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Original Article

Selective and potent agonists for estrogen receptor beta derived from molecular refinements of salicylaldoximes

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ABSTRACT

In a continuing effort to improve the subtype selectivity and agonist potency of estrogen receptor β (ERβ) ligands, we have designed and developed a thus far unexplored structural series obtained by molecular refinements of *monoaryl*-substituted salicylaldoximes (*Salaldox B*). The most interesting compounds in this series (**2c**, **d**) show remarkably high ERβ-binding affinities, with K_i values reaching the sub-nanomolar range ($K_i = 0.38$ nM for **2c** and 0.57 nM for **2d**), and have very high levels of ERβ-subtype selectivity. Both compounds show a potent full agonist character on ERβ ($EC_{50} = 0.23$ nM for **2c** and 1.3 nM for **2d**). Furthermore, **2d** shows a remarkable functional subtype selectivity, with a β/α transcription potency ratio 50-fold higher than that of estradiol.

1. Introduction

Since the discovery of estrogen receptor beta (ERβ) in 1996 [1], much effort has been devoted to the search for molecules that are able to selectively interact with this receptor [2,3]. Such ERβ-selective agonists hold promise for the treatment of diverse diseases, such as rheumatoid arthritis and inflammatory diseases [4], certain cancers [5,6], endometriosis [7], as well as cardiovascular [8] and CNS pathologies [9]. The therapeutic potential of ERβ-selective compounds appears to be particularly favourable because the beneficial effects of stimulation through ERβ would be expected to be free from undesired proliferative effects on breast and uterus, which are mediated largely by the other receptor subtype, ERα [10,11].

ERβ, as well as ERα, belong to the superfamily of nuclear receptors that act as ligand-regulated transcription factors [12]. Their amino acid sequence shows 59% identity in the ligand binding domain (LBD), although the differences within the ligand binding cavities consist of only two, conservative amino acid substitutions. In fact, of the 23 amino acid residues that line the ligand binding cavities of the two ERs (within 4 Å of the ligand), only two are different: Leu384 and Met421 of ERα are replaced by Met336 and Ile373, respectively, in ERβ [1]. These slight modifications and other minor alterations of tertiary structure make the volume of the ERβ-binding pocket smaller than the one in ERα and somewhat different in shape. Due to the lack of a pronounced difference between the two receptor subtypes, the design and development of molecules that selectively bind to and activate ERβ is not a trivial task, although there are now several pharmacophore models for the kind of molecular frameworks more likely to engender ER subtype selectivity [3].

We recently reported on two restricted series of monoaryl-substituted salicylaldoximes bearing a *para*-hydroxylated aryl substituent at either position 4 (*Salaldox A*) [13,14] or 5 (*Salaldox B*) [14] of the central ring. These were inspired by a consolidated pharmacophore model originally developed for indazole derivatives [15] (Fig. 1). These compounds derive from our original observation that one of the two phenol groups typically present in non-steroidal ER-ligands could be isosterically replaced by a six-membered pseudocycle, formed by an intramolecular H-bond involving the phenol and the oxime nitrogen atom, which had led

Abbreviations: ER, estrogen receptor; ERα, estrogen receptor subtype alpha; ERβ, estrogen receptor subtype beta; LBD, ligand binding domain; RBA, relative binding affinity; RTP, relative transcriptional potency; TLC, thin-layer chromatography; CG, conjugated gradient.

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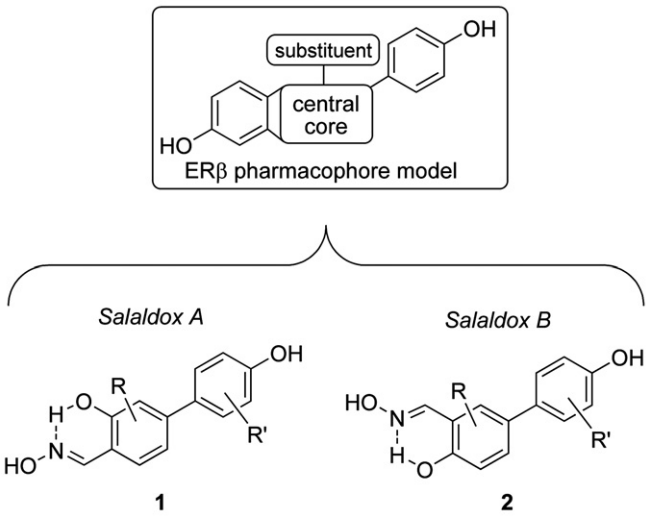
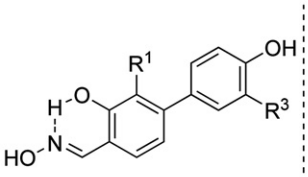


Fig. 1. Structural derivation of the two series of salicylaldoximes, *Salaldox A* (**1**) and *Salaldox B* (**2**), from the ERβ pharmacophore model.

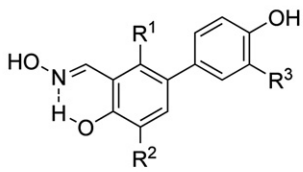
us to initially develop several diaryl-substituted salicyl- [16] and anthranyl-aldoximes [17] as non-subtype-selective ER-ligands.

We initially obtained highly selective ERβ-ligands belonging to the *Salaldox A* series (Fig. 2) by progressively refining the substitution pattern (compounds **1a**, **c**, and **d**) [13,14], and we confirmed the detrimental effect of a second aryl substituent on ERβ binding (**1b**) [16]. However, even the best *Salaldox A* members proved to be only partial agonists for ERβ, since their maximal activation values, compared to estradiol, were of 60% for **1c** and 85% for **1d** [13,14]. Moreover, the beta-selectivities shown by these two compounds in functional assays were considerably less than their ERβ-selectivities in binding assays, probably because of differences in the manner in which the ERα- and ERβ-ligand complexes interact with various cellular coregulators, which can act as modulators of ligand potency.



1a-d

compound	R^1	R^3
1a	H	H
1b	*p*-OH-Ph	H
1c	Cl	H
1d	Cl	F



2a-g

compound	R^1	R^2	R^3
2a	H	H	H
2b	*p*-OH-Ph	H	H
2c	Cl	H	H
2d	Cl	H	F
2e	Cl	CH_3	H
2f	CH_3	Cl	H
2g	CH_3	Cl	F

Fig. 2. Reference salicylaldoximes (**1a**–**d**, **2a**) and new derivatives (**2b**–**g**) designed as ERβ-selective agonists.

