Investigations on the Effects of Basic Side Chains on the Hormonal Profile of (4*R*,5*S*)/(4*S*,5*R*)-4,5-Bis(4-hydroxyphenyl)-2-imidazolines

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Received June 16, 2004

Basic side chains determine the pharmacology of selective estrogen receptor modulators such as tamoxifen or raloxifene. In this study we tried to turn the hormonal profile of (4*R*,5*S*)/(4*S*,5*R*)-4,5-bis(4-hydroxyphenyl)-2-imidazolines from agonistic to antagonistic by introduction of a dimethylaminoethane, a piperidin-1-ylethane, or a pyrrolidin-1-ylethane side chain into one of the 4-hydroxyphenyl rings. The compounds were tested for agonistic and antagonistic activity on hormone sensitive, ERα-positive MCF7-2a cells, stably transfected with the plasmid ERE_{wtc}luc and on U-2 OS cells transiently transfected with plasmids encoding for ERα (pSG5-ERα) or ER*β* (pSG5-ER*β* FL) as well as the reporter plasmid $(ERE)_2luc^+$. Despite the presence of a basic side chain, the majority of the 4,5-diaryl-2-imidazolines showed agonistic effects. The most active compound, (4*R*,5*S*)/(4*S*,5*R*)-4-(2-chloro-4-(2-piperidin-1-ylethoxy)phenyl)-5-(2,6-dichloro-4-hydroxyphenyl)-2-imidazoline (**5a**), achieved at ERα an EC_{50} value of 0.085 μM and at ER*β* an $EC_{50} = 0.40$ μM. High antagonistic properties only possessed the C2 ethyl substituted compounds **2a** and **4a**. (4*R*,5*S*)/(4*S*,5*R*)-2-Ethyl-4-(4-hydroxyphenyl)-5-(4-(2-piperidin-1-ylethoxy)-phenyl)-2-imidazoline (**2a**) reduced the effect of estradiol at ERα strongly with $IC_{50} = 0.038$ μM, while its antagonistic properties at ER*β* were distinctly lower ($IC_{50} = 9.00$ μM), probably due to the partial agonistic effects ($EC_{50} = 0.50$ μM).

Introduction

Selective estrogen receptor modulators (SERMs) show tissue selective agonistic or antagonistic effects. SERMs such as tamoxifen (TAM) and raloxifene (RAL), which are used in the therapy of hormone dependent breast cancer, consist of a nonsteroidal core equipped with a basic side chain at a phenolic hydroxy group. This aminoethoxy moiety changes the hormonal profile from estrogen-like to antiestrogen.[1]

Despite their high benefit, TAM and RAL are associated with a series of dose-limited side effects, e.g. an increased risk of thromboembolism or exacerbation of hot flushes.[2] Therefore, there is a great interest in the development of new, well-tolerable SERMs.

Several attempts were made to obtain SERMs by a structural modification of steroid hormones.[3] Thus, the introduction of a 9-[(4,4,5,5,5-pentafluoropentyl)sulfinyl]-nonyl chain at the 7α-position (faslodex) or a dimethylaminoethoxyphenyl residue at the C11 atom (RU 39 411) of estradiol (E2) led to the hormonal profile of antiestrogens.[4,5] In contrast to faslodex, RU 39411 represents a partial antiestrogen, as its estrogenic activity was demonstrated by its strong ability to induce synthesis of the progesterone receptor in MCF-7 cells.[6]

A structural feature of many nonsteroidal SERMs is a bicyclic core containing a phenolic hydroxy group with a basic side chain and an aryl ring emanating from the core. This class includes benzothiophenes (e.g. RAL), indoles (e.g. pipendoxifene),[7] and tetrahydronaphthalenes (e.g. lasofoxifene).[8]

Further compounds with very interesting pharmacological properties are five-membered heterocycles, such as tetrasubstituted pyrazoles, furans, and pyrroles, developed by the group of Katzenellenbogen.[9,10] Compounds out of these classes showed high affinity to the ER with pronounced subtype selectivity. Addition of basic side chains to pyrazole compounds resulted in potent antagonists without estrogenic side effects.[11,12]

In our group, we developed halide-substituted (4*R*,5*S*)/(4*S*,5*R*)-4,5-bis(4-hydroxyphenyl)-2-imidazolines as ER agonists. These compounds possess a spatial structure quite different from conventional estrogens but showed transcriptional activity in ERα-positive MCF-7-2a cells.[13] Now we tried to change their hormonal profile to antiestrogenic by introduction of a dimethylaminoethane, a piperidin-1-ylethane, or a pyrrolidin-1-ylethane side chain into one of the 4-hydroxyphenyl rings.

Chemistry

Synthesis. (4*R*,5*S*)/(4*S*,5*R*)-4,5-Bis(4-hydroxyphenyl)-2-imidazolines with various substitution pattern in the aromatic rings were obtained from the corresponding 1,2-bis(4-methoxyphenyl)-1,2-diaminoethanes by reaction with triethylorthoformate or triethylorthopropionate and subsequent ether cleavage with BBr_3.[14,15] Subsequently, the 2-imidazolines were dissolved in a sodium ethanolate solution, treated with the appropriate 2-chloroethylamine derivative, and heated to reflux. The resulting crude products (see Table 1) were purified by column chromatography on neutral Al_2O_3 with different mixtures of CH_2Cl_2/CH_3OH.

Structural Characterization. The structures of the 2-imidazolines **1**−**5** were evaluated in previous papers.[14,15] NMR spectroscopical studies indicated a planar imidazoline ring with pseudoaxial arrangement of the aryl rings. This led to symmetrical molecules (**1**−**4**) with

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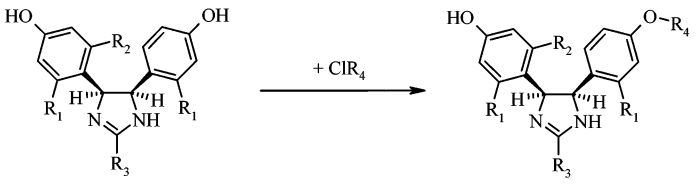
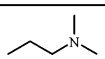
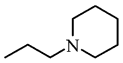
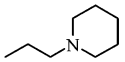
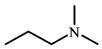
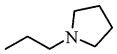
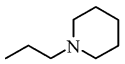
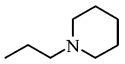
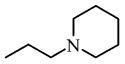
10.1021/jm040855c CCC: $30.25
Published on Web 12/18/2004

Table 1. Effects of 4,5-Diaryl-2-imidazolines on the Luciferase Expression in MCF-7-2a Cells Stably Transfected with the Plasmid $ERE_{wtc}luc$ or in U-2 OS Cells Transiently Transfected with Plasmids Encoding for ERα (pSG5-ERα) or ERβ (pSG5-ERβ FL) and the Reporter Plasmid $(ERE)_2luc^+$



1 - 5 1a,b; 2a; 3a-c; 4a; 5a

					MCF-7-2a cells		ERα				ERß			
	R_1	R_2	R_3	R_4	Activation [%]	Inhibition of E2[a]	Activation [%]		Inhibition of E2[b] [%]		Activation [%]		Inhibition of E2[b] [%]	
					at 1μM		at 10μM	EC_{50} [μM]	at 10μM	IC_{50} [μM]	at 10μM	EC_{50} [μM]	at 10μM	IC_{50} [μM]
1	H	H	H	H	0	0								
1a	H	H	H		19	4	74	3.47	38	-	36	-	0	-
1b	H	H	H		4	0	16	-	3	-	26	-	12	
2	H	H	Et	H	6	0								
2a	H	H	Et		0	0	0	-	66	0.038	95	0.50	57	9.00
3	Cl	H	H	H	58	0								
3a	Cl	H	H		10	4	101	1.50	0	-	75	3.55	4	-
3b	Cl	H	H		-5	3	123	1.82	0	-	105	0.47	30	-
3c	Cl	H	H		0	0	85	1.70	0	-	60[c]	0.60	0	-
4	Cl	H	Et	H	6	0								
4a	Cl	H	Et		0	3	10	-	34	-	92	2.80	75	6.00
5	Cl	Cl	H	H	112	0								
5a	Cl	Cl	H		4	13	98	0.085	19	-	103	0.40	0	-

[a] E2 concentration, 1 nM. [b] E2 concentration, 0.03 nM for ERα and 0.30 nM for ERβ. [c] Impeded agonist and reached its maximal effect already at 1 μM (57% activation).

only one set of resonances for the aromatic and benzylic protons in the ^1H and ^{13}C NMR spectra. On the other hand, **5** represents an asymmetrical molecule because of the different substitution pattern in the aromatic rings. The coupling constant between the benzylic protons (^3J = 13.3 Hz) correlated very well with the above-mentioned planar structure.

