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1-Carbamoylalkyl-2-phenylindoles: Relationship Between Side Chain Structure and Estrogen Antagonism

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The 2-phenylindole system has proved to be a versatile structure for the design of potent antiestrogens, especially when functional groups have been introduced into the alkyl side chain in position 1. In analogy to steroidal structures such as ICI 164,384 a number of 2-phenylindoles with carbamoylalkyl and aminoalkyl side chains were synthesized. They bind to the calf uterine estrogen receptor with relative binding affinities between 2.1 and 21 (estradiol = 100). The antiestrogenic effect of these compounds was demonstrated by the inhibition of transcriptional activity which was measured in a new luciferase assay with the EREwtc luc as reporter plasmid. The derivative with a methyl-*n*-propyldodecanamide side chain (4h) antagonized the effect of estradiol (10^{-9} M) completely at concentrations of 10^{-7} M and higher. As a sensitive model for quantification of estrogenic and antiestrogenic effects *in vitro* we used HeLa-cells cotransfected both with the reporter plasmid and estrogen receptor expression vectors HEG0 and HE0. In cells transfected with these vectors transcriptional activity was strongly dependent on side chain structure. With mutated receptors we were able to show that this activity was mainly due to TAF-1 whereas TAF-2 remained silent. When we studied the effect of some of the new compounds *in vivo* using the mouse uterine weight assay, we observed a correlation between transcriptional activity in transfected HeLa cells and estrogenic effects in mice. Two of the 1-carbamoylalkyl-2-phenylindoles (4f, 4h) proved to be "pure" antiestrogens both *in vitro* and *in vivo*. In estrogen-sensitive MCF-7 breast cancer cells, they strongly inhibit cellular growth. Some of the IC_{50}-values were close to 10^{-8} M.

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INTRODUCTION

The endocrine therapy of breast cancer has become an established treatment for patients whose tumor tissues contain estrogen receptors. In most countries, the only antiestrogen on the market for this indication is tamoxifen. Two close analogs of tamoxifen are toremifene [1] which is already available in some countries and droloxifene [2]. Unfortunately, about 40% of the patients do not respond to the antiestrogen treatment despite the presence of estrogen receptors in the malignant tissue [3]. The reason for this failure of therapy is not yet known. One possibility lies in the nature of the estrogen receptor which can lose its function by mutation [4] as described for the androgen receptor [5] or in other mechanisms of drug resistance. It might be also due to the incomplete antagonism and/or weak agonism of tamoxifen. This drug exhibits weak estrogenic activity both in man [6] and rat [7]; in the mouse, it acts as pure estrogen [8]. Some of the side effects of tamoxifen such as tumor flares [9] and vaginal cornification [6] are thought to be a consequence of the agonist (estrogenic) activity of the drug [10]. A higher incidence of endometrium cancer following long term treatment with tamoxifen is discussed [11] and might be associated with an estrogenic effect. On the other hand, the hormonal activity of this drug possibly relieves osteoporosis [12] and other symptoms of estrogen withdrawal in women. To our knowledge, all other non-steroidal antiestrogens in clinical use or trials e.g. chlomiphene, toremifene and droloxifene also show estrogenic effects. This applies not only to triphenylethylene derivatives but also to the 2-phenylindole zindoxifene, that we have designed for the treatment of hormone-dependent mammary and prostatic carcinomas [13, 14].

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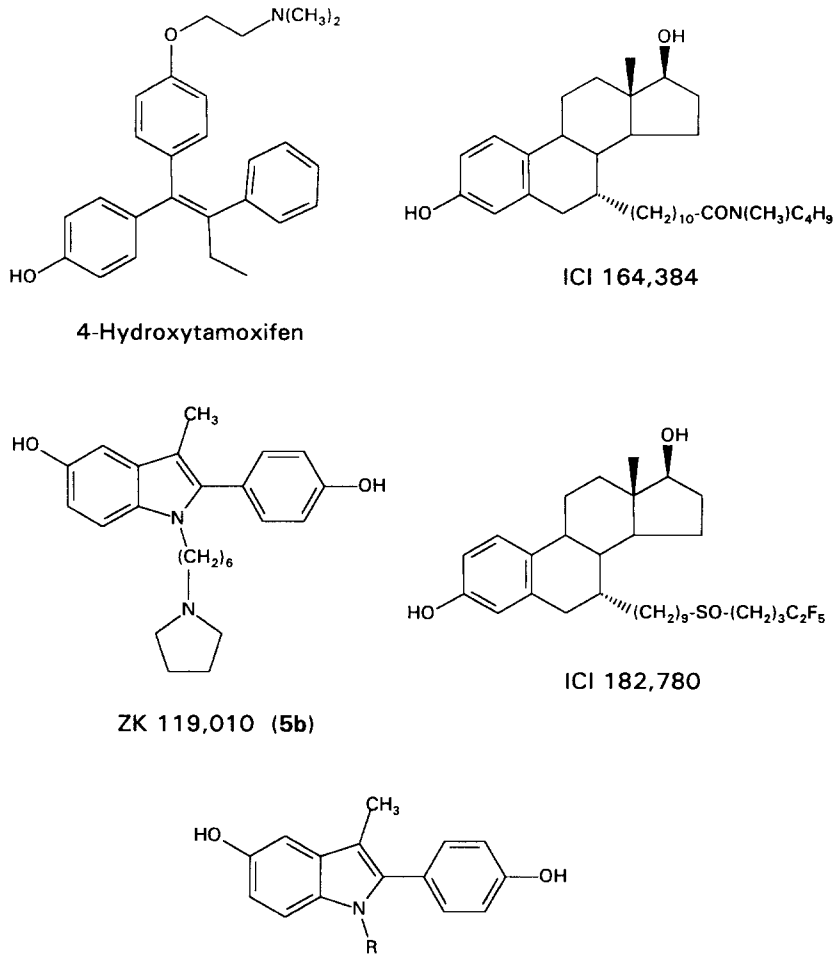

Fig. 1. Chemical structures of compounds used in this study.

The first antiestrogen (ICI 164,384) without any estrogenic activity in experimental systems was described by Wakeling and Bowler [15]. It possesses an estradiol based structure with a side chain in position 7α which includes an amide function (Fig. 1). Our present studies are focussed on non-steroidal estrogen antagonists that derive from 2-phenylindole [16]. When we modified the side chain in the 2-phenylindole system we found that the introduction of an amino function increased the antagonistic activity of this structure considerably [17]. Compound ZK 119,010 (Fig. 1) emerged from these investigations and was shown to be devoid of significant agonistic activity in mice [17]. However, some other endocrine studies especially those in rats revealed weak estrogenic effects at high doses [18]. Therefore we carried out further modifications of the side chain structure in order to obtain antiestrogens which are devoid of estrogenic activity in all relevant test systems. Compounds of this type are usually described as *pure* antiestrogens in pharmacological terms.

Extensive studies at ICI [19] with steroidal derivatives have shown the importance of an amide function in the side chain and the relevance of the distance between the steroid and the functional group. The overall length of the side chain should cover 14 methylene groups plus the amide function which should be separated from the C-7 atom by 4 or 10 methylene groups. With these results in mind we synthesized a number of 2-phenylindole derivatives with aminoalkyl and carbamoylalkyl side chains and determined their binding affinities for the estrogen receptor. Usually, the endocrine activity of new antiestrogens is determined in animal experiments such as mouse (rat) uterus weight test. The increase in uterus weight reflects the end point of the biological response to a molecular signal on the DNA level. We decided to shift the point of detection to one of the early steps in the sequence of molecular and biological events following the hormonal stimulation. The transcription of estrogen-regulated genes is one of the crucial responses. We measured the activation of transcription in a new assay in HeLa-cells cotransfected with expression vectors for estrogen receptors and a reporter plasmid harboring the luciferase

gene from *Photinus pyralis* under the control of an estrogen responsive element (ERE) [20]. The mouse uterus weight test was used to validate the *in vitro* results. The estrogen receptor-mediated antitumor activity of the new compounds was determined in estrogen-sensitive MCF-7 breast cancer cells.

