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Invesgations on the Influence of Halide Substituents on the Estrogen Receptor Interaction of 2,4,5-Tris(4-hydroxyphenyl)imidazoles

Previously, we reported on the synthesis and estrogen receptor (ER) interaction of imidazoles, which had to be 1-alkyl-4,5-bis(2-halo-4-hydroxyphenyl) substituted for a high relative binding affinity (RBA > 1 %). This led to the assumption that a shielding of the polar heterocyclic system is a prerequisite for ER binding. In continuation of this study we synthesized 2,4,5-tris(4-hydroxyphenyl)imidazoles with Cl- or F-atoms in the *ortho*-positions of the aromatic rings and evaluated whether they mediate sufficient hydrophobicity for ER interaction. 2-(2,6-Dichloro-3/4-hydroxyphenyl)-4,5-bis(2-halo-4-hydroxyphenyl)imidazoles were synthesized by reaction of the respective methoxy-substituted benzil with either the 2,6-dichloro-4-methoxy- or the 2,6-dichloro-3-methoxybenzaldehyde in ammonium acetate solution. The required ether cleavage was performed subsequently with BBr_3. In the competition experiment with [^{3}H]estradiol the imidazoles with the a C2-standing (2,6-dichloro-4-hydroxyphenyl) ring showed an RBA > 0.02 %, but did not activate the luciferase gene in estrogen receptor positive MCF-7-2a breast cancer cells stably transfected with the plasmid ERE_{wtc}luc. In the test for antagonistic potency only the 2-(2,6-dichloro-4-hydroxyphenyl)-4,5-bis(4-hydroxyphenyl)imidazole **3** antagonized the effects of 1 nM estradiol slightly. From these data, it can be concluded that a C2-standing 2,6-dichloro-4-hydroxyphenyl ring is not appropriate to optimize the ER interaction of 4,5-(4-hydroxyphenyl)imidazoles.

Keywords: Estrogenic activity; Luciferase assay; Estrogen receptor binding; 2,4,5-triarylimidazoles

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Introduction

The estrogen receptor (ER) is a ligand- inducible nuclear transcription factor for the mediation of the effects of estrogen steroid hormones [1]. Through binding to the ligand binding domain (LBD) of the ER the hormones initiate in many tissues of the reproduction system a cascade of molecular and biochemical effects [2]. Furthermore, the ER also plays an important role in bone density maintenance [3–5], regulation of the blood/lipid profile [4–8], and brain function [9, 10]. Therefore, many scientific groups used the ER as a target for the discovery of selectively acting new drugs (Scheme 1).

The recently solved X-ray structures [11–13] of the LBD of ERα co-crystallized with agonists (e.g., estradiol (**E2**), diethylstilbestrol (**DES**)) and antagonists (e.g., raloxifene (**RAL**), 4-hydroxytamoxifen (**4OHT**) are helpful tools for the design of compounds with ER affinity.

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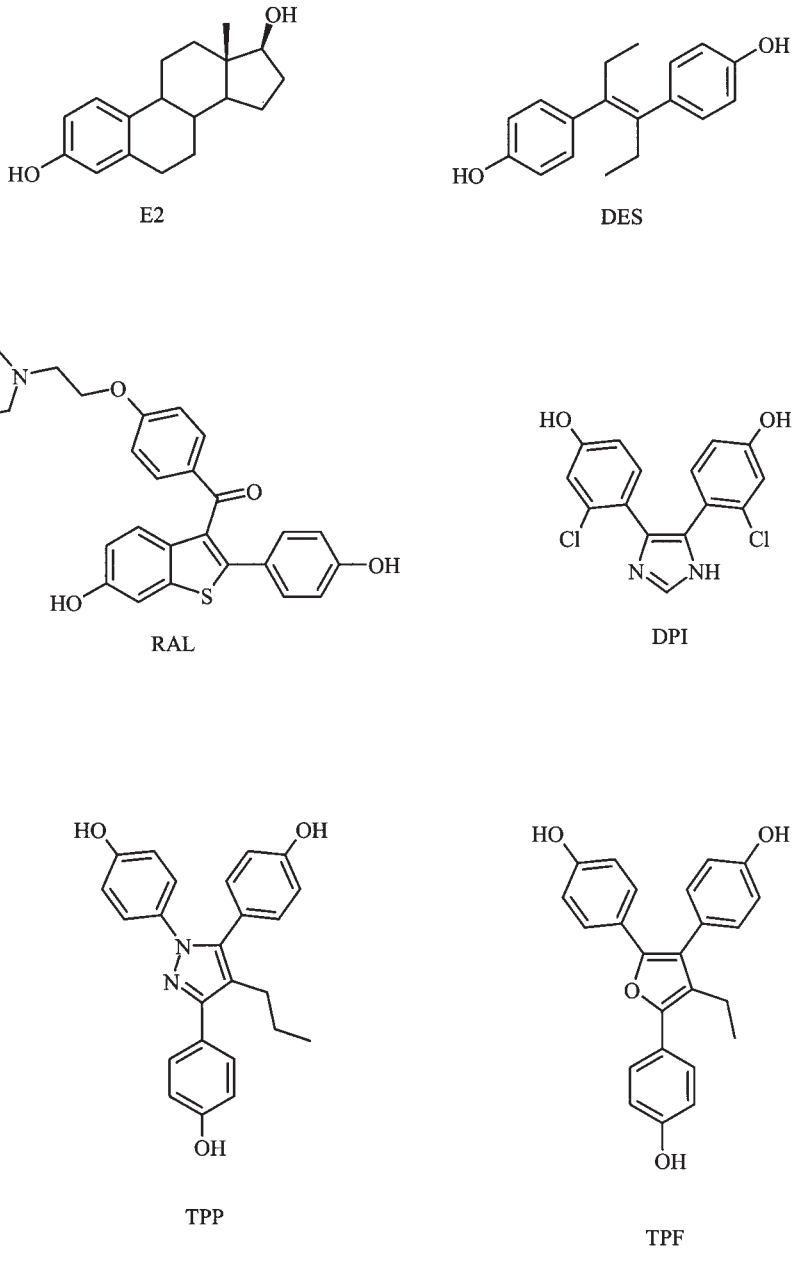
E2 and **DES** are oriented in the LBD in such a way that the OH groups build H-bridges to Glu353, Arg 394, and His 524. Lipophilic residues of amino acids in the LBD are in van der Waals contacts with the hydrophobic core of the molecules and stabilize the ER/drug conjugate. **4OHT** and **RAL** occupy the same central core of the LBD, and the basic side chains are oriented in a narrow side pocket building an H-bridge to Asp351. This anchorage fixes **RAL** in a plane conformation.

