2-Phenylindole-linked Nitroimidazoles as Potential Radiosensitizers for Estrogen Receptor Positive Tumors

Sebastian Erber and Erwin von Angerer*

Institut für Pharmazie, Universität Regensburg, Universitätsstraße 31, D-8400 Regensburg

Received August 9, 1989

5-Hydroxy-2-(4-hydroxyphenyl)-3-methylindole was linked to nitroimidazoles by tetramethylene and hexamethylene spacer groups. Derivatives with a C_6-spacer (**4c** and **4d**) exhibited high binding affinities for the estrogen receptor (RBA = 3.5; estradiol: 100), a prerequisite for a selective uptake by estrogen receptor positive tumors. Both compounds inhibited the growth of hormone-sensitive human MCF-7 mammary tumor cells at concentrations $> 5 \times 10^{-6}$ M, presumably due to the weak antiestrogenic effect observed in the mouse uterine weight test.

Nitroimidazol-verknüpfte 2-Phenylindole als potentielle Radiosensibilisatoren östrogenrezeptor-positiver Tumoren

5-Hydroxy-2-(4-hydroxyphenyl)-3-methylindol wurde über eine Tetramethylen- bzw. Hexamethylengruppe mit Nitroimidazolen verknüpft. Die Derivate mit einer C_6-Spacergruppe (**4c** und **4d**) zeigten eine hohe Affinität zum Östrogenrezeptor (RBA = 3.5; Östradiol: 100), womit eine Voraussetzung für die selektive Aufnahme durch östrogenrezeptorpositive Tumoren gegeben war. Beide Verbindungen hemmen das Wachstum hormonsensitiver menschlicher MCF-7-Mammatumorzellen in Konzentrationen $> 5 \times 10^{-6}$ M, vermutlich aufgrund der schwachen antiöstrogenen Wirkung, die im Uterusgewichtstest an der Maus beobachtet wurde.

A major problem in radiotherapy of solid tumors is the presence of hypoxic tumor cells in those parts of the tumor which are not sufficiently supplied with oxygen. This population of cells has a reduced rate of proliferation and is less sensitive to radiation than normal cells[1]. When tumor cells in the peripheral part of the tumor are killed by radiation, the hypoxic cells can be supplied with oxygen and nutrients and start growing again[2]. In order to overcome this resistance a number of nitroheterocycles were studied for their ability to "substitute" the oxygen in the hypoxic cells. Most of the studies with radiosensitizing agents were performed with nitroimidazole derivatives like metronidazole[3,4]. A major disadvantage of these drugs is the lack of specificity for tumor tissues and, therefore, a rather high toxicity for normal tissues. For a selective accumulation of the radiosensitizer tumor-oriented agents are needed.

One approach to reach this goal is the development of radiosensitizing molecules which are bound to antibodies directed to tumor cells[5]. Another possibility is the synthesis of nitroheterocycles linked to a carrier with affinity for steroid hormone receptors known to be present in many mammary, prostatic and ovarian carcinomas. In this paper, we report on the synthesis of nitroimidazole derivatives linked to 5-hydroxy-2-(4-hydroxyphenyl)-3-methylindole by spacer groups of varying length, their binding affinity for the estrogen receptor and biological activity.

Chemistry

The anion generated from the methoxy substituted 2-phenylindole **1**[6] with NaH was reacted with an excess of the 1,ω-dibromoalkane to afford the 1-(ω-bromoalkyl) derivatives **2a-c** (Fig. 1). In the next step, the ether functions were cleaved with BBr_3. The phenolic compounds were converted into the acetates **3a-c** which can be purified easily. 4-Nitroimidazole and 2-methyl-5-nitroimidazole respectively were reacted with the ω-bromoalkyl compound in the presence of NaOMe as base and a catalytic amount of NaI. Reaction of the bromobutyl and bromohexane afforded the corresponding indole-linked imidazoles **4a-d** in good yield. Since either of the two N-atoms of the imidazole can act as nucleophile, the formation of two isomers is possible. HPLC analysis of the reaction products revealed that only one isomer was formed. The position 4 for the nitro group was assigned by the *Nuclear Overhauser* Effect: Irradiation of **4b** at the frequence of the methylen group fixed to the imidazole led to a dramatic increase of the signal for the imidazole hydrogen. The ester functions of the 2-phenylindole were hydrolysed during the reaction and isolation of the products.