The same asymmetry results from alkylation of one phenolic group in **1**−**4**. The benzylic protons of **1a,b** and **2a** are diastereotopically split with coupling constant of 12.1 Hz (**1a,b**) and 11.7 Hz (**2a**), respectively. Interestingly, the spectra of the derivatives with *o*-chlorine substituents (**3a**−**c**, **4a**) exhibit only one singlet resonance for the methine protons, although all other signals indicate asymmetric molecules.

The basic side chain at **5a** can be located either at the 2-chloro-4-hydroxyphenyl or at the 2,6-dichloro-4-hydroxyphenyl ring, resulting in different compounds. However, we isolated only one of the isomers, whose structure was clarified by ^1H NMR spectroscopy.

The spectra of **5a** showed signals at $\delta = 6.63$ and 6.86 comparable to those of a 2,6-dichloro-4-hydroxyphenyl ring (δ = 6.58 and 6.81) as well as at $\delta = 6.94-6.99$ (H3′ and H5′) and $\delta = 7.46-7.57$ (H6′) comparable to that of a 2-chloro-4-methoxyphenyl ring ($\delta = 6.87-6.91$ and δ = 7.50). Therefore, **5a** was assigned as (4*R*,5*S*)/

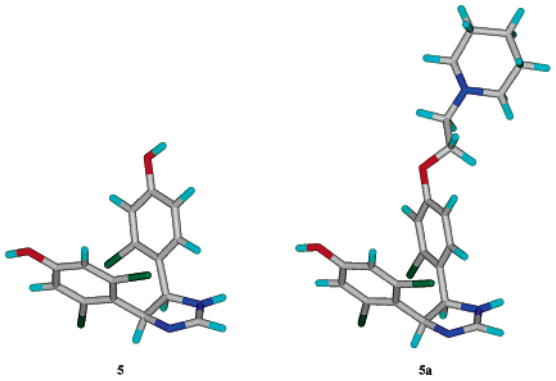


Figure 1. Calculated low-energy structure of the 2-imidazolines **5** and **5a**.

(4*S*,5*R*)-4-(2-chloro-4-(piperidin-1-ylethoxy)phenyl)-5-(2,6-dichloro-4-hydroxyphenyl)-2-imidazoline.

The spectroscopical studies were confirmed by computational methods using Sybyl 6.7. The theoretical calculations showed that the introduction of the basic side chains did not change the spatial structure of the 2-imidazoline ring. It remains planar (Figure 1). The aryl rings are pseudoaxially arranged and the benzylic protons take a dihedral angle between 0° and 15°. The O−O distance amounts to about 6.5 Å. The low-energy conformations of the 2-imidazolines **5** and **5a** are shown in Figure 1.

Biology

Biological Properties. The relative binding affinity (RBA value) of the side-chain-bearing 2-imidazolines could not be calculated, because they were not able to displace [^{3}H]E2 from the ER in concentrations <10 μM. Nevertheless, the hormonal profile was evaluated in luciferase assays using MCF-7-2a cells stably transfected with the plasmid ERE$_{wtc}$luc[4] or U-2 OS cells transiently transfected with plasmids encoding for ERα (pSG5-ERα) or ERβ (pSG5-ERβ FL) as well as the reporter plasmid (ERE)$_2$luc$^+$.

Activity at the MCF-7-2a Cell Line. The results at the MCF-7-2a cell line of the compounds lacking a basic side chain were discussed previously.[14,15] In brief, the configuration and the substituents in the aromatic rings control the hormonal activity. 4*R*,5*S*/4*S*,5*R*-Configurated compounds are by far more active than their congeners. Chlorine substituents in the ortho-positions of the aromatic rings mediate a sufficient hydrophobicity for van der Waals contacts in the ligand-binding domain. Finally, 4-hydroxy groups are essential for hormonal activity. The respective O-methyl derivatives are inactive. The most active compound in this series was the 2-imidazoline **5** with an EC$_{50}$ of 0.065 μM. The introduction of different basic side chains into **1**−**5** reduced the activity at MCF-7-2a cells distinctly. Only **1a** showed low induction of luciferase expression (activation at 1 μM, 19%; see Table 1). Furthermore, the influence of all compounds on the effect of E2 (10^{-9} M) was insignificant (see Table 1).

Agonistic Properties at ERα and ERβ. Nevertheless, the 4,5-diaryl-2-imidazolines were evaluated for subtype selectivity at U-2 OS cells, because this assay is more sensitive to estrogens (E2: EC$_{50}$ (ERα) = 2.5 × 10^{-12} M; EC$_{50}$ (ERβ) = 1.0 × 10^{-11} M) and antiestrogens (RAL: IC$_{50}$ (ERα) = 4.00 × 10^{-10} M; IC$_{50}$ (ERβ) = 8.0 × 10^{-8} M) than that using MCF-7-2a cells (E2: EC$_{50}$ = 4.0 × 10^{-11} M; RAL: IC$_{50}$ = 3.5 × 10^{-9} M; see Figure 2).

(4*R*,5*S*)/(4*S*,5*R*)-4-(4-(2-Dimethylaminoethoxy)phenyl)-5-(4-hydroxyphenyl)-2-imidazoline (**1a**) was agonistically active at ERα (EC$_{50}$ = 3.47 μM), while the respective piperidine derivative **1b** was nearly inactive (activation at 10 μM, 16%; see Table 1). 2,2′-Dichloro substituents in the aromatic rings enhanced the luciferase expression independent of the kind of basic side chain (**3a**−**c**: EC$_{50}$ = 1.50−1.82 μM). A further Cl-atom in one of the aromatic rings of **3c** resulted in the most active compound, **5a** (EC$_{50}$ = 0.085 μM; see also Figure 3), while the introduction of an ethyl chain at the C2 atom of **3c** led to a complete loss of agonistic properties (see **4a** in Table 1).

At ERβ, **1a** and **1b** had low activity and activated the luciferase activity at 10 μM to 36% and 26%, respectively. 2,2′-Dichloro substitution of the aromatic rings increased the hormonal potency dependent on the kind of side chain: **3a** (EC$_{50}$ = 3.55 μM) < **3b** (EC$_{50}$ = 0.47 μM) ≈ **3c** (EC$_{50}$ = 0.60 μM). Compound **3c** represented an impeded agonist that reached its maximal effect of 60% already at a concentration of 1 μM. A further Cl-atom in one of the aromatic rings did not change the agonistic potency (**5a**: EC$_{50}$ = 0.40 μM) but changed the hormonal profile to that of a full agonist. In contrast to ERα, the C2 ethyl-substituted compounds **2a** (EC$_{50}$ = 0.50 μM) and **4a** (EC$_{50}$ = 2.80 μM) were agonistically active at ERβ.

Antagonistic properties at ERα and ERβ. Among the new compounds, only the C2 ethyl derivatives are able to antagonize effectively the maximal effect of E2 (ERα at 3.0 × 10^{-11} M and ERβ at 3.0 × 10^{-10} M). (4*R*,5*S*)/(4*S*,5*R*)-2-Ethyl-4-(4-hydroxyphenyl)-5-(4-(2-piperidin-1-ylethoxy)phenyl)-2-imidazoline (**2a**) reduced the effect of E2 at ERα strongly with IC$_{50}$ = 0.038 μM, while its antagonistic properties at ERβ (IC$_{50}$ = 9.00 μM) were diminished by its agonistic effects (see Figure 3). Introduction of chlorine substituents in the aromatic rings resulting in **4a** strongly decreasing the antiestrogenic potency at ERα (inhibition of E2 at 10 μM, 34%) but prevented the E2 antagonistic effects at ERβ (IC$_{50}$ = 6.00 μM). It should be noted that very low antagonistic properties were evaluated for **1a** at ERα (inhibition at 10 μM, 38%) and for **3b** at ERβ (inhibition at 10 μM, 30%). RAL was evaluated as reference and showed antagonistic effects at both receptor subtypes (see Figure 2) with selectivity for ERα.