We later identified the simplest member of the *Salaldox B* class (**2a**) as a promising beta-selective ligand and, notably, also as the first ERβ *full* agonist among our oxime derivatives [14]. Nonetheless, despite its high ERβ-binding preference, compound **2a** also experienced a loss of beta-selectivity in transcriptional assays, most likely for the same reasons reported above for compounds **1c** and **1d**.

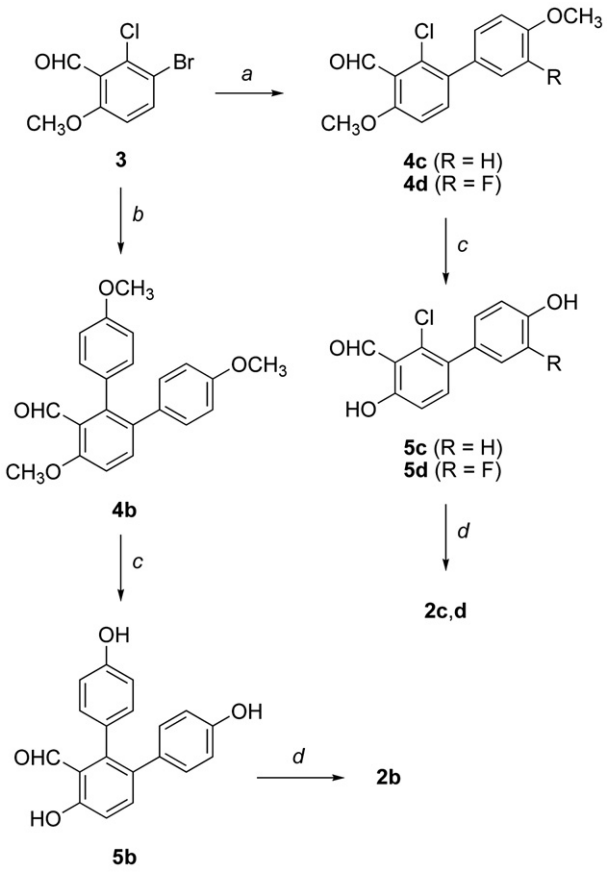
To overcome these limitations, we have continued our efforts to obtain compounds that can both bind and activate ERβ in a highly efficient and selective fashion. To this purpose, we herein report a logical extension of our structural optimization process to the *Salaldox B* class, using some of the same molecular interventions that proved successful in the *Salaldox A* series of derivatives studied previously, combined with new insights from molecular modeling. In the process, we prepared a focused series of analogs of **2a** through which we have investigated the effect of introducing a 6-chloro atom in the central ring (**2c**) and an additional 3′-F-group in the aryl substituent (**2d**). We also introduced relatively small substituents (such as methyl and chlorine) in position 3 of the central ring, a place for molecular variations that has so far been unexplored within these salicylaldoxime derivatives. We first introduced a 3-methyl group (**2e**) and then inverted the respective 3/6-positions of the methyl and chlorine groups, such as in **2f** and in its 3′-fluoro-substituted analog **2g**. Finally, we obtained compound **2b** as a *Salaldox B* analog of compound **1b**, to verify whether the same structural restrictions in the *Salaldox A* series would also apply to this new series of ERβ-ligands.

2. Chemistry

The synthesis of compounds **2b**–**d** started from 3-bromo-2-chloro-6-methoxybenzaldehyde (**3**) [18], as shown in Scheme 1. When compound **3** was submitted to a single cycle of Pd-catalyzed cross-coupling reaction under classical Suzuki conditions [19] (specifically, *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) in the presence of 1.2 equivalent of the boronic acid, 4-methoxyphenylboronic or 3-fluoro-4-methoxyphenylboronic acid, it selectively formed the corresponding monoaryl-substituted adducts **4c**, **d**, respectively. In fact, these conditions resulted in the chemo-selective replacement of only the bromine atom of **3**, with the chloro group remaining intact. On the other hand, the repetition of two reaction cycles under the same Suzuki conditions, using a total of 3.2 equivalents of boronic acid, afforded mostly the diaryl-substituted intermediate **4b**. The resulting adducts were treated with boron tribromide to obtain *O*-demethylated salicylaldehydes **5b**–**d**, which were then condensed with hydroxylamine hydrochloride, thus yielding final salicylaldoximes **2b**–**d**.

The synthetic route to methyl/chlorine-substituted oximes **2e**–**g** was slightly longer, as shown in Scheme 2. Commercially available phenols **6e**, **f** underwent *O*-allylation upon treatment with allyl bromide. Claisen rearrangement of the resulting ethers **7e**, **f** at 210 °C yielded *o*-allyl-phenols **8e**, **f**. The terminal double bond of **8e**, **f** was shifted to the internal position by alkaline isomerisation with potassium *tert*-butoxide in DMSO at 55 °C, to give β-methylstyrene derivatives **9e**, **f** as *E*-diastereomers. Oxidative cleavage of the styrene-type double bond with sodium periodate in the presence of catalytic amounts of osmium tetroxide yielded aldehydes **10e**, **f**, which were then treated with bromine in glacial acetic acid, affording mono-brominated salicylaldehyde derivatives **11e**, **f**.

At this point, we initially tried to carry out a cross-coupling reaction of **11e**, **f** with the appropriate boronic acid, to effect the replacement of the bromine atom with a suitable aryl substituent.