Unexpectedly, the bromooctane derivative did not react with the imidazole but formed the cyclic ether **5**. The formation can be rationalized by an intramolecular nucleophilic attack of the phenolate. The ring closure via the oxygen at the phenyl ring was confirmed by NMR spectroscopy showing only one phenolic proton with a chemical shift which is characteristic for the 5-hydroxyindole system.

Results and Discussion

The binding affinities for the estrogen receptor were measured by a competitive binding assay with 17β-[^{3}H]estradiol. Calf uterine cytosol was used as receptor source and the dextran coated charcoal (DCC) method applied[6]. The relative binding affinities (RBA) are given as the ratio of the molar concentrations of 17b-estradiol and indole required to decrease the receptor bound radioactivity by 50%, multiplied by 100.

All of the nitroimidazole derivatives bind to the estrogen receptor (Table 1). However, there is a strong difference in affinity between derivatives with a C_4-spacer group (**4a** and **b**) and those with a hexamethylene group (**4c** and **d**). The

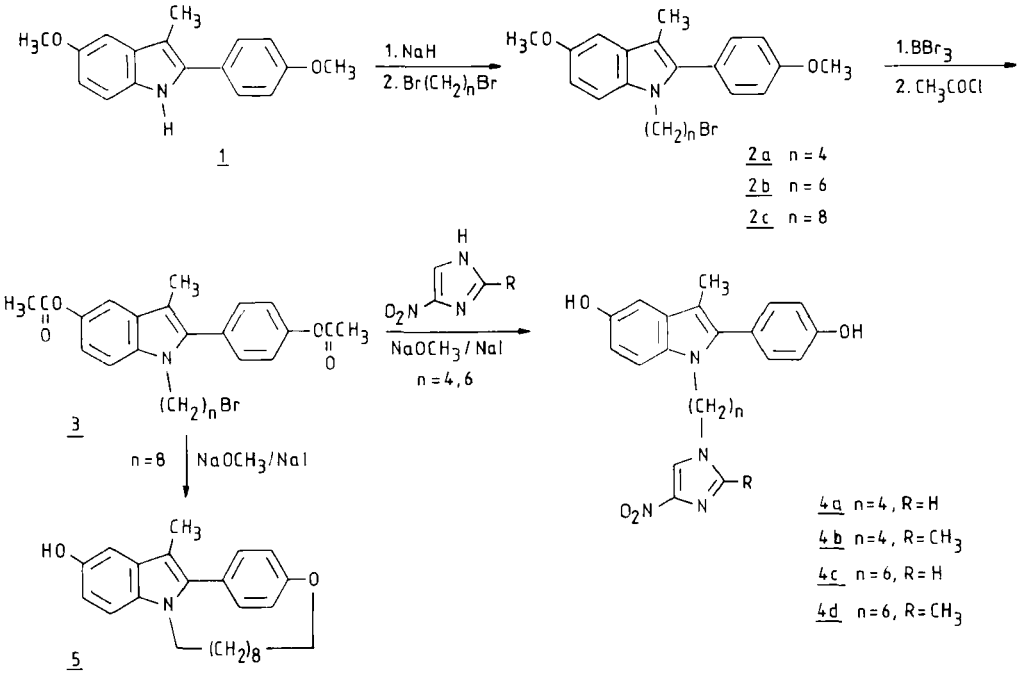
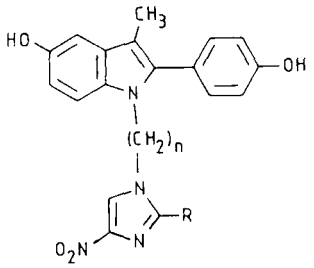


Fig. 1: Synthesis of 4-nitroimidazole linked 2-phenylindole derivatives.

Tab 1: Relative binding affinities (RBA) of the synthesized nitroimidazole derivatives **4a-d.**



comp.	n	R	RBA*[%]
4a	4	-methyl	0.11
4b	4	-H	0.25
4c	6	-methyl	3.5
4d	6	-H	3.5

* Relative binding affinities for the calf uterine estrogen receptor = ratio of molar concentrations of 17β-estradiol (E_2) and inhibitor required to decrease the amount of bound [^{3}H]E_2 by 50%, x 100.