Discussion

The aim of this study was the design of new ER antagonists by introduction of basic side chains into 4,5-bis(4-hydroxyphenyl)-2-imidazolines. Compounds out of this class with halogen substituents in the aromatic rings are ER agonists in MCF-7-2a cells and were assigned as type II estrogens with an unusual binding mode in the LBD of the ERα.[13,14]

It was supposed that these compounds are attached at the same binding cave like classical type I estrogens (e.g. E2 and diethylstilbestrol), but contact Asp 351 instead of His 524 in the LBD of ERα. Asp 351 is an important amino acid located in a side pocket of the LBD and preferably contacted by the basic side chains

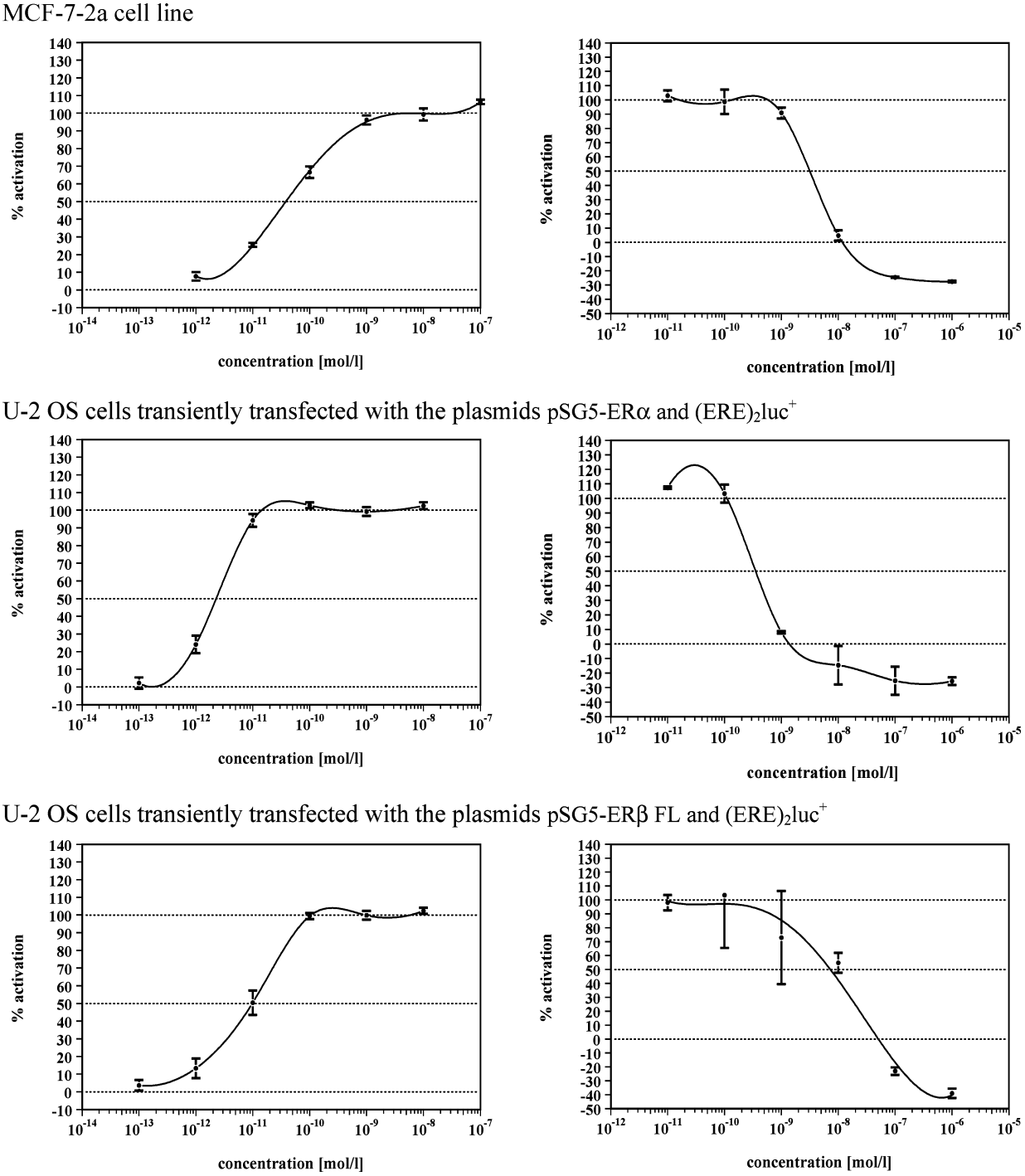


Figure 2. Agonistic (left) and the antagonistic (right) effects of E2 and RAL.

of SERMs such as 4-hydroxytamoxifen (4-OHT), RAL, and RU 39 411.[1,16,17] The resulting H-bond leads to a conformational change of the ER molecule, a reposition of helix 12, normally oriented over the binding cave after binding of agonists, and prevents the formation of a transcriptional competent conformation of the activation function 2 (AF2).

However, we demonstrated for 4-OHT and related compounds that a dimethylaminoethoxy side chain is not exclusively a prerequisite for antagonistic effects. The hormonal profile is also determined by the kind of C2-alkyl chain. The 1,1-bis(4-hydroxyphenyl)-2-phenyl-but-1-ene showed nearly the same effects on the MCF-7-2a cell line as 4-OHT. Reduction or elongation of the C2-alkyl chain and hydrophilic terminal groups decreased the activity.[18−20] Therefore, it can be assumed that high antagonistic properties demand van der Waals contacts to hydrophobic amino acids located around the ethyl chain in the LBD/4-OHT conjugate.

Similar results were obtained by Aliau et al.,[21] who evaluated the binding mode of RU 39 411 at ERα by theoretical methods. They prepared a model of the LBD/RU complex for ERα and used the crystal structure of the LBD/RAL conjugate as template. After molecular dynamic simulation, they detected great similarity to E2 as well as to RAL. The N-atom of the basic side chain is neighbored to Asp 351 (2.84 Å) and the 17β-OH group contacts His 524 at a distance of 2.91 Å. If an interaction