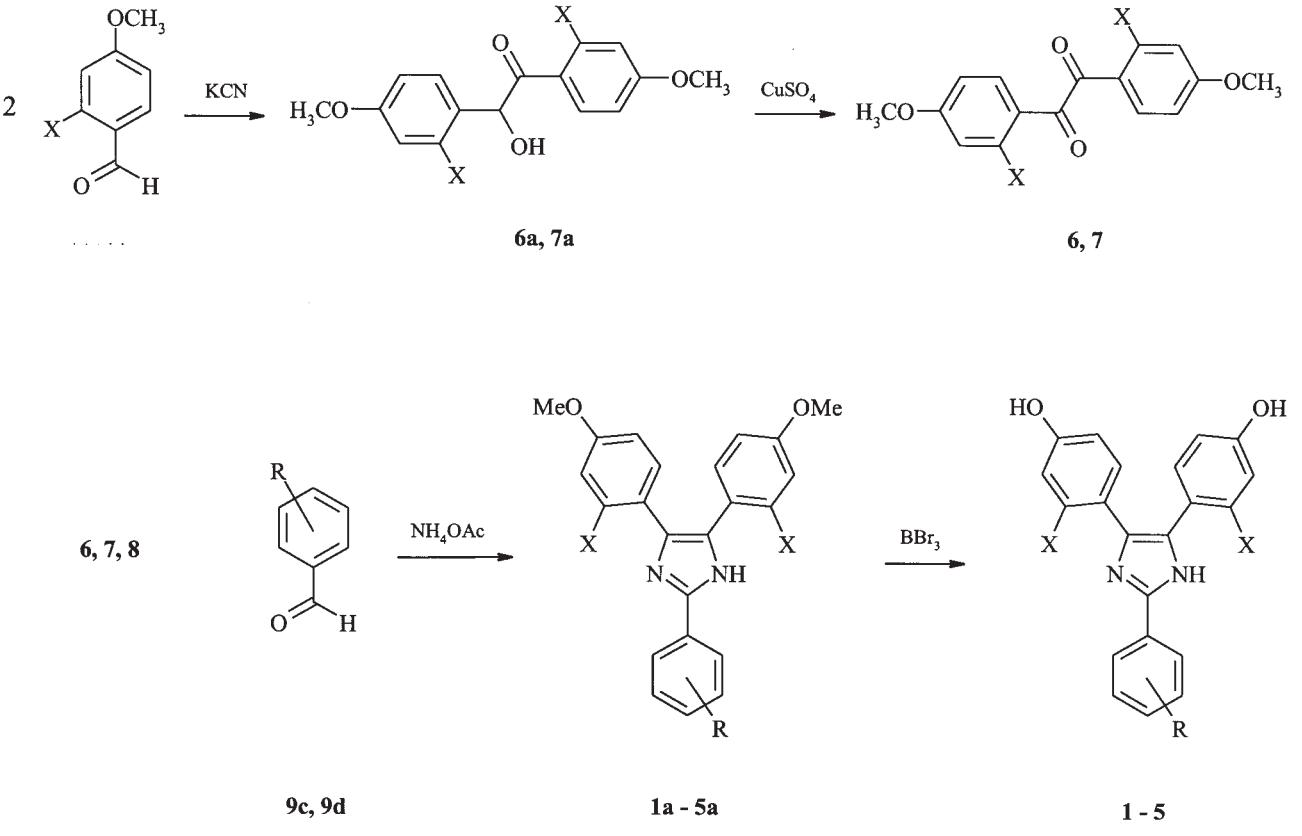
The crystal structures confirm the assumption that especially flat and rigid molecules interact with the ER. Following this strategy, the carbon atoms of the ethyl group of tamoxifen (**TAM**) were linked back to the proximate phenyl ring to reduce the flexibility. Resulting diphenylindenes [14], diphenyldihydro- or diphenyltetrahydronaphthalene derivatives [15–17], or **TAM** analogs with larger rings were subjected to intensive structure activity studies [18]. Further investigated heterocycles are benzofurans [19], benzothiophenes [20], benzopyrans [21], and indoles [22]. In each case, the fused bicyclic system is thought to mimic the A/B ring of **E2**.



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

Rigid molecules with very interesting pharmacological properties are tetrasubstituted pyrazoles, furans, thiophenes, and pyrroles developed by the group of J. A. Katzenellenbogen [23, 24]. ER binding and transcriptional activation studies showed that several members of these series possess high ER subtype selectivity and can be used as lead structures for the design of selective estrogen receptor modulators (SERM).

In our own studies, we selected the 1-alkyl-4,5-bis(2-halo-4-hydroxyphenyl)imidazole for the design of novel ER agonists [25]. The 1-ethyl-4,5-bis(2-chloro-4-hydroxy phenyl)imidazole showed a relative binding affinity of 1.26 %. The significance of an alkyl substituent at the 1-position for ER binding and gene activation of 4,5-bis(4-hydroxyphenyl)imidazoles was already confirmed by Fink et al. [26]. However, this effect depends on an additional 4-OH-phenyl ring in the 2-position. The 2,4,5-tris(4-hydroxyphenyl)-1-propylimidazole was identified as most active compound with an RBA = 0.62 %. The authors interpreted the relatively low binding affinity with the high inherent polarity of the heterocyclic system and the high dipole moment.



Scheme 2. Synthesis of the triarylimidazoles **1**–**5** (X = H, F, Cl; R = 2,6-Cl_2,3-OH/3-OCH_3; 2,6-Cl_2,4-OH/4-OCH_3).

In an earlier study, we evaluated the influence of halide substituents in the aromatic rings of estrogenically active 1,2-bis(4-hydroxyphenyl)ethylenediamines, which were designed as carrier molecules for metal complexes [27, 28]. These compounds interact with the ER and exert estrogenic effects if at least one phenyl ring possesses a 2,6-dichloro-3/4-hydroxy substitution pattern. The *ortho*-standing substituents shield the hydrophilic amino groups allowing hydrophobic contacts in the LBD of the ER. These positive results prompted us to shield the imidazole core by introduction of a 2,6-dichloro-3/4-hydroxyphenyl ring into the 2-position. The resulting 2-(2,6-dichloro-3/4-hydroxyphenyl)-4,5-bis(2-halo-4-hydroxyphenyl)imidazoles were evaluated for ER binding as well as for agonistic and antagonistic properties in MCF-7-2a cells stably transfected with the plasmid ERE_{wtc}luc.

Results

Synthesis and structural characterization

The synthesis of 2,4,5-triarylimidazoles started from benzils (**6**, **7**, **8**), which can be obtained by benzoin condensation of respectively substituted benzaldehydes and oxidation with $CuSO_4$ (Scheme 2). The subsequent ring formation was performed with the 2,6-dichloro-3/4-methoxybenzaldehydes **9c** and **9d** in a modified Radziszewski reaction [29–31]. Ether cleavage with BBr_3 yielded the imidazoles **1**–**5**.

The heterocyclic skeleton represents an aromatic system in which the substituents at the C atoms are located in the heterocyclic plane. Figure 1 shows the energetically preferred conformations of the 4,5-bis(2-chloro-4-hydroxyphenyl)-2-(2,6-dichloro-4-hydroxyphenyl)imidazole **5** and the 4,5-bis(2-chloro-4-hydroxyphenyl)imidazole **DPI**. The arrangement of the aryl rings in 4,5-position (diarylethene pharmacophor) is independent of the C2 aryl substituent. The torsion angles amount to 6.9° (**5**) and 5.8° (**DPI**) and the distance of the OH groups is nearly identical (8.8 and 8.9 Å). The 4-OH group in the 2-phenyl ring of **5** is arranged in a distances of 12.9 Å to those in the 4,5-standing rings.