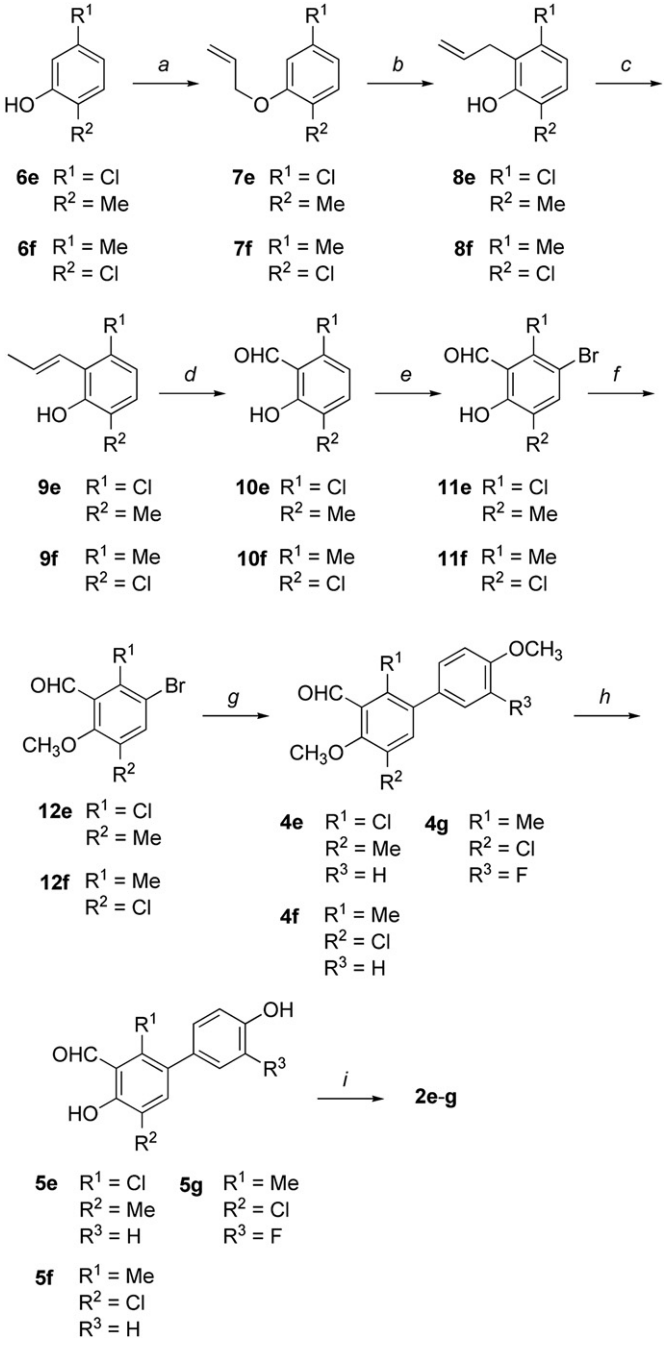
Scheme 1. Synthesis of salicylaldoximes **2b**–**d**. Reagents and conditions: (a) 4-MeO-$C_6H_4B(OH)_2$ or 3-F-4-MeO-$C_6H_4B(OH)_2$ (1.2 eq), $Pd(OAc)_2$, PPh_3, aqueous 2 M Na_2CO_3, 1:1 toluene/EtOH, 100 °C, 16 h; (b) 2 times: 4-MeO-$C_6H_4B(OH)_2$ (1.6 eq), $Pd(OAc)_2$, PPh_3, aqueous 2 M Na_2CO_3, 1:1 toluene/EtOH, 100 °C, 16 h; (c) BBr_3, CH_2Cl_2, −78 to 0 °C, 1 h; (d) $NH_2OH{\cdot}HCl$, EtOH–H_2O, 50 °C, 5 h.

Unfortunately, we were not able to perform this reaction efficiently, probably because of the bidentate chelating effect that the free vicinal OH/CHO groups of the salicylaldehyde portion have on both the boron atom of the boronic acid and on the palladium catalyst of the cross-coupling reaction, thus diverting the reactants from the correct reaction pathway during the Suzuki step. Therefore, we first protected the phenol hydroxyl with a methyl group, by reaction with iodomethane and potassium carbonate in acetone, and then submitted the resulting anisole derivatives **12e**, **f** to the Suzuki coupling reaction. This time the reaction worked nicely, and monoaryl adducts **4e**–**g** were so obtained. Final steps included *O*-demethylation with BBr_3, to give intermediates **5e**–**g**, and subsequent condensation with hydroxylamine hydrochloride, which afforded final oximes **2e**–**g**.

All the oximes (**2b**–**g**) were obtained as single *E*-diastereoisomeric forms, presumably because the intramolecular hydrogen bond, which can only form in the *E*-isomer, contributes to the oxime stability. This selectivity had already been demonstrated for other oxime analogs previously reported [13,14,16,17], and it was confirmed here by the chemical shift values of the oxime protons of all the new products, which were always found downfield from 8 ppm ($\delta \geq 8$, see Section 7) [20].

3. Estrogen receptor binding assays

ERα- and ERβ-binding affinities of new oximes **2b**–**g** were determined by a radiometric competitive binding assay, using



Scheme 2. Synthesis of salicylaldoximes **2e**–**g**. Reagents and conditions: (a) allyl bromide, K_2CO_3, acetonitrile, 80 °C; (b) neat, 180 °C; (c) *t*-BuOK, DMSO, 55 °C; (d) OsO_4, $NaIO_4$, dioxane-H_2O; (e) Br_2, AcOH, RT; (f) MeI, K_2CO_3, acetone, RT; (g) 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; (h) BBr_3, CH_2Cl_2, −78 to 0 °C, 1 h; (i) $NH_2OH{\cdot}HCl$, EtOH–H_2O, 50 °C, 5 h.

methods that have been previously described [21,22]. The relative binding affinity (RBA) values for the newly reported compounds, together with those previously obtained for compounds **1a**–**d** and **2a** [13,14,16b], are summarized in Table 1. RBA values are reported as percentages (%) of that of estradiol, which is set at 100% (Entry 1).

We first analyzed some relevant results obtained previously with the *Salaldox A* series (Table 1, Entries 2–5): It turns out that the simplest member of this class (**1a**) is already a very ERβ-selective ligand (RBA β/α ratio = 79), although its binding affinity for the

Table 1
Relative binding affinities[a] of compounds of the *Salaldox A* (**1a**–**d**) and *Salaldox B* (**2a**–**g**) series for the estrogen receptors α and β.

Entry	Ligand	hERα (%)	hERβ (%)	β/α ratio
1	Estradiol	(100)	(100)	1
Salaldox A Series				
2	**1a**[b]	0.007 ± 0.001	0.55 ± 0.11	79
3	**1b**[c]	0.92 ± 0.04	0.35 ± 0.01	0.38
4	**1c**[b]	0.065 ± 0.016	4.21 ± 0.66	65
5	**1d**[d]	0.11 ± 0.03	7.01 ± 1.00	64
Salaldox B Series				
6	**2a**[d]	0.064 ± 0.016	2.64 ± 0.62	41
7	**2b**	88.4 ± 18.1	101 ± 2	1.1
8	**2c**	4.46 ± 0.60	130 ± 25	29
9	**2d**	1.88 ± 0.30	87.1 ± 15.0	46
10	**2e**	0.074 ± 0.006	0.64 ± 0.09	8.6
11	**2f**	1.47 ± 0.04	15.8 ± 3.5	11
12	**2 g**	0.39 ± 0.04	7.90 ± 0.40	20