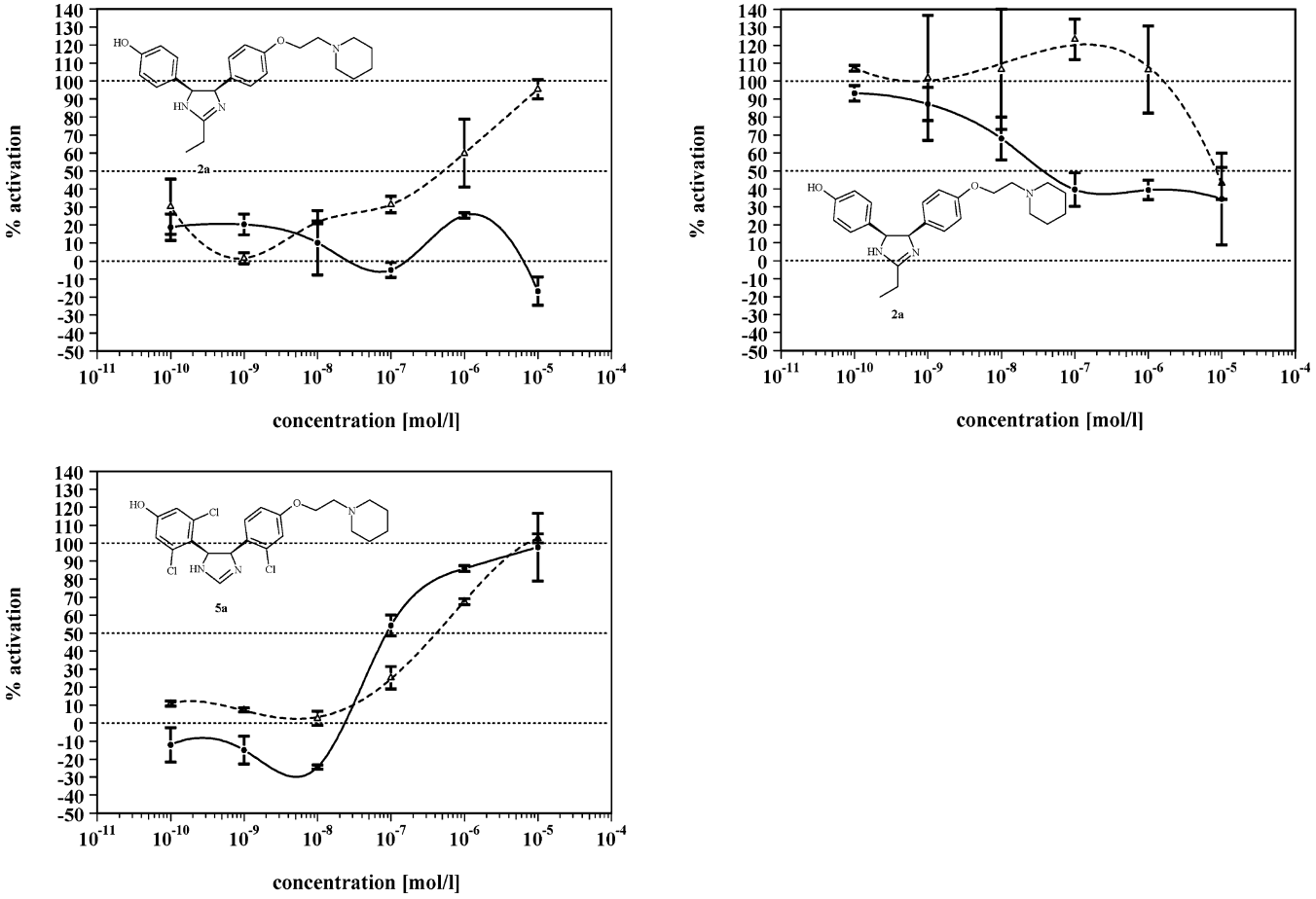


Figure 3. Agonistic (left) and the antagonistic (right) effects of the 2-imidazolines **2a** and **5a** at ERα (solid line) and ERβ (dashed line).

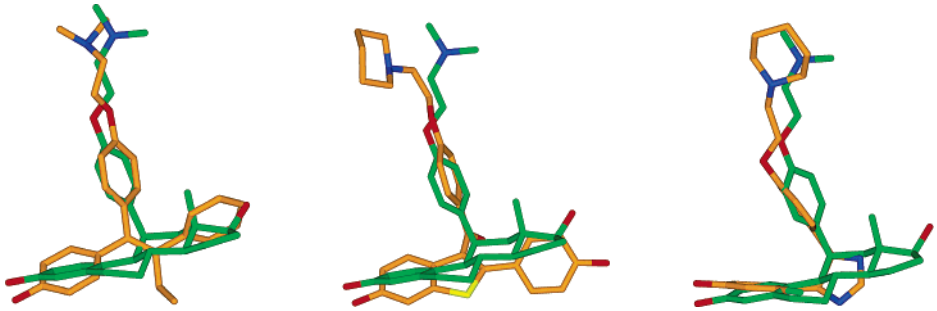


Figure 4. Superposition of 4-OHT (left), RAL (middle), and the 2-imidazoline **5a** (right) with RU 39 411.

of the 11β substituent with Asp 351 exists, the H-bond to His 524, however, plays only a negligible or moderate role in the mediation of antagonistic properties, as demonstrated in mutagenesis and DEPC-treatment experiments.

These findings prompted us to equip type II estrogens out of the 2-imidazoline class with basic side chains. We assumed that a basic side chain at the phenolic ring located in the hydrophobic side pocket should turn the hormonal profile from agonistic to antagonistic.

However, contrary to our assumption, we detected at ERα but also at ERβ mainly agonistic effects. The hormonal potency increased with the number of chlorine atoms in the aromatic rings. As most active compound, the (4*R*,5*S*)/(4*S*,5*R*)-4-(2-chloro-4-(2-piperidin-1-ylethoxy)-phenyl)-5-(2,6-dichloro-4-hydroxyphenyl)-2-imidazoline (**5a**) showed an EC_{50} = 0.085 μM. At ERβ, **5a** was considerably less active (EC_{50} = 0.40 μM). This can be a consequence of a slightly smaller LBD of ERβ. It has been found that sterically demanding ligands display therefore selectivity for ERα.

At both ER subtypes no antagonistic properties were detected for **5a**, despite the high structural analogy of the essential pharmacophor to that of 4-OHT, RAL, and RU 39 411, as documented by superposition experiments presented in Figure 4.

If the 2,6-dichloro-4-hydroxyphenyl ring is superimposed with the benzothiophene core of RAL or the A/B ring of RU 39 411, the basic side chains will take comparable spatial orientation. A contact to Asp351 is therefore very likely.

Jordan et al.[22−24] recently showed that this amino acid is critically important for the estrogen-like actions of SERMs at ERα. They hypothized that this effect results from an insufficient shielding of the charge at Asp351. Compared to 4-OHT, the antiestrogenic side chain of

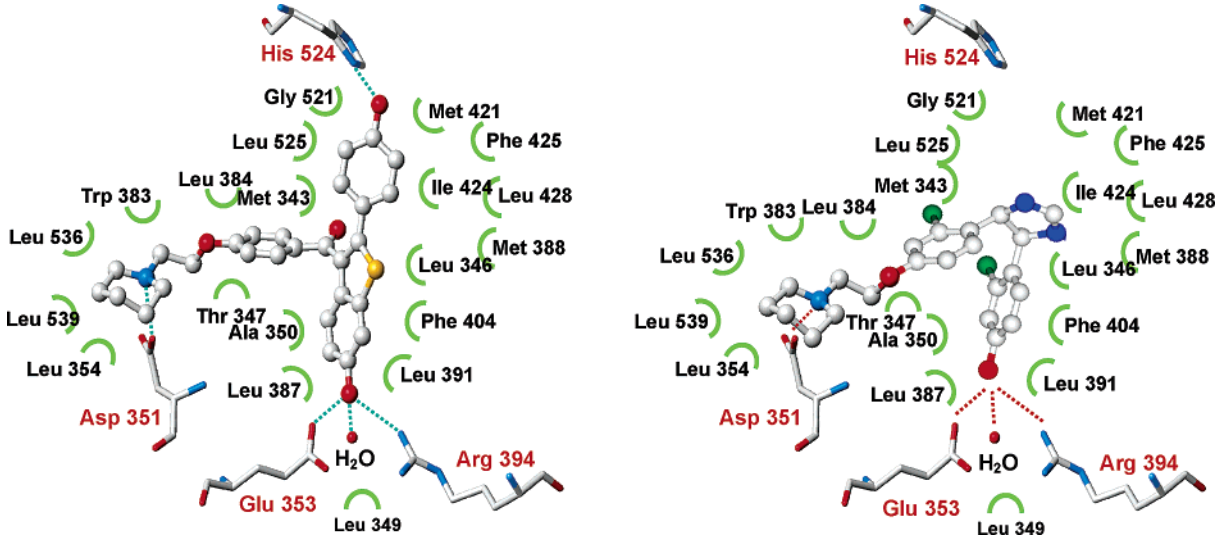


Figure 5. Theoretical binding mode of RAL (left) and the 2-imidazoline **5a** (right).

RAL shields Asp351, thereby silencing all of the estrogen-like properties of the ER complex. This interaction requires an optimal position of the core molecule in the LBD.

From these findings we deduced that the imidazolines are too small to cause an "antiestrogenic orientation" in the binding cave (see Figure 5). Therefore, we prepared the C2 ethyl derivatives **2a** and **4a** and evaluated their agonistic and antagonistic profile at ERα and ER*β*. The (4*R*,5*S*)/(4*S*,5*R*)-2-ethyl-4-(4-hydroxyphenyl)-5-(4-(2-piperidin-1-ylethoxy)phenyl)-2-imidazoline (**2a**) represented a pure antiestrogen and reduced the effect of E2 at ERα strongly (IC_{50} = 0.038 μM). Chlorine atoms in the 2-positions of the aromatic rings (**4a**) led to a loss of activity. Interestingly, both compounds showed antagonistic potency at ER*β*, which was however reduced by their partial agonistic properties (see Table 1).