RBA values of **4c** and **d** exceed the ones of **4a** and **b** by one order of magnitude. This observation is in accordance to results obtained with 2-phenylindole derivatives with amino functions in the side chain exhibiting a maximum of affinity for C-6-spacer groups[7]. In comparison to the analoguous 1-pentyl-2-phenylindole lacking the substituent in the side chain (RBA = 2.5[6]), the affinity of compounds with a C4-alkyl chain is greatly reduced. Obviously, the nitro-heterocycle in these derivatives interferes with the hormone binding site.

The cytostatic activity of the nitroimidazoles was determined in vitro using two different human breast cancer cell lines: ER negative MDA-MB 231 and ER positive MCF-7 cells. No inhibitory effect was observed with hormone-independent MDA-MB 231 cells (data not shown). With hormone-sensitive MCF-7 cells, only derivatives with a hexamethylene spacer showed a significant inhibition of cellular growth, whereas compounds with a shorter alkyl chain were inactive due to the low affinity for the estrogen receptor (Table 2).

Tab. 2: Effect of **4a-d** and Tamoxifen on the Growth of the Hormon-dependent MCF-7 Cell Line.

	T/C[a] [%]			
comp.	1 x 10^{-7} M[b]	1 x 10^{-6} M[b]	5 x 10^{-6} M[b]	1 x 10^{-5} M[b]
Tam	56.5 ± 16.3[c]	59.1 ± 14.1[c]	33.6 ± 10.3[c]	-5.7 ± 3.4[c]
4a	122.6 ± 44.0	142.5 ± 45.9	111.4 ± 37.3	97.3 ± 34.2
4b	108.4 ± 23.0	111.8 ± 18.7	84.9 ± 17.1	79.3 ± 15.8
4c	92.2 ± 18.0	89.3 ± 18.7	77.5 ± 15.2	40.8 ± 9.4[c]
4d	83.8 ± 21.3	82.3 ± 21.9	68.4 ± 16.5[c]	46.4 ± 11.0[c]

[a] Ratio of optical densities of test and control wells; initial density = 0%; mean of 16 wells ± SD.

[b] Inhibitor concentration [mol/l] in the incubation medium.

[c] Significant difference between control and test values, $p < 0.01$.

The endocrine activity of the imidazoles **4c** and **d** was determined in the mouse uterine weight test. No significant estrogenic effect was observed at doses between 1 and 125 µg/animal. At the highest dose, both compounds showed a weak but significant inhibition of the estrone stimulated uterine growth (Table 3).

Tab. 3: Estrogenic and Antiestrogenic Activity of **4c** and **4d** in the Immature Mouse Uterine Weight Test.

	uterotrophic test		antiuterotrophic test[a]	
comp.	dose, μg[b]	rel. ut. weight[c]	rel. ut. weight[c]	inhibn, %
Kontr.	-	15.4 ± 3.2	15.4 ± 3.2	
ßstron	0.4	51.7 ± 5.8	51.7 ± 5.8	
4c	1	17.1 ± 2.4		
	5	13.4 ± 4.4	49.7 ± 5.0	5.4
	25	13.5 ± 2.2	48.3 ± 8.9	9.4
	125	14.0 ± 2.8	40.9 ± 5.8	29.7[d]
4d	1	15.4 ± 4.8		
	5	14.6 ± 2.3	52.9 ± 8.8	-
	25	17.9 ± 7.7	47.9 ± 9.1	10.5
	125	16.0 ± 4.7	43.9 ± 7.1	21.4[e]

[a] Simultaneous administration of test compound and 0.4 μg of estrone/animal/day.
[b] Dose per animal, administered at 3 consecutive days sc.
[c] Relative uterine weight = uterus dry weight [mg]/body weight [g] x 100, determined 24 h after the last injection; mean of 6 animals ± SD.
[d] Significant, $p < 0.01$
[e] Significant, $p < 0.025$

The aim of this study was to find out whether the 2-phenylindole structure is suitable for the development of radiosensitizers with binding affinity for the estrogen receptor. When 4-nitroimidazole and 2-methyl-5-nitroimidazole were linked to the indole nitrogen by a hexamethylene group, compounds with high binding affinities were obtained. The RBA-values are similar to those of drugs used in endocrine therapy (e.g. Tamoxifen; RBA = 2). The cytostatic activity in human MCF-7 breast cancer cells can be rationalized by the weak antiestrogenic effect observed in immature mice. Studies on the radiosensitizing potency of these conjugates will be subject of a forthcoming publication.

