Structural Evolutions of Salicylaldoximes as Selective Agonists for Estrogen Receptor β

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The bioisosteric replacement of the phenol ring, a signature functional group of most estrogen receptor (ER) ligands, with a hydrogen-bonded pseudocyclic ring, led to the development of a novel class of nonsteroidal ER-ligands based on a salicylaldoxime template. A series of structural modifications were applied to selected molecules belonging to the monoaryl-salicylaldoxime chemical class in an attempt to improve further their ERβ-selective receptor affinity and agonist properties. Among several modifications, the best results were obtained by the simultaneous introduction of a *meta*-fluorine atom into the *para*-hydroxyphenyl substituent present in the 4-position of salicylaldoxime, together with the insertion of a chloro group in the 3-position of the central scaffold. The resulting compound showed the best affinity (K_i = 7.1 nM) and selectivity for ERβ over ERα. Moreover, in transcription assays, it proved to be a selective and potent ERβ-full agonist with an EC_{50} of 4.8 nM.

Introduction

The effects of estrogen hormones are mediated by the estrogen receptor subtypes alpha (ERα) and beta (ERβ[a]), which are ligand-regulated transcription factors.[1] Since the discovery of the ERβ subtype,[2] studies have pointed out the existence of up to five different ERβ isoforms (ERβ1−5) that arise from alternative splicing of the last exon coding for ERβ.[3,4] However, the originally cloned 59 kDa ERβ1 isoform is the only fully functional isoform and, therefore, is the isoform referred to simply as ERβ.[3,4]

For many years, ERα was considered the only target for estrogen-related therapies, which were based on either the agonist properties of certain drugs, such as ones for hormone replacement therapy (HRT), osteoporosis, and hot flushes, or on antagonist properties such as those exhibited by antitumor agents effective against hormone-sensitive breast cancer. In many cases, the use of selective estrogen receptor modulators (SERMs) has offered advantages for both types of therapies.[5,6]

The more recently discovered receptor, ERβ, has inspired research efforts devoted to distinguishing the biological effects of estrogen action through each of the two ER-subtypes. Some ERβ-selective agonists have become clinical candidates in several therapeutic areas such as for the treatment of prostate hyperplasia and cancer,[7] bone loss,[8] arthritis, and intestinal inflammation.[9] Notably, the beneficial effects resulting from selective ERβ-stimulation are free from the undesired ERα-mediated proliferative effects on breast and uterus, and this constitutes a great advantage for the prospective therapeutic use of such drugs.

The amino acid sequence of ERα and ERβ show 59% amino acid sequence identity in the ligand binding domain (LBD), but analysis of the ligand binding cavities of the two subtypes reveals only minor differences. In fact, there are only two amino acid substitutions: Leu384 and Met421 of ERα are respectively replaced by Met336 and Ile373 in ERβ.[2] Overall, the ERβ binding pocket has a smaller volume than that of ERα and there are also slight differences in the shape of these cavities due to amino acid residues lining the cavity borders. This high level of similarity between the ERα and ERβ binding pockets has made the development of highly subtype-selective, ERβ agonists particularly challenging. Nevertheless, the number of novel chemical classes that demonstrate selective affinity for ERβ continues to increase.

In recent years, we have developed several diaryl-substituted salicylaldoxime[10,11] and anthranylaldoxime[12,13] derivatives that are able to bind to the ERs with high affinity. These compounds contain a six-membered pseudocycle, formed by an intramolecular H-bond, that was designed to isosterically replace the steroid phenolic A ring, which is required in ER ligands.[14] In spite of the good binding affinity shown by some compounds in these classes, a satisfactory level of subtype binding selectivity was never obtained.

Later, an ERβ pharmacophoric model,[15] based on a structural analysis of many nonsteroidal ERβ-selective ligands, inspired the development of our initial series of monoaryl-substituted salicylaldoximes,[16] whose simplest member (**1**) is shown in Figure 1. Compound **1** showed a good level of ERβ selectivity in the receptor binding assay, although its absolute affinity was not as high as desired. We then introduced various substituents into the 3-position of **1**, such as a chlorine atom, a methyl, or a cyano group. Binding assays showed that the 3-chloro-substituted derivative **2** (Figure 1) possessed the best properties in terms of ERβ-binding affinity and β-subtype selectivity.[16] Transcriptional assays showed that compound **2** is a partial agonist on ERβ, but unfortunately, its functional activity is much less ERβ selective than its binding affinity.[16]

For this reason, we started to consider several possible molecular variations that could afford new monoaryl-subsituted salicylaldoximes possessing good ERβ-binding affinity and selectivity, like that of compound **2**, but would at the same time

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[a] Abbreviations: ER, estrogen receptor; ERα, estrogen receptor subtype alpha; ERβ, estrogen receptor subtype β; HRT, hormone replacement therapy; SERMs, selective estrogen receptor modulators; LBD, ligand binding domain; RBA, relative binding affinity; RTP, relative transcriptional potency; TLC, thin-layer chromatography; CG, conjugated gradient.

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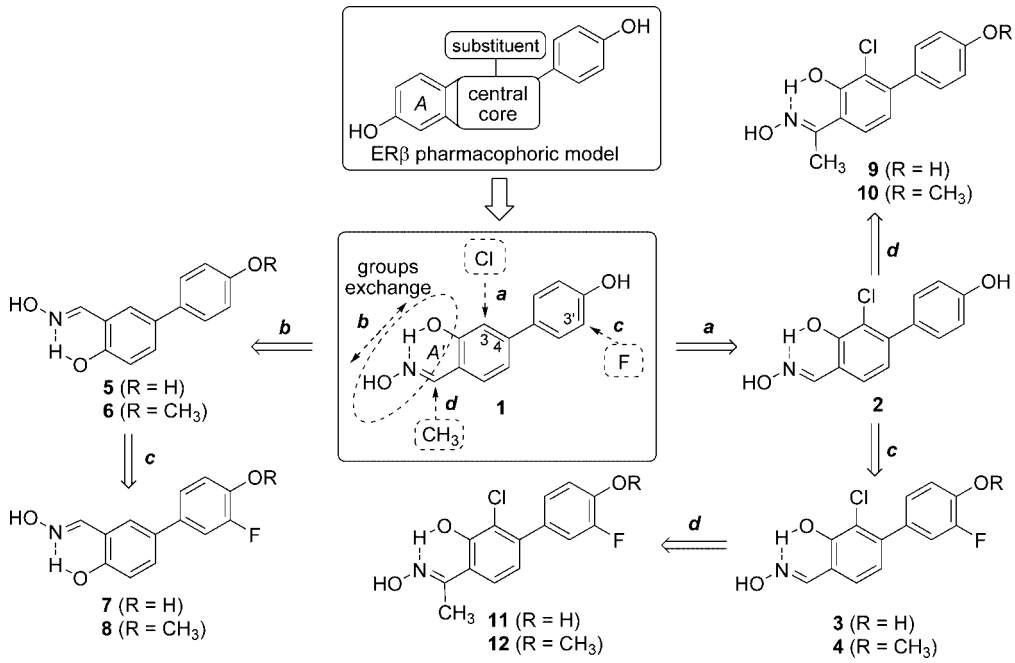
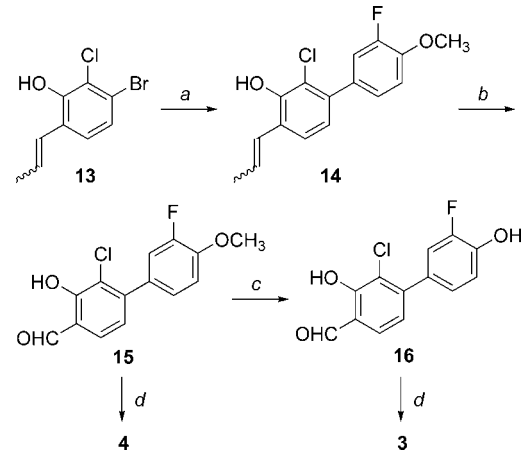


Figure 1. Compounds **2**−**12** deriving from structural evolutions (*a*−*d*) of monoaryl-substituted salicylaldoxime **1**.

have improved selectivity as ERβ-agonists in transcriptional activity assays. To accomplish this, we planned a series of structural evolutions that we applied to the simplest member of this class of compounds (**1**); these can be summarized as follows (Figure 1): (*a*) introduction of a chlorine atom into the 3-position of the central scaffold (already exploited for the development of **2**,[16] now also combined with other modifications), (*b*) exchange of the relative positions of the phenol OH and oxime groups, (*c*) introduction of a *meta*-fluorine atom into the peripheral 4-aryl substituent, and (*d*) introduction of a methyl group on the oxime carbon atom. The choice of the combinations of the various structural modifications was mainly dictated by the synthetic accessibility of the resulting target molecules. These structural evolutions have led to the development of new hydroxyphenyl-substituted oximes (R═H: **3**, **5**, **7**, **9**, and **11**) and their *O*-methylated counterparts (R═CH_3: **4**, **6**, **8**, **10**, and **12**), which were synthesized and submitted to biological assays to determine whether any of these modifications would be beneficial in terms of selective ERβ-ligand binding and agonist properties.

