO<sub>2</sub>(CH<sub>2</sub>)<sub>2</sub>), 3.27–3.59 (m, 2H, -OCH<sub>2</sub>-), 4.50 (s, 2H, Ar-CH<sub>2</sub>O-), 6.63–7.40 (m, 11H, Ar-H). 5-Hydroxy-2-(4-hydroxyphenyl)-3-methyl-1-[3-[5-(pentylsulfonyl)-pentyloxymethyl]-phenyl]-indole (**10b**): m.p. (CHCl<sub>3</sub>) 74–75°C. C<sub>32</sub>H<sub>39</sub>NO<sub>5</sub>S (549.7); <sup>1</sup>H-NMR 0.68–2.10 (m, 15H, -(CH<sub>2</sub>)<sub>3</sub>-, -(CH<sub>2</sub>)<sub>3</sub>CH<sub>3</sub>), 2.25 (s, 3H, Ar-CH<sub>3</sub>), 2.72–3.26 (m, 6H, SO<sub>2</sub>(CH<sub>2</sub>)<sub>2</sub>, -OCH<sub>2</sub>), 4.32 (s, 2H, Ar-CH<sub>2</sub>O-), 5.36 (s, br, 1H, -OH), 6.45 (s, br, 1H, -OH), 6.5–7.35 (m, 11H, Ar-H); MS m/z (%) 550 (24; [MH]<sup>+</sup>); 549 (66; M<sup>+</sup>); 414 (11; [M-SO<sub>2</sub>C<sub>5</sub>H<sub>11</sub>]<sup>+</sup>); 344 (26; [M-(CH<sub>2</sub>)<sub>5</sub>SO<sub>2</sub>C<sub>5</sub>H<sub>11</sub>]<sup>+</sup>); 328 (46; [M-O(CH<sub>2</sub>)<sub>5</sub>SO<sub>2</sub>C<sub>5</sub>H<sub>11</sub>]<sup>+</sup>). 5-Acetoxy-2-(4-acetoxyphenyl)-3-methyl-1-[4-[4-(pentylsulfonyl)-butoxymethyl]phenyl]-indole (**11a**): colorless resin; C<sub>35</sub>H<sub>41</sub>NO<sub>7</sub>S (619.8); <sup>1</sup>H-NMR 0.69–2.19 (m, 13H, -(CH<sub>2</sub>)<sub>2</sub>-, -(CH<sub>2</sub>)<sub>3</sub>CH<sub>3</sub>), 2.24 (s, 3H, Ar-CH<sub>3</sub>), 2.36 (s, 6H, CH<sub>3</sub>CO<sub>2</sub>-), 2.76–3.16 (m, 4H, SO<sub>2</sub>(CH<sub>2</sub>)<sub>2</sub>), 3.25–3.61 (m, 2H, -OCH<sub>2</sub>), 4.48 (s, 2H, Ar-CH<sub>2</sub>O-), 6.75–7.49 (m, 11H, Ar-H). 5-Acetoxy-2-(4-acetoxyphenyl)-3-methyl-1-[3-[5-(pentylsulfonyl)-pentyloxymethyl]-phenyl]-indole (**11b**): m.p. (EtOH/hexane) 57°C; C<sub>36</sub>H<sub>43</sub>NO<sub>7</sub>S (633.8); <sup>1</sup>H-NMR 0.91 (t, <sup>3</sup>J=7 Hz, 3H, -CH<sub>3</sub>), 1.23–1.67, 1.75–1.93 (m, 12H, -(CH<sub>2</sub>)<sub>3</sub>-, -(CH<sub>2</sub>)<sub>3</sub>CH<sub>3</sub>), 2.28 (s, 3H, Ar-CH<sub>3</sub>), 2.34 (s, 3H, CH<sub>3</sub>CO<sub>2</sub>-), 2.36 (s, 3H, CH<sub>3</sub>CO<sub>2</sub>-), 2.87–3.01 (m, 4H, SO<sub>2</sub>(CH<sub>2</sub>)<sub>2</sub>), 3.35 (t, <sup>3</sup>J=6 Hz, 2H, -OCH<sub>2</sub>-), 4.43 (s, 2H, Ar-CH<sub>2</sub>O-), 6.90 (dd, <sup>3</sup>J=9 Hz, <sup>4</sup>J=2 Hz, 1H, indole-H<sup>6</sup>), 7.01, 7.19 (AA'BB', <sup>3</sup>J=9 Hz, 4H, Ar-H), 7.09 (d, <sup>4</sup>J=2 Hz, 1H, indole-H<sup>4</sup>), 7.05–7.37 (m, 5H, Ar-H). 5-Hydroxy-2-(4-hydroxyphenyl)-3-methyl-1-[4-[5-(pentylsulfonyl)pentyloxy]butyl]indole (**22**): oil; C<sub>29</sub>H<sub>41</sub>NO<sub>5</sub>S (515.7); <sup>1</sup>H-NMR (ppm) = 0.67–2.23 (m; 19H; -(CH<sub>2</sub>)<sub>2</sub>-, -(CH<sub>2</sub>)<sub>3</sub>-, -(CH<sub>2</sub>)<sub>3</sub>CH<sub>3</sub>); 2.10 (s; 3H; Ar-CH<sub>3</sub>); 2.76–3.40 (m; 8H; O(CH<sub>2</sub>)<sub>2</sub>, SO<sub>2</sub>(CH<sub>2</sub>)<sub>2</sub>); 3.96 (t; <sup>3</sup>J=7 Hz; 2H; ArN-CH<sub>2</sub>-); 6.62–7.36 (m; 7H; Ar-H).

