Investigations of New Lead Structures for the Design of Selective Estrogen Receptor Modulators

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Heterocyclic derivatives of (*R*,*S*)/(*S*,*R*)-1-(2-chloro-4-hydroxyphenyl)-2-(2,6-dichloro-4-hydroxyphenyl)ethylenediamine (**L1)** were synthesized and tested for estrogen receptor binding. The selection of the heterocycles was based on theoretical consideration. (2*R*,3*S*)/(2*S*,3*R*)-2-(2-Chloro-4-hydroxyphenyl)-3-(2,6-dichloro-4-hydroxyphenyl)piperazine **2**, (4*R*,5*S*)/(4*S*,5*R*)-4-(2-chloro-4-hydroxyphenyl)-5-(2,6-dichloro-4-hydroxyphenyl)-2-imidazoline **3**, and 4-(2-chloro-4-hydroxyphenyl)-5-(2,6-dichloro-4-hydroxyphenyl)imidazole **4** possess a spatial structure with neighboring aromatic rings as is realized in hormonally active [1,2-diphenylethylenediamine]-platinum(II) complexes. The 1,2-diphenylethane pharmacophor, however, cannot adapt an antiperiplanar conformation to interact with the estrogen receptor (ER) comparable to synthetic (e.g., diethylstilbestrol (**DES**)) or steroidal (e.g., estradiol (**E2**)) estrogens. Due to the different spatial structures, the heterocycles cause only a marginal displacement of **E2** from its binding site (relative binding affinity (RBA) < 0.1%). Nevertheless, unequivocally ER mediated gene activation was verified on the MCF-7-2a cell line. Imidazoline **3** as the most active compound reached the maximum effect of **E2** (100% activation) in a concentration of 5×10^{-7} M, while piperazine **2** and imidazole **4** activate luciferase expression only in a small but significant amount of 20% and 27%, respectively. We therefore assigned these heterocyclic compounds to a second class of hormones (type-II-estrogens), which are attached at the ER at different amino acids than **DES** or **E2** (type-I-estrogens).

Introduction

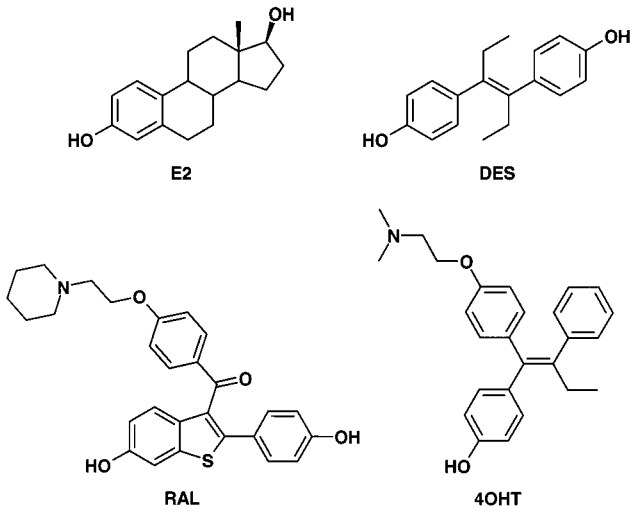
Estrogens are endocrine regulators of the male and female reproductive systems such as the mammary gland, uterus, ovary, testis, and prostate. They also play an important role in so-called nontarget tissue, e.g., bone, liver, or in the cardiovascular system, where estrogens have certain protective effects.[1−3]

All effects are mediated by at least two estrogen receptors (ERα and ERβ), which have different patterns of tissue expression.[4,5] ERα and Erβ are transcription factors located in cell nuclei and are activated by the binding of hormones.[6,7] While the binding site of hormones was only speculative during the past decades, X-ray analyses of ERα and ERβ/drug conjugates are now available.[8−10]

From the crystallographic data, it can be deduced that the binding mode of the drugs in the ligand binding domain (LBD) of ERα and ERβ is nearly the same, although the LBDs of the receptors exhibit a homology of only 47%.

The crystal structures show that **E2** and **DES** (formula, see Chart 1) are fixed in the LBD by H-bonds to the γ-carboxylate of Glu353 (305) and to the guandinium group of Arg394 (346) (the numbers in parentheses represent the number of amino acids in the LBD of ERβ). For agonistic effects, the second hydroxy group of **E2** or **DES** has to form a hydrogen bond with the imidazole ring of His524 (475). The hormone binding leads to a change of the three-dimensional structure of the ERs, a dimerization of ER/drug conjugates, and the translocation of the dimers into the nuclei of the cells. This mode of action was used to achieve a selective accumulation of cisplatin derivatives in the nuclei of hormone sensitive tumor cells.