EXPERIMENTAL

Synthesis

Melting points were determined on a Büchi 510 apparatus and are uncorrected. Solid compounds were analyzed for C, H, and N within ±0.4% of the calculated values. IR and [^{1}H]-NMR spectra were consistent with the assigned structures. The synthesis of ZK 119,010 (= **5b**) has been described previously [17]. The amides **4a–h** were prepared by alkylation of the corresponding methoxy-substituted 1H-2-phenylindole **1** with the respective ω-bromoalkanoic amides **2** and subsequent ether cleavage by boron tribromide. The amino derivatives **5a**, **5c** and **5f** were obtained by reduction of **4a**, **4c** and **4f**, respectively with lithium aluminium hydride (Fig. 2).

General procedure for the introduction of amide side chains

Under a nitrogen atmosphere, 5-methoxy-2-(4-methoxyphenyl)-3-methylindole **1** [16] (14 mmol) in 80 ml of dry DMF was added slowly to an ice-cold suspension of sodium hydride (20 mmol) in 20 ml of dry DMF. After stirring for 20 min at 0°C, a solution of ω-bromoalkanoic amide **2** (14 mmol) in 60 ml of DMF was added slowly to the indole anion. After stirring for 2 h at room temperature, water was added to destroy the excess of sodium hydride. The product was extracted with EtOAc. The organic layer was washed with water and dried ($MgSO_4$). After evaporation of the solvent *in vacuo* the residue was purified by column chromatography (SiO_2; CH_2Cl_2/EtOAc mixtures).

The following compounds were obtained by this method: 1-[4-(*n*-decyl-methylcarbamoyl)-butyl]-5-methoxy-2-(4-methoxyphenyl)-3-methylindole (**3a**); yellow oil; 71% yield, $C_{33}H_{48}N_2O_3$ (520.8); ^{1}H-NMR ($CDCl_3$): δ (ppm) = 0.65–2.22 (m; 25 H; —$(CH_2)_2$—, —$(CH_2)_8CH_3$), —CH_2CO—), 2.15 (s; 3H; CCH_3), 2.77, 2.80 (s; 3H; —$CON(CH_3)$—), 2.99–3.42 (m; 2H; —$CON(CH_2)$—), 3.85 (s; 6H; —OCH_3), 3.97 (t; ^3J = 7 Hz; 2H; $ArNCH_2$—), 6.71–7.41 (m; 7H; ArH); 5-methoxy-2-(4-methoxyphenyl)-3-methyl-1-[5-(pyrrolidinocarbonyl)pentyl]indole (**3b**) m.p. 125–127°C; 85% yield; $C_{27}H_{34}N_2O_3$ (434.7); ^{1}H-NMR ($CDCl_3$): δ (ppm) = 1.10–2.31 (m; 12H; —$(CH_2)_4$—, —CH_2—CH_2—), 2.22 (s; 3H; CCH_3), 3.32 (t, ^3J = 7 Hz; 2H; —$CON(CH_2)$—), 3.46 (t, ^3J = 7 Hz; 2H; —$CON(CH_2)$—); 3.92 (s; 6H; —OCH_3), 4.02 (t; ^3J = 7 Hz; $ArNCH_2$—), 6.74–7.45 (m; 7H; ArH); 1-[5-(*n*-butyl-methylcarbamoyl)-pentyl]-5-methoxy-2-(4-methoxyphenyl)-3-methylindole (**3c**); yellow oil; 80% yield, $C_{28}H_{38}N_2O_3$ (450.6); ^{1}H-NMR ($CDCl_3$): δ (ppm) = 0.71–2.25 (m; 15H; —$(CH_2)_3$—, —$(CH_2)_2CH_3$, —CH_2CO—), 2.12 (s; 3H; CCH_3), 2.79, 2.83 (s; 3H; —$CON(CH_3)$—), 3.00–3.40 (m; 2H; —$CON(CH_2)$—), 3.87 (s; 6H; —OCH_3), 3.96 (t; ^3J = 7 Hz; 2H; $ArNCH_2$—), 6.76–7.48 (m; 7H; ArH); 1-[7-(*n*-heptyl-methylcarbamoyl)-heptyl]-5-methoxy-2-(4-methoxyphenyl)-3-methylindole (**3d**); amber oil; 76% yield, $C_{33}H_{48}N_2O_3$ (520.8); ^{1}H-NMR ($CDCl_3$): δ (ppm) = 0.67–1.85 (m; 23H; —$(CH_2)_5$—, —$(CH_2)_5CH_3$), 2.02–2.44 (m; 2H; —CH_2CO—), 2.19 (s; 3H; CCH_3), 2.90, 2.94 (s; 3H; —$CON(CH_3)$—), 3.06–3.49 (m; 2H; —$CON(CH_2)$—), 3.90 (s; 6H;

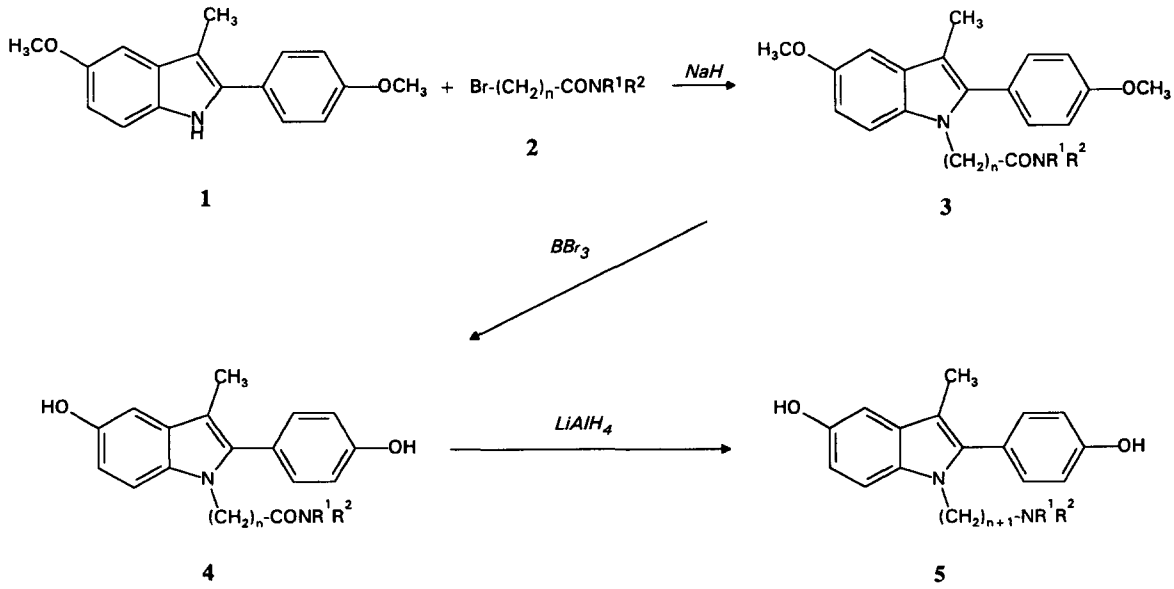

Fig. 2. Synthesis of 1-(carbamoylalkyl)- and 1-(aminoalkyl)-5-hydroxy-2-(4-hydroxyphenyl)-3-methylindoles.