[a] Determined by a competitive radiometric binding assay with [^{3}H]estradiol; preparations of purified, full-length human ERα and ERβ (Invitrogen, PanVera) were used; see Section 7. 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 ERβ 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. [13].
[c] See Ref. [16b].
[d] See Ref. [14].

beta-receptor was rather modest (0.55%). Insertion of a chlorine into the 6 position of the central scaffold, as in compound **1c**, preserved the high selectivity level (RBA β/α ratio = 65) and markedly increased the ERβ-binding affinity (4.21%) [13]. By contrast, insertion of a second *para*-hydroxyphenyl substituent in this scaffold produces a compound (**1b**) whose affinity for ERβ was dramatically reduced (0.35%) [16b], thus confirming the importance of the *monoaryl*-substitution motif within this class. Being inspired by a few very successful examples reported in the literature, such as biphenylcarbaldehyde oxime derivatives [23] and benzoxazole ERB-041 [24], we introduced a fluorine atom in the 3′-position of the *para*-hydroxyphenyl group of **1c**, and the compound thus obtained (**1d**) possessed a higher affinity for ERβ (7.01%) than its non-fluorinated counterpart, together with a similar subtype selectivity (RBA β/α ratio = 64).

We then turned to analysis of the binding affinity of members of the *Salaldox B* series. Although this series was designed by a completely different structural modification of **1a**, which involved an inversion of the respective positions of the hydroxy and oxime groups of the salicylaldoxime scaffold, we were surprised to find that the simplest member of *Salaldox B* series (**2a**) had a 5-fold higher ERβ-binding affinity (2.64%) compared to **1a** (Table 1, Entries 2 and 6). Most importantly, the ERα binding affinity of **2a** remained quite low (0.064%), so that this compound retained a notable beta-selectivity (RBA β/α ratio = 41) [14]. This promising behavior of the "progenitor" member of the *Salaldox B* series (**2a**) indicated that this series merited further exploration.

Among the newly synthesized *Salaldox B* derivatives (Table 1, Entries 7–12), compound **2c**, possessing a chlorine atom in the central scaffold, displays an outstanding affinity for ERβ, with a RBA value of 130% (corresponding to a K_i of 0.38 nM), and a robust beta-selectivity (RBA β/α ratio = 29). It should be noted that the affinity of **2c** for ERβ is significantly higher than that of estradiol itself. Compound **2d**, derived from an addition of a *meta*-fluorine in the *para*-hydroxylated aryl substituent, has a considerably higher ERβ-selectivity (RBA β/α ratio = 46) than **2c**, thus confirming that this kind of structural modification often leads to an improved preference for the beta-subtype, as was seen before in the *Salaldox A* series [14]. The binding affinity of **2d** for ERβ is also remarkably high, as shown by its 87% RBA value (corresponding to a K_i of 0.57 nM). By contrast, the addition of a methyl group to the central ring of compound **2c** results in a compound (**2e**) that has a dramatic drop in affinity for both receptor subtypes (RBA = 0.074% for ERα and 0.639% for ERβ). A marked recovery of binding properties is obtained, however, when the relative positions of the methyl and chlorine substituents of **2e** are reversed, as shown by the good affinity values associated with compound **2f** (RBA = 1.47% for ERα and 15.8% for ERβ), although the beta-selectivity is not as high as desired (RBA β/α ratio = 11). Here again, introduction of a *meta*-fluorine atom into the 4-hydroxyphenyl substituent of **2f**, leading to **2g** (RBA = 0.392% for ERα and 7.90% for ERβ), effects a 2-fold increase of ERβ-selectivity (RBA β/α ratio = 20). Finally, the single *Salaldox B* member bearing two *para*-hydroxyphenyl substituents (**2b**) is the only one not showing any appreciable preference for the beta-subtype (RBA β/α ratio = 1.1), although its affinities for both receptors (RBA = 88.4% for ERα and 101% for ERβ) are remarkably higher than those of its *Salaldox A* analog **1b**, reaching values that are surprisingly close to those of estradiol. This last result further supports our original hypothesis that the *monoaryl*-substitution motif is a strict prerequisite for obtaining good ERβ-selectivity in this type of salicylaldoxime derivative [13,14].

4. Molecular modeling

An automated computational analysis of the newly synthesized compounds was performed to try to rationalize their binding properties. Docking of the ligands into ERα and ERβ (PDB codes 2I0J and 2I0G, respectively) was carried out using AUTODOCK 4.0 software [25]. Fig. 3A and B displays the docking results for *diaryl*-substituted compound **2b** into both ERs. In agreement with its similar affinity for both receptor subtypes, this compound shows the same interactions in the ERα and ERβ ligand binding pockets. The *para*-hydroxyl group on the aryl substituent *distal* to the oxime function is involved in an H-bond network, which includes (ERα residues in parentheses) E305 (E353), R346 (R394), and a water molecule. The *para*-hydroxyl of the aryl substituent *proximal* to the oxime function group forms an H-bond with T299 (T347). Finally, the pseudocycle/oxime system forms an H-bond with H475 (H524). All of these supposedly strong interactions confirm the very high binding affinities that **2b** has for both ERα and ERβ.

The docking of *monoaryl*-substituted compound **2c** (Fig. S1B, Supplementary data) and **2d** (Fig. 3D) into ERβ produces results substantially similar to those previously obtained with their non-halogenated analog **2a** [14], and highlights that: (1) the pseudocycle/oxime system is engaged in the H-bond network of the E305-R346-water system; and (2) the OH of the *p*-hydroxyphenyl ring forms a H-bond with T299. In both compounds, the chlorine atom is inserted into a pocket delimited by A302, W335, M336, and L339. It should be noted that this orientation is completely different from that previously found in the docking analysis of *Salaldox A* derivatives such as **1c** and **1d** into ERβ-LBD, which instead place their *p*-hydroxyphenyl substituent in the H-bond network of the E305-R346-water system and the pseudocycle/oxime portion forming a H-bond with H475 [14]. It is interesting to note that the strong interaction between the 4′-hydroxyl of the ligands and T299 is possible only in the ERβ-LBD cavity, because only in this ER subtype is there enough space for the phenol group to reach the OH of T299 by occupying an area close to M336. The same does not happen in ERα, where the methionine (M336 in ERβ) is replaced by a bulkier and less flexible leucine (L384 in ERα) [26], causing a completely different disposition of the two compounds in this receptor subtype. In fact, compared to what happens in ERβ, in ERα **2c** (Fig. S1A, Supplementary data) and **2d**