The C2 ethyl chain can mediate antagonistic properties either by a better orientation in the LBD or by an enhanced hydrophobicity at the imidazoline core. The relevance of the C2 phenyl ring as well as the C2 ethyl chain for the antiestrogenic properties of tamoxifen was already demonstrated during the past decades.[25,26]

Therefore, we will study the influence of substituents at the N-atoms and the C2 atom of the 2-imidazoline core on the antagonistic properties in a further structure−activity relationship study.

Conclusion

4,5-Bis(4-hydroxyphenyl)-2-imidazolines with basic side chains are type II estrogens, which activate ERα and ER*β*. (4*R*,5*S*)/(4*S*,5*R*)-4-(2-Chloro-4-(2-piperidin-1-ylethoxy)phenyl)-5-(2,6-dichloro-4-hydroxyphenyl)-2-imidazoline (**5a**), as most active compound, shows an EC_{50} = 0.085 μM at ERα and an EC_{50} = 0.40 μM at ER*β*. To cause antagonistic effects, the imidazoline core has to be ethyl-substituted at C2. The most potent antagonist, (4*R*,5*S*)/(4*S*,5*R*)-2-ethyl-4-(4-hydroxyphenyl)-5-(4-(2-piperidin-1-ylethoxy)phenyl)-2-imidazoline (**2a**), is a pure antiestrogen (IC_{50} = 38 nM) at ERα, while its antiestrogenic potency at ER*β* is diminished by agonistic side effects.

Materials and Methods

General Procedures. IR spectra (KBr pellets) were collected with a Perkin-Elmer model 580 A. ^{1}H NMR spectra were determined with a Bruker ADX 400 spectrometer at 400 MHz (internal standard, TMS). EI-MS spectra were gathered with CH-7A-Varian MAT (70 eV) and Kratos MS 25 RF (80 eV) instruments. Luminescence measurement was done with a Microlumat LB 96 P (EG & G Berthold, Germany). Elemental analyses were carried out at the Microlaboratory of the Free University Berlin. All computational graphics were built using SYBYL 6.7 (Tripos Inc. 1699 South Hanley Rd., St. Louis, MO 63144). Geometry optimization was carried out using the MM3 force field within the program, running on an INDY workstation.

Syntheses. (4*R*,5*S*)/(4*S*,5*R*)-4,5-Bis(4-hydroxyphenyl)-2-imidazolines **1**−**5** were synthesized as described earlier.[14,15]

General Procedure for the Introduction of a Basic Side Chain into 4,5-Diaryl-2-imidazolines. A 3.00 mmol portion of sodium was cut in small pieces and carefully added to 25 mL of dry ethanol. The solution was stirred until the sodium was completely dissolved. Subsequently, 1.00 mmol of the 2-imidazoline and 1.00 mmol of the appropriate alkyl chloride in 5 mL of dry ethanol were added. The solution was heated to reflux for 5 h. After cooling to room temperature, ethereal HCl was added and the solution was filtrated. The solvent was evaporated and the crude product was purified by column chromatography on neutral Al_2O_3.

(4*R*,5*S*)/(4*S*,5*R*)-4-(4-(2-Dimethylaminoethoxy)phenyl)-5-(4-hydroxyphenyl)-2-imidazoline (1a) was prepared from (4*R*,5*S*)/(4*S*,5*R*)-4,5-bis(4-hydroxyphenyl)-2-imidazoline hydrobromide **1** (1.00 mmol, 335.2 mg) and 2-chloroethyldimethylamine hydrochloride (1.00 mmol, 144.1 mg). Purification: column chromatography with chloroform/methanol 9/1. Yield: 0.30 mmol (118.3 mg), 25%, green oil. IR (KBr, cm^{-1}): $\tilde{\nu}$ = 3600−2500 s, br (OH), 1632 m, 1517 m, 1248 m. MS (EI, 60 °C): *m/z* (%) = 325 (36) [$M^{+\bullet}$], 122 (43), 72 (53), 58 (100). ^{1}H NMR ([D$_6$]-DMSO): δ = 2.78 (s, 6H, NC*H*$_3$), 3.47 (t, ^3J = 5.0 Hz, 2H, OCH$_2$C*H*$_2$N), 4.23 (t, ^3J = 5.0 Hz, 2H, OC*H*$_2$CH$_2$N), 5.62 (d, ^3J = 12.1 Hz, 1H, ArC*H*), 5.66 (d, ^3J = 12.1 Hz, 1H, ArC*H*), 6.52 (AA′*BB*′, ^3J = 8.5 Hz, 2H, Ar′*H*-3, Ar′*H*-5), 6.69−6.85 (*m*, 4H, Ar*H*-3, Ar*H*-5, Ar′*H*-2, Ar′*H*-6), 6.96 (*AA*′BB′, ^3J = 8.6 Hz, 2H, Ar*H*-2, Ar*H*-6), 8.95 (s, 1H, N=C*H*-N), 9.47 (s, 1H, ArO*H*, exchangeable by D$_2$O), 10.42 (br, 1H, N*H*, exchangeable by D$_2$O), 10.81 (s, 1H, N*H*, exchangeable by D$_2$O), 10.85 (s, 1H, N*H*, exchangeable by D$_2$O). Anal. ($C_{19}H_{23}N_3O_2$·2HCl·4H$_2$O) C, H, N.

(4*R*,5*S*)/(4*S*,5*R*)-4-(4-Hydroxyphenyl)-5-(4-(2-piperidin-1-ylethoxy)phenyl)-2-imidazoline (1b) was prepared from (4*R*,5*S*)/(4*S*,5*R*)-4,5-bis(4-hydroxyphenyl)-2-imidazoline hydrobromide **1** (1.00 mmol, 335.2 mg) and 1-(2-chloroethyl)-

piperidine hydrochloride (1.00 mmol, 184.1 mg). Purification: column chromatography with chloroform/methanol 9/1. Yield: 0.13 mmol (62.4 mg), 13%, brown oil. IR (KBr, cm^{-1}): $\bar{\nu}$ = 3600−2500 s, br (OH), 1620 s, 1515 s, 1454 m, 1246 s, 1181 m. MS (EI, 170 °C): *m/z* (%) = 419 (2) [$M^{+\bullet}$], 128 (18), 98 (17), 84 (100). ^{1}H NMR ([D$_6$]-DMSO): δ = 1.28−1.46 (m, 1H, NCH$_2$-CH$_2$C*H*$_2$CH$_2$CH$_2$N), 1.60−1.73 (m, 1H, NCH$_2$CH$_2$C*H*$_2$CH$_2$-CH$_2$N), 1.73−1.88 (m, 4H, NCH$_2$C*H*$_2$CH$_2$C*H*$_2$CH$_2$N), 2.83−3.12 (m, 2H, NC*H*$_2$CH$_2$CH$_2$CH$_2$C*H*$_2$N), 3.37−3.51 (m, 4H, NC*H*$_2$-CH$_2$CH$_2$CH$_2$C*H*$_2$N, OCH$_2$C*H*$_2$N), 4.28 (t, ^3J = 5.0 Hz, 2H, OC*H*$_2$CH$_2$N), 5.62 (d, ^3J = 12.1 Hz, 1H, ArC*H*), 5.66 (d, ^3J = 12.1 Hz, 1H, ArC*H*), 6.53 (AA′*BB*′, ^3J = 8.5 Hz, 2H, Ar′*H*-3, Ar′*H*-5), 6.78 (AA′*BB*′, ^3J = 8.5 Hz, 2H, Ar′*H*-2, Ar′*H*-6), 6.80 (*AA*′BB′, ^3J = 8.6 Hz, 2H, Ar*H*-3, Ar*H*-5), 6.96 (*AA*′BB′, ^3J = 8.6 Hz, 2H, Ar*H*-2, Ar*H*-6), 8.95 (s, 1H, N=C*H*-N), 9.47 (s, 1H, ArO*H*, exchangeable by D$_2$O), 10.39 (br, 1H, N*H*, exchangeable by D$_2$O), 10.81 (s, 1H, N*H*, exchangeable by D$_2$O), 10.85 (s, 1H, N*H*, exchangeable by D$_2$O). Anal. (C$_{22}$H$_{27}$N$_3$O$_2$·2HCl·3H$_2$O) C, H, N.