—OCH_3), 3.97 (t; $^3J = 7$ Hz; 2H; $ArNCH_2$—), 6.76–7.48 (m; 7H; ArH); 5-methoxy-2-(4-methoxyphenyl)-3-methyl-1-[9-(methyl-*n*-pentylcarbamoyl)-nonyl]-indole (**3e**); yellow oil; 64% yield; $C_{33}H_{48}N_2O_3$ (520.8); ^{1}H-NMR ($CDCl_3$): δ (ppm) = 0.67–1.89 (m; 23H; —$(CH_2)_7$—, —$(CH_2)_3CH_3$), 2.07–2.44 (m; 2H; —CH_2CO—), 2.18 (s; 3H; CCH_3), 2.89, 2.93 (s; 3H; —$CON(CH_3)$—), 3.06–3.50 (m; 2H; —$CON(CH_2)$—), 3.87 (s; 6H; —OCH_3), 3.96 (t; $^3J = 7$ Hz; 2H; $ArNCH_2$—), 6.74–7.45 (m; 7H; Ar-H); 1-[10-(*n*-butyl-methylcarbamoyl)-decyl]-5-methoxy-2-(4-methoxyphenyl)-3-methylindole (**3f**); amber oil; 75% yield; $C_{33}H_{48}N_2O_3$ (520.8); ^{1}H-NMR ($CDCl_3$): δ (ppm) = 0.83–2.51 (m; 25H, —$CH_2CH_2CH_3$, —$(CH_2)_9CO$—), 2.24 (s; 3H, CCH_3), 2.99 (s; 3H, —$CONCH_3$), 3.30 (t, $^3J = 7$ Hz; 2H, —$CONCH_2$—), 3.93 (s; 6H, —OCH_3), 4.03 (t, $^3J = 7$ Hz; 2H, $ArNCH_2$—), 6.78–7.70 (m; 7H; ArH); 5-methoxy-2-(4-methoxyphenyl)-3-methyl-1-(10-pyrrolidinocarbonyldecyl)-indole (**3g**); yellow oil; 86% yield; $C_{32}H_{44}N_2O_3$ (504.7); ^{1}H-NMR ($CDCl_3$): δ (ppm) = 0.86–2.47 (m; 22H; —$(CH_2)_9CO$—, —CH_2—CH_2—), 2.19 (s; 3H; CCH_3), 3.21–3.62 (m; —$CON(CH_2)_2$), 3.88 (s; 6H; —OCH_3), 3.95 (t; $^3J = 7$ Hz; $ArNCH_2$—), 6.74–7.45 (m; 7H; ArH); 5-methoxy-2-(4-methoxyphenyl)-3-methyl-1-[11-(*n*-propyl-methylcarbamoyl)-undecyl]-indole (**3h**); amber oil; 72% yield; $C_{33}H_{48}N_2O_3$ (520.8); ^{1}H-NMR ($CDCl_3$): δ (ppm) = 0.65–1.94 (m; 23H; —$(CH_2)_9$—, —CH_2CH_3), 2.17 (s; 3H; CCH_3), 2.00–2.46 (m; 2H; —CH_2CO—), 2.88, 2.93 (s; 3H; —$CON(CH_3)$—), 3.04–3.49 (m; 2H; —$CON(CH_2)$—), 3.88 (s; 6H; —OCH_3), 3.93 (t; $^3J = 7$ Hz; $ArNCH_2$—), 6.70–7.41 (m; 7H; ArH).

General procedure for the ether cleavage

Under a nitrogen atmosphere, 2.5 mmol of the methoxy derivative **3** in 50 ml of dry CH_2Cl_2 was added to 10 mmol of boron tribromide in 50 ml of dry CH_2Cl_2 at −20°C. After stirring for 2 h at room temperature, sat. $NaHCO_3$ solution was added slowly with cooling. When the vigorous reaction had ceased the mixture was extracted several times with EtOAc. The organic layer was washed with water, dried ($MgSO_4$) and the solvent removed *in vacuo*. The products were purified by column chromatography (SiO_2; CH_2Cl_2/EtOAc 2:1).

The following compounds were used in this study: 1-[4-(*n*-decyl-methylcarbamoyl)butyl]-5-hydroxy-2-(4-hydroxyphenyl)-methylindole (**4a**); amorphous powder; 91% yield; $C_{31}H_{44}N_2O_3$ (492.7); ^{1}H-NMR ($CDCl_3$): δ (ppm) = 0.80–2.30 (m; 25H; —$(CH_2)_2$—, —$(CH_2)_8CH_3$; —CH_2CO—), 2.11 (s; 3H; CCH_3), 2.80, 2.88 (s; 3H; —$CON(CH_3)$—), 3.05–3.44 (m; 2H; —$CON(CH_2)$—), 3.95 (t; $^3J = 7$ Hz; 2H; $ArNCH_2$—), 6.61–7.35 (m; 7H; ArH); 5-hydroxy-2-(4-hydroxyphenyl)-3-methyl-1-[5-(pyrrolidinocarbonyl)pentyl]-indole (**4b**); m.p. 187–190°C; 85% yield; $C_{25}H_{30}N_2O_3$ (476.7); ^{1}H-NMR (DMSO-d_6): δ (ppm) = 0.76–2.18 (m; 12H; —$(CH_2)_4CO$—, —CH_2CH_2—), 2.03 (s; 3H; CCH_3), 3.05–3.46 (m; 4H; —$CON(CH_2)_2$), 3.95 (t; $^3J = 7$ Hz; $ArNCH_2$—), 6.53–7.36 (m; 7H; ArH); 1-[5-(*n*-butyl-methylcarbamoyl)pentyl]-5-hydroxy-2-(4-hydroxyphenyl)-methylindole (**4c**); m.p. 153–154°C (MeOH); 82% yield; $C_{26}H_{34}N_2O_3$ (422.5); ^{1}H-NMR (CD_3OD): δ (ppm) = 0.80–1.80 (m; 13H; —$(CH_2)_3$—, —$(CH_2)_2CH_3$), 2.11 (s; 3H; CCH_3), 2.15 (t, $^3J = 7$ Hz; 2H; —$COCH_2$—), 2.83, 2.88 (s; 3H; —$CON(CH_3)$—), 3.04–3.44 (m; 2H; —$CON(CH_2)$—), 3.96 (t; $^3J = 7$ Hz; 2H; $ArNCH_2$—), 6.63–7.30 (m; 7H; ArH); 1-[7-(*n*-heptyl-methylcarbamoyl)-heptyl]-5-hydroxy-2-(4-hydroxyphenyl)-3-methylindole (**4d**); greenish oil; 76% yield; $C_{31}H_{44}N_2O_3$ (492.7); ^{1}H-NMR ($CDCl_3$): δ (ppm) = 0.67–1.84 (m; 23H; $(CH_2)_5$—, —$(CH_2)_5CH_3$), 2.00–2.48 (m; 2H; —CH_2CO—), 2.14 (s; 3H; CCH_3), 2.94 (s; 3H; —$CON(CH_3)$—), 3.05–3.50 (m; 2H; —$CON(CH_2)$—), 3.91 (t; $^3J = 7$ Hz; 2H; $ArNCH_2$—), 6.63–7.30 (m; 7H; ArH); 5-hydroxy-2-(4-hydroxyphenyl)-3-methyl-1-[9-(methyl-*n*-pentylcarbamoyl)-nonyl]-indol (**4e**); yellow oil; 91% yield; $C_{31}H_{44}N_2O_3$ (492.7); ^{1}H-NMR ($CDCl_3$): δ (ppm) = 0.70–1.97 (m; 23H; —$(CH_2)_7$—, —$(CH_2)_3CH_3$), 2.13 (s; 3H; CCH_3), 2.33 (t $^3J = 6$ Hz; 2H; —CH_2CO—), 2.95 (s; 3H; —$CON(CH_3)$—), 3.06–3.54 (m; 2H; —$CON(CH_2)$—), 3.88 (t; $^3J = 7$ Hz; 2H; $ArNCH_2$), 6.56–7.24 (m; 7H; ArH); 1-[10-(*n*-butyl-methylcarbamoyl)-decyl]-5-hydroxy-2-(4-hydroxyphenyl)-3-methylindole (**4f**); yellow oil; 80% yield; $C_{31}H_{44}N_2O_3$ (492.7); ^{1}H-NMR ($CDCl_3$): δ (ppm) = 0.80–2.55 (m; 25H, —$CH_2CH_2CH_3$, —$(CH_2)_9CON$—), 2.20 (s; 3H, CCH_3), 3.02 (s; 3H, —$CONCH_3$), 3.10–3.55 (m; 2H —$CON(CH_2)$—), 4.04 (t, ^3J—7 Hz, 2H, $ArNCH_2$—), 6.74–7.54 (m; 7H; ArH); 5-hydroxy-2-(4-hydroxyphenyl)-3-methyl-1-[10-(pyrrolidinocarbonyl)decyl]-indole (**4g**); m.p. 67–69°C 88% yield; $C_{30}H_{40}N_2O_3$ (476.7); ^{1}H-NMR ($CDCl_3$): δ (ppm) = 0.82–2.49 (m; 22H; —$(CH_2)_9CO$—, —CH_2CH_2—), 2.15 (s; 3H; CCH_3), 3.30–3.71 (m; 4H; —$CON(CH_2)_2$), 3.95 (t; $^3J = 7$ Hz; $ArNCH_2$—), 6.65–7.33 (m; 7H; ArH); 5-hydroxy-2-(4-hydroxyphenyl)-3-methyl-1-[11-(*n*-propyl-methylcarbamoyl)-undecyl]-indole (**4h**); yellow oil; 95% yield; $C_{31}H_{44}N_2O_3$ (492.7); ^{1}H-NMR ($CDCl_3$): δ (ppm) = 0.72–2.02 (m; 23H; —$(CH_2)_9$—, —CH_2CH_3), 2.17 (s; 3H; CCH_3), 2.40 (t; $^3J = 7$ Hz; 2H; —CH_2CO—), 2.97, 3.00 (s; 3H; —$CON(CH_3)$—), 3.09–3.58 (m; 2H; —$CON(CH_2)$—), 3.97 (t; $^3J = 7$ Hz; 2H; $ArNCH_2$—), 5.31 (s, br; 1H; —OH), 6.66–7.35 (m; 7H; ArH), 8.69 (s; br; 1H; —OH).