#### Materials and reagents for bioassays

[<sup>3</sup>H]17β-Estradiol was purchased from New England Nuclear (Dreieich, Germany); all other biochemicals including tamoxifen were obtained from

Sigma (Munich, Germany). 4-Hydroxytamoxifen was a gift from Professor P. W. Jungblut, Hannover (Germany). ICI 182,780 was generously provided by Dr M. R. Schneider, Berlin (Germany). Hormone-sensitive human MCF-7 breast cancer cells and hormone-independent human MDA-MB 231 breast cancer cells were obtained from the American Type Culture Collection (ATCC), human HeLa cervix carcinoma cells from the European Collection of Animal Cell Cultures. MCF-7/2a cells with the reporter construct integrated in the genome had been cloned in the authors' laboratory [10]. The luciferase reporter plasmid EREwtc luc harboring the luciferase gene from *Photinus pyralis* under the control of an estrogen response element (ERE) was synthesized from pGEM/luc (Promega) and EREwtc [12] (generously provided by Dr Klein-Hitpaß, Essen, Germany) in the authors' laboratory as described [13]. The estrogen receptor expression vector HEG0 [14] was generously provided by Professor P. Chambon, Strasbourg. HBS buffer was prepared from a solution of 32.0 g NaCl, 1.48 g KCl, 0.5 g Na<sub>2</sub>HPO<sub>4</sub>·2H<sub>2</sub>O, 4.0 g D-(+)-glucose, 20.0 g HEPES in 200 ml of water, adjusted to pH 7.5 with NaOH, which was diluted by a factor of 10 and adjusted to pH 7.08.

#### Estrogen receptor binding assay

For the determination of relative binding affinities (RBA), the previously described procedure was applied with modifications [11]. The 500 μl-incubation mixture comprised 5 nM [<sup>3</sup>H]17β-estradiol [added in 100 μl Tris-buffer (0.01 M, pH 7.5), supplemented with EDTA (0.01 M) and NaN<sub>3</sub> (0.003 M)], 10<sup>-9</sup> to 10<sup>-5</sup> M competing ligand (in 100 μl buffer), 100 μl of calf uterine cytosol and buffer. The mixture was incubated for 18 h at 4°C, after which 0.5 ml of dextran-coated charcoal (DCC) slurry (0.8% charcoal Norit A and 0.008% dextran in buffer) was added to the tubes and the contents were mixed. The tubes were incubated for 90 min at 4°C and then centrifuged at 700 × g for 10 min to pellet the charcoal. An aliquot (100 μl) of the supernatant was removed and radioactivity was determined by liquid scintillation spectrometry after addition of 3 ml of Quickszint 212 (Zinsser). Nonspecific binding was calculated using 5 μM 17β-estradiol as the competing ligand. Radioactivity was plotted as a function of the log concentration of the competing ligand in the assay. RBA was calculated as the ratio of the molar concentrations of estradiol and test compound required to decrease the amount of bound radioactivity by 50%, multiplied by 100.

#### Estimation of estrogenic activity in transiently transfected HeLa cells

HeLa cells were grown in Dulbecco's modified Eagle medium supplemented with 5% FCS, 100 U penicillin, 100 μg streptomycin and 150 mg L-gluta-

