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Synthesis, Structural Evaluation, and Estrogen Receptor Interaction of 4,5-Bis(4-hydroxyphenyl)imidazoles

4,5-Bis(4-hydroxyphenyl)imidazoles with 2,2′-H (**1**), 2,2′-F (**2**), 2,2′-Cl (**3**), and 2,2′6-Cl (**4**) substituents in the aromatic rings were synthesized by oxidation of the respective methoxy-substituted (*R,S*)/(*S,R*)-4,5-diaryl-2-imidazolines with MnO_2 and subsequent ether cleavage with BBr_3. *N*-alkylation of **1** and **3** with ethyl iodide yielded the compounds **5** and **6**. The imidazoles were characterized by NMR spectroscopy and tested for estrogen receptor binding in a competition experiment with [^{3}H]estradiol using calf uterine cytosol. Gene activation was verified in a luciferase assay using estrogen receptor positive MCF-7-2a cells stably transfected with the plasmid ERE_{wtc}luc. All halide substituted imidazoles competed with estradiol for the binding site at the estrogen receptor. The *N*-ethyl derivative **6** showed the highest relative binding affinity of 1.26 %. Treatment of MCF-7-2a cells, however, did not lead to gene activation. The relative activation of **6** amounted only to 10 % at 1µM compared to **E2** (100 %).

Keywords: Estrogenic activity; Luciferase assay; Estrogen receptor binding; 4,5-Diarylimidazoles

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Introduction

Estrogens are hormones that regulate numerous biological effects in male and female [1–6]. The effects are mediated by the estrogen receptor (ER). The attachment of drugs causes a conformational change of the receptor, dimerisation of the resulting ER/drug-conjugates, and subsequent binding to the "Estrogen Response Element" (ERE) of the DNA [7]. This cascade finally leads to gene activation. For the design of new selective estrogen receptor modulators (SERM), a knowledge of the binding mode is essential. While for decades the binding mode of estradiol (**E2**) and related agonists, e.g. diethylstilbestrol (**DES**), but also of antagonists, e.g. raloxifene (**RAL**) or 4-hydroxytamoxifen (**4OHT**) (formulae, Chart 1), was speculative, the X-ray structures of the ligand binding domain (LBD) of the ER co-crystallized with these drugs are now available [8–10]. It could be shown that agonists and antagonists are primarily H-bonded to the carboxylate of Glu 353, the guanidinium residue of Arg 394, and a water molecule. A second essential anchor to cause estrogenic activity is His 524. Due to this interaction, helix 12 as part of the LBD is oriented over the binding cavity, and protects the drug from the environment. The antagonistic effects of **RAL** and **4OHT** result from the orientation of the basic side chain in a narrow side pocket. H bonding of the piperidine (**RAL**) or dimethylamino (**4OHT**) nitrogen to Asp 351 changes the position of the helix 12, which prevents gene activation.

In a previously published structure activity study we identified (*R,S*)/(*S,R*)-configurated 2,3-diarylpiper-

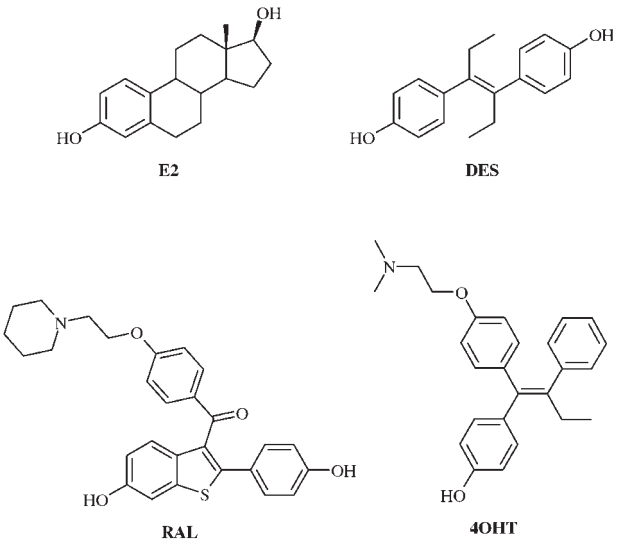

Chart 1. The structural formulae of compounds referred to in the text.

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azines and 4,5-diaryl-2-imidazolines as new lead structures for the design of SERM [11]. We could show that the molecules must bear two hydroxy groups and hydrophobic substituents in the aromatic rings. In this class of compounds, the essential H-bridge to His 524 can be replaced by another H-bridge, presumably to Asp 351. The pharmacophoric 1,2-diarylethane moiety [12] takes in (*R,S*)/(*S,R*)-2,3-diarylpiperazines a synclinal (dihedral angle about 40–60°, see **7** in Figure 1) and in (*R,S*)/(*S,R*)-4,5-diaryl-2-imidazolines an eclipsed conformation (pseudo axially oriented aromatic rings: dihedral angle about 0–10° see **8** in Figure 1) [13, 14]. Since the spatial structure of the latter induces significantly higher gene activation, we investigated the ER binding properties of 4,5-diarylimidazoles, since at the heterocyclic ring the pharmacophor is fixed in a stable *cis*-configuration comparable to the conformation in imidazolines. The influence of the substitution pattern in the aromatic rings on the ER binding and the gene activation in MCF-7-2a cells is described.

Synthesis

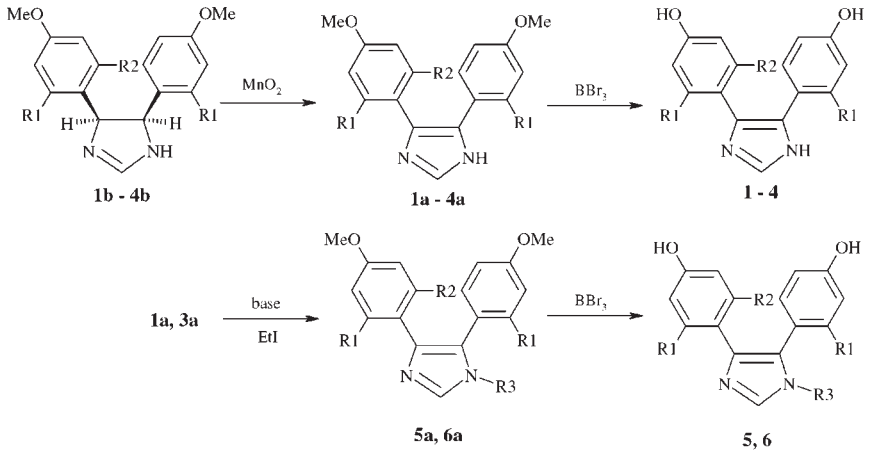
4,5-Diarylimidazoles were synthesized by oxidation of 4,5-diaryl-2-imidazolines [14] with MnO_2 (Scheme 1) analogously to the method described by Martin et al. [15].

Since by this reaction the asymmetric character of the molecules is lost, imidazoles could be generated either from (*R,S*)/(*S,R*)- or (*R,R*)/(*S,S*)-configurated 2-imidazolines. However, the (*R,R*)/(*S,S*)-configurated compounds cannot be oxidized, since the aromatic rings, located above and below the heterocyclic plane hinder the contact of the manganese dioxide.