General procedure for the reduction

Under a nitrogen atmosphere, 2.60 mmol of the amide **4** in 80 ml of dry THF was added slowly to a boiling suspension of 7.00 mmol of $LiAlH_4$ in 50 ml of dry THF. After 1 h, the mixture was cooled to room temperature and stirring was continued overnight. The excess of $LiAlH_4$ was destroyed by cautious addition of water and sat. $NaHCO_3$-solution. The mixture was extracted with EtOAc. The organic layer was washed

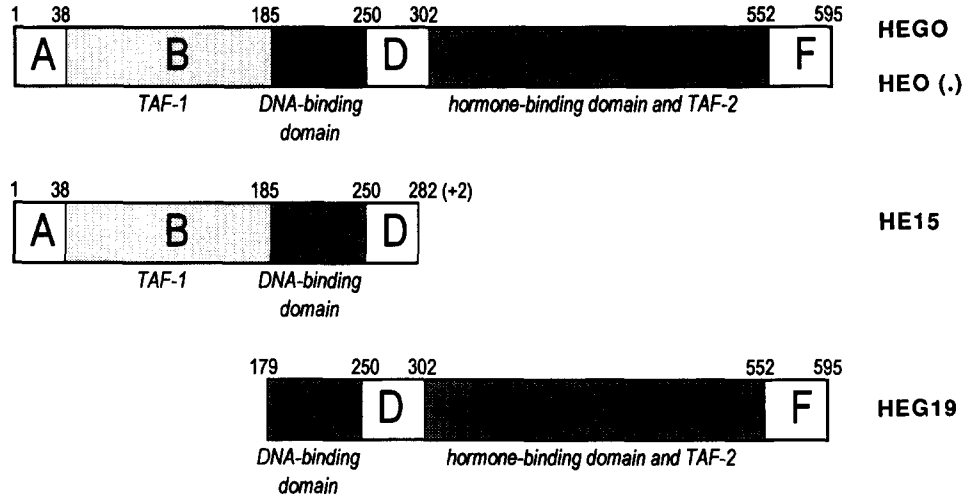

Fig. 3. Functional domains of the human estrogen receptor (HEG0) and mutants HE0, HE15, and HEG19 used in the transcription activation assays. HE0 differs from the wildtype receptor by a glycine/valine exchange in position 400.

with water and dried ($MgSO_4$). After removal of the solvent, the residue was purified by column chromatography (SiO_2; EtOAc/MeOH mixtures).

The following compounds were synthesized by this method: 1-[5-(*n*-decyl-methylamino)-pentyl]-5-hydroxy-2-(4-hydroxyphenyl)-3-methylindole (**5a**); brownish oil; 53% yield; $C_{31}H_{46}N_2O_2$ (478.7); ^{1}H-NMR (CD_3OD): δ (ppm) = 0.70–1.70 (m; 25H; —$(CH_2)_8CH_3$,—$(CH_2)_3$—), 1.95–2.42 (m; 4H, —$N(CH_2)_2$—), 2.08 (s; 3H; CCH_3), 2.10 (s; 3H; —NCH_3), 3.94 (t, ^3J = 7 Hz; 2H, $ArNCH_2$—), 6.48–7.24 (m; 7H, ArH); 1-[6-(*n*-butyl-methylamino)-hexyl]-5-hydroxy-2-(4-hydroxyphenyl)-3-methylindole (**5c**); amber oil; 66% yield; $C_{26}H_{36}N_2O_2$ (408.5); ^{1}H-NMR (CD_3OD): δ (ppm) = 0.58–1.76 (m; 15H; —$CH_2CH_2CH_3$,—$(CH_2)_4$—), 1.88–2.63 (m; 4H; —$N(CH_2)_2$—), 2.12 (s; 3H, CCH_3), 2.20 (s; 3H, —NCH_3), 4.98 (t, ^3J = 7 Hz; 2H; $ArNCH_2$—), 6.50–7.51 (m; 7H, ArH); 1-[11-(*n*-butyl-methylamino)-undecyl]-5-hydroxy-2-(4-hydroxyphenyl)-3-methy lindole (**5f**); brownish oil; 57% yield; $C_{31}H_{46}N_2O_2$ (478.7); ^{1}H-NMR (CD_3OD): δ (ppm) = 0.77–1.80 (m; 25H; —$(CH_2)_2CH_3$,—$(CH_2)_9$—), 1.90–2.60 (m; 4H; —$N(CH_2)_2$—), 2.14 (s; 3H; CCH_3), 2.23 (s; 3H; NCH_3), 4.00 (t, ^3J = 7 Hz; 2H; $ArNCH_2$—), 6.66–7.37 (m; 7H; ArH).