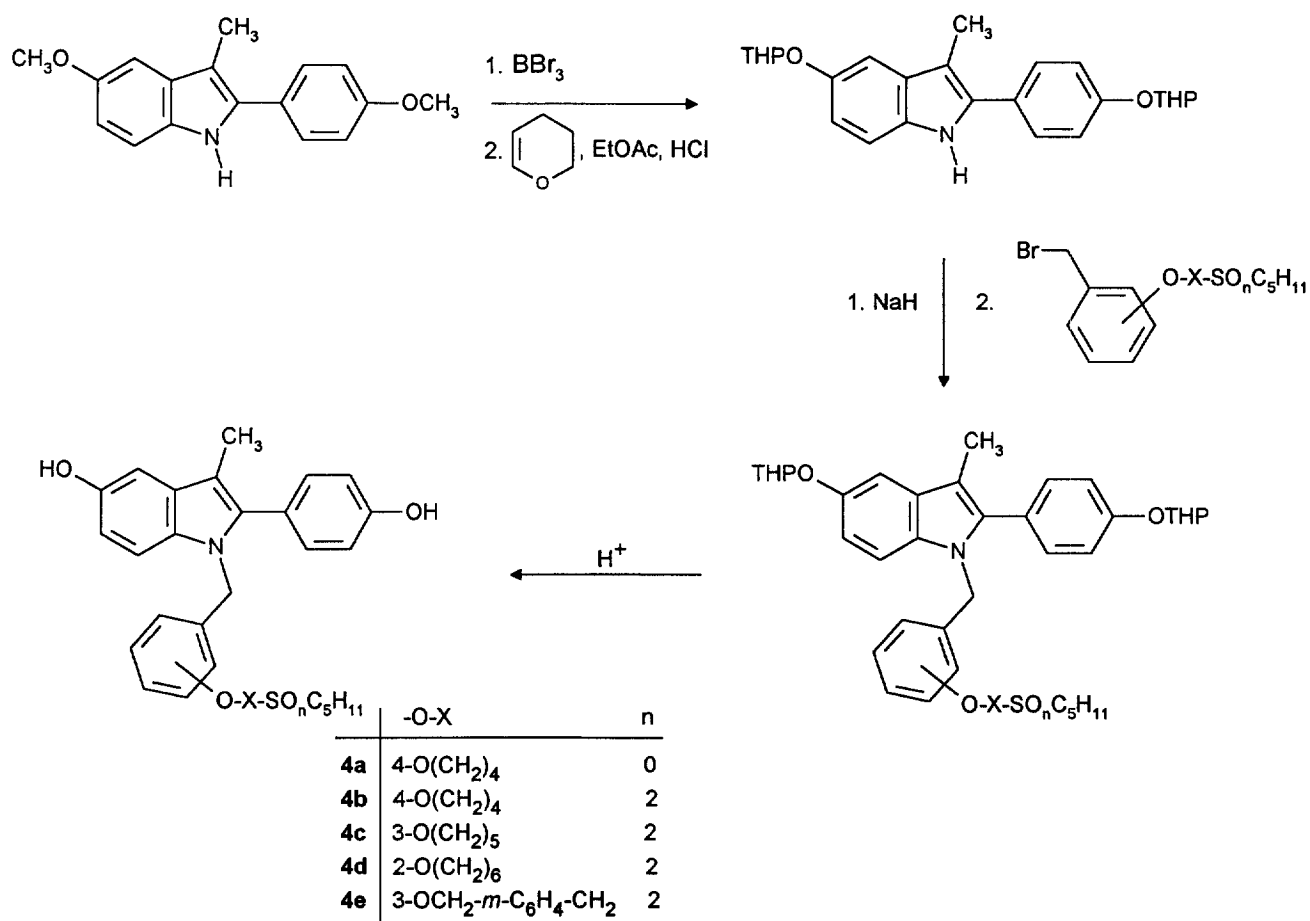
mine in 500 ml of medium without phenol red. Approximately 1 week before the start of the experiment, cells were cultivated with medium supplemented with 10% dextran/charcoal-treated fetal calf serum (ct-FCS). Transient transfections were performed in six-well plates by the calcium phosphate/DNA coprecipitation method as described previously [9]. Cells were seeded at a titer of  $1.5 \times 10^5$ /well and grown for 24 h before transfection with DNA ( $2 \mu\text{g}$  EREwtc luc plus  $0.1 \mu\text{g}$  HEG0). Estradiol and antiestrogens were added to the cells simultaneously with DNA. 4 h after the addition of DNA solution, medium was removed and cells were washed with 2 ml of PBS, followed by treatment with glycerol (15% in PBS) for 2 min. After washing twice with PBS, fresh medium containing the test substances was added. Cells were harvested 18 h after transfection. Before harvesting, cells were washed twice with PBS. Cell lysis buffer (200  $\mu\text{l}$ , pH 7.8) containing 5 mM TRIS-phosphate, 0.4 mM dithioerythritol, 0.4 mM 1, 2-diaminocyclohexane-*N,N,N',N'*-tetraacetate, 10% glycerol and 1% Triton X-100 was added to each well. After 20 min at room temperature, cells were collected with a rubber

policeman, cleared by centrifugation and stored at  $-20^\circ\text{C}$ .