Chart 1



For this purpose, 1,2-diarylethylenediamine carrier ligands were derived from the nonsteroidal estrogen **DES**, which, depending on the configuration and the substitution pattern of the aromatic rings, possess hormonal effects.[11] One of the most active compounds is (*R*,*S*)/(*S*,*R*)-1-(2-chloro-4-hydroxyphenyl)-2-(2,6-dichloro-4-hydroxyphenyl)ethylenediamine **L1** (see Scheme 1), which shows the profile of an impeded estrogen. In solution, **L1** covers, by rotation around the ethane axis,

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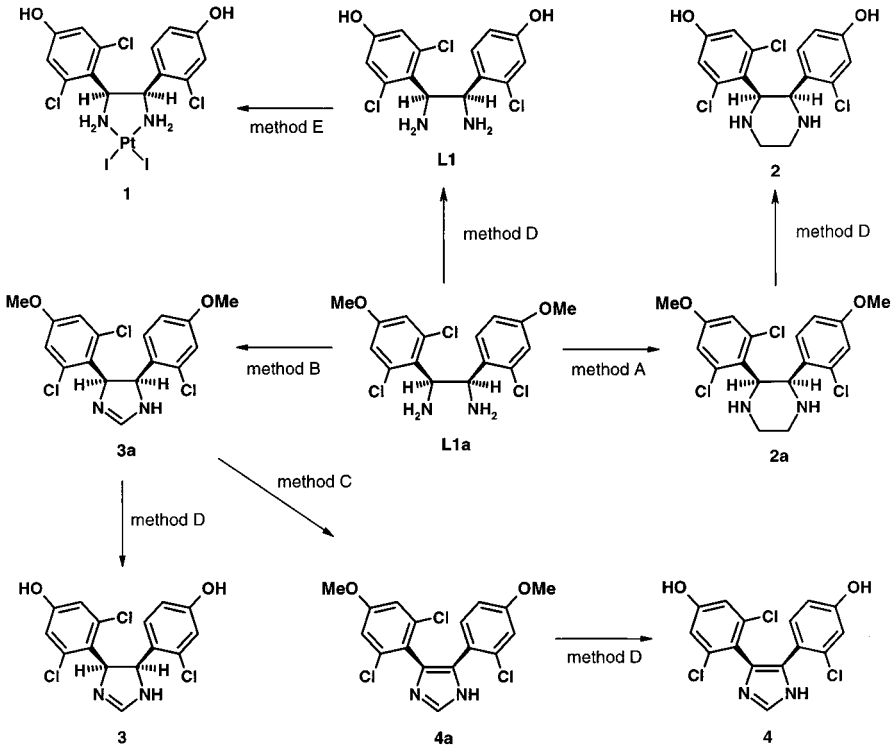
Scheme 1



three low-energy conformations, with synclinally and antiperiplanarly standing aromatic rings. In the latter, the 1,2-diphenylethane moiety, which is the essential pharmacophor, shows great similarity to the *E*-stilbene structure in **DES**. A comparable attachment at the receptor can therefore be assumed.

The hormonal activity changed to that of a true estrogen by coordination to platinum. In the [(*R*,*S*)/(*S*,*R*)-1-(2-chloro-4-hydroxyphenyl)-2-(2,6-dichloro-4-hydroxyphenyl)ethylenediamine]diiodoplatinum(II) complex **1**, the aromatic rings are forced in a synclinal position at the five-membered chelate ring.[12] The spatial structure of the pharmacophor is therefore different from those of **L1** or **E2** and requires other interactions with the ER to achieve gene activation. To evaluate the responsible structure for these effects, heterocyclic compounds related to complex **1** were synthesized and tested for receptor affinity.

Results and Discussion

The selection of the heterocycles with a 1,2-diphenylethylenediamine partial structure used in this study was based on theoretical considerations. The spatial structure determined by Tripos Sybyl 6.6 (Tripos Force Field, Gasteiger−Hückel charges) should be similar to complex **1**. 2,3-Diphenylpiperazine **2**, 4,5-diphenyl-2-imidazoline **3**, and 4,5-diphenylimidazole **4** were selected from the possible derivatives (see Scheme 1). 2-Imidazoline **3** possesses a planar N−C═N unit comparable to the N−Pt−N-region in **1**, and the aromatic rings are arranged in vicinity. In piperazine **2**, the 1,2-diphenylethane pharmacophor adapts a conformation with a stable orientation of the aromatic rings in equatorial/axial position as realized in complex **1**. The structure of imidazole **4** is quite planar and also corresponds to the *cis*-stilbene structure in **4OHT** (formula, see Chart 1).

Table 1. Structural Characteristics of the Investigated Compounds

	complex **1**	piperazine **2**	imidazoline **3**	imidazole **4**
arrangement of the aromatic rings	axially/equatorially	axially/equatorially	pseudo-axially	in plane of the heterocycle
torsion angle between the aromatic rings	58.4°	47.9°	2.8°	5.7°
O−O distance of the hydroxy groups	7.8 Å	7.1 Å	5.1 Å	8.9 Å

As synthon for the syntheses of the heterocyclic compounds (Scheme 1), the (*R*,*S*)/(*S*,*R*)-configurated 1,2-diarylethylenediamine **L1a** is available.[11] To obtain piperazine **2a**, **L1a** has to react with oxalic acid diethylester and subsequently with BH_3·THF (method A). The synthesis of 2-imidazoline **3a** was achieved by heating a solution of **L1a** with a catalytical amount of HCl_{conc} in triethylorthoformate (method B). Oxidation of **3a** with activated MnO_2 yielded the imidazole **4a** (method C). The ether cleavage to obtain the compounds **L1**, **2**, **3**, and **4** was performed by using BBr_3 (method D).