Materials and reagents for bioassays

[^{3}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 Dr P. W. Jungblut, Hannover (Germany). ICI 164,384 and ICI 182,780 were generously provided by Dr M. R. Schneider, Berlin (Germany). The luciferase reporter plasmid EREwtc luc harboring the luciferase gene from *Photinus pyralis* under the control of an ERE was synthesized from pGEM/luc (Promega) and EREwtc [21] (generously provided by Dr Klein-Hitpaß, Essen, Germany) in the authors' laboratory as described previously [22]. The estrogen receptor expression vectors HE0 [23], HEG0 [24], HE15 [25] and HEG19 [26] (Fig. 3) were 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 $Na_2HPO_4 \cdot 2H_2O$, 4.0 g D-(+)-glucose, 20.0 g HEPES in 1 l water, adjusted to pH 7.5 with NaOH, which was diluted by a factor of 10 and adjusted to pH 7.08.

Estradiol receptor binding assay

For the determination of relative binding affinities (RBA), the previously described procedure was applied with modifications [16]. The 500 μl-incubation mixture comprised 5 nM [^{3}H]17β-estradiol [added in 100 μl Tris-buffer (0.01 M, pH 7.5), supplemented with EDTA (0.01 M) and NaN_3 (0.003 M)], 10^{-9} to 10^{-5} 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). Non-specific binding was calculated using 5 μM 17β-estradiol as competing ligand. Radioactivity was plotted as a function of the log concentration of 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.

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

Hormone-sensitive human MCF-7 breast cancer cells were obtained from American Type Culture Collection (ATCC, Rockville, MD, U.S.A.). Cells were grown in improved Minimal Essential Medium (MEM), as modified by Richter *et al.* [27] (Biochrom, Berlin, Germany), supplemented with glutamine (0.3 g/l), gentamycin (60 mg/l) and 10% DCC-treated newborn calf serum (ctNCS) (Gibco). The serum was sterilized through a 0.20 μm filter (Sartorius, Göttingen, Germany) and stored at -20°C. Cells were grown in a humidified incubator in 5% CO_2 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_2 at 37°C, medium was replaced by one containing the test-compound. 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 phosphate buffered saline (PBS); 100 μl/well]. After incubation for 4–7 days, 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 addition of 100 μl of ethanol (70%), plates were gently shaken for 2 h. Optical density of each well was measured in a microplate autoreader EL 309 (Bio-tek) at 578 nm.

Luciferase assay in transfected MCF-7 human breast cancer cells

MCF-7 cells used for transfection were grown in Dulbecco's Modified Essential Medium (DMEM) supplemented with 10% fetal calf serum (FCS), 100 U penicillin, 100 μg streptomycin and 150 mg L-glutamine in 500 ml of medium without phenol red. Shortly before confluence, the medium was removed and cells were washed with 10 ml of PBS. Cells were gently shaken for a few seconds with trypsin–EDTA solution (4 ml) and after removal of the solution incubated for 2 min at 37°C. After addition of 10 ml of medium, the cell suspension (0.5 ml per well) was transferred to 6-well plates containing 2 ml of medium. Cells were grown until the density of the monolayer was about 50% (1–2 days) before 2 μg of the reporter plasmid EREwtc luc per well were added. For a successful transfection it is necessary to generate a very fine precipitate of the DNA by subsequent dilution with 45% water, 5% 2.5 M $CaCl_2$ and 50% HBS buffer and continuous shaking. After 20 min at room temperature an opalescent solution should be obtained.

After addition of the 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 with PBS, fresh medium containing the test substances was added. The maximum of luciferase expression was reached 18 h after addition of the transfection solution. At that time, medium was removed and cells were washed with PBS. Cell lysis and quantification of luminescence was performed according to the procedure described in luciferase assay system E1500 of PROMEGA (Serva, Heidelberg, Germany). Luminescence was measured in a luminometer Lumat LB 9501 (Berthold, Wildbad, Germany) as relative light units (RLU) which were converted into fg luciferase by a calibration curve. Protein concentration was determined by the Bradford method [28].

Luciferase assay in transfected HeLa-cells

HeLa-cells were grown as described for MCF-7 cells. One week before the start of the experiment, the medium was replaced by one containing DCC-treated FCS (ctFCS). The method for transfection was the same as described for MCF-7 cells except that estrogen receptor expression vectors HE0, HEG0, HE15 and HEG19, respectively were used together with the reporter plasmid EREwtc luc.

Mice uterine weight tests

Immature female mice (20 days old, of the NMRI strain) from Charles River Wiga (Sulzfeld, Germany), were randomly divided into groups of 6 to 10 animals. To determine estrogenic activity, compounds were dissolved in olive oil (100 μl/animal) and injected subcutaneously on 3 consecutive days. Control animals received the vehicle alone. 24 h after the last injection, the animals were killed by cervical dislocation and weighed. Uteri were dissected free of fat and fixed in Bouin solution (saturated aqueous picric acid 40% formaldehyde glacial acetic acid 15:5:1, by vol.) for 2 h. Uteri were freed from connective tissue, washed with ethanol, dried at 100°C for 18 h, and weighted. The relative uterus weight was calculated by the formula: uterine dry weight (mg)/body weight (g), multiplied by 100.

To determine the antiestrogenic activity, injections contained a standard dose (0.4 μg) of estrone and increasing doses of the compounds. The inhibition (%) of the estrone-stimulated uterine growth was estimated by the formula: $100 - [(W_{S,T} - W_V)/(W_S - W_V) \times 100]$ ($W_{S,T}$ = rel. uterus weight of animals treated with estrone standard (0.4 μg) + test compound; W_V = rel. uterus weight of control animals; W_S = rel. uterus weight of animals treated with estrone standard).

RESULTS

Estrogen receptor binding affinity

All new derivatives were first tested for their ability to bind to the estrogen receptor. As in previous studies, we used the calf uterine cytosol as a convenient source of estrogen receptors. The RBA values obtained with calf estrogen receptors are generally somewhat lower than those from other species. Therefore, various reference compounds were included for comparison. The RBA values of the carbamoylalkyl derivatives ranged from 2.1 to 7.0 (Table 1). The comparison of **4f** with the steroidal derivative ICI 164,384 with the same side chain reveals similar binding affinities. When the carbamoyl group was converted into the amino function, the RBA values increased considerably. These values are close to that of hexestrol and higher than that of 4-hydroxytamoxifen (Table 1). The comparison of the binding affinities of the steroidal structures for the calf estrogen receptor with those for the mouse estrogen receptor expressed in *Spodoptera frugiperda* showed the expected reduction due to the different species (ICI 164,384: 5.6 vs 23; ICI 182,780: 6.2 vs 17 [29]).

Transcription activation

Estrogen antagonist without agonistic activity ("pure" antiestrogens) should completely suppress the transcription of estrogen-regulated genes. Thus, we studied first the effect of our new compounds on transcription in cells transfected with the reporter plasmid EREwtc Luc [20]. In these cells, the luciferase gene from *Photinus pyralis* is under control of an ERE. The activation of transcription of this gene gives rise to the synthesis of luciferase which can be quantified by measuring the luciferase catalyzed emission of light. The obvious choice of cells for these experiments were cells that express estrogen receptors such as MCF-7 human breast cancer cells.