Scheme 1. Synthesis of Salicylaldoximes **3** and **4**[a]



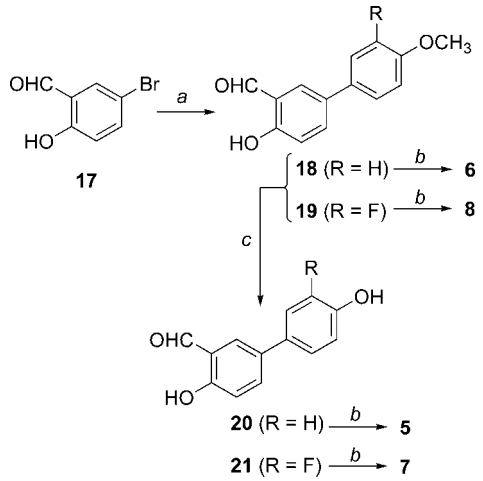
[a] Key: (a) 3-*F*-4-MeO-$C_6H_4B(OH)_2$, $Pd(OAc)_2$, PPh_3, aqueous 2 M Na_2CO_3, 1:1 toluene/EtOH, 100 °C, 16 h; (b) OsO_4, $NaIO_4$, dioxane-H_2O, 2 h; (c) BBr_3, CH_2Cl_2, −78 to 0 °C, 1 h; (d) $NH_2OH\cdot HCl$, MeOH-H_2O, 50 °C, 5 h.

Results and Discussion

Synthetic Chemistry. The synthesis of 3-chloro-substituted salicylaldoximes bearing a *meta*-fluorine atom on the 4-aryl substituent (**3** and **4**) followed a synthetic path similar to one we had used for the synthesis of oxime **2** (Scheme 1).[16] A 9:1 *E/Z*-diastereoisomeric mixture of 3-bromo-2-chloro-6-(prop-1-enyl)phenol (**13**), prepared in three steps from 3-bromo-2-chlorophenol as previously reported,[16] was submitted to palladium-catalyzed cross coupling with 3-fluoro-4-methoxyphenylboronic acid. In this case, the use of classic Suzuki conditions, namely, in situ formation of $Pd(PPh_3)_4$ by reaction of palladium acetate with a 5-fold excess of triphenylphosphine with an aqueous base and conventional heating at 100 °C overnight, guaranteed the chemoselective replacement of the bromine atom only, with the chloro group remaining intact. Subsequent oxidative cleavage of the olefin double bond of the resulting biphenyl derivative **14** was achieved with two equivalents of sodium periodate in the presence of catalytic amounts of osmium tetroxide. The resulting salicylaldehyde (**15**) was treated with boron tribromide to obtain *O*-demethylated aldehyde **16**, which was then condensed with hydroxylamine hydrochloride, thus yielding the final oxime **3**. Methoxy-substituted salicylaldoxime **4** was obtained by direct reaction of aldehyde **15** with hydroxylamine hydrochloride.

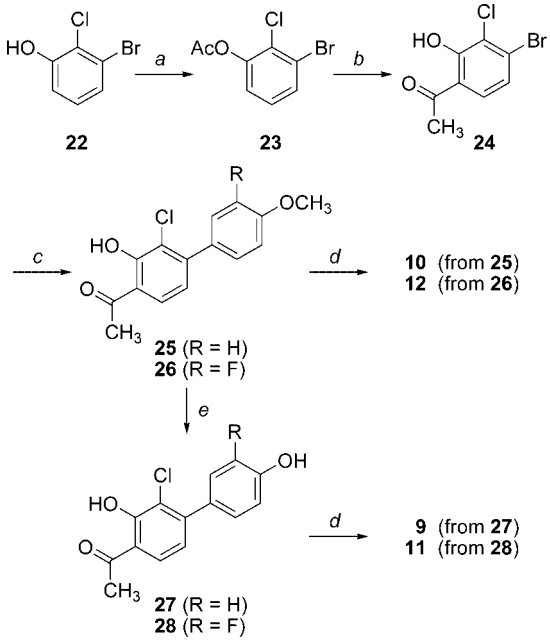
Salicylaldoximes **5**−**8**, derived by the formal exchange of the positions of the OH and CHO groups, were synthesized as shown in Scheme 2. Commercially available 5-bromosalicylaldehyde (**17**) was submitted to a Pd-catalyzed cross coupling reaction with either 4-methoxyphenylboronic acid to give biaryl derivative **18**, or with 3-fluoro-4-methoxyphenylboronic acid, for the preparation of analogue **19**. Subsequent *O*-demethylation with BBr_3 afforded free phenols **20** from **18**, and **21** from **19**. Final condensation with hydroxylamine hydrochloride yielded final oximes **5** and **7**. The preparation of *O*-methylated oximes **6** and **8** was obtained by direct condensation of *O*-Me-substituted aldehydes **18** and **19**, respectively, with $NH_2OH\cdot HCl$.

Scheme 2. Synthesis of Salicylaldoximes **5**−**8**[a]



[a] Key: (a) 4-MeO-$C_6H_4B(OH)_2$ or 3-*F*-4-MeO-$C_6H_4B(OH)_2$, $Pd(OAc)_2$, PPh_3, aqueous 2 M Na_2CO_3, 1:1 toluene/EtOH, 100 °C, 16 h; (b) $NH_2OH\cdot HCl$, MeOH−H_2O, 50 °C, 5 h; (c) BBr_3, CH_2Cl_2, −78 to 0 °C, 1 h.

Scheme 3. Synthesis Salicylaldoximes **9**−**12**[a]



[a] Key: (a) (i) NaOH, $nBu_4N^+HSO_4^-$ (cat.), dioxane, (ii) acetyl chloride, dioxane, RT, 1 h; (b) $AlCl_3$, 130 °C, 4 h neat; (c) 4-MeO-$C_6H_4B(OH)_2$ or 3-*F*-4-MeO-$C_6H_4B(OH)_2$, $Pd(OAc)_2$, PPh_3, aqueous 2 M Na_2CO_3, 1:1 toluene/EtOH, 100 °C, 16 h; (d) $NH_2OH\cdot HCl$, MeOH−H_2O, 50 °C, 5 h; (e) BBr_3, CH_2Cl_2, −78 to 0 °C, 1 h.

The synthesis of ketoximes **9**−**12** starts from 3-bromo-2-chlorophenol **22**, which was prepared following a previously reported route (Scheme 3).[17] Phenol **22** was esterified with acetyl chloride under phase-transfer conditions in the presence of base and, upon heating to 130 °C with aluminum trichloride, the resulting acetate **23** underwent a Fries rearrangement to give the *ortho*-acetyl compound **24**. Biaryl derivatives **25** and **26** were obtained from *o*-acetylphenol **24** by a Pd-catalyzed cross-coupling reaction with 4-methoxyphenylboronic or 3-fluoro-4-methoxyphenylboronic acid, respectively. BBr_3-promoted *O*-demethylation afforded compounds **27** and **28**, which were treated with hydroxylamine hydrochloride to give, respectively, the final ketoximes **9** and **11**. Treatment of methoxy-substituted ketones **25** and **26** afforded oximes **10** and **12**.

Table 1. Relative Binding Affinities[a] of Compounds **1**−**12** for the Estrogen Receptors α and *β*

ligand	hERα (%)	hER*β* (%)	*β*/α ratio
estradiol	(100)	(100)	1
1[b]	0.007 ± 0.001	0.553 ± 0.110	79
2[b]	0.065 ± 0.016	4.21 ± 0.66	65
3	0.114 ± 0.030	7.01 ± 1.00	62
4	0.006 ± 0.001	0.011 ± 0.001	1.8
5	0.064 ± 0.016	2.64 ± 0.62	41
6	0.003 ± 0.001	0.011 ± 0.003	3.7
7	0.021 ± 0.001	0.970 ± 0.110	46
8	0.007 ± 0.001	0.013 ± 0.001	1.9
9	0.012 ± 0.004	0.123 ± 0.030	10
10	<0.001	0.002 ± 0.001	
11	0.006 ± 0.001	0.077 ± 0.010	13
12	<0.001	0.002 ± 0.001	

[a] Determined by a competitive radiometric binding assay with [^{3}H]estradiol; preparations of purified, full-length human ERα and ER*β* (PanVera) were used; see Experimental Section. Values are reported as the mean ± the range or SD of 2 or more independent experiments; the K_d for estradiol for ERα is 0.2 nM and for ER*β* is 0.5 nM. K_i values for the new compounds can be readily calculated by using the formula: $K_i = (K_d[\text{estradiol}]/\text{RBA}) \times 100$. [b] See ref 16.