The authors would like to thank *M. Beer* for technical assistance and the Deutsche Forschungsgemeinschaft (SFB 234) for financial support.

Experimental Part

Mp: Büchi 510 apparatus (uncorr.).- ^{1}H-NMR Spectra: Varian EM 360L spectrometer.- Mass Spectra: Varian MAT CH 5.- Column Chromatography: Kieselgel 60 (Merck).- Elemental Analyses: Mikroanalytisches Laboratorium, Univ. Regensburg.- Temp. in °C.

General Procedure for the N-Alkylation of 5-Methoxy-2-(4-methoxyphenyl)-3-methylindole

With ice cooling, 2.4 g (0.1 mol) of NaH (80% in paraffin) were suspended in 200 ml of dry DMF and stirred for 15 min, followed by dropwise addition of 75 mmol of 5-methoxy-2-(4-methoxyphenyl)-3-methylindole[6] in dry DMF. After stirring for 45 min, this solution was added slowly to an ice-cold solution of 0.1 mol 1,ω-dibromoalkane in 120 ml of dry DMF. After removal of the cooling bath, the mixture was stirred for 4 h at room temp. before it was poured into ice water. The aqueous solution was extracted three times with ether. The combined org. layers were washed with water and dried over $MgSO_4$. The solvent was removed i. vac. and the residue purified by column chromatography (CH_2Cl_2) and crystallized from EtOH to afford colorless crystals.

1-(4-Bromobutyl)-5-methoxy-2-(4-methoxyphenyl)-3-methylindole (**2a**)

Yield 54%, mp. 55-57°.- $C_{21}H_{24}NO_2Br$ (402.3) Calcd. C 62.7 H 6.01 N 3.5 Found C 62.6 H 5.84 N 3.4.- ^{1}H-NMR ($CDCl_3$): δ = 1.50-1.80 (m; 4H, $-(CH_2)_2-$); 2.20 (s; 3H; $-CH_3$); 3.16 (t; J = 6 Hz; 2H, $-CH_2Br$); 3.89 (s; 3H, $-OCH_3$); 3.91 (s; 3H, $-OCH_3$); 4.03 (t; J = 7 Hz; 2H, $-N(CH_2)-$); 6.93 (dd; J_1 = 9 Hz; J_2 = 2 Hz; 1H, ArH); 7.09 (d; J = 2 Hz; 1H, ArH); 7.28 (d; J = 9 Hz; 1H, ArH); 7.03, 7.36 (AA'BB'; J = 9 Hz; 4H, ArH).

1-(6-Bromohexyl)-5-methoxy-2-(4-methoxyphenyl)-3-methylindole (**2b**)

Yield 61%, mp. 54-56°.- $C_{23}H_{28}NO_2Br$ (430.4) Calcd. C 64.2 H 6.55 N 3.2 Found C 63.9 H 6.50 N 3.0.- ^{1}H-NMR ($CDCl_3$): δ = 1.00-1.95 (m; 8H, $-(CH_2)_4-$); 2.19 (s; 3H, $-CH_3$); 3.26 (t; J = 6 Hz; 2H, $-CH_2Br$); 3.88 (s; 6H, $-OCH_3$); 3.98 (t; J = 7 Hz; 2H, $-N(CH_2)-$); 6.83 (dd; J_1 = 9 Hz; J_2 = 2 Hz; 1H, ArH); 7.02 (d; J = 2 Hz; 1H, ArH); 7.20 (d; J = 9 Hz; 1H, ArH); 6.97, 7.38 (AA'BB'; J = 9 Hz; 4H, ArH).