Table 1. Binding affinities of 1-substituted 5-hydroxy-2-(4-hydroxyphenyl)-3-methylindoles and reference compounds for the estrogen receptor

Compound	R[a]	RBA[b]
4a	$-(CH_2)_4-CO-N(CH_3)-C_{10}H_{21}$	2.1
4b	$-(CH_2)_5-CO-NC_4H_8$	7.0
4c	$-(CH_2)_5-CO-N(CH_3)-C_4H_9$	4.3
4d	$-(CH_2)_7-CO-N(CH_3)-C_7H_{15}$	3.5
4e	$-(CH_2)_9-CO-N(CH_3)-C_5H_{11}$	2.2
4f	$-(CH_2)_{10}-CO-N(CH_3)-C_4H_9$	7.0
4g	$-(CH_2)_{10}-CO-NC_4H_8$	3.7
4h	$-(CH_2)_{11}-CO-N(CH_3)-C_3H_7$	2.1
5a	$-(CH_2)_5-N(CH_3)-C_{10}H_{21}$	4.9
5b (ZK 119,010)	$-(CH_2)_6-NC_4H_8$	21
5c	$-(CH_2)_6-N(CH_3)-C_4H_9$	17
5f	$-(CH_2)_{11}-N(CH_3)-C_4H_9$	15
Tamoxifen		0.36
4-Hydroxytamoxifen		6.8
ICI 164,384		5.6
ICI 182,780		6.2
Hexestrol		22

[a]R refers to the general structure in Fig. 1.
[b]Relative binding affinities for the calf uterine estrogen receptor; value for 17β-estradiol = 100.

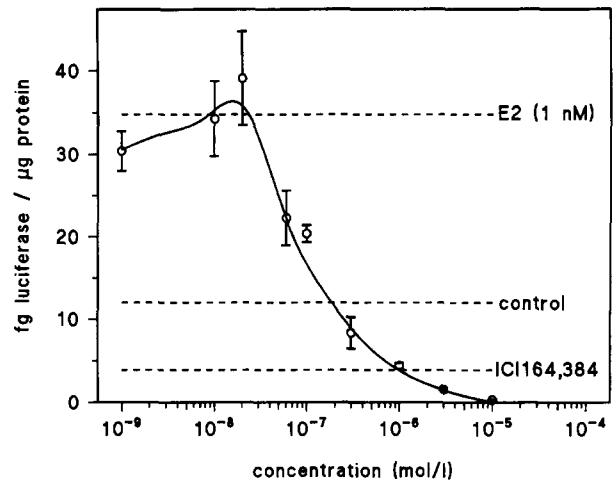

Fig. 4. Inhibition of estradiol (1 nM)-stimulated transcription of EREwtc luc in MCF-7 by increasing concentrations of compound 4h, measured by luciferase activity. ICI 164.384 (1 µM) served as reference compound, untreated FCS without drug as control value.

When MCF-7 cells were transfected with the reporter plasmid, luciferase activity was considerably increased by estradiol at concentrations of 10^{-11} M and higher [20]. This effect can be abolished by simultaneous addition of an antiestrogen such as ICI 164,384 (10^{-6} M) or the 2-phenylindole derivative **4h** (Fig. 4). The antagonistic activity of these agents led to a decrease of the amount of luciferase formed below the control level. When we treated transfected MCF-7 cells with antiestrogen in the absence of estradiol, we found no significant difference of luciferase activity between 4-hydroxytamoxifen, ICI 164,384, ZK 119,010 and DCC-treated control serum [30]. Obviously, weak estrogenic effects are obscured by other factors and can not be detected. After completion of this manuscript, Demirpence *et al.* [31] reported on a rather similar assay in which MCF-7 cells stably transfected with a luciferase reporter plasmid were used to characterize a number of potent estrogens.

Since it was not possible to differentiate between partial antagonists and those which are devoid of agonist activity in MCF-7 cells, we modified our system and used receptor negative HeLa cells cotransfected with expression vectors for the estrogen receptor and the reporter plasmid. Both, the vector for the wild type human estrogen receptor HEG0 [24] and the HE0 vector [32] which is characterized by a glycine/valine exchange in position 400 [33] were used in these experiments (Fig. 3). In HeLa cells, cotransfected with the expression vector HEG0 for the human estrogen receptor and the reporter plasmid, luciferase activity was stimulated by estradiol at 10^{-9} M and higher (Fig. 5). Figure 6 summarizes the effect of various

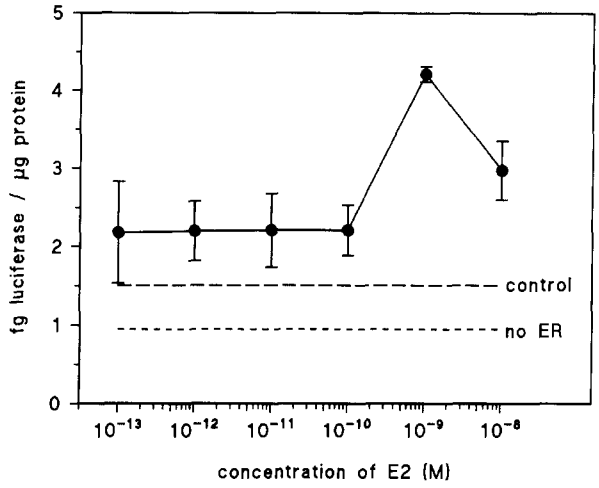

Fig. 5. Effect of 17β-estradiol on luciferase expression in HeLa-cells cotransfected with the reporter plasmid EREwtc luc and the expression vector HEG0 for the human estrogen receptor (ER). Control = DCC-treated FCS.

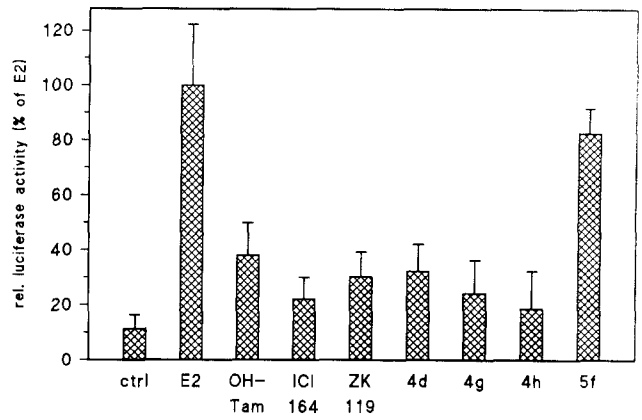

Fig. 7. Transcriptional activity of 17β-estradiol (E2, 10 nM), 4-hydroxytamoxifen (OHTam, 1 µM), ICI 164,384 (1 µM), ZK 119,010 (=5b, 1 µM), and compounds 4d, 4g, 4h, and 5f (1 µM) in HeLa cells cotransfected with the reporter plasmid EREwtc luc and the estrogen receptor expression vector HE0. Values are means of 3 independent experiments ±SD.

2-phenylindole derivatives on the estrogen receptor mediated transcription of the luciferase gene in HeLa cells at a concentration of 10^{-6} M. Two distinct classes of compounds can be recognized: some show transcriptional activity which others lack. The lowest luciferase levels were recorded for the steroidal antiestrogens ICI 164,384 and ICI 182,780 (Fig. 6). Similar values were found for the 1-carbamoylalkyl-2-phenylindoles **4b**, **4d**, **4g**, and **4h**. The partial antagonist 4-hydroxytamoxifen and some of the 2-phenylindole derivatives strongly activated luciferase expression.