All the aldoximes (**3**−**8**) were obtained as single *E*-diastereoisomers because only these isomers can form the stabilizing intramolecular hydrogen bonds. This selectivity had already been verified for other oxime analogues previously reported,[10−13,16] and it was confirmed by the ^{1}H NMR chemical shift value (δ) of the oxime proton, which is always found downfield from 8 ppm ($\delta \geq 8.45$ ppm, see Experimental Section).[18] The *E*-configuration of ketoximes **9**−**12** was correlated with the chemical shift of the methyl protons, which is in the range of 2.35−2.41 ppm for these four compounds; by contrast, ketoximes of acetophenones with *Z*-configurations show chemical shifts for the methyl protons that are less than 2.0 ppm.[19] Further evidence comes from the ^{13}C NMR spectra, which showed δ values of the methyl carbon atoms in the range of 10.78−11.00 ppm (δ = 11.00 (**9**), 10.94 (**10**), 10.78 (**11**), 10.78 (**12**) ppm), consistent with values found for *E*-diastereoisomers of aromatic ketoximes; the *Z* isomers typically have chemical shifts around 21−22 ppm.[19]

Estrogen Receptor Binding Assays. ERα and ER*β* binding affinities of oximes **3**−**12** were determined by a radiometric competitive binding assay, using methods that have been previously described.[20,21] The relative binding affinity (RBA) values for the newly reported compounds, together with those previously obtained for compounds **1** and **2**,[16] are summarized in Table 1. RBA values are reported as percentages (%) of that of estradiol, which is set at 100%.

As we had already experienced in the past,[16] and in accordance with the pharmacophore model (Figure 1),[15] *para*-methoxyphenyl-substituted oximes (**4**, **6**, **8**, **10**, and **12**) did not show appreciable levels of affinity for either receptor subtype. Among the *para*-hydroxyphenyl-substituted compounds, the simplest member of this class (compound **1**) had already shown a remarkable level of ER*β*-selectivity (ER*β*/α = 79), although its absolute affinity for ER*β* was low (RBA = 0.55%).[16] The first structural evolution (type *a*, Figure 1) applied to compound **1**, was the introduction of a chlorine atom into the 3-position of the central scaffold, leading to compound **2**. This caused an 8-fold increase in binding affinity and, at the same time, maintained a good level of ER*β*-selectivity (ER*β*/α = 65).[16] Oxime **5**, derived by exchange of the relative positions of the phenol and oxime groups (structural modification type *b*), showed a 5-fold increase in ER*β*-binding affinity when com-

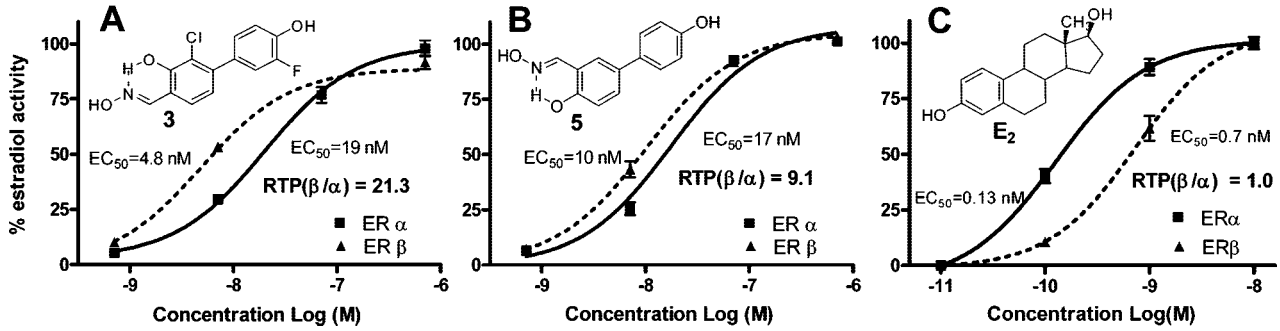


Figure 2. Dose−response curves for transcriptional activation by compound **3** (A), compound **5** (B), and estradiol (C) through ERβ (dashed line) and ERα (solid line). Human endometrial cancer (HEC-1) cells were transfected with expression vectors for ERα or ERβ and an $(ERE)_2$-pS2-luc reporter gene and were treated with estradiol, compound **3**, or compound **5** at the concentrations indicated. Luciferase activity was expressed relative to β-galactosidase activity from an internal control plasmid. The maximal activity with 1 nM E_2 was set at 100%. Values are the mean of duplicate determinations. EC_{50} values give absolute potencies. The ERβ/ERα relative transcriptional potency (RTP(β/α)) ratios are calculated as explained in the text.

pared to **1**, together with the persistence of a notable level of ERβ-selectivity (ERβ/α = 41). Oximes **3** and **7** were obtained by modification type *c*, namely, the introduction of a *meta*-fluorine atom into the 4-hydroxyphenyl substituent on compounds **2** and **5**, respectively. This kind of substituent is also present in one of the best ERβ-selective ligands, ERB-041,[22a,b] which is being studied clinically for the treatment of various kinds of inflammatory pathologies, including rheumatoid arthritis. Moreover, a beneficial effect of the *m-F* group on ERβ-affinity was also observed on biphenylcarbaldehyde oxime derivatives previously reported.[22c] This modification, however, was not very effective in improving the biological properties of compound **5** because the resulting compound **7** showed a drop (almost 3-fold) in ERβ-binding affinity. On the other hand, the same modification on compound **2** leading to oxime **3** resulted in an increase in ERβ binding affinity; this reached the best value in this series of compounds, a RBA value of 7.0% relative to estradiol, which corresponds to an absolute binding affinity (K_i) of 7.1 nM. Fortunately, a high level of subtype-selectivity was also preserved (ERβ/α = 62). Finally, the last kind of modification (type *d*), which introduces a methyl group into the oxime carbon atom of compound **2**, leading to product **9**, or of compound **3**, leading to **11**, did not have any positive effect in terms of ERβ-binding affinity and selectivity.

Transcription Assays. Compounds **3** and **5**, showing the highest binding affinity and selectivity for ERβ, were submitted to further biological testing to assess their pharmacological character. They were assayed for transcriptional activity through both receptor subtypes, together with estradiol for reference. Reporter gene transfection assays were conducted in human endometrial (HEC-1) cells, using expression plasmids for either full-length human ERα or ERβ and an estrogen-responsive luciferase reporter gene system.[23]

In our previous studies, compound **2** proved to be an ERβ partial agonist in these assays, having a maximum activity of 60% relative to estradiol and an EC_{50} value of 11 nM. However, it also efficiently stimulated ERα, with an EC_{50} of 26 nM. This resulted in low ERβ-selectivity in terms of transcriptional potency (ERβ/α = 2.4) compared to the selectivity of its binding affinity (ERβ/α = 65).[16] It should be noted that reduced ERβ-selectivity in terms of transcriptional potency vs binding affinity may be ascribed to differences in the manner in which the ERα- and ERβ-ligand complexes interact with various cellular co-regulators, which can act as modulators of ligand potency.

Newly synthesized compound **3**, which differs from **2** by the presence of an additional F-atom, showed much more pronounced agonist properties in these assays, with a maximum ERβ-activation of ≥85% when compared to estradiol (Figure 2A); furthermore, **3** proved to be more potent than **2**, with an EC_{50} of 4.8 nM, a value close to its absolute affinity K_i (7.1 nM) found in the binding assay (section above). This compound also had a better β-selective profile than **2**, because its activation of ERα has an EC_{50} value of 19 nM. Compound **5** was also a potent ERβ-agonist, with an EC_{50} of 10 nM and a maximum activation approaching that of estradiol (∼100%), although the ERβ selectivity level for this compound was lower than for **3** (Figure 2B).

To facilitate comparisons of the ER subtype *transcriptional potencies* of our compounds with their ER subtype *binding affinities*, we converted the EC_{50} values from the transcription assays to relative transcriptional potency (RTP) values, which were calculated as $RTP = EC_{50}^{(estradiol)}/EC_{50}^{(ligand)} \times 100$ (RTP, estradiol = 100). The RTPs provide a measure of transcriptional potency relative to that of estradiol and thus are appropriate to compare with their binding affinities, which are also measured relative to estradiol by the competitive radiometric binding assays. Estradiol has a 2.5-fold preference in favor of ERα in terms of binding (K_d [ERα] = 0.2 nM vs [ERβ] = 0.5 nM) and a 5.4-fold preference in terms of transcriptional potency (EC_{50} [ERα] = 0.13 nM vs [ERβ] = 0.70 nM). By these metrics, compound **3** has an RBA(β/α) ratio of 62 and an RTP(β/α) ratio of 21.3, and compound **5** has an RBA(β/α) ratio of 41 and an RTP(β/α) ratio of 9.1. Thus, measured relative to estradiol, much of the ERβ affinity preference of these compounds is, indeed, preserved in their ERβ transcriptional potency preference.