For *N*-alkylation, the acidic character of the imidazole ring is utilized. The reaction with bases increases the nucleophilic character of the imidazole nitrogens and *N*-alkylation with ethyl iodide can be achieved. While alkylation of imidazole **1 a** was performed nearly quantitatively with sodium ethanolate, the same reaction failed with the 4,5-bis(2-chloro-4-methoxyphenyl)imidazole **3 a**. Only the use of sodium hydride to convert **3 a** into a defined sodium salt and the subsequent reaction with ethyl iodide yielded *N*-ethyl-4,5-bis(2-chloro-4-methoxyphenyl)imidazole **6 a** in satisfactory amounts. The hydroxy substituted imidazoles **1** to **6** were generated by ether cleavage with BBr_3.

Structural evaluation

The imidazole moiety represents a planar aromatic ring system, which forces all substituents into one plane. In Figure 1 a low energy conformation of 4-(2-chloro-4-hydroxyphenyl)-5-(2,6-dichloro-4-hydroxyphenyl)imid-



R_1	R_2	R_3	compound
H	H	H	**1b, 1a, 1**
F	H	H	**2b, 2a, 2**
Cl	H	H	**3b, 3a, 3**
Cl	Cl	H	**4b, 4a, 4**
H	H	ethyl	**5a, 5**
Cl	Cl	ethyl	**6a, 6**

Scheme 1. Synthesis of 4,5-diarylimidazoles.

azole **4** is shown as an example. The *cis*-configurated aromatic rings (dihedral angle: 5.8°) are twisted and oriented to the imidazole ring at angles of 70.0° (2-chloro-4-hydroxyphenyl ring) and 40.1° (2,6-dichloro-4-hydroxyphenyl ring). The O–O distance amounts to 8.9 Å.

The structural characterization of the imidazoles was performed by NMR spectroscopy. In the ^{1}H NMR spectra of the 4,5-bis(4-hydroxyphenyl)imidazole **1** taken in [D6]-DMSO (Figure 2) the N*H* signal is broad and weak indicating a proton exchange reaction between the nitro-

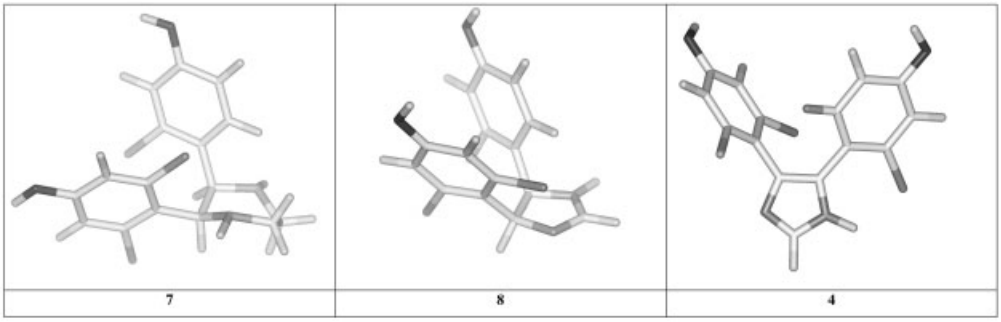


Figure 1. Low energy conformations of the 2-chloro-4-hydroxy/2′,6′-dichloro-4′-hydroxy substituted 2,3-diarylpiperazine **7**, 4,5-diaryl-2-imidazoline **8** and 4,5-diarylimidazole **4**.

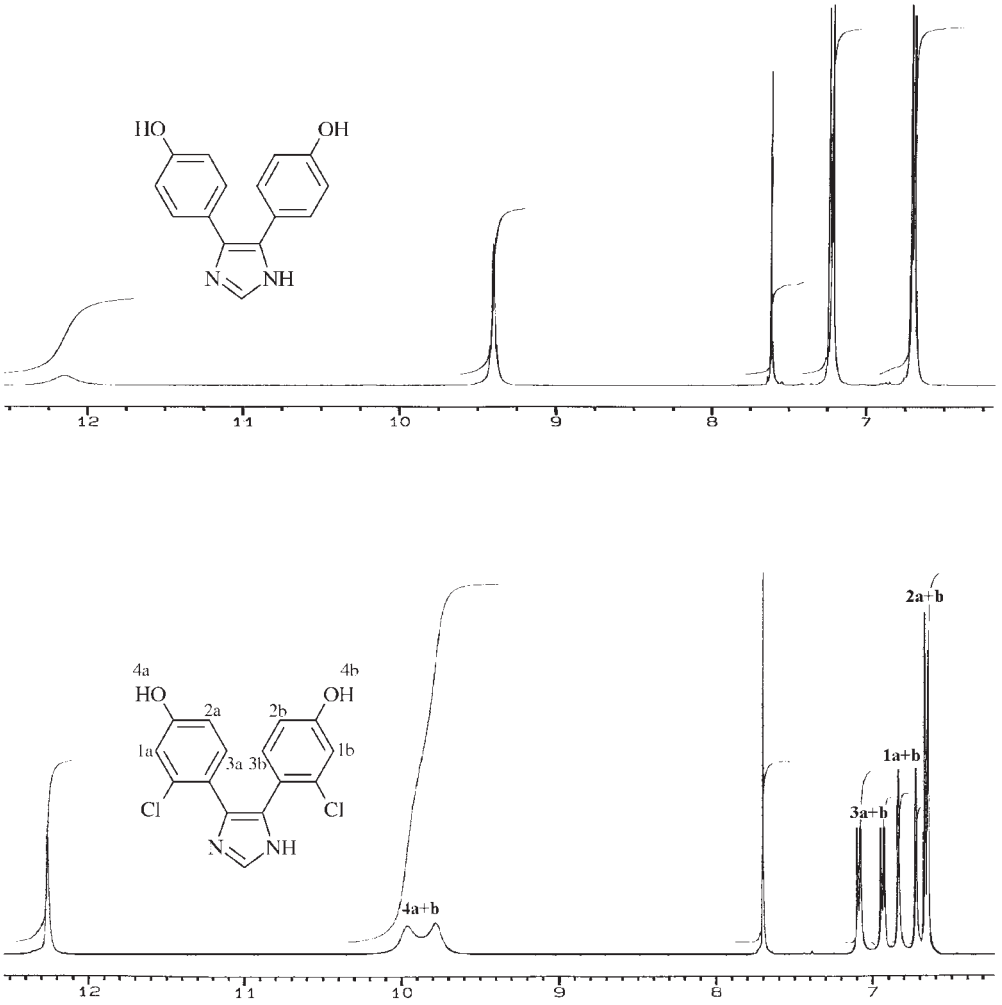


Figure 2. 400 MHz ^{1}H NMR spectra of the imidazoles **1** and **3** in [D6]-DMSO.