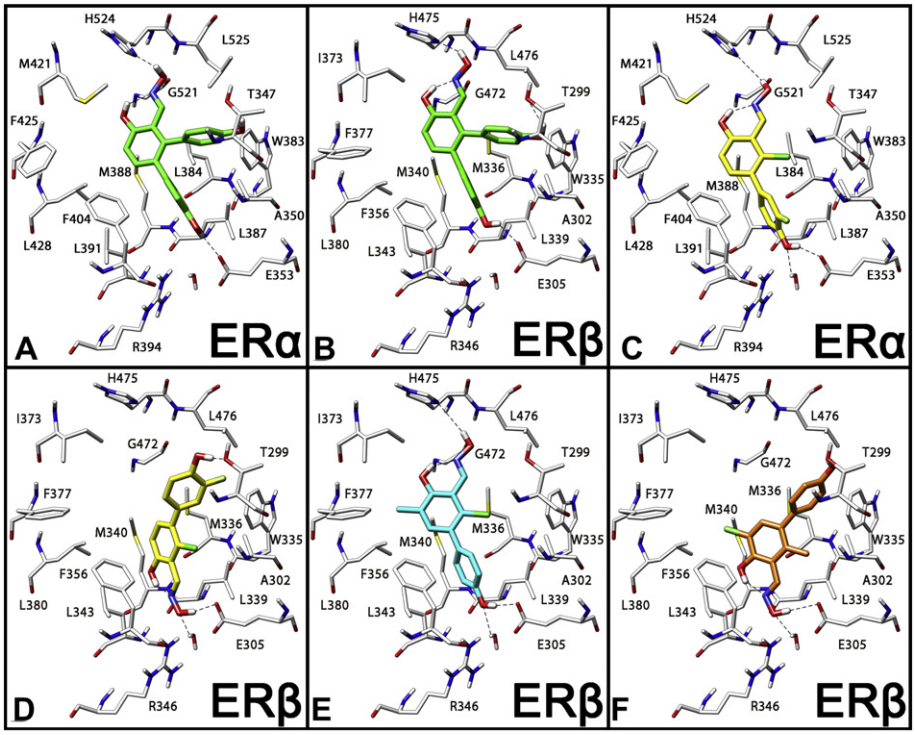


Fig. 3. Docking analysis into ERα and ERβ: (A) docking of **2b** (green) into ERα; (B) docking of **2b** (green) into ERβ; (C) docking of **2d** (yellow) into ERα; (D) docking of **2d** (yellow) into ERβ; (E) docking of **2e** (light blue) into ERβ; (F) docking of **2f** (orange) into ERβ. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

(Fig. 3C) are turned upside down such that their phenol 4′-OH group is involved in the H-bond network with E353, R394, and a water molecule, and their pseudocycle/oxime system forms a H-bond with H524.

The addition of a methyl group in the position *para* to the chloro-substituent of the central ring, as in **2e** (Fig. 3E), is not tolerated in the same low energy conformation assumed by **2c** and **2d** in ERβ, because the binding region in the proximity of M340 is not large enough to tolerate the presence of a CH_3. This causes the orientation of **2e** to become rotated by 180° in the ERβ-binding pocket, resulting in a complex that is less stable than those with **2c** or **2d**. Thus, **2e** has a dramatically reduced binding affinity for ERβ. On the other hand, if the position of the two CH_3/Cl central substituents is interchanged, the resulting compounds, **2f** (Fig. 3F) and **2g** (Fig. S2, Supplementary data), have a preferred conformation in ERβ that is the same as the one found for compounds **2c** and **2d**, because the methyl substituent of **2f** and **2g** can now be accommodated in the pocket delimited by A302, W335, M336, and L339, while the chlorine atom, being smaller than the methyl group present in an analogous position of **2e**, now fits nicely in the pocket close to M340. These factors result in the good binding affinity that both **2f** and **2g** have for ERβ, although their values are not better than those found for **2c** and **2d**.

It should be noted that in our modeling studies, the analyzed ligands were simply docked into a single, rigid version of the receptor, and we did not computationally evaluate the possible fit-induced effects. This kind of approach requires the use of a flexible receptor and, hence, is much more computationally intensive than rigid receptor docking. At present, the main docking software programs are able to take into account the flexibility of only a small number of residues, making the possibility of evaluating the flexibility of a binding site very difficult. For these reasons, we used the ERβ–**2d** complex as a test set for a two-layer QM/MM calculation using Gaussian09 to verify the reliability of our docking results [27]. The ERβ–**2d** complex obtained from the docking studies was energy-minimized and then subjected to QM/MM, which has been so far used with good success by many authors in the field of drug design to find correct interactions within biological systems [28]. In these calculations, the zone of highest interest is treated quantum mechanically, while the rest of the system is treated by classical mechanics, thus reducing computational expenses. We used the ONIOM module of Gaussian09, using the B3LYP chemical model for the quantum mechanics (QM) system [29], with a 6-31G++** basis set and the Amber force field (parm96) for the molecular mechanics (MM) system. The QM system consisted of the ligand, the structural water molecule, and the side chains of T299, E305 and R346. Fig. 4 shows the superimposition between the starting ERβ–**2d** complex and the QM/MM-optimized one. The ligand maintained the interactions with T299 and the E305-R346-water system, showing a Root Mean Square Deviation (RMSD) from the starting structure of only 0.3 Å. Also, the binding site residues showed only small movements, with an RMSD of 0.7 Å.

5. Transcription assays

ERβ-selective ligands **2b**–**d**, displaying the highest levels of binding affinity and selectivity for ERβ, as well as previously reported reference compounds **1c**, **1d** and **2a**, 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 [30].