For comparison, we also studied the effect of a few compounds in HeLa cells transfected with the HE0 vector (Fig. 7). In this model, the agonist component

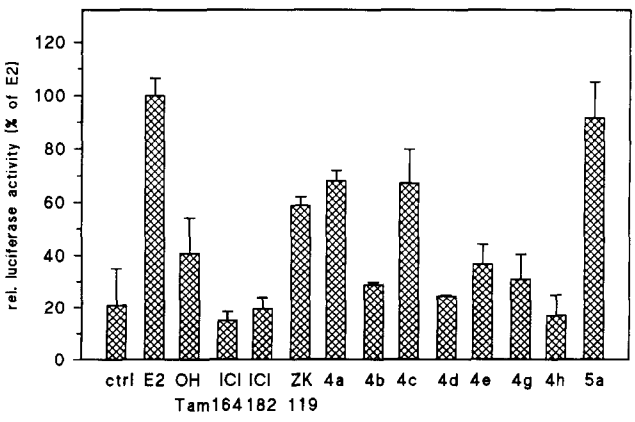

Fig. 6. Transcriptional activity of 17β-estradiol (E2, 10 nM), 4-hydroxytamoxifen (OHTam, 1 µM), ICI 164,384 (ICI 164, 1 µM), ICI 182,780 (ICI 182, 1 µM), and various 2-phenylindole derivatives [4a–e, 4g, 4h, 5a, ZK 119,010(=5b), 1 µM] in HeLa cells cotransfected with the reporter plasmid EREwtc luc and the expression vector HEG0 for the human estrogen receptor. Values are means of 3 independent experiments ± SD.

of 4-hydroxytamoxifen and ZK 119,010 was less pronounced. The results for the more potent antagonists are in good agreement with those obtained with the wildtype receptor. Since the transfection of the HeLa cells is only transient, a large number of transfection experiments with various compounds as ligand for the estrogen receptor have been performed. The reproducibility of results was usually in the range of SD especially in respect to those antiestrogens which are devoid of agonist activity.

One major issue of these investigations was to find out which structural features of the side chain are necessary to suppress transcriptional activity of estrogen-regulated genes completely. For a complete blockade, both transactivating functions TAF-1 and TAF-2 have to be inactive. In order to study this in detail, we used not only the expression vector for the wildtype estrogen receptor (HEG0) but also the specific deletion mutants HE15, which lacks both the hormone binding domain and the transcription activating function 2 (TAF-2), and HEG19, which is devoid of TAF-1 (Fig. 3). With the wildtype receptor, two of the antiestrogens were completely inactive in stimulating luciferase activity: ICI 164,384 and the 2-phenylindole 4f with the identical undecanamide side chain (Fig. 8). All derivatives with an amino group in the side chain—this also applies to tamoxifen—showed transcription activation. The experiment with the estrogen receptor mutant (HEG19) lacking the TAF-1 domain showed that this activity is only mediated by TAF-1 which has been described as constitutive activity [24]. Except estradiol, none of these compounds exhibited TAF-2 mediated luciferase expression. The mutant vector HE15 which harbors TAF-1 and the DNA-binding domain activated transcription independently from ligand used.

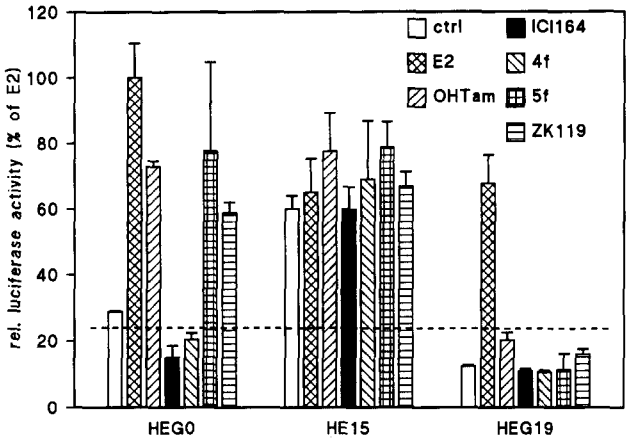

Fig. 8. Transcriptional activity of 17β-estradiol (E2, 10 nM), 4-hydroxytamoxifen (OH-Tam, 1 μM), ICI 164.384 (1 μM), ZK 119.010 (1 μM), and compounds 4f and 5f (1 μM) in HeLa cells cotransfected with EREwtc luc plasmid and expression vectors for the human wildtype estrogen receptor HEG0 and deletion mutants HE15 and HEG19. Values are means of 3 independent experiments ± SD. The dashed line represents the mean value of luciferase activity in cells not transfected with estrogen receptor expression vectors.

Endocrine activity in mice

The results shown above demonstrate that the regulation of transcription is a very sensitive instrument to detect small alterations in the side chain structure of ligands bound to the estrogen receptor. The basic structure, steroid or 2-phenylindole, seems to play a less important role. For the further development of these compounds it was necessary to establish a relationship between transcriptional activity and endocrine properties in animals. Therefore, we submitted a number of these 2-phenylindole derivatives to the mouse uterine weight test in order to determine their estrogenic and antiestrogenic profiles. As in previous studies, we used estrone as reference estrogen in these *in vivo* experiments [17].

On the molecular level, the 2-phenylindole **4f** was found to be an antagonist without any estrogenic activity whereas the reduced derivative **5f** acted as a partial antagonist. Dose–response curves from the mouse uterine weight tests are fully in accordance with these observations: no estrogenic activity with **4f** but weak estrogenicity with **5f** (Fig. 9). Compound **4d** appears to be a border-line case: no increase of luciferase activity in HEG0-transfected cells but some effect in the HE0 system. When we analyzed the uterotrophic effect, we detected some agonistic activity at high doses together with an incomplete antagonism (Fig. 10). Derivative **4h** was devoid of any estrogenic effects in the mouse uterus weight test as expected from the luciferase assay (Fig. 10). Since **4h** also suppresses estrogen-stimulated uterine growth completely it can be considered as a "pure" antiestrogen in all respects and a good candidate for further development.

Effect on MCF-7 cell growth

The aim of these studies is the development of new antiestrogens without estrogenic side effects for the therapy of estrogen-dependent malignancies such as breast cancer. A widely used *in vitro* model for the evaluation of cytostatics acting via the estrogen receptor are human MCF-7 breast cancer cells. All compounds of this study strongly inhibited cellular growth in a dose-dependent manner (Figs 11 and 12). The IC_{50}-values of the most active derivatives **4f**, **4e**, **4h** and **5f** were in the range of $1–5 \times 10^{-8}$ mol/l (Fig. 11). Their cytostatic activities are similar to that of 4-hydroxytamoxifen and somewhat lower than those of the steroidal derivatives ICI 164,384 and ICI 182,780. When the carbamoyl function was reduced to an amino group the inhibitory effect increased slightly. The assumption that the antitumor activity in MCF-7 cells

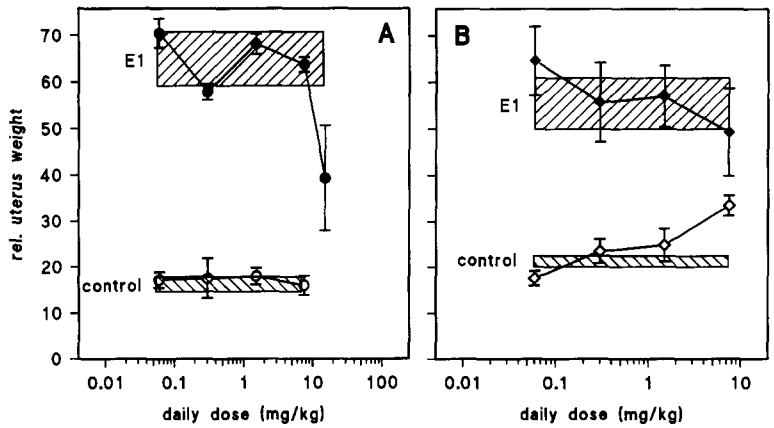

Fig. 9. Estrogenic and antiestrogenic activity of 4f (A) and 5f (B) in the mouse uterine weight test. Animals were injected daily with the drug alone (-○-) or together with a standard dose of 0.4 μg estrone/animal (-●-) for 3 days. Rel. uterus weight [=uterus dry weight (mg)/body weight (g) × 100] was determined 24 h after the last injection; mean of 6 animals ± SD. Hatched boxes indicate values for control and estrone-treated animals ± SD.