^{1}H-NMR spectroscopic studies revealed that the NH-proton at the heterocyclic ring of **5** is fixed in DMSO solution. In contrast, the heating of a DMSO solution of **DPI** to

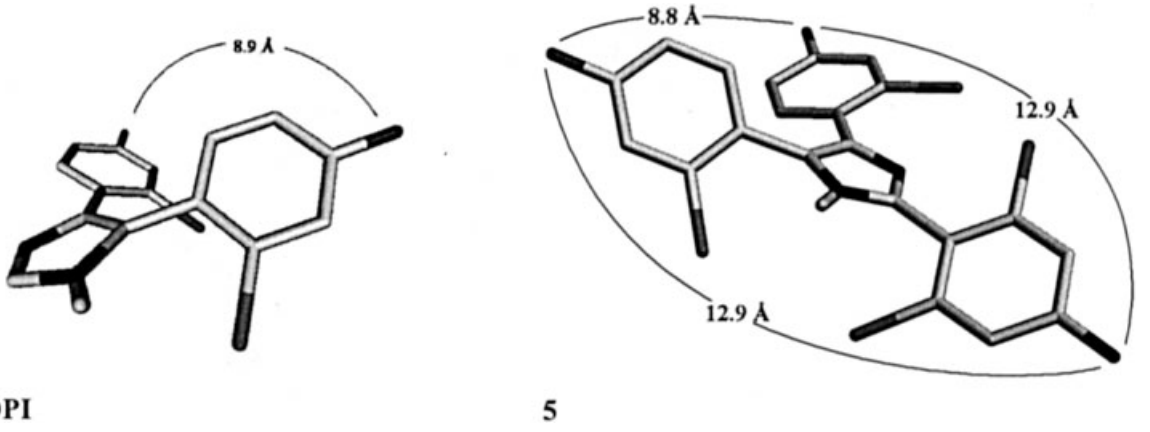
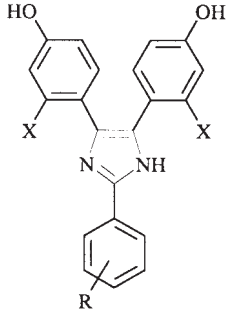


Figure 1. Comparison of the spatial structures of the 4,5-bis(2-chloro-4-hydroxyphenyl)imidazole **DPI** (left) and 4,5-bis(2-chloro-4-hydroxyphenyl)-2-(2,6-dichloro-4-hydroxyphenyl)imidazole **5** (right).

Table 1. Relative binding affinity and agonistic/antagonistic effects of the 2,4,5-triarylimidazoles **1** to **5** in the luciferase assay.



	X	R	RBA[a] [%]	Rel. activation of luciferase expression [% of **E2**] at 10^{-6} M *–OH derivatives*	Rel. activation of luciferase expression [% of **E2**] at 10^{-6} M *–OCH_3 derivatives*	Inhibition [%] of the **E2** effect (1 nM) at 10^{-6} M *–OH derivatives*
1	H	4-OH	<0.02	3	*5*	14
2	H	2,6-Cl_2, 3-OH	<0.02	2	*8*	13
3	H	2,6-Cl_2, 4-OH	0.02	3	*5*	28
4	F	2,6-Cl_2, 4-OH	0.03	3	*0*	22
5	Cl	2,6-Cl_2, 4-OH	0.19	7	*1*	15
DPI			0.11	8	*–3*	n.d.[b]

[a] Relative binding affinity (RBA), % = $[E_2]/[I] \times 100$; $[E_2]$ and [I] are the molar concentrations of nonradioactive E_2 and inhibitor required to decrease the bound radioactivity by 50 %; E_2 = 17β-estradiol. Mean value of 3 determinations.
[b] Not determined.

413 K led to proton exchange reactions and therefore to an equivalence of the nitrogens. This effect was observed for **5** only in acidic solution.

Biological activity

The affinity to the binding site of **E2** was quantified in a competition experiment using calf uteri cytosol and [^{3}H]-**E2** [32]. The relative binding affinity (RBA, **E2** = 100 %) is the proportion of the molar concentrations of unlabeled **E2** and the used inhibitor required to reduce the receptor binding of [^{3}H]-**E2** to 50 %.

As listed in Table 1 only the imidazoles **3**, **4**, and **5** competed with **E2** for its binding site. The RBA = 0.19 % of the most active compound, the 4,5-bis(2-chloro-4-hydroxyphenyl)-2-(2,6-dichloro-4-hydroxyphenyl)imidazole **5** showed that a 2-standing 2,6-dichloro-4-hydroxyphenyl ring increased the ER binding only marginally (**DPI**: RBA = 0.11 %).

The gene activation resulting from the ER binding was evaluated concentration dependent (10^{-10} M to 10^{-5} M) in a luciferase assay using MCF-7-2a cells. The ER-positive human breast cancer cells are stably transfected with the reporter plasmid ERE_{wtc}luc. The binding of ER/drug dimers at the estrogen response elements (ERE) of the plasmid activates the luciferase gene. The quantification of the luciferase expression allows a prediction of the agonistic potency, the inhibition of the **E2** induced activation correlates with the antagonistic effects of the compounds [33].

The effects on the MCF-7-2a cell line were low. None of the compounds activated the gene expression despite the enhanced hydrophobicity due to the 2-standing 2,6-dichloro-3/4-hydroxy substituted phenyl ring. The **E2** (1 nM) stimulated luciferase expression was only reduced significantly by **3** in the concentrations of 10^{-6} M (28 %) and 10^{-5} M (38 %).

Discussion

Planar heterocyclic rings are well known as potential lead structures for the design of SERM. Especially imidazoles, pyrazoles and related compounds were subjects of structure activity studies [23, 14, 26, 34]. Fink et al. [26] investigated the ER-interaction of the 4,5-bis(4-hydroxyphenyl)imidazole (RBA < 0.001 %) and showed that a third phenol ring in position 2 (RBA = 0.007 %) and an additional ethyl chain at the N(1) atom (RBA = 0.38 %) increased the relative binding affinity.

In our studies, we demonstrated [25] that 4,5-bis(4-hydroxyphenyl)imidazoles bind to the ER with high affinity, if the *ortho*-positions of the aromatic rings are Cl-substituted. In this series, the 1-ethyl-4,5-bis(2-chloro-4-hydroxyphenyl)imidazole possessed the highest RBA with 1.26 %. O-methylation prevented the ER binding. This indicates that 4,5-diarylimidazoles are attached to the LBD analogously to **E2** or **DES** by a combination of hydrophobic contacts and H-bridges.

The 1,2-diarylethene pharmacophor of the 4,5-diarylimidazoles is arranged in a Z-stilbene-like structure. An anchorage in the LBD comparable to **DES** or **E2** is impossible. Therefore, the hypothetical binding model for type II estrogens [35] must be used for 4,5-diarylimidazoles, since no crystal structures of the LBD occupied with to these compounds are available.