Molecular Modeling. Docking studies were undertaken to explore the interaction of the newly synthesized compounds with the ERα and ERβ ligand binding sites. Figure 3A shows the docking of compound **3** into ERβ, and it shows a binding mode very similar to that already reported for 4′-hydroxybiphenylcarbaldehyde oxime derivatives,[22c] and for compound **2**,[16] a compound from which it differs by only a fluorine atom in position 3′. In this model, the pseudocycle/oxime system forms H-bonds with H475 and the backbone carbonyl of G472; the chlorine atom is inserted into the lipophilic pocket delimited by A302, W335, M336, and L339, while the phenol OH substituent is involved in a H-bond network that includes E305, R346, and a water molecule. The presence of the fluorine substituent results in an increase of ERβ affinity, but the docking studies do not reveal any important interactions for this group. However, as reported by Malamas and co-workers in their

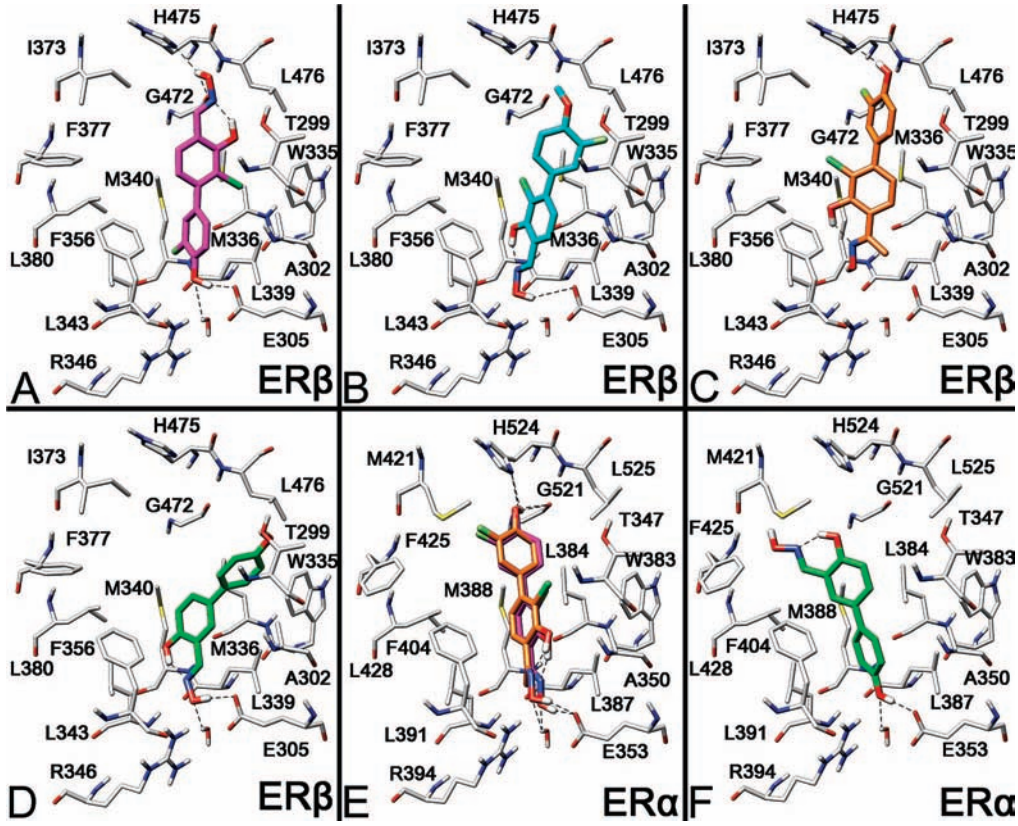


Figure 3. Docking analysis of ligands into ERβ and ERα: (A) docking of **3** (magenta) ERβ, (B) docking of **4** (cyan) into ERβ, (C) docking of **11** (orange) into ERβ, (D) docking of **5** (green) into ERβ, (E) docking of **3** and **11** into ERα, (F) docking of **5** into ERα.

analysis of ERB-041 binding,[22b] the repulsion between the fluorine atom of compound **3** and the carbonyl oxygen of L339 results in a shift of the molecule by about 0.7 Å; this allows a stronger interaction of the pseudocycle/oxime system with G472 and H475, while the greater distance between the phenol OH group and the E305−R346−water system is balanced by the higher polarization of the phenol OH group due to the fluorine substituent.

The substitution of the phenol OH with a methoxy group results in about a 600-fold reduction in ERβ affinity (compound **4**). As shown in Figure 3B, this substitution causes the ligand to invert its position in the binding pocket with respect to compound **3**, leading to the loss of the interactions with G472 and H475.

The introduction of a methyl group into the oxime carbon atom of compound **3** results in about a 90-fold reduction in ERβ affinity (compound **11**). We have already reported an analysis of the cavities surrounding reference compound **2** in the ERβ binding site,[16] and this analysis indicated the presence of two empty voids around the central aromatic ring, while no space was found around the pseudocycle/oxime unit. This was confirmed by a docking analysis of **11**, as shown in Figure 3C. Because of the lack of any void in this region, addition of the methyl substituent onto the oxime carbon atom results in an inversion of ligand orientation with respect to compound **3**. In this new orientation, a H-bond forms between the phenol OH substituent and H475, but there is a loss of all the interactions between the ligand and the E305−R346−water system.

Finally, compound **5**, in which there has been an exchange on the relative positions of the phenol and oxime groups in compound **1**, experiences about a 5-fold increase in ERβ-binding affinity. The docking results showed that the pseudocycle/oxime system was able to interact with the E305−R346−water system, whereas the shift of the *p*-phenol ring from C4 to C5 results in a loss of the H-bond with H475 and G472, but formation of a new H-bond with T299 (Figure 3D). This different interaction may explain the good ERβ binding affinity of this compound, and it is also consistent with the lack of beneficial effects by the introduction of a 3′-fluoro substituent (cf. **5** and **7**) that was found in going from compound **2** to **3**. It is of note that the strong interaction between the 4′-hydroxyl of **5** and T299 is possible only with ERβ because only in this ER subtype is there enough space for the phenol group in the area close to M336. Thus, the phenol OH group comes in close proximity to the alcohol group of T299; the same does not happen in ERα, where the methionine (M336 in ERβ) is replaced by a bulkier leucine (L384 in ERα), causing a completely different disposition of compound **5** in this receptor subtype (Figure 3F).

In general, all the compounds tested proved to be weaker ligands with ERα. As already reported,[16] the compounds having a Cl substituent in the central aromatic ring, such as compound **3**, show, in ERα, the phenol OH interacting with H524 and the pseudocycle/oxime system forming H-bonds with the E353−R394−water system (see **3** and **11**, Figure 3E). As shown in Figure 3A, the Cl-substituent of **3** in ERβ is able to interact with the lipophilic pocket delimited by A302, W335, M336, and L339, whereas in ERα the replacement of M336 by L384 restricts the size of this cavity. This steric restriction causes compound **3** to bind to ERα in a reversed orientation and with lower affinity.

As for compound **5**, characterized by exchange of the relative positions of the phenol and oxime groups (Figure 3F), its phenolic OH interacts in the ERα binding site with the E305−R346−water system, while the pseudocycle/oxime sys-

tem does not show any important interactions, confirming its low affinity for this subtype, and thus its high beta-selectivity.