1-(8-Bromooctyl)-5-methoxy-2-(4-methoxyphenyl)-3-methylindole (**2c**)

Yield 47%, mp. 32-33°.- $C_{25}H_{32}NO_2Br$ (458.4) Calcd. C 65.5 H 7.04 N 3.1 Found C 65.4 H 6.91 N 3.0.- ^{1}H-NMR ($CDCl_3$): δ = 1.02-1.97 (m; 12H, $-(CH_2)_6-$); 2.19 (s; 3H, $-CH_3$); 3.34 (t; J = 6 Hz; 2H, $-CH_2Br$); 3.89 (s; 6H, $-OCH_3$); 3.97 (t; J = 7 Hz; 2H, $-N(CH_2)-$); 6.86 (dd; J_1 = 9 Hz; J_2 = 2 Hz; 1H, ArH); 7.03 (d; J = 2 Hz; 1H, ArH); 7.22 (d; J = 9 Hz; 1H, ArH); 6.98, 7.30 (AA'BB'; J = 9 Hz, 4H, ArH).

General Procedure for the Ether Cleavage and Acetylation

A solution of the methoxy-substituted 2-phenylindole (4.0 mmol) in dry CH_2Cl_2 (10 ml) was cooled with ice. Under N_2, a solution of 8.0 mmol BBr_3 in 5 ml of dry CH_2Cl_2 was added slowly. After 30 min the cooling bath was removed and the mixture stirred for 2 h at room temp.. The mixture was cooled with ice and treated with a solution of $NaHCO_3$ until the vigorous reaction ceased. The org. layer was separated and the aqueous phase extracted three times with EtOAc. The combined org. layers were washed with water and dried ($MgSO_4$). After the solvent had been removed, the dark residue was treated with AcCl and refluxed for 1 h. The mixture was poured onto ice and extracted three times with CH_2Cl_2. The combined org. layers were washed with water and dried ($MgSO_4$). After evaporation of the solvent, the remaining residue was chromatographed (CH_2Cl_2) and crystallized from EtOH, except for **3c** being an oil.

5-Acetoxy-2-(4-acetoxyphenyl)-1-(4-bromobutyl)-3-methylindole (**3a**)

Yield 59%, mp. 87-89°.- $C_{23}H_{24}NO_4Br$ (458.4) Calcd. C 60.3 H 5.27 N 3.1 Found C 60.1 H 5.21 N 2.9.- ^{1}H-NMR ($CDCl_3$): δ = 1.50-1.83 (m; 4H, $-(CH_2)_2-$); 2.19 (s; 3H, $-CH_3$); 2.34 (s; 6H, $-COCH_3$); 3.20 (t; J = 6 Hz; 2H, $-CH_2Br$); 4.05 (t; J = 7 Hz; 2H, $-N(CH_2)-$); 6.96 (dd; J_1 = 9 Hz; J_2 = 2 Hz; 1H, ArH); 7.15-7.50 (m; 6H, ArH).

5-Acetoxy-2-(4-acetoxyphenyl)-1-(6-bromohexyl)-3-methylindole (**3b**)

Yield 64%, mp. 85-86°.- $C_{25}H_{28}NO_4Br$ (486.4) Calcd. C 61.7 H 5.80 N 2.9 Found C 61.7 H 6.03 N 2.8.- ^{1}H-NMR ($CDCl_3$): δ = 1.00-1.90 (m; 8H, $-(CH_2)_4-$); 2.17 (s; 3H, $-CH_3$); 2.34 (s; 6H, $-COCH_3$); 3.30 (t; J = 6 Hz; 2H, $-CH_2Br$); 4.03 (t; J = 7 Hz; 2H, $-N(CH_2)-$); 6.92 (dd; J_1 = 9 Hz; J_2 = 2 Hz; 1H, ArH); 7.13-7.74 (m; 6H, ArH).

5-Acetoxy-2-(4-acetoxyphenyl)-1-(8-bromooctyl)-3-methylindole (**3c**)

Yield 59%, colorless oil.- $C_{27}H_{32}NO_4Br$ (514.5).- ^{1}H-NMR ($CDCl_3$): δ = 0.98-1.90 (m; 12H, $-(CH_2)_6-$); 2.19 (s; 3H, $-CH_3$); 2.36 (s; 6H, $-COCH_3$); 3.37 (t; J = 7 Hz; 2H, $-CH_2Br$); 4.01 (t; J = 7 Hz; 2H, $-N(CH_2)-$); 6.97 (dd; J_1 = 9 Hz; J_2 = 2 Hz; 1H, ArH); 7.18-7.53 (m; 6H, ArH).