In contrast to type I estrogens (**E2**, **DES**, etc.), which contact His524, type II estrogens of the piperazine or imidazoline series seem to be attached to Asp351 in the hydrophobic side pocket. Lipophilic parts of the molecules, especially *ortho*-Cl substituents are in van der Waals contacts with lipophilic residues of amino acids and enhance the gene expression. It is assumed that, due to this anchorage, the type II estrogens do not activate the activation function AF2, but use the novel AF2b site [36] that includes acidic surface amino acids on a repositioned helix 12, an exposed aspartate at position 351 and AF1.

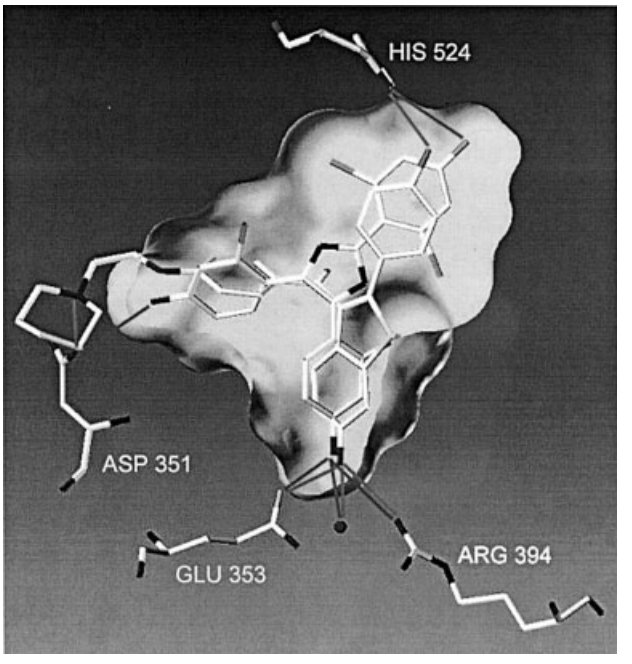


Figure 2. Superposition of the 4,5-bis(2-chloro-4-hydroxyphenyl)-2-(2,6-dichloro-4-hydroxyphenyl)imidazole **5** and raloxifene in the LBD of the estrogen receptor.

In this structure activity study, we tried to optimize the ER interaction of 4,5-bis(4-hydroxyphenyl)imidazoles by introduction of a 2,6-dichloro-4-hydroxyphenyl or 2,6-dichloro-3-hydroxyphenyl ring into the 2-position of the heterocyclic ring. In analogy to 1,2-diarylethylenediamines [27, 28], this substitution pattern guarantees the shielding of the electron rich imidazole core by the *ortho*-Cl substituents and the contact to His524. As demonstrated in Figure 1, the distances between the hydroxy groups located in the 4,5- and the 2,4/2,5-standing aromatic rings amount to 8.8 Å and 12.9 Å, respectively. The latter is nearly identical to that of **DES**.

Two orientations are possible in the LBD due to the symmetric character of the molecules. The first one based on our model for the ER binding of type II estrogens at which the 2,6-dichloro-4-hydroxy-substituted aromatic ring contacts His524 and the 4,5-diarylimidazole moiety is attached to Glu353, Arg394, and Asp351. This orientation correlates very well with that of **RAL** (Figure 2).

Fink et al. [26] investigated in a structure/activity study novel templates for ER ligands and prepared several triphenol imidazole and pyrazole derivatives. They showed that compounds of both classes interact with the ER and postulated an orientation in the LBD comparable to our model.

Recently, a second binding mode was suggested for tris(4-hydroxyphenyl)-substituted five membered heterocycles. 3-Ethyl-2,4,5-tris(4-hydroxyphenyl)furan (**TPF**, Scheme 1) and 4-propyl-1,3,5-tris(4-hydroxyphenyl)-pyrazole (**TPP**, Scheme 1) are oriented in the LBD in such a way that the 2,4-bis(4-hydroxyphenyl)furan or the 3,5-bis(4-hydroxyphenyl)pyrazole moiety mimics the E-stilbene partial structure of **DES** as well as the steroidal skeleton of **E2**. In this case the N(1)- or C(5)-standing phenol ring cannot be oriented in the narrow side pocket, but can make a hydrogen bond with the OH group of Thr347, which is the only polar residue in the ligand binding pocket. This binding mode was made responsible for high affinity selectivity for ERα, which increases 408 times for **TPP** and 48 times for **TPF**.

Generally, the imidazoles **1**–**5** can be attached in the same way in the LBD. If the contact to Thr347 plays an important role in gene activation, it would cause effects in the luciferase assay on MCF-7-2a cells, because these are hormone dependent breast cancer cells with an ERα content of about 95 %.

However, the imidazoles **1** to **5** possessed no agonistic properties, and antagonistic effects were determined only for **3** in concentrations of 10^{-6} M (28 %) and 10^{-5} M (38 %).

Conclusion

In this structure activity relationship study we showed that *ortho*-Cl substituents in the C2-standing phenyl ring are not appropriate to optimize the ER interaction of 2,4,5-tris(4-hydroxyphenyl)imidazoles causing gene activation. Nevertheless, due to its low but significant antagonistic profile in MCF-7-2a cells, **3** represents an interesting lead structure for the design of SERM with antagonistic effects in tumor cells. Therefore, we focused our attention on the effects of alkyl substituents on the hormonal properties of tris(4-hydroxyphenyl)imidazoles.

Acknowledgements

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Experimental

General procedures

IR spectra (KBr pellets): Perkin Elmer Model 580 A (Shelton, USA). ^{1}H-NMR: Bruker ADX 400 spectrometer at 400 MHz (internal standard: TMS) (Rheinstetten, Germany). Elemental analyses: Microlaboratory of Free University of Berlin.

EI-MS-spectra: CH-7A-Varian MAT (70eV) (Palo Alto, USA) or Kratos MS 25 RF (80eV) (New York, USA). 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 from within the SYBYL program, running on an INDY workstation. Liquid Scintillation Counter: 1450 MicrobetaTM Plus (Wallac, Turku, Finland). Microlumat: LB 96 P (Berthold Technologies, Bad Wildungen, Germany).

Syntheses

The benzaldehydes **9 a** to **9 d** were synthesized as described earlier [28], 4,4′-dimethoxybenzil and 4-methoxybenzaldehyde are commercially available.

General procedure for the synthesis of 2,2′-dihalo-4,4′-dimethoxybenzoines

A solution of 2-halo-4-methoxybenzaldehyde (20.0 mmol) and KCN (5.00 mmol) in 15 mL of ethanol/water (60 %) was heated to reflux for 12 h. Further 5.00 mmol KCN were added after 3, 6, and 9 h. After the reaction completed, the solution was allowed to cool to room temperature (RT). 30 mL of water were added and it was extracted with CH_2Cl_2. The organic layer was separated, washed 3 times with water, dried over Na_2SO_4, and evaporated. The crude product was purified by chromatography on silica gel with diethyl ether/ligroine (1 + 3).