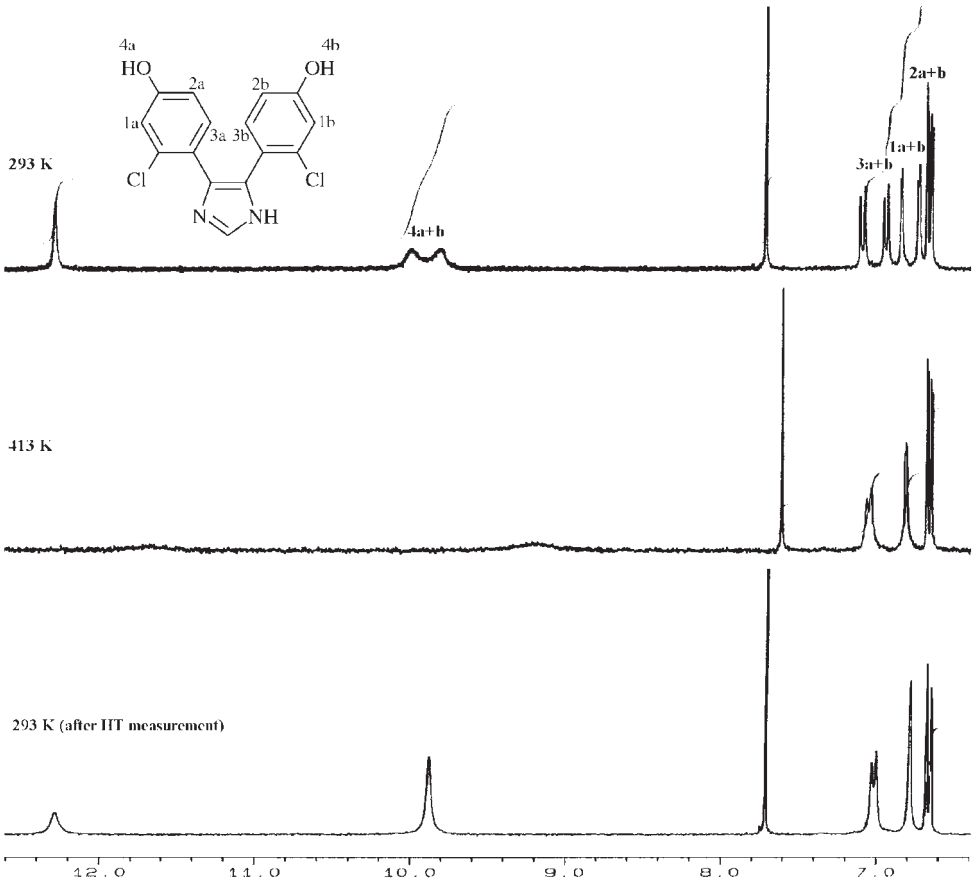


Figure 3. 300 MHz ^{1}H NMR spectra of the imidazole **3** at room temperature (A), at high temperature (B) and after HT measurement (C); solvent [D6]-DMSO.

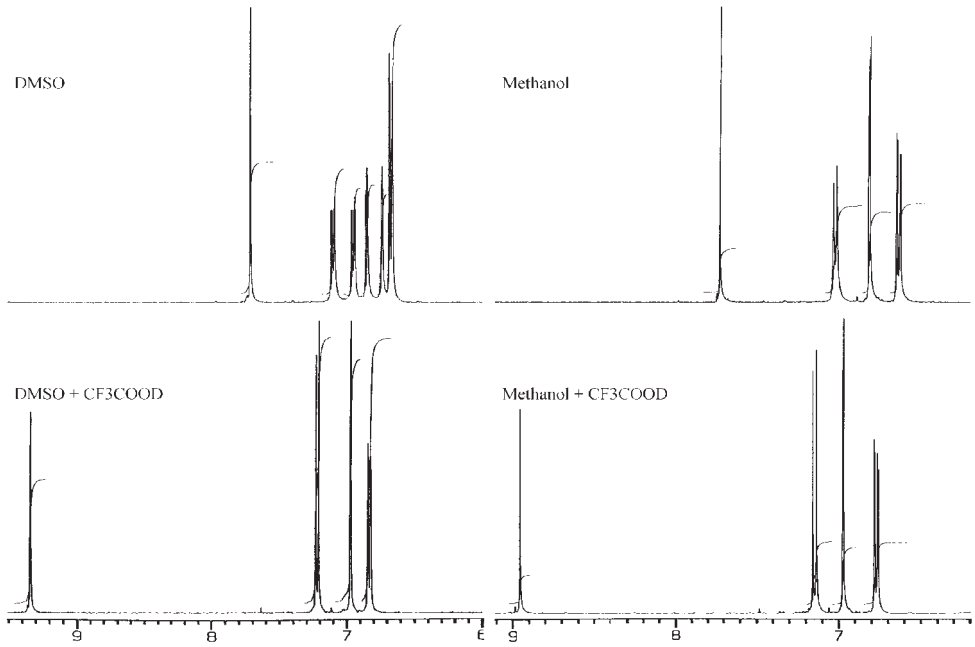


Figure 4. 400 MHz ^{1}H NMR spectra of the imidazole **3** in various solvents.

gens [16]. Hereby, the 4- and 5-position of the imidazole become chemically equivalent and only one set of resonances appears in the spectra.

The substituents in the 2-positions of the aromatic rings double the resonances. In the spectra of the 4,5-bis-(2-halo-4-hydroxyphenyl)imidazoles **2** and **3** (**3** see Figure 2) two signal groups for the protons 1 a + b, 3 a + b, and 4 a + b are present. The protons H2a and H2b are isochronous. This splitting could either be the consequence of a hindered rotation of the aromatic rings or an asymmetric imidazole moiety due to a stably bound N*H*.

To gain further information, high temperature measurements (HT) were performed (Figure 3). If rotational isomerism exists, the signals should coalesce and during the cooling down to room temperature the equilibrium between both isomers should take place once more, or the most stable isomer should originate.

Indeed, the signals H1a + b at 6.73 and 6.84 ppm, H3a + b at 6.94 and 7.09 ppm, and H4a + b at 9.79 and 9.97 ppm in the spectra of **3**, coalesce during heating to 413 K, respectively. After HT measurement the spectra show signals at 6.78 ppm (H1a + b), 7.01 ppm (H3a + b), and 9.89 ppm (H4a + b). These resonances cannot be assigned to one of the set of resonances in the start spectra (293 K) but represent the average of the respective signals. The same effect was detected by temperature dependent measurement of ^{13}C NMR spectra (data not shown), so it can be concluded that this effect is not the result of a restricted rotation of the aromatic rings.

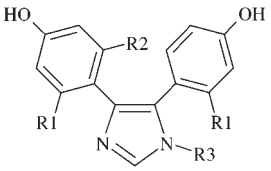
In the start spectra of **3** the N*H* resonance is sharp but shows an increase in width and flatness after HT measurement (see Figure 3). This indicates the presence of tautomeric and proton exchange processes. Addition of CF_3COOD to the [D6]-DMSO solution fastens the process and the splitting collapses (Figure 4). Furthermore, in [D4]-methanol there is only one set of signals for H1a + b, H3a + b, and H4a + b, respectively (see Figure 4), which indicates an equivalence of the 4- and 5-position in **3**. These findings show that a stable N*H* position or a very slow proton reaction in DMSO led to an asymmetric molecule structure.