Luciferase was assayed using the Promega kit according to manufacturer's protocol. The luminescence of 30  $\mu\text{l}$  samples and 100  $\mu\text{l}$  Promega assay solution was measured in a luminometer Lumat LB 9501 (Berthold, Wildbad, Germany). Luminescence (in relative light units, RLU) was integrated over 10 s. The background was approximately 250 RLU/10s. With samples from *Photinus pyralis* (Boehringer, Mannheim, Germany) a linear correlation between luminescence (RLU/10s) and the amount of luciferase was established in the range from 0.0001 to 30  $\mu\text{g}$  enzyme with 3000 RLU referring to 10 fg luciferase. Measurements were corrected for the protein content of the samples quantified according to Bradford [15] using bovine serum albumin as standard. Unless stated otherwise, average values and the deviations of three independent measurements are shown.

#### Quantification of antiestrogenic activity in stably transfected MCF-7/2a cells

The protocol for transient transfectants was used with modifications. The addition of DNA and treatment with glycerol was omitted. Untreated cells were



Scheme 1. Synthesis of 1-benzyl-2-phenylindoles **4a-e** (THP = tetrahydropyran-2-yl).

seeded 24 h prior to the addition of estradiol alone or in combination with an antagonist. The incubation period was 50 h.

#### Determination of cytostatic activity in MCF-7 human breast cancer cells

Hormone-sensitive human MCF-7 breast cancer cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD). Cells were grown in EMEM, supplemented with sodium pyruvate (110 mg/ml), gentamycin sulfate (50 mg/l), NaHCO<sub>3</sub> (2.2 g/l) and 10% FCS (Gibco). The serum was sterilized through a 0.20 μm filter (Sartorius, Göttingen, FRG) and stored at -20°C. Cells were grown in a humidified incubator in 5% CO<sub>2</sub> at 37°C and harvested with 0.05% trypsin-0.02% EDTA in 0.15 M NaCl. At the start of the experiment, the cell suspension was transferred to 96-well microplates (100 μl/well). After growing them for 3 days in a humidified incubator with 5% CO<sub>2</sub> at 37°C, the medium was replaced by one containing the test compound and 10% dextran-charcoal (DCC) treated FCS to avoid interference with steroidal hormones in the serum. Control wells (16/plate) contained 0.1% of DMF that was used for the preparation of the stock solution. The initial cell density was determined by addition of glutaric dialdehyde (1% in PBS; 100 μl/well). After incubation for 4-7 days, the medium was removed and 100 μl of glutaric aldehyde in PBS (1%) were added for fixation. After 15 min, the solution of aldehyde was decanted. Cells were stained by treating them for 25 min with 100 μl of an aqueous solution of crystal violet (0.02%). After decanting, cells were washed several times with water to remove adherent dye. After the addition of 100 μl of ethanol (70%), plates were gently shaken for 2 h. The optical density of each well was measured in a microplate autoreader EL 309 (Bio-tek) at 578 nm.

#### Determination of cytostatic activity in MDA-MB 231 cells

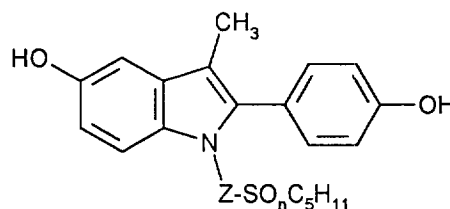
The protocol for MCF-7 cells was used with modifications: cells were grown in McCoy 5A medium supplemented with 5% FCS. The period of incubation was reduced to 2-3 days.

## RESULTS

### Estrogen receptor binding affinity

One of the major goals of this study was to elaborate which orientation of the side chain that carries the functional group would be the most favorable one in respect to the binding to the estrogen receptor. All of the hydroxy derivatives bind to the calf uterine estrogen receptor with RBA values ranging from 1.1 to 8.4% of 17β-estradiol (Table 1). In the 1-benzyl series, the affinity gradually decreased from the *para*-

Table 1. Binding affinities of 1-benzyl-2-phenyl- and 1,2-diphenylindoles for the estrogen receptor