By combining spectroscopical and molecular modeling methods, the structures of the new drugs were determined. The coupling constants show an arrangement of the benzylic protons at an angle of 0−20° (J = 12 Hz) in 2-imidazoline **3** and 35−55° (J = 4.9 Hz) in piperazine **2**. These structures were refined by energy minimization using Tripos Sybyl 6.6. In the low-energy conformation, **2** exists in a twist conformation, which is 4.7 kJ/mol lower in energy than the chair conformation. The 2,6-dichloro-4-hydroxyphenyl residue is equatorially arranged comparable to complex **1** (Table 1). In imidazoline **3**, the planarity of the N−C═N bond defines the position of the aromatic rings. At the energy minimum, the aromatic rings are pseudoaxially oriented

Table 2. Estrogen Receptor Binding and Luciferase Expression in MCF-7-2a Cells

compd	relative binding affinity (% RBA)	luciferase expression % activation at 10^{-6} M
L1a	0.00	0.00
L1	1.50	98
1	0.43	81
2a	0.00	3
2	<0.02	20
3a	0.00	6
3	0.08	112
4a	0.00	6
4	0.10	27

with a torsion angle of 2.8° (Table 1). The spatial structure of the 1,2-diphenylethane corresponds to an orientation during the interconversion of the five-membered platinum chelate ring of **1**. In imidazole **4**, the phenyl rings are fixed in a *Z*-stilbene partial structure with a torsion angle of 5.7°.

The relative binding affinity to the ER (RBA) was determined for all compounds in a competition experiment using calf uteri cytosol. In this test, which does not distinguish ERα and ER*β* binding, the amount of radiolabeled **E2** displaced from the receptor is quantified. The RBA value is therefore an indication for the tendency of the drug to interact at the same place (site) as **E2**.

The MCF-7-2a cell line was used to quantify the ligand-induced gene activation. These estrogen receptor containing mammary carcinoma cells were stably transfected with the plasmid ERE_{wtc}luc. The plasmid contains the "estrogen response element" (ERE) of the DNA as the enhancer sequence and a reporter sequence, which codes for luciferase. The binding of ER/drug conjugate dimers leads to the expression of luciferase, which correlates very well with the estrogenic potency of the drug.[13]

All new heterocyles show much lower binding affinities (Table 2) than that of **E2** (RBA **1**: 0.43%; **2**: 0.08%; **3**: 0.02%; **4**: 0.10%, **E2**: 100%). None of the compounds can displace **E2** from its binding site more than 0.1%. Nevertheless, on the MCF-7-2a cell line unequivocally ER mediated gene activation was verified (Table 2). (*R*,*S*)/(*S*,*R*)-1-(2-Chloro-4-hydroxyphenyl)-2-(2,6-dichloro-4-hydroxyphenyl)ethylenediamine **L1** at a concentration of 10^{-6} M led to an activation of the luciferase expression of 98%. This correlates very well with the RBA value of 1.50% and the hormonal profile of an impeded estrogen. Coordination to platinum decreases the RBA to 0.43%, while the gene expression in MCF-7-2a was nearly unchanged. Among the new heterocycles, imidazoline **3** was the most active compound and was even more active than **1** (Figure 1). Compound **3** reached the maximum effect of **E2** at a concentration of 5×10^{-7} M, whereas piperazine **2** and imidazole **4** activate luciferase expression to 20% and 27%, respectively.

These results indicate that the spatial structure of the 1,2-diphenylethane pharmacophor determines the activity of the drugs. There are two preferred conformations for the interaction with the ER to induce gene activation. An antiperiplanar arrangement of the 1,2-diphenylethane comparable to **DES** or Hexestrol makes an attachment at the ER analogously to **E2** possible (Figure 2). Due to the anchorage at the same amino acids at the ER, a displacement of [^{3}H]**E2** from its binding site is possible (high RBA values). These estrogens were therefore classified as type-I-estrogens.

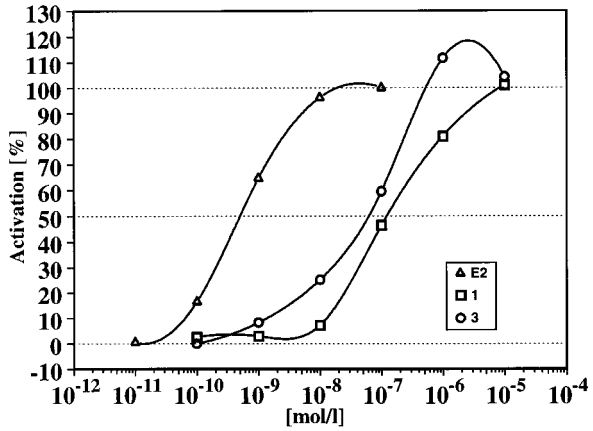


Figure 1. Activation (%) of luciferase expression in MCF-7-2a cells by estradiol (**E2**) complex **1** and 2-imidazoline **3**.