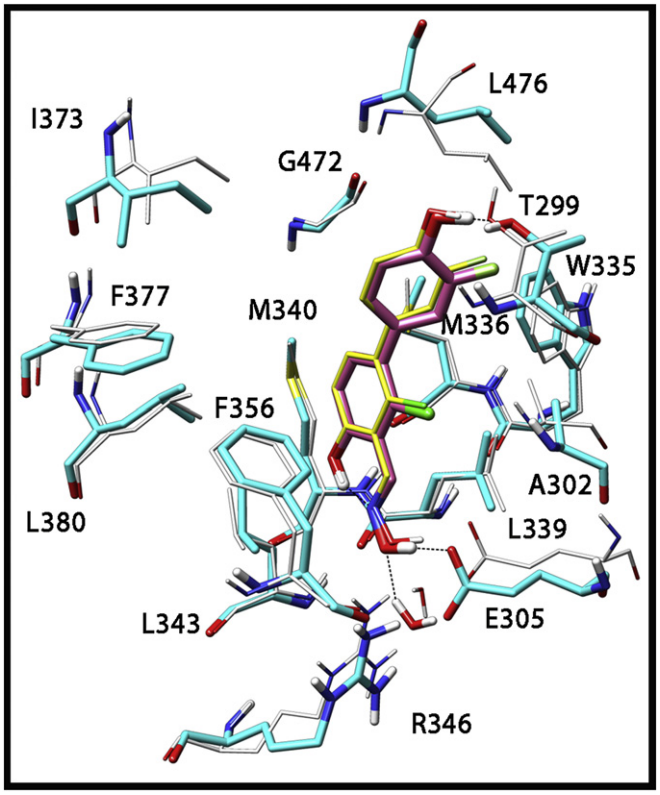


Fig. 4. QM/MM simulation of the ERβ–**2d** complex. Superimposition between the QM/MM results (binding site and ligand coloured light blue and pink, respectively) and the structure resulting from the docking calculations (binding site and ligand coloured white and yellow, respectively). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

The first representative examples of the previously reported *Salaldox A* series (Table 2, Entries 1–2), compound **1c**, reached only partial activation of ERβ (E_{MAX} = 60%) [13], whereas its "fluorinated" analog **1d** displayed a general improvement in all functional properties, such as potency (EC_{50} = 4.8 nM), maximal effect (E_{MAX} = 85%), and subtype selectivity (β/α EC_{50}-selectivity ratio = 4.0) [14]. In any case, these *Salaldox A* members could only be classified as *partial* agonists, because none of them reached maximal activation of transcription (normalized to that resulting from 100 nM estradiol). By contrast, the first example of the *Salaldox B* series, compound **2a**, behaved as a full agonist on both receptor subtypes, but its functional beta-selectivity was not satisfactory (Entry 3).

Among the newly synthesized *Saladox B* ligands (Table 2, Entries 4–6), *diaryl*-substituted derivative **2b**, which proved to be non-subtype-selective in binding assays, shows a similar lack of selectivity in these transcriptional assays, where it shows only a slight preference for ERα (EC_{50} = 0.30 nM, E_{MAX} = 90%) vs. ERβ (EC_{50} = 0.50 nM, E_{MAX} = 80%). *Monoaryl*-substituted derivatives **2c** and **2d** show preferential activation of ERβ rather than ERα, consistent with their ERβ-binding selectivities, and, similar to their "progenitor" **2a**, both cause full activation of transcription. In particular, the single insertion of a chlorine atom in the central ring of **2a** generates a full agonist (**2c**) having a >40-fold increased potency on ERβ and reaching a sub-nanomolar EC_{50} value (0.23 nM) and a slightly better beta-selectivity than **2a**. The combined addition of the central chlorine group and the 3′-fluorine atom in the pendant aryl substituent, as in compound **2d**, results in a jump in the selectivity of ERβ-activation (β/α EC_{50}-selectivity ratio = 6.5), together with a substantial preservation of full agonist potency (EC_{50} = 1.3 nM).

Table 2
Transcription potencies of ERβ-ligands of the *Salaldox A* (**1c**, **d**) and *Salaldox B* (**2a**–**d**) series through human estrogen receptors α and β.[a]

Entry	Ligand	*h*ERα activation		*h*ERβ activation		β/α EC_{50}-selectivity ratio
		EC_{50} (nM)[b]	E_{MAX} (%)[c]	EC_{50} (nM)[b]	E_{MAX} (%)[c]	
Salaldox A Series						
1	**1c**[d]	26	80	11	60	2.4
2	**1d**[e]	19	95	4.8	85	4.0
Salaldox B Series						
3	**2a**[e]	17	100	10	100	1.7
4	**2b**	0.30	90	0.50	80	0.6
5	**2c**	0.58	100	0.23	100	2.5
6	**2d**	8.4	100	1.3	100	6.5
7	Estradiol	0.09	100	0.72	100	0.12

[a] Human endometrial cancer (HEC-1) cells, transfected with expression vectors for ERα or ERβ and an $(ERE)_2$-pS2-luc reporter gene, were treated with the indicated compound and resulting luciferase activity was measured as expression of transcription (see Section 7).
[b] Half-maximal effective concentration.
[c] Maximal effect normalized to the activity with 100 nM estradiol, which was set at 100%.
[d] See Ref. [13].
[e] See Ref. [14].

Overall, as we observed in the past [13,14], there is a general reduced ERβ-selectivity in terms of transcriptional potency vs. binding affinity. This apparent discrepancy may be attributed to the fact that the receptor–ligand complex is present by itself in the binding assays, whereas in the cellular transcription assays it is engaged in various interactions with the many coregulators present in the cell; these receptor–coregulator binding interactions can act as additional modulators of ligand potency [31]. It should be noted that both **2c** and **2d** are by far more beta-selective full agonists than is estradiol, which has EC_{50} values of 0.72 nM on ERβ vs. 0.09 nM on ERα (Table 2, Entry 7); one of them (**2c**) is even more potent on ERβ than estradiol itself.

In order to make a better comparisons of the ER subtype *transcriptional potencies* of our derivatives with their ER subtype *binding affinities*, we converted the EC_{50} values from the functional 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 give a measure of transcriptional potency *relative* to that of estradiol and, therefore, are suitable factors to be used in comparisons with their binding affinities, which are also measured relative to estradiol. In our present assays, estradiol has a 2.5-fold preference in favour of ERα in terms of binding (K_d [ERα] = 0.2 nM vs. [ERβ] = 0.5 nM) and a 8-fold preference in terms of transcriptional potency (EC_{50} [ERα] = 0.09 nM vs. [ERβ] = 0.72 nM). This seems to be caused by the fact that, when stimulated by estradiol, ERα is a more potent transcription activator than ERβ in inducing cellular responses [32]. We report herein the RTP values of our most potent ERβ-agonists **2b**–**d** and that of estradiol, together with their dose–response curves for transcriptional activation (Fig. 5). According to these metrics, compound **2c** has an RBA(β/α) ratio of 29 (Table 1) and an RTP(β/α) ratio of 20 (Fig. 5, Panel C), and compound **2d** has an RBA(β/α) ratio of 46 (Table 1) and an RTP(β/α) ratio of 52 (Fig. 5, Panel D). Hence, measured relative to estradiol, the ERβ affinity preference of these compounds is, indeed, preserved in their ERβ transcriptional potency preference.