Biological activity

The evaluation of the relative binding affinity (RBA) was performed in the competition experiment with [^{3}H]estradiol ([^{3}H]-**E2**) [17]. Additionally, a luciferase assay was used to obtain information about the agonistic effects on molecular level [18]. For this purpose, ER-positive MCF-7 mammary carcinoma cells were stably transfected with the plasmid ERE_{wtc}luc. After binding of hormonally active compounds to the ER, the resulting ER/drug conjugates interact after dimerization with the ERE at the plasmid and activate the reporter sequence which codes for luciferase. The expression of luciferase correlates very well with the estrogenic potency of the drug [19].

With the exception of the 4,5-bis(4-hydroxyphenyl)imidazole **1** and its alkyl derivative **5** all other compounds were able to compete with **E2** for its binding site (Table 1). Introduction of 2-F or 2-Cl substituents in the 4-hydroxyphenyl rings increased the RBA from < 0.02 % (**1**) to 0.05 % (**2**) and 0.11 % (**3**). Further enhancement of the binding affinity by introduction of substituents in the aromatic ring failed. However, *N*-alkylation of **3** increased the RBA value enormously to RBA = 1.26 %.

Table 1. Relative binding affinity and relative activation in the luciferase assay of the imidazoles **1** to **6**.



	R_1	R_2	R_3	RBA [%]	Rel. activation [%] at 1 µM –OH derivatives	Rel. activation [%] at 1 µM *–OCH_3 derivatives*
1	H	H	H	< 0.02	3	7
2	F	H	H	0.05	2	3
3	Cl	H	H	0.11	8	–3
4	Cl	Cl	H	0.10	27	6
5	H	H	ethyl	< 0.02	4	6
6	Cl	H	ethyl	1.26	10	5

Although the estrogen receptor binding of imidazoles was verified, the luciferase expression could not be activated in high amounts. While the imidazoles **1** (rel. activation: 3 %), **2** (rel. activation: 2 %), and **3** (rel. activation: 8 %) are completely inactive at 1µM (rel. activation of **E2**: 100 %), the threefold Cl-substituted compound **4** represents, with 27 %, the most active compound of this series. Interestingly, *N*-alkylation of **3** did not change the estrogenic activity although the RBA value was more than 10 times higher. O-Methylation of **4** led to a complete loss of activity and confirmed the relevance of free hydroxy groups for the receptor binding.

Discussion

(*R,S*)/(*S,R*)-configurated 1,2-diarylethylenediamines derived from the synthetic estrogen **DES** were used as carrier ligands for the cytostatic metal complex cisplatin. Dependent on the substitution pattern, the resulting [1,2-diarylethylenediamine]platinum(II) complexes possess high estrogenic potency [20–22]. Interestingly, the spatial structures of the molecules differ strongly from that of known steroidal or synthetic estrogens. In contrast to the parent compound **DES**, the 1,2-diarylethane pharmacophor takes on a synclinal conformation in hormonally active complexes and can not be attached to identical binding sites at the ER.

To gain a better insight into the binding mode, we synthesized drugs with comparable spatial structure [11]. In a preliminary study we fixed the 1-(2-chloro-4-hydroxyphenyl)-2-(2,6-dichloro-4-hydroxyphenyl)ethane pharmacophor as 2,3-diarylpiperazine and 4,5-diaryl-2-imidazoline [11].

The (*R,S*)/(*S,R*)-2,3-diarylpiperazine **7** takes on several low energy chair and twist conformations, with the 2,6-dichloro-4-hydroxyphenyl ring being equatorially arranged and the neighbouring 2-chloro-4-hydroxyphenyl ring standing axially in each energetically preferred structure [11, 13]. The dihedral angle between the aromatic rings varies from 39.4° to 47.9° with O–O distances of 6.5 Å–7.1 Å.

In the (*R,S*)/(*S,R*)-4,5-diaryl-2-imidazoline **8**, the heterocyclic ring is planar and the phenyl substituents are pseudo axially oriented in a dihedral angle of 2.8° [11, 14]. The 1,2-diphenylethane moiety is therefore arranged in a *Z*-stilbene-like structure.

In a previously published structure-activity study, we investigated the binding affinity and the hormonal activity of Cl/OH-substituted stilbenes. Unfortunately, data from the *Z*-configurated 1-(2-chloro-4-hydroxyphenyl)-2-(2,6-dichloro-4-hydroxyphenyl)ethene are not available due to its low stability [12]. Under the *in vitro* condition, a fast conversion into the *E*-isomer takes place. However, if the hydroxy group in the 1-phenyl ring is shifted into the 3- or 5-position, the *Z*-isomers are stable enough for testing. In the competition experiment with [^{3}H]-**E2** the *Z*-isomers are half as active as the *E*-isomer and possess the hormonal profile of partial estrogens [12]. In estrogenically active 1,2-bis(4-acetoxyphenyl)-1-phenylbut-2-enes the *Z*-1,2-bis(4-hydroxyphenyl)ethene structure is also realized. A comparison of the results obtained with the pure *E*- and *Z*-isomers indicates identical activity in the luciferase assay (activation about 60 % at 1 µM) [23]. These positive results prompted us to fix the 1,2-diphenylethane pharmacophor in a planar 4,5-diarylimidazole structure. The aromatic rings are fixed in the heterocyclic plane and the O–O distance amounts to 8.8 Å–8.9 Å.

Compared to the piperazines and 2-imidazolines, the imidazoles could displace **E2** from its binding site. The RBA value increased with the number of hydrophobic substituents in the aromatic rings. 4-(2-Chloro-4-hydroxyphenyl)-5-(2,6-dichloro-4-hydroxyphenyl)imidazole **4** displaced **E2** from the LBD to 0.1 %. *N*-Ethylation of **3** results in the most active compound (**6**) in this series. Imidazole **6** showed an RBA value of 1.26 %. *O*-Methylation led to a complete loss of binding affinity. These results led to the assumption that imidazoles are bound at the LBD by a combination of hydrophobic contacts and H bridges. Interestingly, despite the high RBA = 1.26 %, **6** did not activate luciferase expression in MCF-7-2a cells. The non-alkylated compound **3** was even more active and achieved in a concentration of 1 µM a relative activation of 27 %.

4,5-Bis(4-hydroxyphenyl)imidazoles were already synthesized and tested by Fink et al. [24]. In accordance with our results, the alkyl substituents at the 1-position increased the RBA. However, this effect depends on an additional 4-OH-phenyl ring in the 2-position. As most active compound the 2,4,5-tris(4-hydroxyphenyl)-1-propylimidazole possesses an RBA value of 0.62 %.