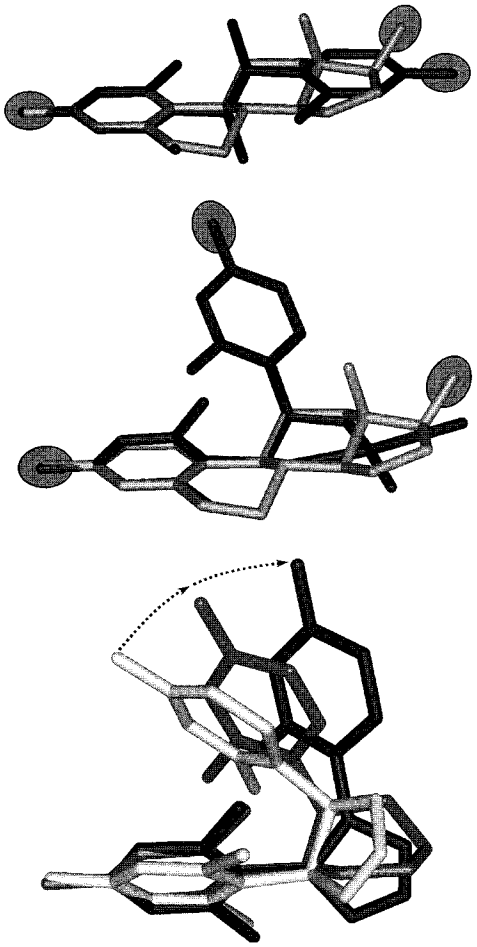


Figure 2. Superposition of **L1** and **E2** (top); **1** and **E2** (middle); and piperazine **2**, 2-imidazolin **3**, and imidazole **4** (bottom).

In complex **1** and the heterocyles **2**−**4**, the 1,2-diphenylethane is fixed in synclinal conformation and an **E2**-like interaction with the ER is impossible (for a superposition of **1** and **E2**, see Figure 2). Since these compounds nevertheless activate luciferase expression, which requires ER binding, their pharmacophor has to occupy other anchors at the ER. We therefore assign these estrogens to a second class of hormones (type-II-estrogens).

The essential part of these type-II-estrogens are synclinally standing aromatic rings with free phenolic hydroxy groups. The respective *O*-methyl derivatives are completely inactive. Dependending on the heterocyclic ring, the O−O distance increases in the series (Figure 2) imidazoline **3** (5.1 Å) < piperazine **2** (7.1 Å) < complex **1** (7.8 Å) < imidazole **4** (8.9 Å).

In imidazole **4**, the O−O distance is fixed due to the *Z*-stilbene partial structure. In piperazine **2**, the stable chair conformation of the six-membered piperazine defines the position of the hydroxy groups. A change of the O−O distance during the ER binding can be excluded. In complex **1**, however, the five-membered chelate ring can undergo an interconversion between a λ- and a δ-conformation. In this case, the O−O distance is reduced from 7.8 Å to about 5.7 Å in the transition state. The same arrangement of the phenyl rings (O−O distance = 5.1 Å) is present in imidazoline **3** and seems to be essential to achieve signal transduction of the ER.

These three-dimensional structures are inconvenient for estrogenic active compounds, strongly differ from type-I-estrogens, and require a binding to the ER at other amino acids. The binding site and the binding mode of the type-I-estrogens **E2**[8,9] and **DES**[9] at the ER as well as of the antagonists **RAL**[8] and **4OHT**[9] (formula, see Chart 1) are available from the crystal structures of the respective drug/ER conjugates. On the basis of these investigations, the hypothesis of the binding mode of estrogenic active drugs published during the last 25 years can be refined.

Already in the 1980s, a phenolic site for **E2** and related estrogens as well as an antiestrogen region in the LBD at which antiestrogens are anchored additionally with their basic side chain (e.g., the alkylaminoethoxy moiety of tamoxifen) were proposed (for a review, see ref 14). Due to missing crystallographic data of the receptor molecule at that time, a lot of structure−activity studies were performed to evaluate the structural prerequisite for agonistic or antagonistic effects (review articles are, e.g., refs 14 and 15). It is well-known that drugs interact with the receptor in a low-energy binding conformation, so X-ray analysis,[16] molecular modeling,[17] and spectroscopic methods[11] were used to determine the spatial structure of ER affinic compounds. On the basis of these data and the results of sequence homology studies[18] with other members of the steroid hormone binding receptor superfamily as well as the results of site-directed mutagenesis experiments of the ER,[19] several models for the molecular structure of the ligand bound ER were developed.[20−22]

The crystallized drug/ER conjugates demonstrated, however, that none of these hypothetical models result in the real structure. Therefore, the crystallographic data of these drug/ER conjugates should be taken as a basis for the interpretation of affinity studies.

The X-ray structures showed that agonists as well as antagonists interact at the LBD via hydrogen bonds from the phenolic hydroxyl to the γ-carboxylate of Glu353, to the guandinium group of Arg394, and to a structurally conserved water molecule (Figure 3). In a second H-bridge, the agonists are fixed in the LBD. Even though the 4′-OH-group of **DES** is located 1.7 Å from the D-ring hydroxyl of **E2**, both molecules are anchored at the imidazole ring of His524. The same amino acids are attached in the **RAL**/LBD complex (Figure 3). The large basic side chain of **RAL** binds to the carboxylate group of Asp351 in a narrow channel that extends outward from the center of the cavity, normally plugged by Val536 in agonist/ER complexes. As **RAL**, **4OHT** induces a conformation of the LBD, which differs in both the secondary as well as the tertiary structural organization from the one driven by agonist binding. Due to the missing second hydroxyl, the molecule is not able for H-binding to His 524[7−9] and interacts in this region only by van der Waals contacts with the receptor (Figure 3). The *p*-[*N*,*N*-dimethylaminoethoxy]phenyl moiety is arranged in the same channel as the side chain of **RAL**. A comparison of the structures of **E2** and **4OHT**/LBD conjugates reveals that the binding mode of the **4OHT** side chain hinders the agonist-induced conformation of helix 12 (H12). While in agonist/ER complexes, H12 is positioned over the ligand-binding pocket it is bound in **RAL**/ER and **4OHT**/ER conjugates to the static region of the coactivator recognition groove; therefore, the activation function 2 (AF2) is blocked. As a consequence, the disposition of H12 by the side chain results in antagonistic effects of **RAL** and **4OHT**.[7−9,23]