Conclusions

Several structural evolutions were effected within a series of monoaryl-substituted salicylaldoximes, starting from the simple member of this class, 3-(4-hydroxyphenyl)salicylaldoxime **1**. The first modification consisted of introduction of a chlorine atom on C(3) of the central scaffold, producing compound (**2**), which previously showed a very high affinity for the ERβ-subtype and a high binding selectivity (β/α ratio 65), although the same compound showed comparable levels of activation of the two receptor subtypes in transcriptional assays. In this work, we extended the types of structural modifications and found that exchange of the positions of the −OH and −CH═NOH groups constituting the hydrogen-bonded salicylaldoxime unit, produced a compound (**5**) with better ERβ-affinity than parent compound **1**, although some β potency selectivity was lost in gene transcription assays. The best combination of the transformations was found with the simultaneous introduction of a Cl group in position 3 and a fluorine atom in the *meta*-position of the 4-aryl-substituent, affording compound **3**, which showed the highest selective β affinity ($K_i = 7.1$ nM) and still preserved a good level of ERβ selectivity in functional assays, where it proved to be a partial agonist with high intrinsic activity on ERβ, with a EC_{50} of 4.8 nM. Docking studies highlighted the characteristics of compounds **3** and **5**, which are important for their high ERβ affinity and ERβ/ERα selectivity. Compound **5** is of particular interest because seems to be able to participate to an unprecedented H-bond with a threonine residue of ERβ. This interaction may be further exploited in the design of more potent ERβ-ligands. Finally, although aldoximes are generally considered to be quite unstable, and to readily undergo hydrolysis, especially under acidic conditions or in biological systems, this is not true for aromatic oximes, whose aqueous hydrolysis rate in buffer solutions is extremely slow even at markedly acidic pH values.[24] As a matter of fact, the most important metabolic pathway of this kind of compounds was shown to be a slow oxidative (and not hydrolytic) cleavage of the oxime portion in liver microsomes, which liberates NO.[24] In our laboratories, we have verified that chemical stability of our final salicylaldoximes in buffer (phosphate buffered saline, pH 7.2) is practically unlimited (>4 weeks). Moreover, cellular assays did not give any indication ascribable to biological instability of these compounds, although more precise metabolic studies are unquestionably necessary. Studies are underway to examine other kinds of structural evolutions, as well as other possible combinations of the modifications applied in the present work, to discover compounds showing improvements in their ERβ-selective agonist potency in gene transcription assays.

Experimental Section

Chemistry. Melting points were determined on a Kofler hot-stage apparatus and are uncorrected. NMR spectra were obtained with a Varian Gemini 200 MHz spectrometer. Chemical shifts (δ) are reported in parts per million downfield from tetramethylsilane and referenced from solvent references. Electron impact (EI, 70 eV) mass spectra were obtained on a HP-5988A mass spectrometer. Where indicated, the elemental compositions of the compounds agreed to within ±0.4% of the calculated value. Chromatographic separations were performed on silica gel columns by flash (Kieselgel 40, 0.040−0.063 mm; Merck) or gravity column (Kieselgel 60, 0.063−0.200 mm; Merck) chromatography. Reactions were followed by thin-layer chromatography (TLC) on Merck aluminum silica gel (60 F_{254}) sheets that were visualized under a UV lamp. Evaporation was performed in vacuo (rotating evaporator). Sodium sulfate was always used as the drying agent. Microwave assisted reactions were run in a CEM microwave synthesizer.

(*E*/*Z*)-2-Chloro-3-(3-fluoro-4-methoxyphenyl)-6-(prop-1-enyl)phenol (14). A solution of $Pd(OAc)_2$ (9 mg, 0.04 mmol) and triphenylphosphine (54 mg, 0.20 mmol) in ethanol (1.7 mL) and toluene (1.7 mL) was stirred at RT under nitrogen for 10 min. After that period, compound **13** (337 mg, 1.36 mmol, 9:1 *E*/*Z* diastereomeric mixture),[16] 1.7 mL of an aqueous 2 M solution of Na_2CO_3, and 3-fluoro-4-methoxyphenylboronic acid (309 mg, 1.76 mmol) were sequentially added. The resulting mixture was heated at 100 °C in a sealed vial under nitrogen overnight. After being cooled to RT, the mixture was diluted with water and extracted with EtOAc. The combined organic phase were dried and concentrated. The crude product was purified by flash chromatography (*n*-hexane/ EtOAc 9:1) to yield **14** as a 9:1 *E*/*Z* diastereomeric mixture (352 mg, 88% yield). ^{1}H NMR ($CDCl_3$, 90:10 *E*/*Z* mixture, asterisk denotes minor isomer peaks) δ (ppm): 1.86* (dd, 3H, $J = 7.2$, 1.8 Hz), 1.94 (dd, 3H, $J = 6.6$, 1.6 Hz), 3.93* (s, 3H), 3.94 (s, 3H), 5.93 (bs, 1H), 6.33 (dq, 1H, $J = 15.9$, 6.6 Hz), 6.70 (dq, 1H, $J = 15.7$, 1.5 Hz), 6.84 (d, 1H, $J = 8.1$ Hz), 7.01 (t, 1H, $J = 8.6$ Hz), 7.12−7.23 (m, 2H), 7.32 (d, 1H, $J = 8.1$ Hz).

3-Chloro-4-(3-fluoro-4-methoxyphenyl)salicylaldehyde (15). A solution of **14** (270 mg, 0.92 mmol) in dioxane (9 mL) was treated with 4.5 mL of water, 449 g of sodium periodate (2.10 mmol), and 0.3 mL of a 2.5% solution of osmium tetroxide in *tert*-butanol (0.02 mmol), and the mixture was stirred at RT for 2 h. Most of dioxane was then removed under vacuum and the mixture was diluted with water and extracted with chloroform. The organic phase was dried and evaporated to afford a crude residue that was purified by flash chromatography (*n*-hexane/EtOAc 9:1) to yield pure **15** (248 mg, 96% yield) as a yellow solid. ^{1}H NMR ($CDCl_3$) δ (ppm): 3.96 (s, 3H), 7.02 (d, 1H, $J = 8.1$ Hz), 7.03−7.09 (m, 1H), 7.19−7.29 (m, 2H), 7.54 (d, 1H, $J = 7.9$ Hz), 9.92 (s, 1H), 11.73 (s, 1H).

3-Chloro-4-(3-fluoro-4-hydroxyphenyl)salicylaldehyde (16). A solution of **15** (170 mg, 0.60 mmol) in anhydrous dichloromethane (7 mL) was cooled to −78 °C and treated dropwise with a 1 M solution of BBr_3 in dichloromethane (1.9 mL), and the resulting solution was stirred at the same temperature for 5 min and at 0 °C for 1 h. The mixture was then diluted with water and extracted with ethyl acetate. The organic phase was dried and concentrated. The crude product was purified by flash chromatography (*n*-hexane/ethyl acetate 7:3) to yield pure **16** (117 mg, 73% yield) as a yellow solid. ^{1}H NMR (acetone-d_6) δ (ppm): 7.12−7.26 (m, 3H), 7.35 (dd, 1H, $J = 12.1$, 1.8 Hz), 7.87 (d, 1H, $J = 8.1$ Hz), 9.09 (d, 1H, $J =$ 1.5 Hz, exchangeable), 10.11 (s, 1H), 11.78 (s, 1H, exchangeable).

(*E*)-3-Chloro-4-(3-fluoro-4-hydroxyphenyl)salicylaldoxime (3). A solution of **16** (90 mg, 0.34 mmol) in ethanol (6.5 mL) was treated with a solution of hydroxylamine hydrochloride (47.3 mg, 0.68 mmol) in water (1.5 mL), and the mixture was heated to 50 °C for 1 h. After being cooled to RT, part of the solvent was removed under vacuum, and the mixture was diluted with water and extracted with EtOAc. The organic phase was dried and evaporated to afford a crude residue that was purified by column chromatography (*n*-hexane/ethyl acetate 7:3) to yield pure **3** (76.8 mg, 80% yield) as a white solid. ^{1}H NMR (acetone-d_6) δ (ppm): 7.02 (d, 1H, $J = 8.1$ Hz), 7.12 (t, 1H, $J = 8.2$ Hz), 7.18 (dd, 1H, $J = 8.2$, 2.0 Hz), 7.28 (dd, 1H, $J = 11.7$, 2.0 Hz), 7.44 (d, 1H, $J = 8.1$ Hz), 8.48 (s, 1H), 8.93 (bs, 1H), 10.94 (s, 1H), 11.00 (bs, 1H). ^{13}C NMR (acetone-d_6) δ (ppm): 114.73, 117.83 (d, $J = 19.2$ Hz), 118.24 (d, $J = 3.7$ Hz), 120.19, 122.52, 126.56 (d, $J = 3.7$ Hz), 129.28, 131.84, 142.31, 145.52 (d, $J = 12.8$ Hz), 151.78 (d, $J = 258$ Hz), 151.87, 154.00. MS *m*/*z* 281 (M^+, 100), 263 ($M^+ - H_2O$, 55). Anal. ($C_{13}H_9ClFNO_3$) C, H, N.