As interpreted by Fink et al. [24] the overall relatively low binding affinity of the imidazoles is due to the high inherent polarity of the heterocyclic system and the high dipole moment. These effects can be overcome by the introduction of hydrophobic Cl substituents in the *ortho* positions of the aromatic rings and *N*-alkylation as shown in this structure activity study. Therefore, we investigated the dependence of the relative binding affinity and gene activation of 2,4,5-tris(4-hydroxyphenyl)imidazole on Cl substituents in the *ortho* positions. The results will be published in a forthcoming paper.

Experimental

General procedures

IR spectra (KBr pellets): Perkin Elmer Model 580 A. ^{1}H NMR: Bruker ADX 400 spectrometer at 400 MHz (internal standard: TMS). Elemental analyses: Microlaboratory of the Free University of Berlin. EI-MS spectra: CH-7A-Varian MAT (70 eV) or Kratos MS 25 RF (80eV). All computational graphics were built using SYBYL 6.6, Tripos Inc., 1699 South Hanley Rd., St. Louis, Missouri, 63144, USA. Geometry optimization was carried out using the Tripos force field within the SYBYL program, running on an INDY workstation. Liquid Scintillation Counter: 1450 Microbeta^{TM} Plus (Wallac, Finland). Microlumat: LB 96 P (EG & G Berthold, Germany).

Syntheses

(1R,2S)/(1S,2R)-4,5-diaryl-2-imidazolines **1 b** to **4 b** were synthesized as described earlier [14].

General procedure for the synthesis of 4,5-diarylimidazoles

To a solution of the respective 4,5-diaryl-2-imidazoline (1.0 mmol) in 20 mL dry $CHCl_3$ 6.00 mmol of MnO_2 were added. After stirring the reaction mixture for 24 h, the manganese dioxide was filtered off. Removal of the solvent left the crude product, which was purified either by column chromatography on silica gel or by recrystallization.

4,5-Bis(4-methoxyphenyl)imidazole (**1 a**)

From (4*R*,5*S*)/(4*S*,5*R*)-4,5-bis(4-methoxyphenyl)-2-imidazoline **1 b**: 1.24 mmol (350 mg). Purification by column chromatography on silica gel with methanol. **Yield**: 1.21 mmol (338 mg), 97 %; colorless powder, mp 178–180 °C. **IR** (KBr): $\bar{\nu}$ = 3416 m, br (NH); 2838 w (OCH_3); 1615 m; 1524 s; 1508 s; 1293 m; 1251 s; 1171 m; 1107 m; 1038 m; 833 s. **^{1}H NMR** ($CDCl_3$): δ = 3.82 (s, 6 H, OCH_3); 6.86 (AA′*BB′*, ^3J = 8.0 Hz, 4 H, Ar*H*-3, Ar*H*-5); 7.42 (*AA′*BB′, ^3J = 8.0 Hz, 4 H, Ar*H*-2, Ar*H*-6); 7.59 (s, 1 H, N=C*H*-N); 9.67 (br, 1 H, N*H*, exchangeable by D_2O).

4,5-Bis(2-fluoro-4-methoxyphenyl)imidazole (**2 a**)

From (4*R*,5*S*)/(4*S*,5*R*)-4,5-bis(2-fluoro-4-methoxyphenyl)-2-imidazoline **2 b**: 2.77 mmol (880 mg). Purification by column chromatography on silica gel with diethyl ether/methanol (4 + 1). **Yield**: 1.59 mmol (503 mg), 58 %; colorless powder, mp 187–189 °C. **IR** (KBr): $\bar{\nu}$ = 3440 s, br (NH); 2840 m (OCH_3); 1627 s; 1577 m; 1525 s; 1480 w; 1425 w; 1293 s; 1241 m; 1193 m; 1148 s; 1102 s; 1035 m; 946 m; 856 m; 813 m. **^{1}H NMR** ($CDCl_3$): δ = 3.81 (s, 6 H, OCH_3); 6.62–6.67 (m, 4 H, Ar*H*-3, Ar*H*-5); 7.25–7.30 (m, 2 H, Ar*H*-6); 7.72 (s, 1 H, N=C*H*-N).

4,5-Bis(2-chloro-4-methoxyphenyl)imidazole (**3 a**)

From (4*R*,5*S*)/(4*S*,5*R*)-4,5-bis(2-chloro-4-methoxyphenyl)-2-imidazoline **3 b**: 1.51 mmol (530 mg). Purification by recrystallization from $CHCl_3$/ligroine. **Yield**: 0.659 mmol (230 mg), 44 %; colorless powder, mp 194–196 °C. **IR** (KBr): $\bar{\nu}$ = 3420 s, br (NH); 2838 m (OCH_3); 1610 s; 1564 m; 1515 s; 1498 s; 1466 s; 1439 m; 1289 s; 1226 s; 1046 s; 963 m; 864 m; 815 m. **MS** (EI, 160 °C): *m/z* (%) = 348 (100) $[M]^{+\cdot}$; 333 (8) $[M–CH_3]^+$; 235 (14); 197 (19); 170 (79). **^{1}H NMR** ($CDCl_3$): δ = 3.79 (s, 6 H, OCH_3); 6.72 (dd, ^3J = 8.6 Hz, ^4J = 2.5 Hz, 2 H, Ar*H*-5); 6.92 (d, ^4J = 2.5 Hz, 2 H, Ar*H*-3); 7.13 (d, ^3J = 8.6 Hz, 2 H, Ar*H*-6); 7.82 (s, 1 H, N=C*H*-N); 9.76 (s, 1 H, N*H*, exchangeable by D_2O).

*4-(2-Chloro-4-methoxyphenyl)-5-(2,6-dichloro-4-methoxyphenyl)imidazole (***4 a**)

From (4R,5S)/(4*S*,5*R*)-4-(2-chloro-4-methoxyphenyl)-5-(2,6-dichloro-4-methoxyphenyl)-2-imidazoline **4 b**: 0.920 mmol (355 mg). Purification by column chromatography on silica gel with ethyl acetate. **Yield**: 0.521 mmol (200 mg), 57 %; colorless powder, mp 76 –78°C. **IR** (KBr): $\bar{\nu}$ = 3433 s, br (NH); 2966 w; 2839 w (OCH_3); 1668 m; 1608 s; 1551 m; 1504 s; 1465 m; 1362 w; 1289 s; 1241 s; 1182 m; 1043 s; 840 m; 805 m. **^{1}H NMR** ($CDCl_3$): δ = 3.78 (s, 3 H, OCH_3); 3.79 (s, 3 H, OCH_3); 6.68 (dd, ^3J = 8.6 Hz, ^4J = 2.5 Hz, 1 H, Ar*H*-5); 6.88 (s, 2 H, Ar′*H*); 6.94 (d, ^4J = 2.5 Hz, 1 H, Ar*H*-3); 7.09 (d, ^3J = 8.6 Hz, 1 H, Ar*H*-6); 7.80 (s, 1 H, N=C*H*-N).