(4*R*,5*S*)/(4*S*,5*R*)-2-Ethyl-4-(4-hydroxyphenyl)-5-(4-(2-piperidin-1-ylethoxy)phenyl)-2-imidazoline (2a) was prepared from (4*R*,5*S*)/(4*S*,5*R*)-2-ethyl-4,5-bis(4-hydroxyphenyl)-2-imidazoline hydrobromide **2** (0.20 mmol, 71.9 mg) and 1-(2-chloroethyl)piperidine hydrochloride (0.20 mmol, 36.8 mg). Purification: column chromatography with chloroform/methanol 9/1. Yield: 0.04 mmol (19.9 mg), 19%, yellow oil. IR (KBr, cm^{-1}): $\bar{\nu}$ = 3600−2500 br, s (OH), 1623 w, 1612 w, 1515 w, 1246 w. MS (EI, 35 °C): *m/z* (%) = 393 (13) [$M^{+\bullet}$], 161 (31), 112 (57), 98 (100). ^{1}H NMR ([D$_6$]-DMSO): δ = 1.29−1.40 (m, 4H, CH$_2$C*H*$_3$, NCH$_2$CH$_2$C*H*$_2$CH$_2$CH$_2$N), 1.60−1.84 (m, 5H, NCH$_2$C*H*$_2$C*H*$_2$C*H*$_2$CH$_2$N), 2.77 (q, ^3J = 7.6 Hz, 2H, C*H*$_2$CH$_3$), 2.86−3.00 (m, 2H, NC*H*$_2$CH$_2$CH$_2$CH$_2$C*H*$_2$N), 3.42−3.51 (m, 4H, OCH$_2$C*H*$_2$N, NC*H*$_2$CH$_2$CH$_2$CH$_2$C*H*$_2$N), 4.27 (t, ^3J = 5.1 Hz, 2H, OC*H*$_2$CH$_2$N), 5.60 (d, ^3J = 11.7 Hz, 1H, ArC*H*), 5.64 (d, ^3J = 11.7 Hz, 1H, ArC*H*), 6.53 (AA′*BB*′, ^3J = 8.5 Hz, 2H, Ar′*H*-3, Ar′*H*-5), 6.70−6.87 (*m*, 4H, Ar*H*-3, Ar*H*-5, Ar′*H*-2, Ar′*H*-6,), 6.97 (*AA*′BB′, ^3J = 8.6 Hz, 2H, Ar*H*-2, Ar*H*-6), 9.43 (s, 1H, ArO*H*, exchangeable by D$_2$O), 10.12 (br, 1H, N*H*, exchangeable by D$_2$O), 10.56 (s, 1H, N*H*, exchangeable by D$_2$O), 10.59 (s, 1H, N*H*, exchangeable by D$_2$O). Anal. (C$_{24}$H$_{31}$N$_3$O$_2$·2HCl·3H$_2$O) C, H, N.

(4*R*,5*S*)/(4*S*,5*R*)-4-(2-Chloro-4-(2-dimethylaminoethoxy)-phenyl)-5-(2-chloro-4-hydroxyphenyl)-2-imidazoline (3a) was prepared from (4*R*,5*S*)/(4*S*,5*R*)-4,5-bis(2-chloro-4-hydroxyphenyl)-2-imidazoline hydrobromide **3** (1.00 mmol, 404.1 mg) and 2-chloroethyldimethylamine hydrochloride (1.00 mmol, 144.1 mg). Purification: column chromatography with chloroform/methanol 14/1. Yield: 0.29 mmol (140.7 mg), 29%, yellow powder. Mp: 271 °C. IR (KBr, cm^{-1}): $\bar{\nu}$ = 3600−2500 br, s (OH), 1608 s, 1573 m, 1541 m, 1501 s, 1465 m, 1438 m, 1400 m, 1289 m, 1255 m, 1237 m, 1044 m. MS (EI, 150 °C): *m/z* (%) = 393 (15) [$M^{+\bullet}$], 156 (17), 72 (58), 58 (100). ^{1}H NMR ([D$_6$]-DMSO): δ = 2.79 (s, 6H, NC*H*$_3$), 3.38−3.47 (br, 2H, OCH$_2$C*H*$_2$N), 4.25−4.34 (m, 2H, OC*H*$_2$CH$_2$N), 5.99 (s, 2H, ArC*H*), 6.59 (dd, ^3J = 8.6 Hz, ^4J = 2.4 Hz, 1H, Ar′*H*-5), 6.69 (d, ^4J = 2.4 Hz, 1H, Ar′*H*-3), 6.85 (dd, ^3J = 8.8 Hz, ^4J = 2.5 Hz, 1H, Ar*H*-5), 6.97 (d, ^4J = 2.5 Hz, 1H, Ar*H*-3), 7.00 (d, ^3J = 8.6 Hz, 1H, Ar′*H*-6), 7.16 (d, ^3J = 8.8 Hz, 1H, Ar*H*-6), 9.00 (s, 1H, N=C*H*-N), 10.14 (s, 1H, ArO*H*, exchangeable by D$_2$O), 10.51 (br, 1H, N*H*, exchangeable by D$_2$O), 10.94 (br, 1H, N*H*, exchangeable by D$_2$O), 11.00 (br, 1H, N*H*, exchangeable by D$_2$O). Anal. (C$_{19}$H$_{21}$Cl$_2$N$_3$O$_2$·2HCl·H$_2$O) C, H, N.

(4*R*,5*S*)/(4*S*,5*R*)-4-(2-Chloro-4-hydroxyphenyl)-5-(2-chloro-4-(2-pyrrolidin-1-ylethoxy)phenyl)-2-imidazoline (3b) was prepared from (4*R*,5*S*)/(4*S*,5*R*)-4,5-bis(2-chloro-4-hydroxyphenyl)-2-imidazoline hydrobromide **3** (1.00 mmol, 404.1 mg) and 1-(2-chloroethyl)pyrrolidine hydrochloride (1.00 mmol, 170.1 mg). Purification: column chromatography with chloroform/methanol 14/1. Yield: 0.24 mmol (129.9 mg), 29%, yellow oil. IR (KBr, cm^{-1}): $\bar{\nu}$ = 3600−2500 s, br (OH), 1608 s, 1499 s, 1292 m, 1241 m, 1045 m. MS (EI, 170 °C): *m/z* (%) = 419 (2) [$M^{+\bullet}$], 128 (18), 98 (17), 84 (100). ^{1}H NMR ([D$_6$]-DMSO): δ = 1.76−1.92 (m, 2H, NCH$_2$C*H*$_2$C*H*$_2$CH$_2$N), 1.93−2.08 (m, 2H, NCH$_2$C*H*$_2$C*H*$_2$CH$_2$N), 2.94−3.14 (m, 2H, NC*H*$_2$CH$_2$CH$_2$C*H*$_2$N), 3.42−3.63 (m, 4H, NC*H*$_2$CH$_2$CH$_2$C*H*$_2$N, OCH$_2$-C*H*$_2$N), 4.29−4.35 (m, 2H, OC*H*$_2$CH$_2$N), 5.99 (s, 2H, ArC*H*), 6.59 (dd, ^3J = 8.6 Hz, ^4J = 2.3 Hz, 1H, Ar′*H*-5), 6.69 (d, ^4J = 2.3 Hz, 1H, Ar′*H*-3), 6.86 (dd, ^3J = 8.7 Hz, ^4J = 2.3 Hz, 1H, Ar*H*-5), 6.97 (d, ^4J = 2.3 Hz, 1H, Ar*H*-3), 7.00 (d, ^3J = 8.6 Hz, 1H, Ar′*H*-6), 7.16 (d, ^3J = 8.7 Hz, 1H, Ar*H*-6), 9.00 (s, 1H, N=C*H*-N), 10.15 (s, 1H, ArO*H*, exchangeable by D$_2$O), 10.86 (br, 1H, N*H*, exchangeable by D$_2$O), 10.95 (br, 1H, N*H*, exchangeable by D$_2$O), 11.00 (br, 1H, N*H*, exchangeable by D$_2$O). Anal. (C$_{21}$H$_{23}$Cl$_2$N$_3$O$_2$·2HCl·2H$_2$O) C, H, N.