It can be deduced that His524 represents an adequate anchor for type-I-estrogens such as **DES** and **E2**. Long side chains placed in the channel outward the binding cavity lead to antagonistic effects.

The compounds presented in this paper are not able to interact as type-I-estrogens with the ER, although they are hormonally active. In **1**−**4**, the 1,2-diphenylethane pharmacophor takes only conformations with neighboring aromatic rings, and Glu353, Arg394, as well as His524 cannot be attached at the same time.

To propose a hypothetical binding mode at the LBD for the type-II-estrogens, the structure of imidazoline **3** was fit to the conformations of **RAL**, **DES**, and **4OHT** obtained from the drug/ER conjugates.

A superposition of the pharmacophors of **RAL** and **4OHT** as well as of the heterocyclic compounds shows (Figure 4) that one phenolic ring is positioned in the direction of the (piperidine-1-yl)ethoxyphenyl and dimethylaminoethoxyphenyl moieties. Due to this structure analogy, an orientation in the binding cave of the receptor comparable to **RAL** and **4OHT** should be possible. To achieve agonistic effects, an attachment in the side pocket is necessary since a hydrogen bridge to His524 is impossible.

In Figure 3, we postulate the interaction of type-II-estrogens with the receptor in the example of the imidazoline **3**. First, **3** is attached like other agonists or antagonists at Glu353, Arg394, and the water molecule, and then a second H-bridge is built in the side pocket. Asp351 seems to be possible as anchorage, since it comes near to the 4-hydroxy group. Lipophilic residues of amino acids are located around the attached molecule in the LBD, which can stabilize the receptor/drug complex by van der Waals interactions. The binding leads to signal transduction of the ER as was determined in the luciferase assay on MCF-7-2a cells.

Type-II-estrogens such as **3** can change the conformation of the ER; however, they are not able to displace **E2** from its binding site. They show only very low RBA values in the competition experiment with [^{3}H]**E2**.