Compound	Z	n	RBA <sup>a</sup>
4a	CH <sub>2</sub> - <i>p</i> -C <sub>6</sub> H <sub>4</sub> -O(CH <sub>2</sub> ) <sub>4</sub>	0	1.7
4b	CH <sub>2</sub> - <i>p</i> -C <sub>6</sub> H <sub>4</sub> -O(CH <sub>2</sub> ) <sub>4</sub>	2	8.4
4c	CH <sub>2</sub> - <i>m</i> -C <sub>6</sub> H <sub>4</sub> -O(CH <sub>2</sub> ) <sub>5</sub>	2	5.8
4d	CH <sub>2</sub> - <i>o</i> -C <sub>6</sub> H <sub>4</sub> -O(CH <sub>2</sub> ) <sub>6</sub>	2	1.5
4e	CH <sub>2</sub> - <i>m</i> -C <sub>6</sub> H <sub>4</sub> -OCH <sub>2</sub> - <i>m</i> -C <sub>6</sub> H <sub>4</sub>	2	2.2
10a	<i>p</i> -C <sub>6</sub> H <sub>4</sub> -CH <sub>2</sub> -O(CH <sub>2</sub> ) <sub>4</sub>	2	4.4
10b	<i>m</i> -C <sub>6</sub> H <sub>4</sub> -CH <sub>2</sub> -O(CH <sub>2</sub> ) <sub>5</sub>	2	1.1
11a <sup>b</sup>	<i>p</i> -C <sub>6</sub> H <sub>4</sub> -CH <sub>2</sub> -O(CH <sub>2</sub> ) <sub>4</sub>	2	2.5
11b <sup>c</sup>	<i>m</i> -C <sub>6</sub> H <sub>4</sub> -CH <sub>2</sub> -O(CH <sub>2</sub> ) <sub>5</sub>	2	0.5
22	(CH <sub>2</sub> ) <sub>4</sub> -O(CH <sub>2</sub> ) <sub>5</sub>	2	3.3
23 (ZK 164,015)	(CH <sub>2</sub> ) <sub>10</sub>	2	5.2
Tamoxifen			0.36
ICI 182,780			6.2

<sup>a</sup>Relative binding affinities for the calf uterine estrogen receptor; value for 17β-estradiol = 100.

<sup>b</sup>Diacetate of 10a.

<sup>c</sup>Diacetate of 10b.

(4b) to the *ortho*-substituted compound (4d). An additional phenyl ring had no positive effect. In the 1-phenyl series, again the *para*-orientation of the substituents provided the best binding conditions. However, affinities of the representatives of this type were somewhat lower than those of the corresponding 1-benzyl compounds.

For synthetic reasons the connecting element between the benzyl fragment and the remaining part of the side chain was an oxygen atom instead of a methylene group. Therefore, we synthesized the indole 22 which also contains an ether function in the corresponding position and used it as additional reference compound. The comparison of the 2-phenylindoles with aromatic side chains with the aliphatic derivatives 22 and 23 showed that the introduction of a phenyl group slightly increased the affinity provided the benzene ring is *para*-substituted.

### In vitro estrogenic activity

Since all of the indole derivatives of this study bind to estrogen receptor with RBA values in the same order of magnitude as the reference drugs tamoxifen (0.36) and ICI 182,780 (6.2) endocrine activity could be expected. An *in vitro* model that had proved to be appropriate for the detection of agonist activity are estrogen receptor negative HeLa cells cotransfected with the luciferase reporter plasmid EREwtc luc and the expression vector HEG0 for the human wild-type