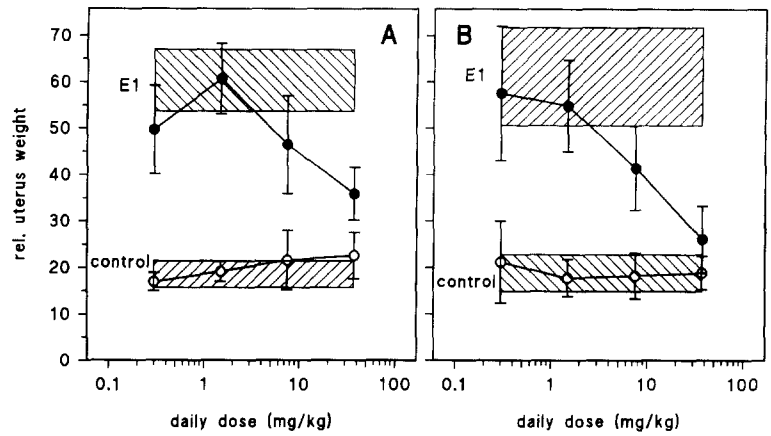

Fig. 10. Estrogenic and antiestrogenic activity of 4d (A) and 4h[(B; for details see Fig. 9).

is receptor mediated is supported by the lack of inhibition in receptor negative MDA-MB 231 human breast cancer cells (data not shown).

DISCUSSION

In this paper we have addressed three major issues: (i) the influence of side chain modifications of non-steroidal structures on the transcription of ERE-regulated genes; (ii) the correlation of transcriptional activity measured in a luciferase assay with endocrine parameters such as the increase of uterus weight; (iii) the estrogen receptor mediated effect on the growth of MCF-7 breast cancer cells. As shown in the laboratories of P. Chambon [25] and others [34], the transcription of estrogen regulated genes can be activated by two functional domains on the estrogen receptor (TAF-1, TAF-2). TAF-2 requires the binding of an estrogen as ligand for its function whereas TAF-1 appears to be constitutive. Studies with various partial estrogen antagonists revealed that their estrogenic activity is solely mediated by TAF-1. In our investigations we have shown that the structure of the side chain (length and functional group) controls the activity of TAF-1 whereas TAF-2 remains silent. The 2-phenylindole **4f** bearing the same side chain as ICI 164,384 and **4h**, where the functional group is shifted by one methylene group completely inhibited transcription. The same observation was made with the steroidal structures ICI 164,384 and ICI 182,780. The

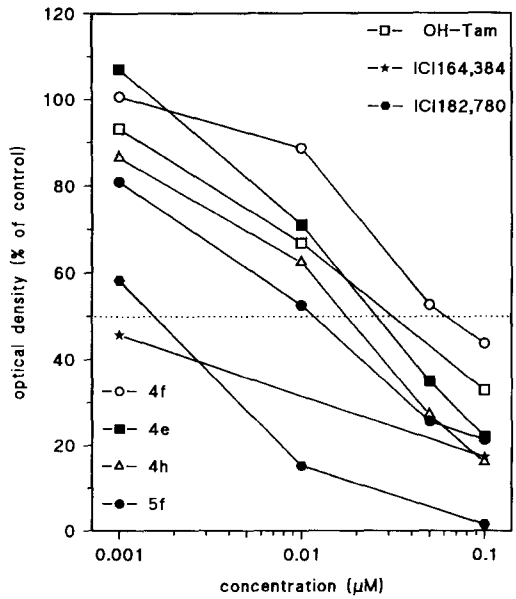

Fig. 11. Effect of 2-phenylindoles 4e, 4f, 4h, and 5f and reference compounds 4-hydroxytamoxifen (OH-Tam), ICI 164,384, and ICI 182,780 on the growth of hormone-sensitive human MCF-7 breast cancer cells, shown as optical densities following crystal violet staining of viable cells. Values are means of 16 replicates; SD are in the range of 10 to 20%.

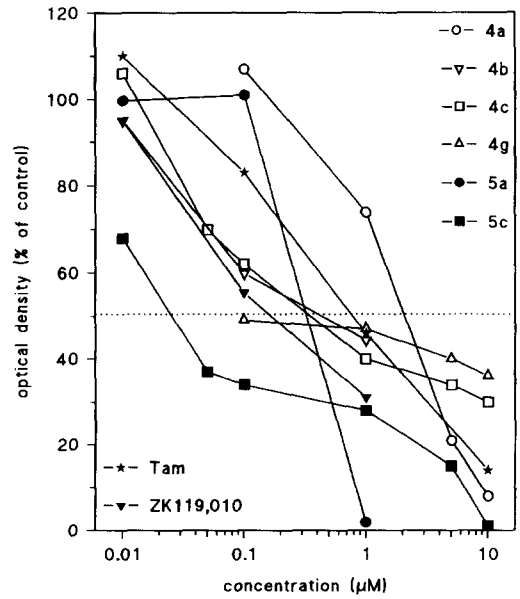

Fig. 12. Effect of 2-phenylindoles 4a, 4b, 4c, 4g, 5a, ZK 119,010 (=5b), 5c, and tamoxifen on the growth of hormone-sensitive human MCF-7 breast cancer cells, shown as optical densities following crystal violet staining of viable cells. Values are means of 16 replicates; SD are in the range of 10 to 20%.

total absence of transactivation can have various reasons: an obvious explanation would be that the receptor has lost its capability to bind to DNA. The DNA binding requires some factors which have not yet been identified [35]. Parker and coworkers explained the lack of activity by the inability of the receptor protein to undergo dimerization which is a prerequisite for an effective interaction with the DNA [22]. An impaired dimerization can be due to a steric hindrance exerted by the long side chain in these molecules. From our results, this explanation appears rather unlikely because compounds with the same length of the side chain can either be active or inactive concerning transcription. We assume that molecules with a distinct side chain structure are able to block both transcription activating functions without interfering with the DNA binding. The structural requirements for the deactivation of TAF-1 appear to be much stricter than those for the blockade of TAF-2. The latter effect leads to partial antagonism.

The 2-phenylindoles **4f** and **4h** which suppressed transcription of the luciferase gene completely showed no estrogenic activity *in vivo*. At higher doses, they were able to antagonize the effect of the simultaneously administered estrogen. Compounds which activated transcription in the luciferase assay—even if the effect is very small—gave rise to a significant estrogenic effect *in vivo* when higher doses of the compound were used. These findings lead to the conclusion that one of the first steps in the design of antiestrogens devoid of estrogenic side effects should be a minimization of transcriptional activity *in vitro* as measured by appropriate techniques such as the luciferase assay applied in this study.

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