(*E*)-3-Chloro-4-(3-fluoro-4-methoxyphenyl)salicylaldoxime (4). Compound **15** (100 mg, 0.36 mmol) was submitted to the same procedure described above for the preparation of **3**. The crude product was purified by flash chromatography (*n*-hexane/ethyl acetate 7:3) to produce pure **4** (68 mg, 62% yield) as a white solid. ^{1}H NMR (acetone-d_6) δ (ppm): 3.99 (s, 3H), 7.02 (d, 1H, $J = 7.9$ Hz), 7.26−7.35 (m, 3H), 7.45 (d, 1H, $J = 8.1$ Hz), 8.49 (s, 1H),

10.95 (s, 1H), 11.02 (s, 1H). ^{13}C NMR (acetone-d_6) δ (ppm): 56.52, 114.07, 117.69 (d, J = 19.2 Hz), 118.10 (d, J = 4.7 Hz), 120.19, 122.48, 126.39 (d, J = 2.7 Hz), 129.29, 132.61, 142.08, 148.31 (d, J = 10.1 Hz), 151.85, 152.30 (d, J = 244 Hz), 154.33. MS m/z 295 (M^+, 100), 277 ($M^+ - H_2O$, 20). Anal. ($C_{14}H_{11}ClFNO_3$) H, N. C: calcd, 56.87; found, 56.37.

5-(4-Methoxyphenyl)salicylaldehyde (18). Commercially available 5-bromosalicylaldehyde (**17**) (500 mg, 2.48 mmol) was submitted to a cross coupling reaction with 4-methoxyphenylboronic acid (503 mg, 3.29 mmol) following the same procedure described above for the preparation of **14**. The crude product was purified by flash chromatography (*n*-hexane/ethyl acetate 7:3) to produce pure **18** (372 mg, 66% yield) as a yellow solid. ^{1}H NMR ($CDCl_3$) δ (ppm): 3.86 (s, 3H), 6.99 (AA′XX′, 2H, J_{AX} = 8.8 Hz, $J_{AA'/XX'}$ = 2.3 Hz), 7.06 (d, 1H, J = 9.2 Hz), 7.48 (AA′XX′, 2H, J_{AX} = 8.8 Hz, $J_{AA'/XX'}$ = 2.4 Hz), 7.71 − 7.76 (m, 2H), 9.97 (s, 1H), 10.96 (s, 1H, exchangeable).

5-(3-Fluoro-4-methoxyphenyl)salicylaldehyde (19). Commercially available 5-bromosalicylaldehyde (**17**) (500 mg, 2.48 mmol) was submitted to a cross coupling reaction with 3-fluoro-4-methoxyphenylboronic acid (551 mg, 3.23 mmol) following the same procedure described above for the preparation of **14**. The crude product was purified by flash chromatography (*n*-hexane/ethyl acetate 7:3) to produce pure **19** (469 mg, 77% yield) as a yellow solid. ^{1}H NMR ($CDCl_3$) δ (ppm): 3.93 (s, 3H), 6.96−7.13 (m, 3H), 7.21−7.33 (m, 1H), 7.68−7.75 (m, 2H), 9.97 (s, 1H), 10.99 (s, 1H, exchangeable).

5-(4-Hydroxyphenyl)salicylaldehyde (20). Compound **18** (200 mg, 0.87 mmol) was submitted to the same procedure described above for the preparation of **16**. The crude product was purified by flash chromatography (*n*-hexane/ethyl acetate 7:3) to produce pure **20** (133 mg, 71% yield) as a yellow solid. ^{1}H NMR ($CDCl_3$) δ (ppm): 5.23 (bs, 1H), 6.92 (AA′XX′, 2H, J_{AX} = 8.4 Hz, $J_{AA'/XX'}$ = 2.4 Hz), 7.05 (d, 1H, J = 9.2 Hz), 7.43 (AA′XX′, 2H, J_{AX} = 8.6 Hz, $J_{AA'/XX'}$ = 2.5 Hz), 7.69−7.74 (m, 2H), 9.96 (s, 1H), 10.96 (s, 1H, exchangeable).

5-(3-Fluoro-4-hydroxyphenyl)salicylaldehyde (21). Compound **19** (300 mg, 0.98 mmol) was submitted to the same procedure described above for the preparation of **16**. The crude product was purified by flash chromatography (*n*-hexane/ethyl acetate 7:3) to produce pure **21** (209 mg, 87% yield) as a yellow solid. ^{1}H NMR ($CDCl_3$) δ (ppm): 5.20 (bs, 1H), 7.04−7.12 (m, 2H), 7.16−7.32 (m, 2H), 7.68−7.72 (m, 2H), 9.97 (s, 1H), 10.99 (s, 1H, exchangeable).

(*E*)-5-(4-Hydroxyphenyl)salicylaldoxime (5). Compound **20** (110 mg, 0.51 mmol) was submitted to the same procedure described above for the preparation of **3**. The crude product was purified by flash chromatography (*n*-hexane/ethyl acetate 6:4) to produce pure **5** (73 mg, 62% yield) as a white solid. ^{1}H NMR (acetone-d_6) δ (ppm): 6.91 (AA′XX′, 2H, J_{AX} = 8.6 Hz, $J_{AA'/XX'}$ = 2.6 Hz), 6.96 (d, 1H, J = 8.6 Hz), 7.46 (AA′XX′, 2H, J_{AX} = 8.4 Hz, $J_{AA'/XX'}$ = 2.7 Hz), 7.51 (dd, 1H, J = 8.6, 2.4 Hz), 7.60 (d, 1H, J = 2.4 Hz), 8.38 (s, 1H), 8.45 (s, 1H, exchangeable), 10.03 (s, 1H, exchangeable), 10.70 (s, 1H, exchangeable). ^{13}C NMR (acetone-d_6) δ (ppm): 116.27, 117.13, 118.30, 127.96, 128.22, 129.02, 131.79, 133.19, 151.43, 156.23, 157.35. MS m/z 229 (M^+, 100), 211 ($M^+ - H_2O$, 40). Anal. ($C_{13}H_{11}NO_3$) H, N. C: calcd, 68.11; found, 67.49.

(*E*)-5-(4-Methoxyphenyl)salicylaldoxime (6). Compound **18** (150 mg, 0.66 mmol) was submitted to the same procedure described above for the preparation of **3**. The crude product was purified by flash chromatography (*n*-hexane/ethyl acetate 7:3) to produce pure **6** (128 mg, 80% yield) as a white solid. ^{1}H NMR (acetone-d_6) δ (ppm): 3.83 (s, 3H), 6.97 (d, 1H, J = 8.4 Hz), 7.00 (AA′XX′, 2H, J_{AX} = 8.8 Hz, $J_{AA'/XX'}$ = 2.7 Hz), 7.53 (dd, 1H, J = 8.6, 2.4 Hz), 7.55 (AA′XX′, 2H, J_{AX} = 8.8 Hz, $J_{AA'/XX'}$ = 2.5 Hz), 7.63 (d, 1H, J = 2.4 Hz), 8.45 (s, 1H), 10.06 (s, 1H, exchangeable), 10.71 (s, 1H, exchangeable). ^{13}C NMR (acetone-d_6) δ (ppm): 55.57, 115.02, 117.44, 118.28, 128.16, 129.11, 129.49, 133.04, 133.32, 152.65, 157.17, 159.85. MS m/z 243 (M^+, 100), 225 ($M^+ - H_2O$, 25), 210 ($M^+ - H_2O - CH_3$, 45). Anal. ($C_{14}H_{13}NO_3$) C, H, N.

(*E*)-5-(3-Fluoro-4-hydroxyphenyl)salicylaldoxime (7). Compound **21** (190 mg, 0.82 mmol) was submitted to the same procedure described above for the preparation of **3**. The crude product was purified by flash chromatography (*n*-hexane/ethyl acetate 6:4) to produce pure **7** (162 mg, 81% yield) as a white solid. ^{1}H NMR (acetone-d_6) δ (ppm): 6.97 (d, 1H, J = 8.6 Hz), 7.00−7.10 (m, 1H), 7.25−7.33 (m, 1H), 7.38 (dt, 1H, J = 12.5, 2.4 Hz), 7.54 (dd, 1H, J = 8.6, 2.4 Hz), 7.65 (d, 1H, J = 2.4 Hz), 8.45 (s, 1H), 8.68 (d, 1H, J = 1.5 Hz, exchangeable), 10.10 (s, 1H, exchangeable), 10.74 (s, 1H, exchangeable). ^{13}C NMR (acetone-d_6) δ (ppm): 114.57 (d, J = 19.2 Hz), 117.49, 118.31, 118.94 (d, J = 2.7 Hz), 123.12 (d, J = 3.7 Hz), 129.15, 129.46, 132.06 (d, J = 1.8 Hz), 133.28 (d, J = 5.5 Hz), 144.68 (d, J = 12.8 Hz), 152.50 (d, J = 239 Hz), 152.56, 157.42. MS m/z 247 (M^+, 100), 229 ($M^+ - H_2O$, 27). Anal. ($C_{13}H_{10}FNO_3$) C, H, N.