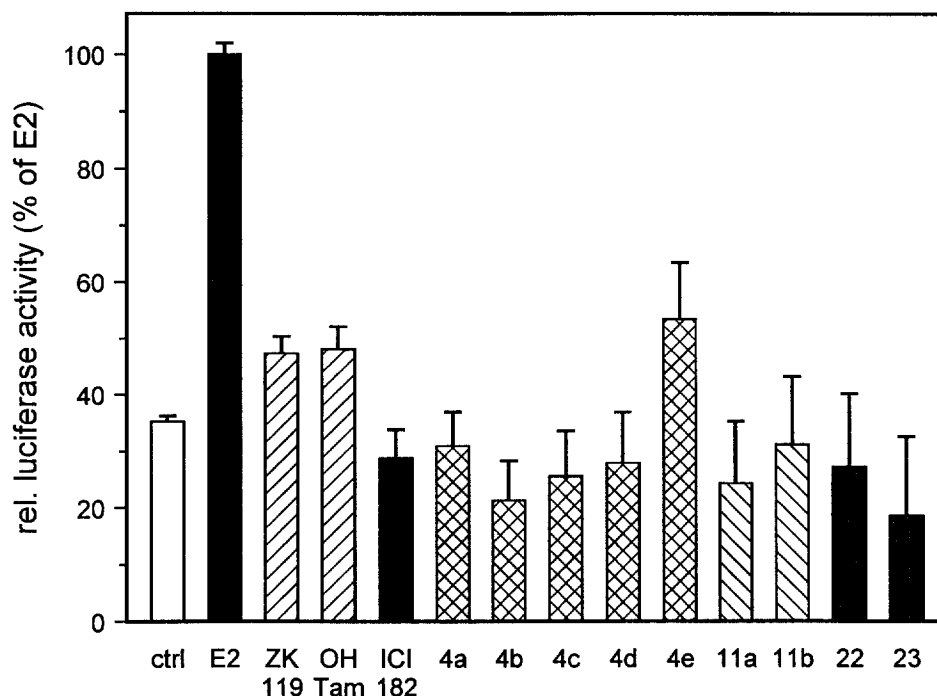


Fig. 1. Luciferase expression in HeLa cells cotransfected with the reporter plasmid EREwtc luc and the estrogen receptor expression vector HEG0, and treated with  $17\beta$ -estradiol (E2, 10 nM), ICI 182,780 (ICI, 1  $\mu$ M), 4-hydroxytamoxifen (OHTam, 1  $\mu$ M), ZK 119,010 (ZK 119, 1  $\mu$ M), and indole derivatives 4a-e, 11a, b, 22 and 23 (1  $\mu$ M). Values are means of three independent experiments  $\pm$  SD.

estrogen receptor. As shown in Fig. 1, partial antagonists such as 4-hydroxytamoxifen and ZK 119,010 [16] stimulated the luciferase expression to a certain extent whereas the value for the pure antiestrogen ICI 182,780 was even below that of the control cells which were only treated with steroid-depleted serum. With one exception (4e) all of the indole derivatives were unable to stimulate luciferase activity when added in a 1  $\mu$ M concentration. In the case of the 1,2-diphenylindoles only the acetates 11a and 11b were tested because of their higher chemical stability. No significant difference between the results obtained with the diacetates and those from the free hydroxy derivatives has been observed in various cellular assays.

#### *In vitro* antiestrogenic activity

The lack of agonist activity could be taken as a hint that these compounds are antagonists. A good model for the estimation antiestrogenic activity are estrogen receptor-positive MCF-7/2a breast cancer cells stably transfected with the reporter construct [10]. Unfortunately, this line proved to be less suitable for the detection of residual estrogenic activities in antiestrogens because stimulatory effects of partial agonists were rather weak in this system (data not shown). Efforts to clone a stably transfected HeLa cell line which expresses both the estrogen receptor and the luciferase under control of an estrogen response element have failed.

When MCF-7/2a cells were treated with combinations of estradiol ( $10^{-8}$  M) and various antagonists in 100-fold higher concentration the luciferase activity measured was very variable (Fig. 2). Some of the indoles (4b, 4c, 22, 23) reduced the luciferase expression to values close to the control value whereas others only partially inhibited the estradiol-stimulated luciferase activity. The compound 11b with a *meta*-substituted phenyl ring linked directly to the indole was inactive in this experiment.

The most active derivative 4b was compared with the steroidal pure antiestrogen ICI 182,780 and indole-based pure antagonist 23 (ZK 164,015) over a wider concentration range (Fig. 3). Its  $IC_{50}$  value ( $8 \cdot 10^{-8}$  mol/l) was between that of ICI 182,780 ( $5 \cdot 10^{-8}$ ) and 23 ( $2 \cdot 10^{-7}$ ). The complete lack of agonist activity and the suppression of estrogen-stimulated luciferase expression characterized 4b as pure antiestrogen in these *in vitro* assays.

#### *Cytostatic activity*

The main goal of our studies is the identification of compounds which might be useful for treatment of hormone-dependent breast cancer. Therefore, we investigated the activity of the new indoles in estrogen receptor positive human MCF-7 breast cancer cells. All of the compounds inhibited the growth of these cells, but the inhibitory effect was strongly dependent on the side chain structure (Fig. 4, Table 2). Strong inhibitors were only found in the 1-benzyl series (4a-