General Procedure for the Alkylation of Nitroimidazoles

A mixture of 3.0 mmol of the alkylbromide **3a-c** in 50 ml of DMF, 3.0 mmol of nitroimidazole, catalytic quantities of NaI (ca. 6 mg) and 3.0 mmol of EtONa in 25 ml MeOH were heated to 110-120° for 4 h. After removal of the condenser, most of the MeOH was allowed to evaporate. Precipitated NaBr was dissolved by the addition of water. The solution was extracted three times with EtOAc, washed with M NaOH, water and dried ($MgSO_4$). After removal of the solvent i. vac., the product was purified by column chromatography (CH_2Cl_2/EtOH 19:1) and crystallized from MeOH/H_2O.

5-Hydroxy-2-(4-hydroxyphenyl)-3-methyl-1-[4-(4-nitroimidazol-1-yl)-butyl]indole (**4a**)

Yield 61%, mp. 214-217°.- $C_{22}H_{22}N_4O_4$ (406.4) Calcd. C 65.0 H 5.46 N 13.8 Found C 65.1 H 5.46 N 13.6.- ^{1}H-NMR (d_6-Aceton): δ = 1.50-1.80 (m; 4H, -$(CH_2)_2$-); 2.11 (s; 3H, -CH_3); 4.02 (t; J = 7 Hz; 2H, -N(CH_2)-$_{Imid.}$); 4.13 (t; J = 7 Hz, 2H, -N(CH_2)-); 6.77 (dd; J_1 = 9 Hz; J_2 = 2 Hz; 1H, ArH); 6.97 (d; J = 2 Hz; 1H, ArH); 7.26 (d; J = 9 Hz; 1H, ArH); 7.00, 7.27 (AA'BB'; J = 9 Hz; 4H, ArH); 7.554 (d; J = 1 Hz; 1H, =CH-$_{Imid.}$); 8.04 (d; J = 1 Hz; 1H, =CH-$_{Imid.}$); 7.68 (s; 1H, -OH); 8.66 (s; 1H, -OH).

5-Hydroxy-2-(4-hydroxyphenyl)-3-methyl-1-[4-(2-methyl-4-nitroimidazol-1-yl)butyl]indole (**4b**)

Yield 73%, mp. 202-204°.- $C_{23}H_{24}N_4O_4$ (420.5) Calcd. C 65.7 H 5.75 N 13.3 Found C 65.3 H 5.49 N 13.0.- ^{1}H-NMR (d_6-Aceton): δ = 1.50-1.72 (m; 4H, -$(CH_2)_2$-); 2.11 (s; 3H, -CH_3); 2.23 (s; 3H, -$(CH_3)_{Imid.}$); 3.89 (t; J = 7 Hz; 2H, -N(CH_2)-$_{Imid.}$); 4.12 (t; J = 7 Hz; 2H, -N(CH_2)-); 6.77 (dd; J_1 = 9 Hz; J_2 = 2 Hz; 1H, ArH); 6.96 (d; J = 2 Hz; 1H, ArH); 7.25 (d; J = 9 Hz; 1H, ArH); 6.98, 7.26 (AA'BB'; J = 9 Hz; 4H, ArH); 7.90 (s; 1H, =$CH_{Imid.}$-); 7.67 (s; 1H, -OH); 8.67 (s; 1H, -OH).

5-Hydroxy-2-(4-hydroxyphenyl)-3-methyl-1-[6-(4-nitroimidazol-1-yl)-hexyl]indole (**4c**)

Yield 75%, mp. 180-181°.- $C_{24}H_{26}N_4O_4$ (434.5) Calcd. C 66.3 H 6.03 N 12.9 Found C 66.1 H 5.87 N 12.5.- ^{1}H-NMR (CD_3OD): δ = 0.84-1.80 (m; 8H, -$(CH_2)_4$-); 2.11 (s; 3H, -CH_3); 3.85 (t; J = 7 Hz; 2H, -N(CH_2)-$_{Imid.}$); 3.95 (t; J = 7 Hz; 2H, -N(CH_2)-); 6.70 (dd; J_1 = 9 Hz; J_2 = 2 Hz; 1H, ArH); 6.88 (d; J = 2 Hz; 1H, ArH); 7.12 (d; J = 9 Hz; 1H, ArH); 6.87, 7.15 (AA'BB'; J = 9 Hz; 4H, ArH); 7.57 (d; J = 1 Hz; 1H, =CH-$_{Imid.}$); 8.00 (d; J = 1 Hz; 1H, =CH-$_{Imid.}$).