This mode of action is confirmed by McGregor Schäfer

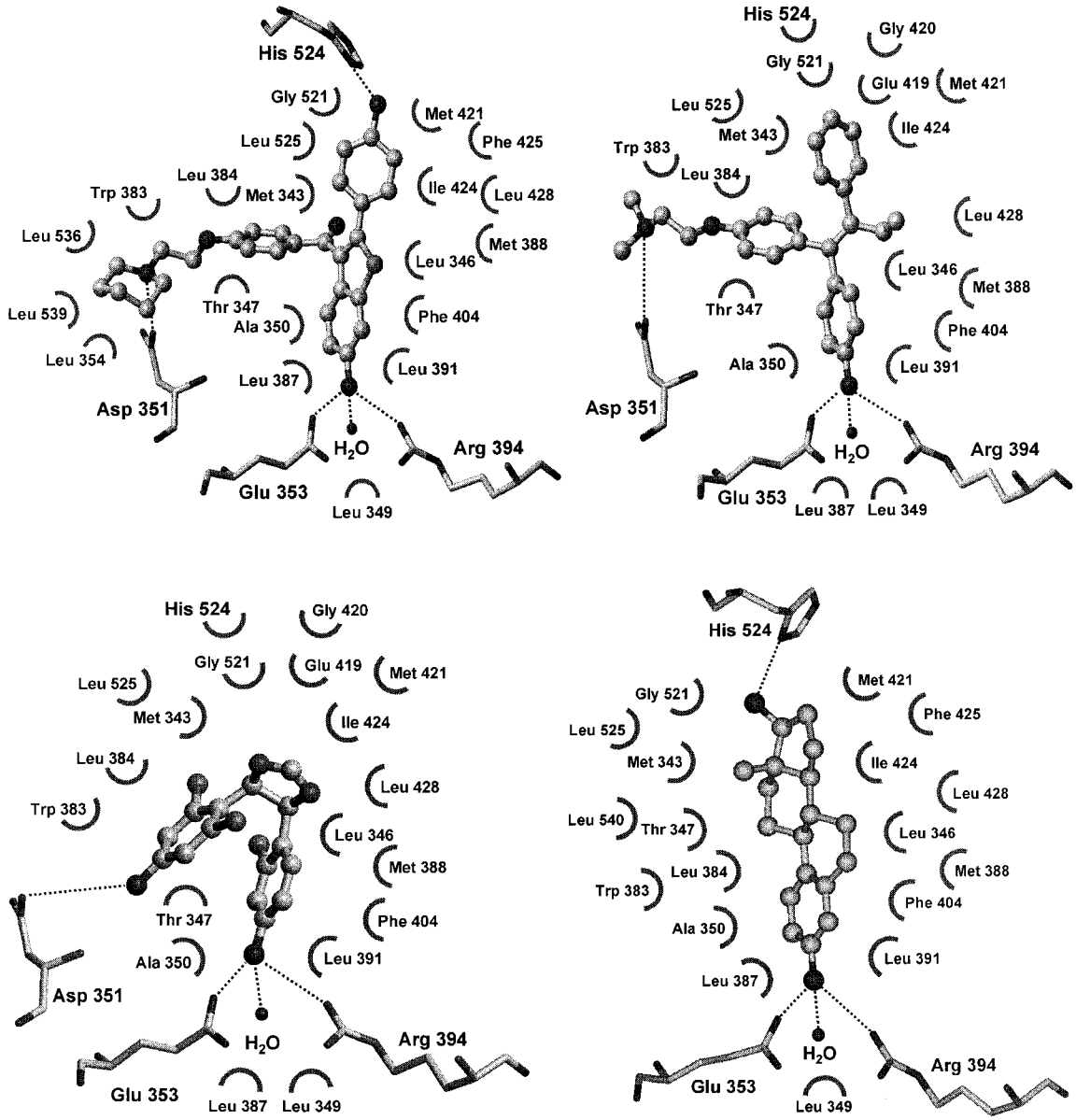


Figure 3. Interaction of **RAL**, **4OHT**, and **E2** with the estrogen receptor (ERα) as well as the postulated attachment of imidazoline **3** to the LBD of ERα.

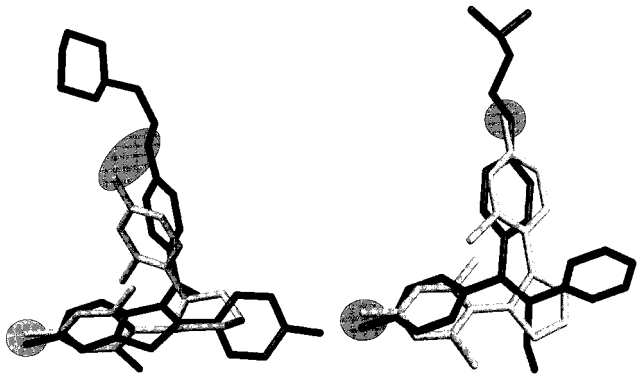


Figure 4. Superposition of **RAL** with piperazine **2** (left) and **4OHT** with imidazole **4** (right).

et al.[24] They investigated the importance of Asp351 for the estrogenic activity of **4OHT**. Substitution of glycine for aspartate at position 351 changed the activity of the **4OHT**/ER complex from estrogen-like to completely antiestrogenic. Therefore, Asp351 presents an alternative anchor for estrogenic molecules (type-II-estrogens).

Conclusion

The results of this paper led to two classes of estrogens:

Type-I-estrogens are derived from **E2** or **DES** and bind analogously to the estrogen receptor.

Type-II-estrogens are related to the (*R*,*S*)/(*S*,*R*)-configurated heterocyclic compounds or platinum(II) complexes and are anchored in part to other amino acids at the estrogen receptor.

Both types of estrogens lead to signal transduction of the ER and can be used as lead structures for the design of new selective estrogen receptor modulators.

Materials and Methods

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 Free University of Berlin; based on the C,

H, and N analyses, all compounds were of acceptable purity (within 0.4% of the calculated values). All computational graphics were built using SYBYL 6.6, Tripos Inc., 1699 South Hanley Rd., St. Louis, MO 63144. Geometry optimization was carried out using the Tripos force field from within the SYBYL program, running on an INDY workstation. Liquid scintillation counter: 1450 Microbeta Plus (Wallac, Finland). Microlumat: LB 96 P (EG & G Berthold, Germany).

Syntheses. (2*R*,3*S*)/(2*S*,3*R*)-1-(2-Chloro-4-methoxyphenyl)-2-(2,6-dichloro-4-methoxyphenyl)ethylenediamine **L1a** and (2*R*,3*S*)/(2*S*,3*R*)-1-(2-chloro-4-hydroxyphenyl)-2-(2,6-dichloro-4-hydroxyphenyl)ethylenediamine **L1** and the respective complex **1** (method E) were synthesized as described previously.[11]