(*E*)-5-(3-Fluoro-4-methoxyphenyl)salicylaldoxime (8). Compound **19** (169 mg, 0.69 mmol) was submitted to the same procedure described above for the preparation of **3**. The crude product was purified by flash chromatography (*n*-hexane/ethyl acetate 7:3) to produce pure **8** (100 mg, 56% yield) as a white solid. ^{1}H NMR (acetone-d_6) δ (ppm): 3.91 (s, 3H), 6.98 (d, 1H, J = 8.4 Hz), 7.15−7.24 (m, 1H), 7.37−7.46 (m, 2H), 7.57 (dd, 1H, J = 8.6, 2.4 Hz), 7.68 (d, 1H, J = 2.4 Hz), 8.46 (s, 1H), 10.11 (s, 1H, exchangeable), 10.74 (s, 1H, exchangeable). ^{13}C NMR (acetone-d_6) δ (ppm): 56.53, 114.57 (d, J = 22.9 Hz), 114.78 (d, J = 7.3 Hz), 117.55, 118.35, 122.88 (d, J = 3.7 Hz), 129.24, 129.53, 131.77 (d, J = 1.8 Hz), 134.02 (d, J = 6.4 Hz), 147.57 (d, J = 11.0 Hz), 152.55, 153.24 (d, J = 243 Hz), 157.57. MS m/z 261 (M^+, 100), 243 ($M^+ - H_2O$, 22), 228 ($M^+ - H_2O - CH_3$, 73). Anal. ($C_{14}H_{12}FNO_3$) C, H, N.

3-Bromo-2-chlorophenyl acetate (23). A solution of **22** (1.8 g, 8.7 mmol)[17] in dioxane (13 mL) was treated with solid NaOH (0.83 g, 21 mmol) and a catalytic amount of tetrabutylammonium hydrogen sulfate (10 mg, 0.028 mmol), and the mixture was stirred under nitrogen at RT for 30 min. A solution of acetyl chloride (0.9 mL, ∼10 mmol) in dioxane (8 mL) was then added with a syringe through a silicon septum. Once the addition was completed, stirring was continued at the same temperature for 1 h. The reaction mixture was then poured into cold (ice) water and extracted with EtOAc. The organic phase was dried and concentrated. The crude product was purified by flash chromatography (*n*-hexane/ethyl acetate 9:1) to yield pure **23** (1.73 g, 79% yield) as a colorless oil. ^{1}H NMR ($CDCl_3$) δ (ppm): 2.36 (s, 3H), 7.10 (d, 1H, J = 8.1, 2.2 Hz), 7.16 (t, 1H, J = 8.2 Hz), 7.53 (dd, 1H, J = 7.5, 2.2 Hz).

6-Acetyl-3-Bromo-2-chlorophenol (24). Compound **23** (400 mg, 1.60 mmol) was treated neat with $AlCl_3$ (278 mg, 2.08 mmol), and the mixture was heated to 130 °C in a sealed vial for 2 h. After cooling to RT, the crude mixture was treated with aqueous 1N HCl and extracted with EtOAc. The organic phase was dried and concentrated. The crude product was purified by flash chromatography (*n*-hexane/ethyl acetate 9:1) to yield pure **24** (252 mg, 64% yield) as an off-white solid. ^{1}H NMR ($CDCl_3$) δ (ppm): 2.65 (s, 3H), 7.22 (d, 1H, J = 8.6 Hz), 7.53 (d, 1H, J = 8.6 Hz).

6-Acetyl-2-chloro-3-(4-methoxyphenyl)phenol (25). Compound **24** (300 mg, 1.40 mmol) was submitted to a cross coupling reaction with 4-methoxyphenylboronic acid (275 mg, 1.80 mmol) following the same procedure described above for the preparation of **14**. The crude product was purified by flash chromatography (*n*-hexane/ethyl acetate 7:3) to produce pure **25** (203 mg, 52% yield) as an off-white solid. ^{1}H NMR ($CDCl_3$) δ (ppm): 2.68 (s, 3H), 3.87 (s, 3H), 6.92 (d, 1H, J = 8.2 Hz), 6.99 (AA′XX′, 2H, J_{AX} = 8.8 Hz, $J_{AA'/XX'}$ = 2.5 Hz), 7.43 (AA′XX′, 2H, J_{AX} = 8.8 Hz, $J_{AA'/XX'}$ = 2.6 Hz), 7.69 (d, 1H, J = 8.2 Hz).

6-Acetyl-2-chloro-3-(3-fluoro-4-methoxyphenyl)phenol (26). Compound **23** (350 mg, 1.41 mmol) was submitted to a cross coupling reaction with 3-fluoro-4-methoxyphenylboronic acid (312 mg, 1.82 mmol) following the same procedure described above for the preparation of **14**. The crude product was purified by flash chromatography (*n*-hexane/ethyl acetate 8:2) to produce pure **26** (208 mg, 50% yield) as an off-white solid. ^{1}H NMR ($CDCl_3$) δ

(ppm): 2.69 (s, 3H), 3.95 (s, 3H), 6.90 (d, 1H, $J = 8.2$ Hz), 7.04 (t, 1H, $J = 8.6$ Hz), 7.18−7.27 (m, 2H), 7.70 (d, 1H, $J = 8.0$ Hz).

6-Acetyl-2-chloro-3-(4-hydroxyphenyl)phenol (27). Compound **25** (155 mg, 0.56 mmol) was submitted to the same procedure described above for the preparation of **16**. The crude product was purified by flash chromatography (*n*-hexane/ethyl acetate 7:3) to produce pure **27** (113 mg, 77% yield) as an off-white solid. ^{1}H NMR ($CDCl_3$) δ (ppm): 2.68 (s, 3H), 5.22 (bs, 1H), 6.88−6.96 (m, 3H), 7.37 (AA′XX′, 2H, $J_{AX} = 8.8$ Hz, $J_{AA'/XX'} = 2.5$ Hz), 7.69 (d, 1H, $J = 8.2$ Hz).

6-Acetyl-2-chloro-3-(3-fluoro-4-hydroxyphenyl)phenol (28). Compound **26** (120 mg, 0.41 mmol) was submitted to the same procedure described above for the preparation of **16**. The crude product was purified by flash chromatography (*n*-hexane/ethyl acetate 8:2) to produce pure **28** (109 mg, 95% yield) as an off-white solid. ^{1}H NMR ($CDCl_3$) δ (ppm): 2.69 (s, 3H), 5.35 (bs, 1H), 6.90 (d, 1H, $J = 8.2$ Hz), 7.08 (t, 1H, $J = 8.1$ Hz), 7.13−7.28 (m, 2H), 7.70 (d, 1H, $J = 8.4$ Hz).

(*E*)-1-(3-Chloro-2-hydroxy-4-(4-hydroxyphenyl)phenyl)ethanone oxime (9). Compound **27** (80 mg, 0.30 mmol) was submitted to the same procedure described above for the preparation of **3**. The crude product was purified by flash chromatography (CH_2Cl_2/MeOH 95:5) to produce pure **9** (70 mg, 84% yield) as a white solid. ^{1}H NMR (CD_3OD) δ (ppm): 2.35 (s, 3H), 6.83 (AA′XX′, 2H, $J_{AX} = 8.8$ Hz, $J_{AA'/XX'} = 2.5$ Hz), 6.87 (d, 1H, $J = 8.2$ Hz), 7.26 (AA′XX′, 2H, $J_{AX} = 8.8$ Hz, $J_{AA'/XX'} = 2.5$ Hz), 7.46 (d, 1H, $J = 8.2$ Hz). ^{13}C NMR (acetone-d_6) δ (ppm): 11.00, 115.58, 119.19, 120.63, 121.63, 126.54, 127.42, 131.31, 147.00, 154.80, 157.92, 158.57. MS *m*/*z* 277 (M^+, 100), 259 ($M^+ - H_2O$, 41). Anal. ($C_{14}H_{12}ClNO_3$) C, H, N.

(*E*)-1-(3-Chloro-2-hydroxy-4-(4-methoxyphenyl)phenyl)ethanone oxime (10). Compound **25** (59 mg, 0.21 mmol) was submitted to the same procedure described above for the preparation of **3**. The crude product was purified by flash chromatography (*n*-hexane/ethyl acetate 7:3) to produce pure **10** (41 mg, 67% yield) as a white solid. ^{1}H NMR (acetone-d_6) δ (ppm): 2.41 (s, 3H), 3.85 (s, 3H), 6.93 (d, 1H, $J = 8.3$ Hz), 7.02 (AA′XX′, 2H, $J_{AX} = 8.8$ Hz, $J_{AA'/XX'} = 2.4$ Hz), 7.41 (AA′XX′, 2H, $J_{AX} = 8.8$ Hz, $J_{AA'/XX'} = 2.3$ Hz), 7.56 (d, 1H, $J = 8.2$ Hz), 10.95 (s, 1H, exchangeable), 12.41 (s, 1H, exchangeable). ^{13}C NMR (acetone-d_6) δ (ppm): 10.94, 55.57, 114.24, 119.35, 120.40, 121.54, 126.54, 131.26, 132.30, 142.97, 155.08, 158.82, 160.27. MS *m*/*z* 291 (M^+, 100), 273 ($M^+ - H_2O$, 30). Anal. ($C_{15}H_{14}ClNO_3$) C, H, N.