2,2′-Difluoro-4,4′-dimethoxybenzoin (**6 a**)

From 2-fluoro-4-methoxybenzaldehyde **9 a**: 8.43 mmol (1.30 g). Yield: 0.811 mmol (250 mg), 19 %; pale yellow oil. IR (KBr): $\bar{\nu}$ = 3456 m, br (OH); 2842 w (OCH_3); 1672 m (C=O); 1616 s; 1508 s; 1444 m; 1271 s; 1158 s; 1121 m; 1031 m; 978 m; 839 m; 756 s. ^{1}H-NMR ($CDCl_3$): δ = 3.74 (s, 3 H, O*C*H_3); 3.81 (s, 3 H, O*C*H_3); 4.50 (s, 1 H, O*H*, exchangeable by D_2O); 5.97 (s, 1 H, Ar*C*H); 6.49 (dd, ^{3}J(H, F) = 13.0 Hz, ^{4}J = 2.5 Hz, 1 H, Ar*H*-3); 6.56 (dd, ^{3}J(H, F) = 11.9 Hz, ^{4}J = 2.4 Hz, 1 H, Ar′*H*-3); 6.60 (dd, ^{3}J = 8.5 Hz, ^{4}J = 2.5 Hz, 1 H, Ar*H*-5); 6.73 (dd, ^{3}J = 8.7 Hz, ^{4}J = 2.4 Hz, 1 H, Ar′*H*-5); 7.07 (dd, ^{3}J = 8.5 Hz, ^{4}J(H, F) = 8.4 Hz, 1 H, Ar*H*-6); 7.92 (dd, ^{3}J = 8.7 Hz, ^{4}J(H, F) = 8.5 Hz, 1 H, Ar′*H*-6).

2,2′-Dichloro-4,4′-dimethoxybenzoin (**7 a**)

From 2-chloro-4-methoxybenzaldehyde **9 b**: 11.7 mmol (2.00 g). Yield: 0.909 mmol (310 mg), 16 %; colorless oil. IR (Film): $\bar{\nu}$ = 3467 m, br (OH); 2840 w (OCH_3); 1688 m (C=O); 1601 s; 1497 s; 1239 s; 1049 s; 975 m; 758 s. ^{1}H-NMR ($CDCl_3$): δ = 3.75 (s, 3 H, O*C*H_3); 3.80 (s, 3 H, O*C*H_3); 4.44 (s, 1 H, O*H*, austauschbar exchangeable by D_2O); 6.27 (s, 1 H, Ar*C*H); 6.72–6.77 (m, 2 H, Ar*H*-5); 6.84 (d, ^{4}J = 2.4 Hz, 1 H, Ar*H*-3); 6.88 (d, ^{4}J = 2.3 Hz, 1 H, Ar*H*-3); 7.10 (d, ^{3}J = 8.6 Hz, 1 H, Ar*H*-6); 7.47 (d, ^{3}J = 8.7 Hz, 1 H, Ar*H*-6).

General procedure for the synthesis of 2,2′-dihalo-4,4′-dimethoxybenzil

$CuSO_4 \times 5\ H_2O$ (10.3 mmol) was dissolved in a mixture of 15.5 mL pyridine and 12.7 mL water. To the blue solution 2.50 mmol of the 2,2′-dihalo-4,4′-dimethoxybenzoine were added and the reaction mixture was heated to 100 °C for 4 h. Subsequently, the green mixture was cooled to RT, poured on

20 mL of water and extracted with CH_2Cl_2. The organic layer was washed twice with 10 mL of 2 N HCl and 20 mL of water. After drying over Na_2SO_4 the solvent was evaporated. The crude product was purified by chromatography on silica gel with diethyl ether/ligroine (1 + 1).

2,2′-Difluoro-4,4′-dimethoxybenzil (**6**)

From 2,2′-difluoro-4,4′-dimethoxybenzoin **6 a**: 0.811 mmol (250 mg). *Yield:* 0.754 mmol (231 mg), 93 %; pale yellow oil. IR (KBr): $\bar{\nu}$ = 2855 w (OCH_3); 1665 m (C=O); 1616 s; 1505 w; 1444 m; 1282 m; 1244 m; 1157 m; 1099 m; 1012 w; 951 w; 846 w. ^{1}H-NMR ($CDCl_3$): δ = 3.88 (s, 6 H, OC*H*$_3$); 6.60 (dd, ^{3}J(H, F) = 12.6 Hz, ^{4}J = 2.3 Hz, 2 H, Ar*H*-3); 6.85 (dd, ^{3}J = 8.4 Hz, ^{4}J = 2.3 Hz, 2 H, Ar*H*-5); 8.02 (dd, ^{3}J = 8.4 Hz, ^{4}J(H, F) = 8.0 Hz, 2 H, Ar*H*-6).

2,2′-Dichloro-4,4′-dimethoxybenzil (**7**)

From 2,2′-dichloro-4,4′-dimethoxybenzoin **7 a**: 0.909 mmol (310 mg). Yield: 0.762 mmol (258 mg), 84 %; yellow oil. IR (KBr): $\bar{\nu}$ = 2842 w (OCH_3); 1663 s (C=O); 1593 s; 1491 s; 1459 m; 1409 m; 1304 s; 1283 s; 1238 s; 1185 m; 1145 w; 1044 s; 895 m; 860 m; 806 m; 760 s; 689 m. ^{1}H-NMR ($CDCl_3$): δ = 3.89 (s, 6 H, OC*H*$_3$); 6.92–6.97 (m, 4 H, Ar*H*-3, Ar*H*-5); 8.06 (d, ^{3}J = 8.4 Hz, 2 H, Ar*H*-6).

General procedure for the synthesis of 2,4,5-triarylimidazole

A solution of the benzil (2.00 mmol), the benzaldehyde (2.00 mmol), and ammonium acetate (16.4 mmol, 1.26 g) in 10 mL of acetic acid was heated to reflux for 12 h. After evaporation of the solvents, 15 mL of water were added and the product was extracted with CH_2Cl_2. The organic layer was washed with Na_2CO_3 solution (5 %) and water and was evapoprated after drying over Na_2SO_4.

2,4,5-Tris(4-methoxyphenyl)imidazole (**1 a**)

From 4,4′-dimethoxybenzil (1.85 mmol (500 mg)) and 4-methoxybenzaldehyde (1.90 mmol (259 mg)). For purification the crude product was recrystallized from ethanol/ligroine. Yield: 0.983 mmol (380 mg), 53 %; colorless powder, mp.: 248–249 °C. IR (KBr): = 3420 m, br (NH); 2998 w; 2838 m (OCH_3); 1617 m; 1520 s; 1502 s; 1293 m; 1247 s; 1177 s; 1032 s; 970 m; 833 s. ^{1}H-NMR ($CDCl_3$): δ = 3.81 (s, 6 H, OC*H*$_3$); 3.83 (s, 3 H, OC*H*$_3$); 6.86 (AA′*BB*′, ^{3}J = 7.0 Hz, 4 H, Ar*H*-3, Ar*H*-5); 6.93 (AA′*BB*′, ^{3}J = 7.8 Hz, 2 H, Ar′*H*-3, Ar′*H*-5); 7.44 (*AA*′BB′, ^{3}J = 7.0 Hz, 4 H, Ar*H*-2, Ar*H*-6); 7.80 (*AA*′BB′, ^{3}J = 7.8 Hz, 2 H, Ar′*H*-2, Ar′*H*-6).