(2*R*,3*S*)/(2*S*,3*R*)-2-(2-Chloro-4-methoxyphenyl)-3-(2,6-dichloro-4-methoxyphenyl)piperazine 2a (Method A). **L1a** (400 mg, 1.06 mmol) and oxalic acid diethylester (1.05 mmol) were dissolved in 20 mL of dioxane and heated under reflux for 72 h. The solvent was evaporated, and the dried crude product was subsequently suspended in 20 mL of dry THF. Under N_2 atmosphere, 5 mL of an 1 M Boran·THF complex was added, and the reaction mixture was heated for 12 h. While cooling in an ice bath it was hydrolyzed with 10 mL of water and 10 mL of half-concentrated HCl. THF was distilled off, and the acidic solution was heated under reflux for an additional hour. After cooling to room temperature, it was washed with diethyl ether, the aqueous layer was alkalized with 20% NaOH, and the product was extracted with dichloromethane. The organic layer was dried over Na_2SO_4 and evaporated. The crude product was purified twice on SiO_2, one after the other, with chloroform/methanol (20 + 1) and diethyl ether/methanol (4 + 1). Yield: 80 mg (19%), yellow oil. ^{1}H NMR ($CDCl_3$): δ = 2.30 (s, 2H, N*H*); 2.75−3.70 (m, 4H, NCH_2C); 3.75 (s, 3H, OCH_3); 3.80 (s, 3H, OCH_3); 4.90 (d, ^3J = 5 Hz, 1H, ArC*H*); 5.10 (d, ^3J = 5 Hz, 1H, ArC*H*); 6.70−7.00 (m, 4H, Ar*H*-3, Ar*H*-5, Ar'*H*); 8.20 (d, ^3J = 8 Hz, 1H, Ar*H*-6). Anal. ($C_{18}H_{19}Cl_3N_2O_2$) C, H, N.

(4*R*,5*S*)/(4*S*,5*R*)-4-(2-Chloro-4-methoxyphenyl)-5-(2,6-dichloro-4-methoxyphenyl)-2-imidazoline 3a (Method B). A solution of **L1a** (376 mg, 1.00 mmol), 0.256 mL of HCl_{conc}, and 2.17 mL of triethylorthoformate was heated under reflux for 72 h. After 36 h, another 1 mL of triethylorthoformate was added to optimize the reaction. The reaction mixture was cooled to room temperature and combined with 20 mL of chloroform. It was extracted with 5 mL of 1 N NaOH and three times with 10 mL of H_2O, respectively. The organic layer was dried over Na_2SO_4 and the solvent was evaporated. Purification was performed by chromatography on silica gel with $CHCl_3$/methanol (20 + 1). Yield: 301 mg (78%), colorless powder. Mp: 63−66 °C. IR (KBr): $\bar{\nu}$ = 3237 w (NH); 2837 w (OCH_3); 1606 s; 1556 m; 1494 m; 1465 m; 1434 m; 1283 m; 1241 m; 1177 m; 1043 s; 840 m; 787 m. MS (EI, 150 °C): *m*/*z* (%) = 384 (18) $[M]^{+\bullet}$; 350 (10); 204 (56); 170 (100); 154 (10); 119 (14); 75 (11). ^{1}H NMR ($CDCl_3$): δ = 3.69 (s, 3H, OCH_3); 3.71 (s, 3H, OCH_3); 5.70 (d, ^3J = 12.4 Hz, 1H, ArC*H*); 6.18 (d, ^3J = 12.4 Hz, 1H, ArC*H*); 6.52 (br, 1H, Ar*H*); 6.63 (d, ^4J = 2.5 Hz, 1H, Ar'*H*-3); 6.66 (dd, ^3J = 8.7 Hz, ^4J = 2.5 Hz, 1H, Ar'*H*-5); 6.73 (br, 1H, Ar*H*); 7.51 (d, ^3J = 8.7 Hz, 1H, Ar'*H*-6); 7.77 (s, 1H, N=C*H*-N). Anal. ($C_{17}H_{15}Cl_3N_2O_2$) C, H, N.

4-(2-Chloro-4-methoxyphenyl)-5-(2,6-dichloro-4-methoxyphenyl)imidazole 4a (Method C). To a solution of 0.920 mmol (355 mg) of 4,5-diaryl-2-imidazoline **3a** in 20 mL of dry $CHCl_3$ was added 6.00 mmol of activated MnO_2, and the suspension was stirred for 24 h. Subsequently, MnO_2 was separated by suction filtration, and the solvent was evaporated. The crude product was purified by chromatography on SiO_2 with acetic ether. Yield: 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, 3H, OCH_3); 3.79 (s, 3H, OCH_3); 6.68 (dd, ^3J = 8.6 Hz, ^4J = 2.5 Hz, 1H, Ar*H*-5); 6.88 (s, 2H, Ar'*H*); 6.94 (d, ^4J = 2.5 Hz, 1H, Ar*H*-3); 7.09 (d, ^3J = 8.6 Hz, 1H, Ar*H*-6); 7.80 (s, 1H, N=C*H*-N). Anal. ($C_{17}H_{13}Cl_3N_2O_2$) C, H, N.

General Procedure for the Ether Cleavage with BBr_3 (Method D). 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 further 48 h. After the reaction mixture was cooled in 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 filtrated and the pH was adjusted to 8 with 2 N HCl, and the precipitate was collected by suction filtration and dried over P_2O_5. Subsequently, the crude product was purified by column chromatography or fractional crystallization.