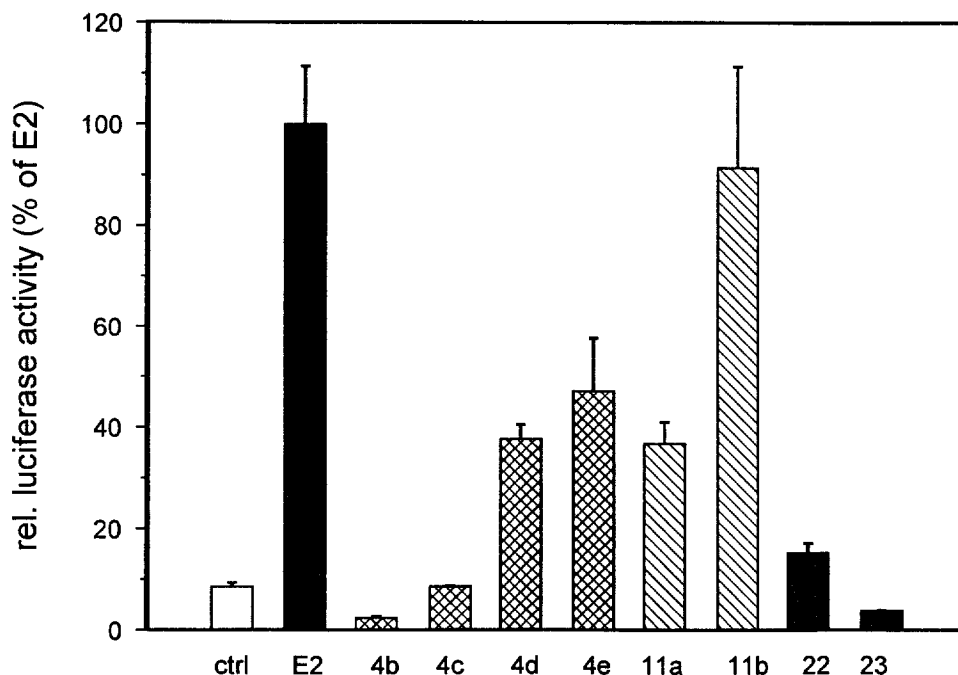


Fig. 2. Luciferase expression in MCF-7/2a cells, stably transfected with the reporter plasmid EREwtc luc and treated with  $17\beta$ -estradiol (E2, 10 nM) alone or in combination with indole derivatives 4a-e, 11a, b, 22 and 23 (1  $\mu$ M). Values are means of three independent experiments  $\pm$  SD.

c) (Fig. 4). Again, the most potent compound was 4b, which was as active as 23 with the aliphatic side chain. However, its cytostatic activity was significantly lower than that of the steroidal antiestrogen ICI 182,780.

All derivatives were also tested in hormone-independent MDA-MB 231 mammary tumor cells in order to detect non-specific cytotoxic action. Since no inhibitory effect was observed at concentrations below

1  $\mu$ M a specific estrogen receptor mediated mechanism for the growth inhibition in MCF-7 cells has to be assumed (data not shown). The only exception was the derivative 4e which showed in MCF-7 cells even at the highest concentration of  $10^{-6}$  M only a minor effect which was comparable to that observed in MDA-MB 231 cells. In this case, an unspecific mode of action is likely.

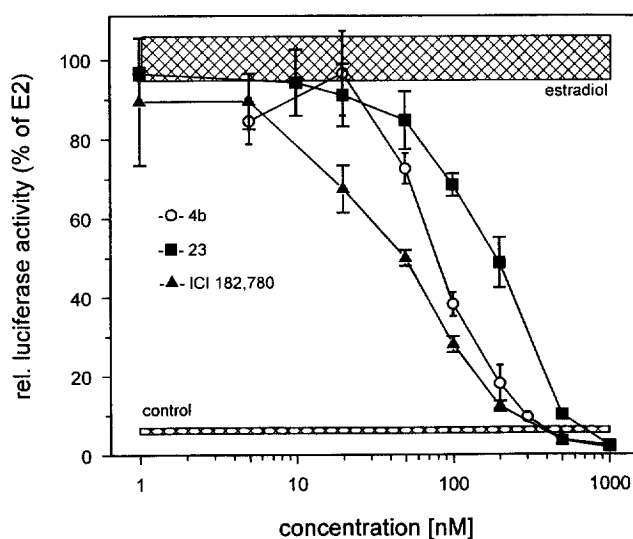


Fig. 3. Luciferase expression in MCF-7/2a cells, stably transfected with the reporter plasmid EREwtc luc and treated with combinations of  $17\beta$ -estradiol (E2, 10 nM) and compounds 4b, 23 and ICI 182,780 at various concentrations. Values are means of three independent experiments  $\pm$  SD.

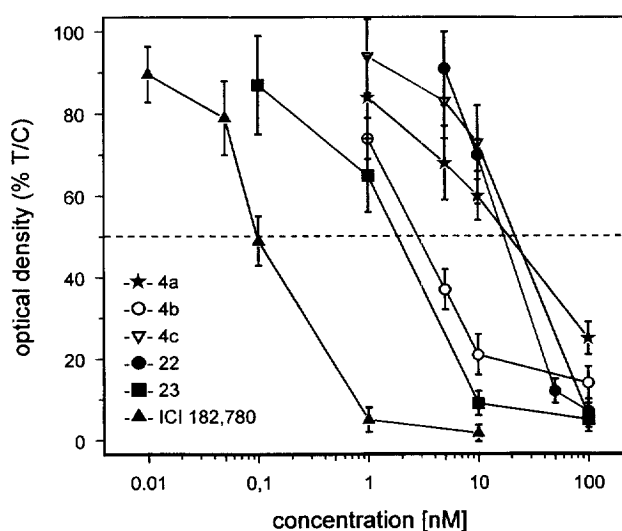


Fig. 4. Effect of 1-benzyl-2-phenylindoles 4a-b, 2-phenylindoles 22 and 23 and ICI 182,780 on the growth of hormone-sensitive human MCF-7 breast cancer cells, shown as optical densities following crystal violet staining of viable cells. Values are means of 16 replicates.