4,5-Bis(4-methoxyphenyl)-2-(2,6-dichloro-3-methoxyphenyl)imidazole (**2 a**)

From 4,4′-dimethoxybenzil (1.85 mmol (500 mg)) and 2,6-dichloro-3-methoxybenzaldehyde **9 c** (1.87 mmol (383 mg)). Purification was performed by column chromatography on silica gel with diethyl ether and ligroine (1 + 1) and subsequent recrystallization from isopropanol/diisopropyl ether. Yield: 1.10 mmol (500 mg), 59 %;, colorless powder, mp.: 126–127 °C. IR (KBr): $\bar{\nu}$ = 3382 m, br (NH); 2936 m; 2834 m (OCH_3); 1615 m; 1567 m; 1518 s; 1491 s; 1459 s; 1294 s; 1249 s; 1177 s; 1060 m; 1032 m; 834 s; 803 m. ^{1}H-NMR ($CDCl_3$): d = 3.82 (s, 6 H, OC*H*$_3$); 3.93 (s, 3 H, OC*H*$_3$); 6.87 (AA′*BB*′, ^{3}J = 8.2 Hz, 4 H, Ar*H*-3, Ar*H*-5); 6.96 (d, ^{3}J = 8.9 Hz, 1 H, Ar′*H*-4); 7.35 (d, ^{3}J = 8.9 Hz, 1 H, Ar′*H*-5); 7.50 (br, 4 H, Ar*H*-2, Ar*H*-6); 9.15 (s, 1 H, N*H*, exchangeable by D_2O).

4,5-Bis(4-methoxyphenyl)-2-(2,6-dichloro-4-methoxyphenyl)-imidazole (**3 a**)

From 4,4′-dimethoxybenzil (1.85 mmol (500 mg)) and 2,6-dichloro-4-methoxybenzaldehyde **9 d** (1.87 mmol (383 mg)). Purification by re-crystallization from $CHCl_3$. Yield: 0.988 mmol (450 mg), 53 %;, colorless powder, mp.: 248–249 °C. IR (KBr): $\bar{\nu}$= 3382 m, br (NH); 2936 m; 2835 m (OCH_3); 1612 s; 1553 s; 1519 s; 1495 s; 1463 s; 1294 s; 1247 s; 1177 s; 1061 m; 1037 s; 971 m; 900 m; 833 s; 804 m. ^{1}H-NMR ($CDCl_3$): δ = 3.80 (s, 3 H, OC*H*$_3$); 3.83 (s, 3 H, OC*H*$_3$); 3.84 (s, 3 H, OC*H*$_3$); 6.82 (AA′*BB*′, ^{3}J = 8.9 Hz, 2 H, Ar*H*-3, Ar*H*-5); 6.90 (AA′*BB*′, ^{3}J = 8.7 Hz, 2 H, Ar*H*-3, Ar*H*-5); 6.95 (s, 2 H, Ar′*H*); 7.38 (*AA*′BB′, ^{3}J = 8.7 Hz, 2 H, Ar*H*-2, Ar*H*-6); 7.57 (*AA*′BB′, ^{3}J = 8.9 Hz, 2 H, Ar*H*-2, Ar*H*-6); 9.11 (s, 1 H, N*H*, exchangeable by D_2O).

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

From 2,2′-difluoro-4,4′-dimethoxybenzil **6** (0.408 mmol (125 mg)) and 2,6-dichloro-4-methoxybenzaldehyde **9 d** (0.410 mmol (84 mg)). Purification by re-crystallization from $CHCl_3$. Yield: 0.342 mmol (168 mg), 84 %; colorless powder, mp.: 234–236 °C. IR (KBr): $\bar{\nu}$ = 2942 w; 2840 w (OCH_3); 1629 s; 1594 s; 1553 m; 1518 m; 1491 s; 1464 m; 1438 m; 1318 m; 1287 s; 1242 m; 1193 m; 1156 s; 1069 m; 1034 m; 945 m; 837 m; 807 m. ^{1}H-NMR ($CDCl_3$): δ = 3.81 (s, 6H, OC*H*$_3$); 3.85 (s, 3 H, OC*H*$_3$); 6.63 (dd, ^{3}J(H, F) = 12.4 Hz, ^{4}J = 2.1 Hz, 2 H, Ar*H*-3); 6.67 (dd, ^{3}J = 8.6 Hz, ^{4}J = 2.1 Hz, 2 H, Ar*H*-5); 6.94 (s, 2 H, Ar′*H*); 7.40 (br, 2 H, Ar*H*-6).

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

From 2,2′-dichloro-4,4′-dimethoxybenzil **7** (0.719 mmol (244 mg)) and 2,6-dichloro-4-methoxybenzaldehyde **9 d** (0.741 mmol (152 mg)). Purification was performed by column chromatography on silica gel with diethyl ether and ligroine (3 + 1). Yield: 0.610 mmol (320 mg), 85 %; colorless powder, mp.: 189–191 °C. IR (KBr): $\bar{\nu}$ = 2940 w; 2840 w (OCH_3); 1610 s; 1555 m; 1508 m; 1481 s; 1436 m; 1288 s; 1227 s; 1186 w; 1060 m; 1038 s; 842 m; 809 m. MS (EI, 220 °C): m/z (%) = 522 (82) $[M]^+$; 507 (6) $[M\text{-}CH_3]^+$; 153 (15). ^{1}H-NMR ($CDCl_3$): δ = 3.79 (s, 6 H, OC*H*$_3$); 3.86 (s, 3 H, OC*H*$_3$); 6.72 (dd, ^{3}J = 8.7 Hz, ^{4}J = 2.3 Hz, 2 H, Ar*H*-5); 6.89 (d, ^{4}J = 2.3 Hz, 2 H, Ar*H*-3); 6.92 (s, 2 H, Ar′*H*); 7.20 (d, ^{3}J = 8.7 Hz, 2 H, Ar*H*-6).

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. BBr_3 (4.5 mmol) in 5 mL of dry CH_2Cl_2 was added at this temperature under N_2 atmosphere. Then the reaction mixture was allowed to warm to room temperature and was stirred for further 48 h. After cooling the reaction mixture with an ice bath, the surplus of BBr_3 was hydrolized three times with methanol and the phenolic product was dissolved in 0.1 N NaOH. The alkaline/water phase was filtrated 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.

2,4,5-Tris(4-hydroxyphenyl)imidazole (**1**)

From 2,4,5-tris(4-methoxyphenyl)imidazole **1 a** (0.621 mmol (240 mg)). Purification was performed by re-crystallization from isopropanol and diisopropyl ether. Yield: 0.569 mmol (196 mg), 92 %; pale grey powder, mp.: 195–198 °C. IR (KBr): $\bar{\nu}$ = 3600–2700 s, br (OH); 2970 w; 1648 m; 1615 s; 1503 s; 1380 m; 1248 s; 1173 s; 1103 m; 836 s. ^{1}H-NMR ([D_6]-DMSO): δ = 6.67

(AA′*BB*′, ^{3}J = 8.5 Hz, 2 H, Ar*H*-3, Ar*H*-5); 6.79 (AA′*BB*′, ^{3}J = 8.4 Hz, 2 H, Ar*H*-3, Ar*H*-5); 6.82 (AA′*BB*′, ^{3}J = 8.7 Hz, 2 H, Ar*H*-3, Ar*H*-5); 7.26 (*AA*′BB′, ^{3}J = 8.4 Hz, 2 H, Ar*H*-2, Ar*H*-6); 7.33 (*AA*′BB′, ^{3}J = 8.5 Hz, 2 H, Ar*H*-2, Ar*H*-6); 7.84 (*AA*′BB′, ^{3}J = 8.7 Hz, 2 H, Ar*H*-2, Ar*H*-6); 9.28 (s, 1 H, ArO*H*, exchangeable by D_2O); 9.57 (s, 1 H, ArO*H*, exchangeable by D_2O); 9.62 (s, 1 H, ArO*H*, exchangeable by D_2O); 12.10 (s, 1 H, N*H*, exchangeable by D_2O).