(2*R*,3*S*)/(2*S*,3*R*)-2-(2-Chloro-4-hydroxyphenyl)-3-(2,6-dichloro-4-hydroxyphenyl)piperazine 2. 2a: 0.212 mmol (85 mg). For ether cleavage the reaction mixture was heated under reflux for 48 h. Purification by chromatography on SiO_2 with diethyl ether/methanol (4 + 1). Yield: 37 mg (47%), colorless powder. Mp: 148−150 °C. IR (KBr): $\bar{\nu}$ = 3600−2900 s, br (OH); 2943 w; 1604 s; 1493 w; 1439 s; 1379 w; 1272 s; 1234 m; 1101 m; 1039 m; 953 m; 901m; 854 m. ^{1}H NMR ([D_4]-methanol): δ = 2.84−2.91 (m, 1H, NCH_2C); 3.00−3.12 (m, 1H, NCH_2C); 3.28−3.42 (m, 1H, NCH_2C); 3.54−3.66 (m, 1H, NCH_2C); 4.82 (d, ^3J = 4.9 Hz, 1H, ArC*H*); 5.02 (d, ^3J = 4.9 Hz, 1H, ArC*H*); 6.58 (d, ^4J = 2.6 Hz, 1H, Ar*H*-3); 6.62 (s, 2H, Ar'*H*-3, Ar'*H*-5); 6.74 (dd, ^3J = 8.7 Hz, ^4J = 2.6 Hz, 1H, Ar*H*-5); 8.10 (d, ^3J = 8.7 Hz, 1H, Ar*H*-6). Anal. ($C_{16}H_{15}Cl_3N_2O_2$) C,H,N.

(4*R*,5*S*)/(4*S*,5*R*)-4-(2-Chloro-4-hydroxyphenyl)-5-(2,6-dichloro-4-hydroxyphenyl)-2-imidazoline 3. 3a: 0.415 mmol (160 mg). The reaction mixture was stirred for 72 h at room temperature. Purification by fractional crystalization from methanol. Yield: 73 mg (49%), colorless powder. Mp: 238−241 °C. IR (KBr): $\bar{\nu}$ = 3600−2700 s, br (OH); 2889 w; 2639 w; 1608 s; 1578 m; 1499 w; 1443 s; 1273 s; 1050 m; 948 m; 904 m; 858 m. MS (EI, 300 °C): *m*/*z* (%) = 356 (27) $[M]^{+\bullet}$; 204 (10); 194 (72); 190 (94); 174 (10); 162 (57); 156 (100); 139 (23). ^{1}H NMR ([D_6]-DMSO): δ = 5.33 (d, ^3J = 12.0 Hz, 1H, ArC*H*); 5.83 (d, ^3J = 12.0 Hz, 1H, ArC*H*); 6.45 (s, 1H, Ar*H*); 6.50 (d, ^4J = 2.1 Hz, 1H, Ar'*H*-3); 6.55 (dd, ^3J = 8.5 Hz, ^4J = 2.1 Hz, 1H, Ar'*H*-5); 6.68 (s, 1H, Ar*H*); 7.28 (s, 1H, N=C*H*-N); 7.37 (d, ^3J = 8.5 Hz, 1H, Ar'*H*-6); 9.68 (br, 2H, ArO*H*). Anal. ($C_{15}H_{11}Cl_3N_2O_2$) C, H, N.

4-(2-Chloro-4-hydroxyphenyl)-5-(2,6-dichloro-4-hydroxyphenyl)imidazole 4. 4a: 0.430 mmol (165 mg). The reaction mixture was stirred for 72 h at room temperature. Purification by chromatography on SiO_2 (diethyl ether/acetone 6 + 1) and subsequent fractional crystalization from chloroform/acetone (ca. 6 + 1). Yield: 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) $[M]^{+\bullet}$; 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, 1H, Ar*H*-5); 6.80 (s, 2H, Ar'*H*-3, Ar'*H*-5); 6.83 (d, ^4J = 2.3 Hz, 1H, Ar*H*-3); 7.01 (d, ^3J = 8.4 Hz, 1H, Ar*H*-6); 7.78 (s, 1H, N=C*H*-N). Anal. ($C_{15}H_9Cl_3N_2O_2$) C, H, N.

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*-hexa-methylpararosaniline (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, MA); phosphate buffered saline (PBS) was prepared by solving 8.0 g NaCl, 0.2 g KCl, 1.44 g $Na_2HPO_4 \cdot 2H_2O$, 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 of trishydroxymethylaminomethan, 0.37224 g of Titriplex III, and 0.19503 g of sodium azide (all from Merck or Fluka) in 1 L of purified 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 described already by Hartmann et al.[25] 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 six to eight appropriate concentrations (300 μL). They were incubated and shaken 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 the mixture was shaken for 90 min at 4 °C and centrifuged, 500 μL of HiSafe3 was mixed with 100 μL of 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). Nonspecific binding was calculated using 2 nmol of 17β-estradiol as the competing ligand. On a semilog plot, the percentage of maximum bound labeled steroid corrected by the nonspecifically 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, the molar concentrations of unlabeled estradiol and of the competitors were determined which reduce the binding of the radioligand by 50%.

$$\mathrm{RBA} = \frac{c_{[^3\mathrm{H}]-\mathrm{estradiol}} \text{ at 50\% inhibition}}{c_{\mathrm{sample}} \text{ at 50\% inhibition}} \times 100\%$$

Luciferase Assay. The pertinent in vitro assay was described earlier by Hafner et al.[26] 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 of cell suspension and 1.5 mL of medium per well) at growing conditions (see above). After 24 h, 20 μL of a stock solution of the test compounds were 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 a 20 min lysation at room temperature, cells were transferred into reaction tubes and centrifugated. Luciferase was assayed using the Promega luciferase assay reagent. A total of 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 percent activation of a 10^{-8} M estradiol control (100%).

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