N-Ethyl-4,5-bis(4-methoxyphenyl)imidazole (**5 a**)

A solution of 2.93 mmol (821 mg) 4,5-bis(4-methoxyphenyl)-imidazole **1 a**, 3.22 mmol (502 mg, 260 µL) ethyl iodide and 3.22 mmol (174 mg) sodium ethanolate in 20 mL of dry 1,4-dioxane was heated to reflux for 12 h. After cooling, 10 mL of 0.1 N NaOH were added and the product was extracted with $CHCl_3$. The organic layer was dried over Na_2SO_4 and the solvent was evaporated. Purification of the crude product was performed by column chromatography on silica gel with diethyl ether/methanol (9 + 1). **Yield**: 1.95 mmol (600 mg), 66 %; pale yellow oil. **IR** (Film): $\bar{\nu}$ = 2935 m; 2836 m (OCH_3); 1613 m; 1576 w; 1502 s; 1344 w; 1267 s; 1177 m; 1104 m; 950 m; 825 s. **^{1}H NMR** ($CDCl_3$): δ = 1.27 (t, ^3J = 7.3 Hz, 3 H, CH_2CH_3); 3.76 (s, 3 H, OCH_3); 3.81 (q, ^3J = 7.3 Hz, 2 H, CH_2CH_3); 3.87 (s, 3 H, OCH_3); 6.76 (AA′*BB′*, ^3J = 8.7 Hz, 2 H, Ar*H*-3, Ar*H*-5); 6.99 (AA′*BB′*, ^3J = 8.5 Hz, 2 H, Ar*H*-3, Ar*H*-5); 7.25 (*AA′*BB′, ^3J = 8.5 Hz, 2 H, Ar*H*-2, Ar*H*-6); 7.41 (*AA′*BB′, ^3J = 8.7 Hz, 2 H, Ar*H*-2, Ar*H*-6); 7.59 (s, 1 H, N=C*H*-N).

N-Ethyl-4,5-bis(2-chloro-4-methoxyphenyl)imidazole (**6 a**)

To a solution of 0.510 mmol (178 mg) 4,5-bis(2-chloro-4-methoxyphenyl)imidazole **3 a** in 20 mL of dry THF 0.550 mmol (22 mg) sodium hydride (60 % in paraffin) and 0.550 mmol (86 mg, 44 µL) ethyl iodide were added and heated to reflux for 4 h. Subsequently, the reaction mixture was hydrolysed with 20 mL of water and the product was extracted with $CHCl_3$. The organic layer was dried over Na_2SO_4 and the solvent was distilled off. **Yield**: 0.498 mmol (188 mg), 98 %; pale yellow oil. **IR** (KBr): $\bar{\nu}$ = 2959 m; 2839 w (OCH_3); 1610 s; 1570 m; 1509 s; 1478 s; 1441 w; 1409 w; 1289 s; 1260 w; 1227 s; 1041 s; 954 m; 870 m; 844 w; 819 m; 754 m. **^{1}H NMR** ($CDCl_3$): δ = 1.31 (t, ^3J = 7.4 Hz, 3 H, CH_2CH_3); 3.71–3.91 (m, 2 H, CH_2CH_3); 3.75 (s, 3 H, OCH_3); 3.80 (s, 3 H, OCH_3); 6.68 (dd, ^3J = 8.5 Hz, ^4J = 2.6 Hz, 1 H, Ar*H*-5); 6.76 (dd, ^3J = 8.5 Hz, ^4J = 2.6 Hz, 1 H, Ar*H*-5); 6.87 (d, ^4J = 2.6 Hz, 1 H, Ar*H*-3); 6.98 (d, ^4J = 2.6 Hz, 1 H, Ar*H*-3); 7.10 (d, ^3J = 8.5 Hz, 1 H, Ar*H*-6); 7.18 (d, ^3J = 8.5 Hz, 1 H, Ar*H*-6); 7.68 (s, 1 H, N=C*H*-N).

General procedure for the ether cleavage with BBr_3

A solution of the methyl ether (1.00 mmol) in 20 mL of dry CH_2Cl_2 was cooled to –60 °C. At this temperature BBr_3 (4.5 mmol) in 5 mL of dry CH_2Cl_2 was added under N_2 atmosphere. Then the reaction mixture was allowed to warm to room temperature and was stirred for a further 48 h. After cooling the reaction mixture with an ice bath, the surplus of BBr_3 was hydrolyzed three times with methanol and the phenolic product was dissolved in 0.1 N NaOH. The alkaline water phase was filtered and the pH was adjusted to 8 with 2 N HCl. The precipitate was collected by suction filtration and dried over P_2O_5. Subsequently, the crude product was purified if necessary by column chromatography or fractional crystallization.

4,5-Bis(4-hydroxyphenyl)imidazole (**1**)

From 4,5-bis(4-methoxyphenyl)imidazole **1 a**: 0.892 mmol (250 mg). **Yield**: 0.674 mmol (170 mg), 76 %; grey powder, mp 307–310 °C. **IR** (KBr): $\bar{\nu}$ = 3600–2800 m, br (OH); 3583 s (NH); 3357 m; 3210 m; 1616 m; 1521 m; 1508 m; 1481 m; 1462 m; 1243 s; 1168 m; 1102 w; 969 w; 835 s. **^{1}H NMR** ([D_6]-DMSO): δ = 6.71 (AA′*BB*′, ^3J = 8.4 Hz, 4 H, Ar*H*-3, Ar*H*-5); 7.24 (*AA*′BB′, ^3J = 8.4 Hz, 4 H, Ar*H*-2, Ar*H*-6); 7.62 (s, 1 H, N=C*H*-N); 9.41 (s, 2 H, ArO*H*, exchangeable by D_2O); 12.15 (s, 1 H, N*H*, exchangeable by D_2O). Anal. ($C_{15}H_{12}N_2O_2$) 252.27: calc.: C 71.42 H 4.79 N 11.10 found: C 71.40 H 4.90 N 11.06.

4,5-Bis(2-fluoro-4-hydroxyphenyl)imidazole (**2**)

From 4,5-bis(2-fluoro-4-methoxyphenyl)imidazole **2 a**: 0.696 mmol (220 mg). **Yield**: 0.663 mmol (191 mg), 95 %; colorless powder, mp 298 °C under decomposition. **IR** (KBr): $\bar{\nu}$ = 3600–2400 s, br (OH); 2813 w; 1630 s; 1596 m; 1524 m; 1459 s; 1304 s; 1258 m; 1154 s; 1119 m; 1100 m; 1058 m; 971 s; 848 s. **MS** (EI, 230 °C): *m/z* (%) = 288 (100) $[M]^{+\cdot}$; 268 $[M–F]^+$ (5); 239 (12). **^{1}H NMR** ([D_6]-DMSO): δ = 6.46 (dd, ^3J(H, F) = 12.1 Hz, ^4J = 1.7 Hz, 1 H, Ar*H*-3); 6.55–6.63 (m, 3 H, Ar*H*-3, 2 x Ar*H*-5); 7.03 (dd, ^3J = 8.8 Hz, ^4J(H, F) = 8.8 Hz, 1 H, Ar*H*-6); 7.26 (dd, ^3J = 8.7 Hz, ^4J(H, F) = 8.7 Hz, 1 H, Ar*H*-6); 7.76 (s, 1 H, N=C*H*-N); 9.79 (s, 1 H, ArO*H*, exchangeable by D_2O); 9.99 (s, 1 H, ArO*H*, exchangeable by D_2O); 12.30 (s, 1 H, N*H*, exchangeable by D_2O). Anal. ($C_{15}H_{10}F_2N_2O_2$) 288.25: calc.: C 62.50 H 3.50 N 9.72 found: C 62.54 H 3.45 N 9.66.