5-Hydroxy-2-(4-hydroxyphenyl)-3-methyl-1-[6-(2-methyl-4-nitroimidazol-1-yl)hexyl]indole (**4d**)

Yield 45%, mp. 133-136°.- $C_{25}H_{28}N_4O_4$ (448.5) Calcd. C 66.9 H 6.29 N 12.5 Found C 66.5 H 6.05 N 12.3.- ^{1}H-NMR (CD_3OD): δ = 0.83-1.80 (m; 8H, -$(CH_2)_4$-); 2.09 (s; 3H, -CH_3); 2.30 (s; 3H, -$(CH_3)_{Imid.}$); 3.75 (t; J = 7 Hz; 2H, -N(CH_2)-$_{Imid.}$); 3.95 (t; J = 7 Hz; 2H, -N(CH_2)-); 6.65 (dd; J_1 = 9 Hz; J_2 = 2 Hz; 1H, ArH); 6.84 (d; J = 2 Hz; 1H, ArH); 7.08 (d; J = 9 Hz; 1H, ArH); 6.85, 7.11 (AA'BB'; J = 9 Hz; 4H, ArH); 7.84 (s; 1H, =CH-$_{Imid.}$).

5-Hydroxy-3-methyl-1,4'-(octamethylene-8-oxy)-2-phenylindole (**5**)

Yield 51%, mp. 204-205°.- $C_{23}H_{27}NO_2$ (349.5) Calcd. C 79.1 H 7.79 N 4.0 Found C 79.1 H 7.66 N 3.7.- MS: m/z = 349 (M^+).- ^{1}H-NMR (d_6-Aceton): δ = 0.60-1.84 (m; 12H, -$(CH_2)_6$-); 2.22 (s; 3H, -CH_3); 3.90 (t; J = 7 Hz; 2H, -N(CH_2)-); 4.36 (t; J = 5.5 Hz; 2H, -OCH_2CH_2-); 6.73 (dd; J1 = 9 Hz; J_2 = 2 Hz; 1H, ArH); 6.95 (d; J = 2 Hz; 1H, ArH); 7.19 (d; J = 9 Hz; 1H, ArH); 7.17, 7.35 (AA'BB'; J = 9 Hz; 4H, ArH); 7.61 (s; 1H, -OH).

Relative Binding Affinities for the Estrogen Receptor

The determination of the RBA values were carried out as described[6], except that 96-well microtiter plates were used instead of 1.5 ml reaction vessels. Thereby, all volumes were reduced to one fifth of the original ones and multichannel pipettes were used.

In vitro determination of cytostatic activity

The hormone-sensitive human MCF-7 breast cancer cell line was obtained from the AMERICAN TYPE CULTURE COLLECTION (Rockville, Md U.S.A.) and cultured as described[8]. At the start of the experiment, the cell suspension was transfered to the 96-well microtiter plates (100 µl/well). After growing them for 2-3 d in a humidified incubator with 5% CO_2 at 37°, medium was replaced by one containing the drug. Control wells (16/plate) contained 0.1% of ethanol, that was used for the preparation of the stock solution. The initial cell density was determined by addition of vinblastin (10^{-7} M). After an incubation time of 3 d, medium was removed and the cells were fixed by adding 100 µl of glutaric aldehyde in PBS (1%). 15 min later, 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, the cells were washed several times with water to remove adherent dye, followed by an addition of 100 µl of ethanol (70%). The plates were gently shaken for 1 h. Optical density of each well was measured in a microplate autoreader EL 309 (Bio-tek) at 578 nm. Data calculation and analysis were performed on an Olivetti M24 PC[9].

Estrogenic and antiestrogenic activity

Estrogenic and antiestrogenic properties were determined by stimulation of the uterine growth or by inhibition of the uterine growth stimulated by estrone, respectively, using the immature mouse uterine weight test. The method used was described[6].

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