Although there are several examples of previously reported ERβ-agonists showing higher subtype selectivities in functional assays (i.e., DPN, SERBA-1, ERB-041) [3], compounds **2c** and **d** are definitely the most ERβ-selective agonists of the whole salicylaldoxime class so far synthesized.

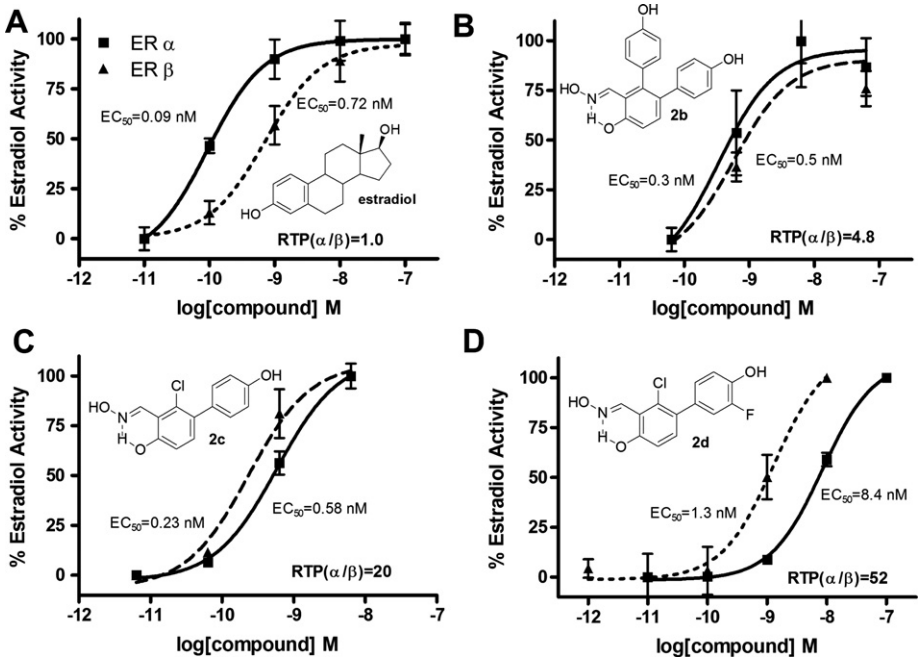


Fig. 5. Dose–response curves for transcriptional activation by estradiol (Panel A), compound **2b** (Panel B), compound **2c** (Panel C) and compound **2d** (Panel D) through ERβ (dashed line) and ERα (solid line). Human endometrial cancer (HEC-1) cells were transfected with expression vectors for ERα or ERβ and an $(ERE)_2$-pS2-luc reporter gene and were treated with estradiol or compound **2b**–**d** at the concentrations indicated. Luciferase activity was expressed relative to β-galactosidase activity from an internal control plasmid. The maximal activity with 100 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.

6. Conclusions

Our present studies confirm that, in contrast to our previously developed *Salaldox A* analogs, which never reached full activation of ERβ (partial agonists), the *monoaryl*-substituted *Salaldox B* derivatives herein reported display full agonist character on ERβ and have much higher binding affinity than their earlier counterparts. In fact, representative compounds **2c** and **2d** have affinities for ERβ comparable to that of estradiol itself, with K_i values in the sub-nanomolar range ($K_i = 0.38$ nM for **2c** and 0.57 nM for **2d**), together with notable levels of selectivity for ERβ over ERα. Most importantly, one of them (**2d**) has a remarkably improved beta-selectivity even in functional assays, which is unprecedented for any of the salicylaldoxime derivatives so far developed. This is demonstrated by ERβ/ERα selective activation by **2d**, which is >50 times higher than that of estradiol, together with a comparable ERβ-agonist potency ($EC_{50} = 1.3$ nM vs. 0.73 nM of estradiol). Curiously, the very same structural modifications that were previously shown to successfully improve selective ERβ-binding affinity and transcriptional activation in the *Salaldox A* series, such as the insertion of a Cl in the central phenyl ring and a *meta*-fluorine in the phenol substituent (compounds **1c** and **1d**), also proved to be beneficial when made at the corresponding positions of this new *Salaldox B* class, as shown by the behavior of compounds **2c** and **2d**. This could have not been easily predicted, because our docking studies indicate that the *Salaldox B* derivatives assume a completely different orientation in ERβ-binding cavity, compared to that found for the other series. As a matter of fact, in these studies, compounds **2c** and **2d** have their oxime portion participating in the H-bond network with residues R346 and E305, and place their peripheral phenol OH in a position where it can establish a strong interaction with a threonine residue (T299). This last interaction is quite unusual for ERβ-agonists, since it has only been reported so far to occur when ligands having antagonist properties interact with the ERβ-binding cavity [33]. Efforts to obtain X-ray structures of complexes of ERβ with these salicylaldoxime derivatives are currently underway and, if successful, should shed further light on the way these compounds interact and activate this intriguing and therapeutically exploitable nuclear receptor.

7. Experimental section

7.1. Chemistry

7.1.1. General

Commercially available chemicals were purchased from Sigma–Aldrich or Alfa Aesar and used without further purification, with the exception of **3**, which was prepared as previously reported [18]. 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 ThermoQuest Finningan (TRACE GCQ plus MARCA) mass spectrometer. Melting points were measured with a Kofler apparatus. Purity was routinely measured by HPLC on a Waters SunFire RP 18 (3.0×150 mm, 5 μm) column (Waters, Milford, MA, www.waters.com) using a Beckmann SystemGold instrument consisting of a chromatography 125 Solvent Module and a 166 UV Detector. Mobile phases: 10 mM ammonium acetate in Millipore purified water (A) and HPLC grade acetonitrile (B). A gradient was formed from 5% to 80% of B in 10 min and held at 80% for 10 min; flow rate was 0.7 mL/min and injection volume was 30 μL; retention times (HPLC, t_R) are given in minutes. HPLC purity of final compounds (**2c**, **d**) was determined by monitoring at 254 and 300 nm and was found in the range 96–99%. 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 reaction were run in a CEM or Biotage microwave synthesizer.