$C_{21}H_{16}N_2O_3$ (344.37): calc.: C 73.24 H 4.68 N 8.13, found: C 73.28 H 4.90 N 8.07.

4,5-Bis(4-hydroxyphenyl)-2-(2,6-dichloro-3-hydroxyphenyl)-imidazole (**2**)

From 4,5-bis(4-methoxyphenyl)-2-(2,6-dichloro-3-methoxyphenyl)imidazole **2 a** (0.659 mmol (300 mg)). Yield: 0.496 mmol (205 mg), 75 %; colorless powder, mp.: 198–202 °C. IR (KBr): $\bar{\nu}$ 3650–2300 s, br (OH); 3620 w; 1617 s; 1568 m; 1521 s; 1440 s; 1394 m; 1243 s; 1168 m; 1102 m; 835 s; 786 m. ^{1}H-NMR ([D_6]-DMSO): δ = 6.68 (AA′*BB*′, ^{3}J = 8.5 Hz, 2 H, Ar*H*-3, Ar*H*-5); 6.78 (AA′*BB*′, ^{3}J = 8.5 Hz, 2 H, Ar*H*-3, Ar*H*-5); 7.09 (d, ^{3}J = 8.8 Hz, 1 H, Ar′*H*-4); 7.24 (*AA*′BB′, ^{3}J = 8.5 Hz, 2 H, Ar*H*-2, Ar*H*-6); 7.31–7.38 (m, 3 H, Ar*H*-2, Ar*H*-6, Ar′*H*-5); 9.38 (br, 3 H, ArO*H*, exchangeable by D_2O); 12.30 (s, 1 H, N*H*, exchangeable by D_2O).

$C_{21}H_{14}Cl_2N_2O_3$ (413.26): calc.: C 61.03 H 3.41 N 6.78, found: C 61.06 H 3.62 N 6.86.

4,5-Bis(4-hydroxyphenyl)-2-(2,6-dichloro-4-hydroxyphenyl)-imidazole (**3**)

From 4,5-bis(4-methoxyphenyl)-2-(2,6-dichloro-4-methoxyphenyl)imidazole **3 a** (0.527 mmol (240 mg)). Yield: 0.484 mmol (200 mg), 92 %; colorless powder, mp.: 264–266 °C. IR (KBr): $\bar{\nu}$= 1650–2500 s, br (OH); 3182 br; 1640 m; 1610 m; 1575 s; 1526 m; 1497 s; 1455 s; 1254 s; 1058 m; 922 m; 834 m. ^{1}H-NMR ([D_6]-DMSO): δ = 6.67 (AA′*BB*′, ^{3}J = 8.4 Hz, 2 H, Ar*H*-3, Ar*H*-5); 6.77 (AA′*BB*′, ^{3}J = 8.3 Hz, 2 H, Ar*H*-3, Ar*H*-5); 6.94 (s, 2 H, Ar′*H*); 7.24 (*AA*′BB′, ^{3}J = 8.3 Hz, 2 H, Ar*H*-2, Ar*H*-6); 7.32 (*AA*′BB′, ^{3}J = 8.4 Hz, 2 H, Ar*H*-2, Ar*H*-6); 9.60 (br, 3 H, ArO*H*, exchangeable by D_2O); 12.24 (s, 1 H, N*H*, exchangeable by D_2O).

$C_{21}H_{14}Cl_2N_2O_3$ (413.26): calc.: C 61.03 H 3.41 N 6.78, found: C 60.99 H 3.47 N 6.89.

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

From 4,5-bis(2-fluoro-4-methoxyphenyl)-2-(2,6-dichloro-4-methoxyphenyl)imidazole **4 a**: (0.187 mmol (92 mg)). Purification was performed by column chromatography on silica gel with diethyl ether and acetone (9 + 1) and subsequent re-crystallization from methanol/acetone (1 + 5). Yield: 0.089 mmol (40 mg), 48 %; colorless powder, mp.: 211–215 °C. IR (KBr): $\bar{\nu}$ = 3650–2400 s, br (OH); 2933 w; 2678 w; 2585 w; 1628 s; 1600 s; 1521 w; 1457 s; 1306 m; 1245 m; 1155 m; 1114 m; 1063 m; 964 m; 849 m; 812 m. MS (EI, 300 °C): m/z (%) = 448 (100) $[M]^{+\bullet}$; 233 (20); 123 (12); 91 (78). ^{1}H-NMR ([D_6]-DMSO): δ = 6.45 (dd, ^{3}J(H, F) = 12.2 Hz, ^{4}J = 2.1 Hz, 1 H, Ar*H*-3); 6.54–6.63 (m, 3 H, Ar*H*-3, 2 × Ar*H*-5); 6.96 (s, 2 H, Ar′*H*); 7.07 (dd, ^{3}J = 8.7 Hz, ^{4}J(H, F) = 8.7 Hz, 1 H, Ar*H*-6); 7.35 (dd, ^{3}J = 8.7 Hz, ^{4}J(H, F) = 8.7 Hz, 1 H, Ar*H*-6); 9.76 (s, 1 H, ArO*H*, exchangeable by D_2O); 9.97 (s, 1 H, ArO*H*, exchangeable by D_2O); 10.64 (s, 1 H, ArO*H*, exchangeable by D_2O); 12.39 (s, 1 H, N*H*, exchangeable by D_2O).

$C_{21}H_{12}Cl_2F_2N_2O_3$ (449.24): calc.: C 56.15 H 2.69 N 6.24, found: C 56.18 H 2.47 N 6.05.