4,5-Bis(2-chloro-4-hydroxyphenyl)imidazole (**3**)

From 4,5-bis(2-chloro-4-methoxyphenyl)imidazole **3 a**: 0.458 mmol (160 mg). Purification by stirring in hot acetone. **Yield**: 0.316 mmol (114 mg), 77 %; colorless powder, mp 295–297 °C. **IR** (KBr): $\bar{\nu}$ = 3600–2300 s, br (OH); 3407 w; 3258 w; 2767 w; 2654 w; 1611 s; 1513 m; 1470 s; 1445 s; 1363 w; 1280 s; 1222 s; 1051 m; 1028 m; 900 s; 861 m; 821 s. **MS** (EI, 250 °C): *m/z* (%) = 320 (100) $[M]^{+\bullet}$; 284 (17) $[M–Cl]^+$; 250 (29) $[M–2Cl]^+$; 221 (15); 139 (14); 125 (14). **^{1}H NMR** ([D_6]-DMSO): δ = 6.66 (dd, ^3J = 8.4 Hz, ^4J = 2.4 Hz, 2 H, Ar*H*-5); 6.73 (d, ^4J = 2.4 Hz, 1 H, Ar*H*-3); 6.84 (d, ^4J = 2.4 Hz, 1 H, Ar*H*-3); 6.94 (d, ^3J = 8.4 Hz, 1 H, Ar*H*-6); 7.09 (d, ^3J = 8.4 Hz, 1 H, Ar*H*-6); 7.70 (s, 1 H, N=C*H*-N); 9.79 (s, br, 1 H, ArO*H*, exchangeable by D_2O); 9.97 (s, br, 1 H, ArO*H*, exchangeable by D_2O); 12.26 (s, 1 H, N*H*, exchangeable by D_2O). Anal. ($C_{15}H_{10}Cl_2N_2O_2$) 321.16: calc.: C 56.10 H 3.14 N 8.72 found: C 56.07 H 3.09 N 8.64.

4-(2-Chloro-4-hydroxyphenyl)-5-(2,6-dichloro-4-hydroxyphenyl)imidazole (**4**)

From 4-(2-chloro-4-methoxyphenyl)-5-(2,6-dichloro-4-methoxyphenyl)imidazole **4 a**: 0.430 mmol (165 mg). The crude product was prepurified by column chromatography on silica gel with diethyl ether/acetone (6 + 1) and subsequent recrystallization from $CHCl_3$ and acetone (6 + 1). **Yield**: 0.225 mmol (80 mg), 52 %; pale yellow powder, mp 220 °C under decomposition. **IR** (KBr): $\bar{\nu}$ = 3600–2700 s, br (OH); 2752 w; 2643 w; 1610 s; 1570 m; 1510 m; 1436 m; 1357 w; 1284 m; 1227 m; 1105 w; 1061 m; 1029 w; 946 m; 899 m; 854 m; 808 m. **MS** (EI, 230 °C): *m/z* (%) = 354 (94) $[M]^{+\cdot}$; 319 (25) $[M–Cl]^+$; 284 (71) $[M–2Cl]^+$; 255 (24); 190 (17); 142 (26); 139 (17). **^{1}H NMR** ([D_4]-methanol): δ = 6.60 (dd, ^3J = 8.4 Hz, ^4J = 2.3 Hz, 1 H, Ar*H*-5); 6.80 (s, 2 H, Ar′*H*-3, Ar′*H*-5); 6.83 (d, ^4J = 2.3 Hz, 1 H, Ar*H*-3); 7.01 (d, ^3J = 8.4 Hz, 1 H, Ar*H*-6); 7.78 (s, 1 H, N=C*H*-N). Anal. ($C_{15}H_9Cl_3N_2O_2$) 355.61: calc.: C 50.66 H 2.55 N 7.88 found: C 50.73 H 2.48 N 7.67.

N-Ethyl-4,5-bis(4-hydroxyphenyl)imidazole (**5**)

From N-ethyl-4,5-bis(4-methoxyphenyl)imidazole **5 a**: 1.51 mmol (467 mg). Purification by recrystallization from acetone. **Yield**: 1.28 mmol (360 mg), 85 %; colorless powder, mp 251–253 °C. **IR** (KBr): $\bar{\nu}$ = 3600–2600 s, br (OH); 2984 w; 1613 s; 1522 s; 1497 s; 1445 m; 1347 m; 1249 s; 1171 m; 1100 m; 961 m; 838 s. **^{1}H NMR** ([D_6]-DMSO): δ = 1.12 (t, ^3J = 7.1 Hz, 3 H, CH_2CH_3); 3.74 (q, ^3J = 7.1 Hz, 2 H, CH_2CH_3); 6.56 (AA′*BB*′, ^3J = 8.3 Hz, 2 H, Ar*H*-3, Ar*H*-5); 6.85 (AA′*BB*′, ^3J = 8.1 Hz, 2 H, Ar*H*-3, Ar*H*-5); 7.11 (*AA*′BB′, ^3J = 8.1 Hz, 2 H, Ar*H*-2, Ar*H*-6); 7.19 (*AA*′BB′, ^3J = 8.3 Hz, 2 H, Ar*H*-2, Ar*H*-6); 7.70 (s, 1 H, N=C*H*-N); 9.21 (s, br, 1 H, ArO*H*, exchangeable by D_2O); 9.66 (s, br, 1 H, ArO*H*, exchangeable by D_2O). Anal. ($C_{17}H_{16}N_2O_2$) 280.33: calc.: C 72.84 H 5.75 N 9.99 found: C 72.84 H 5.85 N 9.89.