(4*R*,5*S*)/(4*S*,5*R*)-4-(2-Chloro-4-(4-hydroxyphenyl)-5-(2-chloro-(2-piperidin-1-ylethoxy)phenyl))-2-imidazoline (3c) was prepared from (4*R*,5*S*)/(4*S*,5*R*)-4,5-bis(2-chloro-4-hydroxyphenyl)-2-imidazoline hydrobromide **3** (1.00 mmol, 404.1 mg) and 1-(2-chloroethyl)piperidine hydrochloride (1.00 mmol, 184.1 mg). Purification: column chromatography with chloroform/methanol 9/1. Yield: 0.27 mmol (149.3 mg), 27%, yellow powder. Mp: 83 °C. IR (KBr, cm^{-1}): $\bar{\nu}$ = 3600−2500 s, br (OH), 1609 m, 1499 m, 1292 m, 1245 m. MS (EI, 140 °C): *m/z* (%) = 365 (11) [$M^{+\bullet}$], 112 (19), 98 (100). ^{1}H NMR ([D$_6$]-DMSO): δ = 1.28−1.45 (m, 1H, NCH$_2$CH$_2$C*H*$_2$CH$_2$CH$_2$N), 1.57−1.72 (m, 1H, NCH$_2$CH$_2$C*H*$_2$CH$_2$CH$_2$N), 1.72−1.85 (m, 4H, NCH$_2$C*H*$_2$CH$_2$C*H*$_2$-CH$_2$N), 2.82−3.01 (m, 2H, NC*H*$_2$CH$_2$CH$_2$CH$_2$C*H*$_2$N), 3.38−3.52 (m, 4H, NC*H*$_2$CH$_2$CH$_2$CH$_2$C*H*$_2$N, OCH$_2$C*H*$_2$N), 4.26−4.40 (m, 2H, OC*H*$_2$CH$_2$N), 5.99 (s, 2H, ArC*H*), 6.59 (dd, ^3J = 8.6 Hz, ^4J = 2.4 Hz, 1H, Ar′*H*-5), 6.68 (d, ^4J = 2.4 Hz, 1H, Ar′*H*-3), 6.85 (dd, ^3J = 8.7 Hz, ^4J = 2.5 Hz, 1H, Ar*H*-5), 6.97 (d, ^4J = 2.5 Hz, 1H, Ar*H*-3), 7.01 (d, ^3J = 8.6 Hz, 1H, Ar′*H*-6), 7.16 (d, ^3J = 8.7 Hz, 1H, Ar*H*-6), 9.00 (s, 1H, N=C*H*-N), 10.12 (s, 1H, ArO*H*, exchangeable by D$_2$O), 10.31 (br, 1H, N*H*, exchangeable by D$_2$O), 10.92 (br, 1H, N*H*, exchangeable by D$_2$O), 10.97 (br, 1H, N*H*, exchangeable by D$_2$O). Anal. (C$_{22}$H$_{25}$Cl$_2$N$_3$O$_2$·2HCl·3H$_2$O) C, H, N.

(4*R*,5*S*)/(4*S*,5*R*)-4-(2-Chloro-4-hydroxyphenyl)-5-(2-chloro-4-(2-piperidin-1-ylethoxy)phenyl)-2-ethyl-2-imidazoline (4a) was prepared from (4*R*,5*S*)/(4*S*,5*R*)-4,5-bis(2-chloro-4-hydroxyphenyl)-2-ethyl-2-imidazoline hydrobromide **4** (0.20 mmol, 86.4 mg) and 1-(2-chloroethyl)piperidine hydrochloride (0.20 mmol, 36.8 mg). Purification: column chromatography with chloroform/methanol 29/1. Yield: 0.04 mmol (23.7 mg), 21%, yellow powder. Mp: 103 °C. IR (KBr, cm^{-1}): $\bar{\nu}$ = 3600−2500 br, s (OH), 1628 m, 1611 m, 1515 w, 1500 w, 1456 w, 1241 m, 1108 w, 1066 w. MS (EI, 50 °C): *m/z* (%) = 461 (3) [$M^{+\bullet}$], 195 (15), 112 (23), 98 (100). ^{1}H NMR ([D$_6$]-DMSO): δ = 1.19−1.43 (m, 4H, CH$_2$C*H*$_3$, NCH$_2$CH$_2$C*H*$_2$CH$_2$CH$_2$N), 1.60−1.88 (m, 5H, NCH$_2$C*H*$_2$C*H*$_2$C*H*$_2$CH$_2$N), 2.77 (q, ^3J = 7.6 Hz, 2H, C*H*$_2$CH$_3$), 2.84−3.02 (m, 2H, NC*H*$_2$CH$_2$CH$_2$CH$_2$C*H*$_2$N), 3.37−3.53 (m, 4H, OCH$_2$C*H*$_2$N, NC*H*$_2$CH$_2$CH$_2$CH$_2$C*H*$_2$N), 4.26−4.41 (m, 2H, OC*H*$_2$CH$_2$N), 5.96 (s, 2H, ArC*H*), 6.60 (dd, ^3J = 8.6 Hz, ^4J = 2.4 Hz, 1H, Ar′*H*-5), 6.67 (d, ^4J = 2.4 Hz, 1H, Ar′*H*-3), 6.84 (dd, ^3J = 8.8 Hz, ^4J = 2.5 Hz, 1H, Ar*H*-5), 6.97 (d, ^4J = 2.5 Hz, 1H, Ar*H*-3), 7.03 (d, ^3J = 8.6 Hz, 1H, Ar′*H*-6), 7.18 (d, ^3J = 8.8 Hz, 1H, Ar*H*-6), 10.10 (s, 1H, ArO*H*, exchangeable by D$_2$O), 10.21 (br, 1H, N*H*, exchangeable by D$_2$O), 10.71 (s, 1H, N*H*, exchangeable by D$_2$O), 10.76 (s, 1H, N*H*, exchangeable by D$_2$O). Anal. (C$_{24}$H$_{29}$Cl$_2$N$_3$O$_2$·2HCl·2H$_2$O) C, H, N.

(4*R*,5*S*)/(4*S*,5*R*)-4-(2-Chloro-4-(2-piperidin-1-ylethoxy)-phenyl)-5-(2,6-dichloro-4-hydroxyphenyl)-2-imidazoline (5a) was prepared from (4*R*,5*S*)/(4*S*,5*R*)-4-(2-chloro-4-hydroxyphenyl)-5-(2,6-dichloro-4-hydroxyphenyl)-2-imidazoline hydrobromide **5** (0.25 mmol, 109.6 mg) and 1-(2-chloroethyl)-piperidine hydrochloride (0.25 mmol, 46.0 mg). Purification: column chromatography with chloroform/methanol 19/1. Yield: 0.05 mmol (25.4 mg), 19%, yellow powder. Mp: 83 °C. IR (KBr, cm^{-1}): $\bar{\nu}$ = 3600−3300 m, br (OH), 2925 s, 2855 m, 1602 m, 1459 w, 1267 w. MS (EI, 65 °C): *m/z* (%) = 467 (1) [$M^{+\bullet}$], 112 (18), 98 (100). ^{1}H NMR ([D$_6$]-DMSO): δ = 1.31−1.50 (m, 1H, NCH$_2$CH$_2$C*H*$_2$CH$_2$CH$_2$N), 1.54−1.88 (m, 5H, NCH$_2$C*H*$_2$C*H*$_2$C*H*$_2$CH$_2$N), 2.84−3.07 (m, 2H, NC*H*$_2$CH$_2$CH$_2$-CH$_2$C*H*$_2$N), 3.38−3.46 (m, 4H, NC*H*$_2$CH$_2$CH$_2$CH$_2$C*H*$_2$N, OCH$_2$-C*H*$_2$N), 4.22−4.41 (m, 2H, OC*H*$_2$CH$_2$N), 5.93 (d, ^3J = 13.4 Hz, 1H, ArC*H*), 6.41 (d, ^3J = 13.4 Hz, 1H, ArC*H*), 6.63 (d, ^4J = 2.5 Hz, 1H, Ar′*H*-5), 6.86 (d, ^4J = 2.5 Hz, 1H, Ar′*H*-3), 6.94−6.99

(m, 2H, Ar*H*-3, Ar*H*-5), 7.53 (d, ^3J = 8.6 Hz, 1H, Ar*H*-6), 8.95 (s, 1H, N=C*H*-N), 10.05 (br, 1H, N*H*, exchangeable by D_2O), 10.74 (s, 1H, ArO*H*, exchangeable by D_2O), 10.91 (br, 1H, N*H*, exchangeable by D_2O), 11.26 (br, 1H, N*H*, exchangeable by D_2O). Anal. ($C_{22}H_{24}Cl_3N_3O_2$·2HCl·H_2O) C, H, N.