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

From 4,5-bis(2-chloro-4-methoxyphenyl)-2-(2,6-dichloro-4-methoxyphenyl)imidazole **5 a** (0.534 mmol (280 mg)). Purification was performed by column chromatography on silica gel with acetone and subsequent re-crystallization from acetone/CH_2Cl_2 (5 + 1). Yield: 0.373 mmol (180 mg), 70 %; colorless powder, mp.: 272–275 °C. IR (KBr): $\bar{\nu}$ = 3600–2600 s, br (OH); 2959 m; 2932 m; 2867 w; 1650 s; 1609 s; 1572 m; 1532 s; 1441 s; 1283 s; 1225 m; 1060 w; 900 m; 856 m; 812 m. **MS** (EI, 400 °C): m/z (%) = 480 (84) $[M]^{+\bullet}$; 446 (10) $[M\text{-}Cl]^+$; 410 (7) $[M\text{-}2\,Cl]^+$; 375 (1) $[M\text{-}3\,Cl]^+$; 265 (11); 231 (31); 139 (36). ^{1}H-NMR ([D_6]-DMSO): δ = 6.67 (dd, ^{3}J = 8.5 Hz, ^{4}J = 2.2 Hz, 2 H, Ar*H*-5); 6.74 (d, ^{4}J = 2.2 Hz, 1 H, Ar*H*-3); 6.85 (d, ^{4}J = 2.2 Hz, 1 H, Ar*H*-3); 6.95 (s, 2 H, Ar′*H*); 6.97 (d, ^{3}J = 8.5 Hz, 1 H, Ar*H*-6); 7.13 (d, ^{3}J = 8.5 Hz, 1 H, Ar*H*-6); 9.76 (s, br, 1 H, ArO*H*, exchangeable by D_2O); 9.97 (s, br, 1 H, ArO*H*, exchangeable by D_2O); 10.64 (s, br, 1 H, ArO*H*, exchangeable by D_2O); 12.34 (s, 1 H, N*H*, exchangeable by D_2O).

$C_{21}H_{12}Cl_4N_2O_3$ (482.15): calc.: C 52.31 H 2.51 N 5.81, found: C 52.11 H 2.21 N 5.56.

Biological methods

Biochemicals, chemicals and materials

Dextran, 17β-estradiol, L-glutamine (L-glutamine solution: 29.2 mg/ml PBS) and Minimum Essential Medium Eagle (EMEM) were purchased from Sigma (Munich, Germany); Dulbecco's Modified Eagle Medium without phenol red (DMEM) from Gibco (Eggenstein, Germany); Bovine calf serum (BCS) from Bio whittaker (Verviers, Belgium); N-Hexamethylpararosaniline (crystal violet) and gentamicin sulfate from Fluka (Deisenhofen, Germany); Glutardialdehyde (25 %) from Merck (Darmstadt, Germany); Trypsin (0.05 %) in ethylenediaminetetraacetic acid (0.02 %) (trypsin/EDTA) from Boehringer (Mannheim, Germany); Penicillin-streptomycin gold standard (10000 IE penicillin/mL, 10 mg streptomycin/mL) and geneticin disulfate (geneticin solution: 35.71 mg/mL PBS) from ICN Biomedicals GmbH (Eschwege, Germany); Norit A (charcoal) from Serva (Heidelberg, Germany); Cell culture lysis reagent (5×) (diluted 1:5 with purified water before use) and the luciferase assay reagent from Promega (Heidelberg, Germany); Optiphase HiSafe3 liquid szintillator from Wallac (Turku, Finland); NET-317-Estradiol[2,4,6,7-^{3}H(N)] (17β-[^{3}H]estradiol) from Du Pont NEN (Boston, Maryland); $CDCl_3$, and [D_6]-DMSO from Aldrich (Steinheim, Germany); Phosphate buffered saline (PBS) was prepared by dissolving 8.0 g NaCl, 0.2 g KCl, 1.44 g $Na_2HPO_4 \times 2\,H_2O$ and 0.2 g KH_2PO_4 (all purchased from Merck or Fluka) in 1000 mL of purified water. TRIS-buffer (pH = 7.5) was prepared by solving 1.211 g trishydroxymethylaminomethan, 0.37224 g Titriplex III and 0.19503 g sodium azide (all from Merck or Fluka) in 1 L of purified water. Deionized water – produced by means of a Millipore Milli-Q Water System, resistance > 18 MΩ. T-75 flasks, reaction tubes and 96-well plates were purchased from Renner GmbH (Dannstadt, Germany).

Estrogen receptor binding assay

The applied method was described already by Hartmann et al. [32] and 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 over

night. To stop the reaction 500 µL of a dextran-charcoal-suspension in TRIS-buffer were 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 semi-log 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, which reduce the binding of the radioligand by 50 %, were determined

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

Cell Lines and Growth Conditions

MCF-7-2a cells were maintained as a monolayer culture at 37 °C in a humidified atmosphere (95 % air, 5 % CO_2) in T-75 flasks using phenol red free DMEM supplemented with penicillin/streptomycin 1 % (V/V), L-glutamine (2 mM), FCS 5 % (V/V) and geneticin solution 0.5 % (V/V) as growth media. Cell line banking and quality control were performed according to the seed stock concept reviewed by Hay [37].

Transcriptional binding assay, Luciferase Aassay

One week before starting the experiment, MCF-7-2a cells were cultivated in DMEM supplemented with L-glutamine, antibiotics, and dextran/charcoal-treated FCS (ct-DMEM, 5 % V/V). Cells from an almost confluent monolayer were removed by trypsinization and suspended in ct-DMEM to approximately 5×10^4 cells/mL. 100 µL of the cell suspension was seeded in the sixty inner wells of a white flat-bottomed 96-well plate (suitable for measuring luminescence). The border wells were filled with 200 µL of isoosmotic liquid (medium, PBS, e.g.) in order to avoid boundary problems. After 24 h, the media was replaced by 180 µL of ct-DMEM and 20 µL of medium containing either **E2** or the test compounds in appropriate amounts to achieve final concentrations ranging from 10^{-7} to 10^{-12} M (**E2**) or 10^{-5} to 10^{-10} M (test compounds). The concentration of the solvent (ethanol abs.) used to prepare stock solutions amounts to 0.1 % (V/V).

After 50 h of incubation under growth conditions, the medium was removed and 50 µL of cell culture lysis reagent was added into each well. The plate was incubated at RT under vigorous shaking (600 rpm, TiMix, Thistle Scientific Uddingston, Glasgow, Scotland). Luciferase was assayed using the Promega luciferase assay reagent. 50 µL of substrate reagent was added into each well and luminescence (in relative light units, RLU) was measured for 10 s by use of a microlumat (Bethold Technologies, Bad Wildungen, Germany). Measurements were corrected correlating the RLU with the cell mass of each sample.

The cell mass was determined in a crystal violet assay [38, 39] in crystal flat-bottomed 96-well plates analogously to the transactivation assay. After incubation for 50 h, the medium was removed and glutaric dialdehyde (1 % in PBS; 100 µL/well) was added for fixation. After 15 min the solution of the aldehyde was replaced by 180 µL PBS / well. The plates were stored at 4 °C until staining. Cells were stained treating them for 30 min with 100 µL of an aqueous solution of crystal violet (0.02 % (m/V)). After decanting, cells were washed several times with water to remove the adherent dye. After addition of 180 µL of ethanol (70 % (v/v)), plates were gently shaken for 4 h. Optical density of each well was measured in a microplate autoreader at 590 nm (Fash scan, Jena Analytik, Jena, Germany).

The estrogenic activity was expressed as % activation of a 10^9 M **E2** control (100 %).

The anti-estrogenic activity was determined by incubation of the MCF-7-2a cells with the test compounds in concentrations from 10^{-5}–10^{-10} M along with a constant concentration of **E2** (10^{-9} M). IC_{50} is the concentration of the compound which is necessary to reduce the effect of **E2** by 50 %.

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