N-Ethyl-4,5-bis(2-chloro-4-hydroxyphenyl)imidazole (**6**)

From *N*-ethyl-4,5-bis(2-chloro-4-methoxyphenyl)imidazole **6 a**: 0.477 mmol (180 mg). Purification by column chromatography on silica gel with acetone. **Yield**: 0.395 mmol (138 mg), 83 %, yellow powder, mp 100–103 °C. **IR** (KBr): $\bar{\nu}$ = 3600–2500 s, br (OH); 2968 w; 2927 m; 2760 w; 2651 w; 1700 m; 1613s; 1572 m; 1512 s; 1444 s; 1345 m; 1288 s; 1224 s; 1129 m; 1069 m; 1045 m; 901 s; 857 m; 823 s. **MS** (EI, 190 °C): *m/z* (%) = 348 (100) $[M]^{+\cdot}$; 319 (5) $[M–C_2H_5]^+$; 313 (17) $[M–Cl]^+$; 284 (17) $[M–Cl–C_2H_5]^+$; 278 (10) $[M–2Cl]^+$; 249 (6) $[M–2Cl–C_2H_5]^+$; 139 (27). **^{1}H NMR** ([D_6]-acetone): δ = 1.27 (t, ^3J = 7.3 Hz, 3 H, CH_2CH_3); 3.77–3.87 (m, 1 H, CH_2CH_3); 3.89–3.99 (m, 1 H, CH_2CH_3); 6.67 (dd, ^3J = 8.4 Hz, ^4J = 2.5 Hz, 1 H, Ar*H*-5); 6.80 (d, ^4J = 2.5 Hz, 1 H, Ar*H*-3); 6.81 (dd, ^3J = 8.4 Hz, ^4J = 2.5 Hz, 1 H, Ar*H*-5); 6.96 (d, ^4J = 2.5 Hz, 1 H, Ar*H*-3); 7.12 (d, ^3J = 8.4 Hz, 1 H, Ar*H*-6); 7.13 (d, ^3J = 8.4 Hz, 1 H, Ar*H*-6); 7.76 (s, 1 H, N=C*H*-N); 8.86 (br, 1 H, ArO*H*, exchangeable by D_2O); 9.21 (br, 1 H, ArO*H*, exchangeable by D_2O). Anal. ($C_{17}H_{14}Cl_2N_2O_2$) 349.22: calc.: C 58.42 H 4.01 N 8.02 found: C 58.24 H 4.22 N 7.91.

Biological methods

Biochemicals, chemicals and materials

Dextran, 17β-estradiol, L-glutamine (L-glutamine solution: 29.2 mg/mL phosphate buffered saline (PBS)), and Minimum Essential Medium Eagle (EMEM) were purchased from Sigma (Munich, Germany); Dulbecco's Modified Eagle Medium without phenol red (DMEM) was from Gibco (Eggenstein, Germany); bovine calf serum (BCS) was from Bio Whittaker (Verviers, Belgium); *N*-hexamethylpararosaniline (crystal violet) and gentamicin sulfate were from Fluka (Deisenhofen, Germany); glutardialdehyde (25 %) was from Merck (Darmstadt, Germany); trypsin (0.05 %) in ethylenediaminetetraacetic acid (0.02 %) (trypsin/EDTA) was from Boehringer (Mannheim, Germany); penicillin-streptomycin gold standard (10 000 IE penicillin/mL, 10 mg streptomycin/mL) and geneticin disulfate (geneticin solution: 35.71 mg/mL PBS) were from ICN Biomedicals GmbH (Eschwege, Germany); norit A (charcoal) was from Serva (Heidelberg, Germany); cell culture lysis reagent (5×) (diluted 1 : 5 with purified water before use) and the luciferase assay reagent were from Promega (Heidelberg, Germany); optiphase HiSafe3 liquid szintillator was from Wallac (Turku, Finland); NET-317-estradiol[2,4,6,7-^{3}H(N)] (17β-[^{3}H]estradiol) was from Du Pont NEN (Boston, Maryland); $CDCl_3$, [D_6]-acetone, [D_4]-methanol and [D_6]-DMSO were from Aldrich (Steinheim, Germany); PBS was prepared by dissolving 8.0 g of NaCl, 0.2 g of KCl, 1.44 g of $Na_2HPO_4 \cdot 2\ H_2O$, and 0.2 g of KH_2PO_4 (all purchased from Merck or Fluka) in 1000 mL of purified water. TRIS-buffer (pH = 7.5) was prepared by dissolving 1.211 g of trishydroxymethylaminomethane, 0.37224 g of Titriplex III and 0.19503 g of sodium azide (all from Merck or Fluka) in 1 L of pu-

rified water. Deionized water was produced by means of a Millipore Milli-Q Water System, resistivity > 18 MΩ. T-75 flasks, reaction tubes, 96-well plates, and 6-well plates were purchased from Renner GmbH (Dannstadt, Germany).

Estrogen receptor binding assay

The applied method was already described by Hartmann et al. [17] and was used with some modifications. The relative binding affinity (RBA) of the test compounds to the estrogen receptor was determined by the displacement of 17β-[^{3}H]estradiol from its binding site. For this purpose, the test compounds were dissolved in ethanol and diluted with TRIS-buffer to 6–8 appropriate concentrations (300 µL). They were incubated shaking with calf uterine cytosol (100 µL) and 17β-[^{3}H]estradiol (0.723 pmol in TRIS-buffer (100 µL); activity: 2249.4 Bq/tube) at 4 °C overnight. To stop the reaction 500 µL of a dextran-charcoal suspension in TRIS-buffer was added to each tube. After shaking for 90 min at 4 °C and centrifugation 500 µL HiSafe3 was mixed with 100 µL supernatant of each sample and the reactivity was determined by liquid scintillation spectroscopy. The same procedure was used to quantify the binding of 17β-[^{3}H]estradiol (0.723 pmol – control). Non-specific binding was calculated using 2 nmol of 17β-estradiol as the competing ligand. On a semilog plot the percentage of maximum bound labelled steroid corrected by the non-specifically bound 17β-[^{3}H]estradiol vs. concentration of the competitor (log-axis) is plotted. At least six concentrations of each compound were chosen to estimate its binding affinity. From this plot those molar concentrations of unlabeled estradiol and of the competitors were determined which reduce the binding of the radioligand by 50 %.

$$\text{RBA} = \frac{c_{[^3\text{H}]\text{–Estradiol}} \text{ at } 50\,\% \text{ inhibition}}{c_{\text{sample}} \text{ at } 50\,\% \text{ inhibition}} \cdot 100\,\%$$

Luciferase assay

The pertinent in vitro assay was described earlier by Hafner et al. [18]. One week before starting the experiment MCF-7-2a cells were cultivated in DMEM supplemented with L-glutamine, antibiotics and dextran/charcoal-treated BCS (ct-BCS, 50 mL/L). Cells from an almost confluent monolayer were removed by trypsinization and suspended to approximately 2.2 × 10^5 cells/mL in the growth medium mentioned above. The cell suspension was then cultivated in six well flat-bottomed plates (0.5 mL cell suspension and 1.5 mL medium per well) at growing conditions (see above). After 24 h 20 µL of a stock solution of the test compounds was added to achieve concentrations ranging from 10^{-5}–10^{-10} M and the plates were incubated for 50 h. Before harvesting, the cells were washed twice with PBS and then 200 µL of cell culture lysis reagent were added into each well. After a 20 min lysation at room temperature cells were transferred into reaction tubes and centrifuged. Luciferase was assayed using the Promega luciferase assay reagent. 50 µL of each supernatant was mixed with 50 µL of substrate reagent. Luminescence (in relative light units, RLU) was measured for 10 s using a microlumat. Measurements were corrected by correlating the quantity of protein (quantified according to Bradford [25]) of each sample with the mass of luciferase. Estrogenic activity was expressed as % activation of a 10^{-8} M Estradiol control (100 %).

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