Biological Methods. Biochemicals, Chemicals, and Materials. L-Glutamine (29.2 mg/mL of PBS) was from Sigma (Munich, Germany); Dulbecco's Modified Eagle Medium without phenol red (DMEM) was from Gibco (Eggenstein, Germany); fetal calf serum (FCS) was from Bio Whittaker (Verviers, Belgium); 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 (35.71 mg/mL of PBS) were from ICN Biomedicals GmbH (Eschwege, Germany); cell culture lysis reagent (5×) (diluted 1:5 with purified water before use) and luciferase assay reagent were from Promega (Heidelberg, Germany); phosphate buffered saline (PBS) was prepared by solving 8.0 g of NaCl, 0.2 g of KCl, 1.44 g of Na_2HPO_4·$2H_2O$, and 0.2 g of KH_2PO_4 (all purchased from Merck or Fluka) in 1000 mL of purified water; dextran and 17β-estradiol were from Sigma (Taufkirchen, Germany), 100 mg glucose forms were from Sigma (Munich, Germany) or PAN (Aidenbach, Germany); fetal bovine serum (FBS) was from Biochrom (Berlin, Germany); *N*-hexamethylparasoaniline (crystal violet) was from Fluka (Deishofen, Germany); glutardialdehyde (25%) (solution for fixation, 1% v/v glutardialdehyde in PBS) and dimethyl sulfoxide (DMSO) were from VW/Merck (Darmstadt, Germany); Norit A (charcoal) was from Serva (Heidelberg, Germany); crystal 96-well plates were from Nunc (Roshilde, Denmark); white 96-well plates were from Packard (Groning, The Netherlands); T175 flasks were from Sarstedt (Nümbrecht, Germany); Fugene6 was from Roche; T-75 flasks, reaction tubes, and six-well plates were purchased from Renner GmbH (Dannstadt, Germany). Deionized water was produced by means of a Millipore Milli-Q Water System (resistivity > 18 MΩ).

Transcriptional Binding Assays. Luciferase Assay with ERα-Positive MCF-7-2a Cells Stably Transfected with the Reporter Plasmid ERE_{wtc}luc. The pertinent in vitro assay was described earlier by Hafner et al.[4] 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-FCS, 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 of cell suspension and 2 mL of medium per well) at growing conditions (see above). After 24 h, 25 μL of a stock solution of the test compounds was added to achieve concentrations ranging from 10^{-5} to 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 was added into each well. After 20 min of lysis at room temperature, cells were transferred into reaction tubes and centrifuged. Luciferase activity was assayed using the Promega luciferase assay reagent. Then, 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[27]) of each sample with the mass of luciferase. Estrogenic activity was expressed as % activation of a 10^{-8} M estradiol control (100%).

To evaluate the antagonistic activity, the cells were incubated with the test compounds in concentrations from 10^{-6} to 10^{-11} M along with a constant amount of estradiol (10^{-9} M). The concentration of the compound that is necessary to reduce the effect of estradiol by 50% is the IC_{50}.

Luciferase Assay with U-2 OS Cells Transiently Transfected with Plasmids Encoding for ERα (pSG5-ERα)) or ERβ (pSG5-ERβ FL) and the Reporter Plasmid $(ERE)_2luc^+$. The U-2 OS cell line, the plasmids encoding for ERα (pSG5-ERα) and ERβ (pSG5-ERβ FL), and the reporter plasmid ($(ERE)_2luc^+$) were kindly provided by Dr. Fritzemeier, Schering AG Berlin (Germany). U-2 OS cells were maintained as a monolayer culture at 37 °C in a humidified atmosphere (7.5% CO_2) in T-75 flasks in DMEM without phenol red supplemented with L-glutamine (8 mM) and 5% (v/v) FCS. Cell line banking and quality control were performed according to the seed stock concept reviewed by Hay.[28] Cells were passaged twice a week seeding the cells at a density of 4×10^5 cells (for 4 days of cultivation) or 4.5×10^5 cells (for 5 days of cultivation) per 75 cm^2 flasks in 15 mL culture media.

The passage of U-2 OS cells before starting the experiment was cultivated in DMEM with glutamine and 5% (v/v) charcoal-treated FCS (ct-DMEM) in 175 cm^2 flasks at a density of 7.8×10^5 cells in 30 mL ct-DMEM. Six hours prior to transfection, cells from an almost confluent monolayer were split and seeded in 10 cm dishes at a density of 1×10^6 cells in 10 mL ct-DMEM. Cells were transiently transfected using Fugene6 as transfection reagent. Typically, 5 μg of reporter plasmid ($(ERE)_2luc^+$) and 0.05 μg of receptor plasmid (pSG5-ERα or pSG5-ERβ FL) were added to the cells. After 18 h, cells were washed once with PBS, harvested, and resuspended in ct-DMEM to approximately 0.5×10^5 cell/mL. All dishes transfected with the same ER-subtype plasmid were pooled to reduce fluctuation of different transfection efficiency. Then, 100 μL of the cell suspension was seeded in the 60 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 in order to avoid boundary problems.

The media was replaced 3 h after seeding by 180 μL of ct-DMEM and 20 μL of medium containing either E2 or test compounds in appropriate amounts to achieve final concentrations ranging from 10^{-8} to 10^{-13} M (E2) or 10^{-5} to 10^{-10} M (test compounds). The concentration of the solvent (DMSO) used to prepare stock solutions amounts to 0.1% (v/v) in the test and serves as a negative control.

For determination of the antiestrogenic activity, the cells were treated as mentioned above. The cells were incubated with the test compounds in concentrations from 10^{-5} to 10^{-13} M along with a constant concentration of E2 (for ERα, 3.0×10^{-11}M; for ERβ, 3.0×10^{-10}M). After 21 h of incubation under growth conditions, the media was removed and 50 μL of passive lysis buffer was added into each well and incubated at room temperature under vigorous shaking (600 rpm, TiMix, Edmund Bühler, Germany).

Luciferase was assayed using the Promega luciferase assay reagent. Substrate reagent (50 μL) was added into each well and luminescence (in relative light units, RLU) was measured for 10 s using a Victor^2 1420 multilable counter (Wallac/Perkin-Elmer). Measurements were corrected correlating the RLU with the cell mass of each sample.

The cell mass was determined in a crystal violet assay[29,30] in crystal flat-bottomed 96-well plates analogously to the transactivation assay. After incubation for 21 h, the medium was removed and glutaric dialdehyde (1% v/v in PBS, 100 μL/well) was added for fixation. After 15 min the solution of the aldehyde was replaced by 180 mL/well PBS. The plates were stored at 4 °C until staining. Cells were stained by treating them for 30 min with 100 μL/well of an aqueous solution of crystal violet (0.02% (m/v)). After decanting, cells were washed twice with water to remove adherent dye. After addition of 180 μL/well of ethanol (70 % (v/v)), plates were gently shaken for 4 h. The optical density of each well was measured in a microplate autoreader at 590 nm (Flash scan, Analytikjena, Germany).

Transactivation activity and quantification of cells mass were both performed in triplicate. The estrogenic activity was expressed as percent activation of the average in the plateau of E2 (100%). The IC_{50} value is the concentration of the compound that is necessary to reduce the effect of E2 by 50% and expresses the antiestrogenic activity.

Acknowledgment. The technical assistance of S. Bergemann and I. Schnautz is acknowledged. The study

presented was supported by Grants Gu285/3-1 and Gu285/3-2 from the Deutsche Forschungsgemeinschaft.

Supporting Information Available: Elemental analyses of side-chain-bearing 2-imidazolines. This material is available free of charge via the Internet at http://pubs.acs.org.

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JM040855C