Table 2. Inhibitory effect of 1-benzyl-2-phenylindoles **4d** and **4e** and 1,2-diphenylindoles **11a** and **11b** on the growth of MCF-7 human breast cancer cells

Compound	T/C <sup>a</sup> (%)		
	1 × 10 <sup>-8</sup> M	1 × 10 <sup>-7</sup> M	1 × 10 <sup>-6</sup> M
<b>4d</b>	97 ± 12	96 ± 11	52 ± 7 <sup>b</sup>
<b>4e</b>	93 ± 6	85 ± 7 <sup>b</sup>	75 ± 7 <sup>b</sup>
<b>11a</b>	99 ± 14	69 ± 17 <sup>b</sup>	24 ± 5 <sup>b</sup>
<b>11b</b>	94 ± 12	95 ± 10	25 ± 6 <sup>b</sup>
OH-Tam <sup>c</sup>	67 ± 16 <sup>b</sup>	33 ± 7 <sup>b</sup>	37 ± 10 <sup>b</sup>

<sup>a</sup>Ratio of optical densities in test wells and control wells after incubation for 4 days; means of 16 replicates ± SD. Values are corrected for initial optical density.

<sup>b</sup>Significant inhibition of cell growth ( $p < 0.01$ ).

<sup>c</sup> 4-Hydroxytamoxifen.

## DISCUSSION

Investigations of several groups including our own one have shown that the side chain determines the pharmacological profile of compounds of estrogen antagonists whereas the basic structure provides most of the binding affinity for the receptor [2]. The functional group which has generally the potential of acting as hydrogen bridge acceptor contributes to the receptor binding as it had been demonstrated by replacing the polar function by a methylene group [8] or a sulfur atom [6]. Although this binding interaction might be less important its implication on the pharmacology of these agents is considerable.

The polymethylene chain that usually links the functional group to the carrier molecule is too flexible to allow speculations on its spacial orientation. Even the distance between both elements cannot be stated precisely because the alkylene chain can form a loop. Since six methylene groups are required for such a loop without strain the incorporation of a phenyl group into the chain should prevent its formation. The binding data of this study clearly showed that an extended orientation is preferred. Since the binding affinities of the 1-benzyl derivatives are higher than those of the 1-phenyl analogues the arrangement of the side chain in the plane of the indole appears to be less favorable.

More important than the RBA values was the effect of the side chain structure on the antiestrogenic potency. It was not unexpected that the derivative with the highest binding affinity (**4b**) also showed the strongest antiestrogenic effect on the luciferase expression in stably transfected MCF-7/2a cells. It exceeded the effect of the reference indole compound **23** (ZK 164,015) and was nearly as active as the estradiol derivative ICI 182,780. In the proliferation assay in estrogen-sensitive MCF-7 cells both 2-phenylindole derivatives **4b** and **23** were equally active. All of the other indole derivatives were considerably less potent despite the fact that the distance between the

functional group in the side chain and the indole nucleus was kept constant and the RBA values were all within the same order of magnitude. Obviously, this cellular assay is very sensitive towards alterations in the spacial orientation of the side chain.

The data from the transcription and proliferation assays suggest that a phenyl ring can be incorporated into the side chain of pure antiestrogens without reducing their potency, provided the aromatic ring is *para*-substituted and a methylene group between the indole nitrogen and the phenyl group can act as a hinge. This observation is in good agreement with the data obtained with steroidal pure antiestrogens. In estradiol both carbon-7 and carbon-11 are sp<sup>3</sup>-configured. Therefore, the side chain in the pure antiestrogen RU 58668 in which the *para*-substituted phenyl group is located in the 11β-position [5] is arranged above the plane of the steroid skeleton whereas the (pentylsulfinyl)nonyl function in ICI 182,780, linked to carbon-7, has the opposite orientation [4]. Therefore, it is feasible that the methylene group in the 1-benzyl-2-phenylindoles mimics one of these tetrahedral carbon atoms in the steroid giving rise to an orientation of the side chain similar to that of the steroidal pure antiestrogens.

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