(*E*)-1-(3-Chloro-2-hydroxy-4-(3-fluoro-4-hydroxyphenyl)phenyl)ethanone oxime (11). Compound **28** (79 mg, 0.28 mmol) was submitted to the same procedure described above for the preparation of **3**. The crude product was purified by flash chromatography (CH_2Cl_2/MeOH 95:5) to produce pure **11** (46 mg, 55% yield) as a white solid. ^{1}H NMR (CD_3OD) δ (ppm): 2.35 (s, 3H), 6.88 (d, 1H, $J = 8.2$ Hz), 6.96 (*pseudo*-t, 1H, $J = 8.5$ Hz), 7.06 (ddd, 1H $J = 8.2$, 2.0, 0.6 Hz), 7.15 (dd, 1H $J = 8.6$, 2.4 Hz), 7.48 (d, 1H, $J = 8.4$ Hz). ^{13}C NMR (CD_3OD) δ (ppm): 10.78, 117.98 (d, $J = 21.1$ Hz), 118.21 (d, $J = 5.5$ Hz), 120.12, 121.03, 121.83, 126.53, 126.60 (d, $J = 3.7$ Hz), 132.30 (d, $J = 6.0$ Hz), 142.41, 145.83 (d, $J = 12.6$ Hz), 152.13 (d, $J = 240$ Hz), 155.14, 158.36. MS *m*/*z* 295 (M^+, 100), 277 ($M^+ - H_2O$, 38). Anal. ($C_{14}H_{11}ClFNO_3$) C, H. N: calcd, 4.74; found, 4.22.

(*E*)-1-(3-Chloro-2-hydroxy-4-(3-fluoro-4-methoxyphenyl)phenyl)ethanone oxime (12). Compound **26** (63 mg, 0.21 mmol) was submitted to the same procedure described above for the preparation of **3**. The crude product was purified by flash chromatography (*n*-hexane/ethyl acetate 7:3) to produce pure **12** (54 mg, 83% yield) as a white solid. ^{1}H NMR (CD_3OD) δ (ppm): 2.35 (s, 3H), 3.92 (s, 3H), 6.89 (d, 1H, $J = 8.4$ Hz), 7.10−7.23 (m, 3H), 7.49 (d, 1H, $J = 8.2$. Hz). ^{13}C NMR (CD_3OD) δ (ppm): 10.78, 56.72, 114.15 (d, $J = 1.8$ Hz), 117.96 (d, $J = 19.2$ Hz), 120.12, 121.01, 121.76, 126.48, 126.55, 133.41 (d, $J = 7.3$ Hz), 142.06 (d, $J = 1.8$ Hz), 148.63 (d, $J = 10.1$ Hz), 152.81 (d, $J = 238$ Hz), 155.30, 158.30. MS *m*/*z* 309 (M^+, 100), 291 ($M^+ - H_2O$, 37). Anal. ($C_{15}H_{13}ClFNO_3$) C, H, N.

Biological Methods. Full-length human ERα and ERβ were obtained from PanVera/Invitrogen (Carlsbad, CA). [^{3}H] Estradiol ([^{3}H]E_2) ([2,4,6,7-^{3}H]estra-1,3,5(10)-triene-3,17β-diol) was obtained from GE Healthcare (Piscataway, NJ) with a specific activity of 70−120 Ci/mmol. Cell culture media were purchased from Gibco BRL (Grand Island, NY). Calf serum was obtained from Hyclone Laboratories, Inc. (Logan, UT), and fetal calf serum was purchased from Atlanta Biologicals (Atlanta, GA). The expression vectors for human ERα (pCMV5-hERα) and human ERβ (pCMV5-ERβ) were as described previously.[25,26] The estrogen responsive reporter plasmid, $(ERE)_2$-pS2-Luc, was constructed by inserting the $(ERE)_2$-pS2 fragment from $(ERE)_2$-pS2-CAT into the *Mlu*I/*Bgl*II sites of pGL3-Basic vector (Promega, Madison, WI). The luciferase assay system was from Promega (Madison, WI). The plasmid pCMVβ-gal (Clontech, Palo Alto, CA), which contains the β-galactosidase gene, was used as an internal control for transfection efficiency.

Estrogen Receptor Binding Assays. Relative binding affinities were determined by competitive radiometric binding assays with 2 nM [^{3}H]E_2 as tracer, as a modification of methods previously described.[20,21] The source of ER was purified full-length human ERα and ERβ purchased from Pan Vera/Invitrogen (Carlsbad, CA). Incubations were done at 0 °C for 18−24 h, and hydroxyapatite was used to absorb the purified receptor−ligand complexes (human ERs).[21] The binding affinities are expressed as relative binding affinity (RBA) values, where the RBA of estradiol is 100%; under these conditions, the K_d of estradiol for ERα is ca. 0.2 nM, and for ERβ 0.5 nM. The determination of these RBA values is reproducible in separate experiments with a CV of 0.3, and the values shown represent the average ± range or SD of 2 or more separate determinations.

Cell Culture and Transient Transfections. Human endometrial cancer (HEC-1) cells were maintained in culture as described.[23] Transfection of HEC-1 cells in 24-well plates used a mixture of 0.35 mL of serum-free MEM medium and 0.15 mL of HBSS containing 5 μL of lipofectin (Life Technologies, Rockville, MD), 20 μL of transferrin (Sigma, St. Louis, MO), 0.2 μg of pCMVβ-galactosidase as internal control, 0.5 μg of the reporter gene plasmid, and 50 ng of ER expression vector. The cells were incubated at 37 °C in a 5% CO_2 containing incubator for 4 h. The medium was then replaced with fresh medium containing 5% charcoal-dextran treated calf serum and the desired concentrations of ligands. Reporter gene activity was assayed at 24 h after ligand addition. Luciferase activity, normalized for the internal control β-galactosidase activity, was assayed as described.[23]

Docking Methods. The crystal structure of ERα (pdb code 2I0J[27]) and ERβ (pdb code 2I0G[27]) was taken from the Protein Data Bank.[28] After adding hydrogen atoms the two proteins complexed with their reference inhibitor were minimized using Amber 9 software[29] and parm03 force field at 300 K. In detail, the complexes were placed in a rectangular parallelepiped water box, an explicit solvent model for water, TIP3P, was used, and the complexes were solvated with a 10 Å water cap. Sodium ions were added as counterions to neutralize the system. Two steps of minimization were then carried out; in the first stage, we kept the protein fixed with a position restraint of 500 kcal/mol/Å^2 and we solely minimized the positions of the water molecules. In the second stage, we minimized the entire system through 5000 steps of steepest descent followed by conjugate gradient (CG) until a convergence of 0.05 Kcal/Å·mol.

The ligands were built using Maestro[30] and were minimized by means of Macromodel[31] in a water environment using the CG method until a convergence value of 0.05 kcal/Å·mol, using the MMFFs force field and a distance-dependent dielectric constant of 1.0.

Automated docking was carried out by means of the AUTODOCK 4.0 program;[32] Autodock Tools[33] was used in order to identify the torsion angles in the ligands, add the solvent model, and assign the Kollman atomic charges to the protein. The ligand charge was calculated using the Gasteiger method. To prevent the loss of the intramolecular H-bond of the pseudocycle/oxime system,

during the docking, we blocked the torsions involved in this intramolecular bond.[13] The regions of interest used by Autodock were defined by considering SERBA-1 into both receptors as the central group; in particular, a grid of 50, 40, and 46 points in the *x*, *y*, and *z* directions was constructed centered on the center of the mass of this compound. A grid spacing of 0.375 Å and a distance-dependent function of the dielectric constant were used for the energetic map calculations.

Using the Lamarckian Genetic Algorithm, the docked compounds were subjected to 100 runs of the Autodock search, using 500000 steps of energy evaluation and the default values of the other parameters. Cluster analysis was performed on the results using an rms tolerance of 1.0 Å, and the best docked conformation was used for the analysis.

All graphic manipulations and visualizations were performed by means of Chimera.[34]

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Supporting Information Available: Combustion analysis data of final compounds **3**−**12**. This material is available free of charge via the Internet at http://pubs.acs.org.

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