*7.1.2. 2-Chloro-4,4′-dimethoxybiphenyl-3-carbaldehyde (**4c**)*

A solution of $Pd(OAc)_2$ (2.9 mg, 0.013 mmol) and triphenylphosphine (16.9 mg, 0.064 mmol) in ethanol (1.0 mL) and toluene (1.0 mL) was stirred at RT under nitrogen for 10 min. After that period, the bromo-aryl precursor **3** [18] (0.43 mmol), an aqueous solution of Na_2CO_3 (1.0 mL, 2 M), and 4-methoxyphenylboronic acid (1.2 equiv) 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 over silica gel. Elution with *n*-hexane/EtOAc 8:2 (R_f = 0.16) afforded **4c** as a yellow solid (70% yield). ^{1}H NMR ($CDCl_3$) δ (ppm): 3.86 (s, 3H, OCH_3), 3.95 (s, 3H, OCH_3), 6.95–6.99 (m, 3H, H5, H3′, H5′), 7.31 (AA′XX′, 2H, J_{AX} = 8.8 Hz, $J_{AA'/XX'}$ = 2.2 Hz, H2′, H6′), 7.45 (d, 1H, J = 8.6 Hz, H6), 10.56 (s, 1H, CHO). Mp: 57–58 °C.

*7.1.3. 2-Chloro-3′-fluoro-4,4′-dimethoxybiphenyl-3-carbaldehyde (**4d**)*

Compound **4d** was prepared by a cross-coupling reaction of **3** with 3-fluoro-4-methoxyphenylboronic acid (1.2 eq), following the same procedure described above for the preparation of **4c**. The crude product was purified by flash chromatography over silica gel. Elution with *n*-hexane/EtOAc 8:2 (R_f = 0.16) afforded **4d** (80% yield) as a yellow solid; ^{1}H NMR ($CDCl_3$) δ (ppm): 3.94 (s, 3H, OCH_3), 3.95 (s, 3H, OCH_3), 6.97 (d, 1H, J = 8.8 Hz, H5), 7.03 (d, 1H, J = 7.7 Hz, H5′), 7.06–7.17 (m, 2H, H2′, H6′), 7.43 (d, 1H, J = 8.8 Hz, H6), 10.55 (s, 1H, CHO). Mp: 73–74 °C.

*7.1.4. 2-Chloro-4,4′-dihydroxybiphenyl-3-carbaldehyde (**5c**)*

A solution of methoxy-substituted aldehyde **4c** (0.12 mmol) in anhydrous dichloromethane (1.5 mL) was cooled to −78 °C and treated dropwise with a solution of BBr_3 in dichloromethane (0.7 mL, 1 M), 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 over silica gel. Elution with *n*-hexane/EtOAc 7:3 (R_f = 0.35) afforded pure **5c** (42% yield) as a yellow solid; ^{1}H NMR ($CDCl_3$) δ (ppm): 6.90 (AA′XX′, 2H, J_{AX} = 8.8 Hz, $J_{AA'/XX'}$ = 2.4 Hz, H3′, H5′), 6.95 (d, 1H, J = 8.9 Hz, H5), 7.27 (AA′XX′, 2H, J_{AX} = 8.5 Hz, $J_{AA'/XX'}$ = 2.6 Hz, H2′, H6′), 7.46 (d, 1H, J = 8.8 Hz, H6), 10.53 (s, 1H, CHO), 12.11 (*exchangeable* s, 1H, OH). Mp: 58–59 °C.

*7.1.5. 2-Chloro-3′-fluoro-4,4′-dihydroxybiphenyl-3-carbaldehyde (**5d**)*

Compound **5d** was prepared from methoxy-substituted aldehyde **4d** by following the same procedure described above for the preparation of **5c**. The crude product was purified by flash chromatography over silica gel. Elution with *n*-hexane/EtOAc 7:3 (R_f = 0.39) afforded pure **5d** (81% yield) as a yellow solid; ^{1}H NMR ($CDCl_3$) δ (ppm): 6.95 (d, 1H, J = 8.8 Hz, H5), 7.04–7.16 (m, 3H, H2′, H5′, H6′), 7.44 (d, 1H, J = 8.8 Hz, H6), 10.52 (s, 1H, CHO), 12.12 (*exchangeable* s, 1H, OH). Mp: 63–64 °C.

*7.1.6. (E)-2-chloro-4,4′-dihydroxybiphenyl-3-carbaldehyde oxime (**2c**)*

A solution of aldehyde **5c** (1.0 mmol) in ethanol (15 mL) was treated with a solution of hydroxylamine hydrochloride (140 mg, 2.02 mmol) in water (3.5 mL), and the mixture was heated to 50 °C for 5 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 over silica gel. Elution with *n*-hexane/EtOAc 6:4 (R_f = 0.48) afforded pure **2c** (99% yield) as a white solid; ^{1}H NMR (CD_3OD) δ (ppm): 6.81 (AA′XX′, 2H, J_{AX} = 8.6 Hz, $J_{AA'/XX'}$ = 2.5 Hz, H3′, H5′), 6.89 (d, 1H, J = 8.6 Hz, H5), 7.17 (AA′XX′, 2H, J_{AX} = 8.6 Hz, $J_{AA'/XX'}$ = 2.5 Hz, H2′, H6′), 7.20 (d, 1H, J = 8.6 Hz, H6), 8.79 (s, 1H, −CH═N−). ^{13}C NMR (CD_3OD) δ (ppm): 115.73, 115.86, 116.22, 131.50, 131.72, 131.98, 132.83, 133.87, 150.27, 157.83, 158.85. MS *m*/*z* 263 (M^+, 15), 155 ($M + H^+ - OH - C_6H_4O$, 100). Mp: 144–146 °C. HPLC, t_R 10.3 min.

*7.1.7. (E)-2-chloro-3′-fluoro-4,4′-dihydroxybiphenyl-3-carbaldehyde oxime (**2d**)*

Compound **2d** was prepared from aldehyde **5d** by following the same procedure described above for the preparation of **2c**. The crude product was purified by flash chromatography over silica gel. Elution with *n*-hexane/EtOAc 7:3 (R_f = 0.35) afforded pure **2d** (93% yield) as a white solid; ^{1}H NMR (acetone-d_6) δ (ppm): 6.96 (d, 1H, J = 8.6 Hz, H5), 7.04–7.19 (m, 3H, H2′, H5′, H6′), 7.30 (d, 1H, J = 8.4 Hz, H6), 8.82 (*exchangeable* bd, 1H, J = 1.3 Hz, 4′-OH), 8.84 (s, 1H, −CH═N−), 10.91 (*exchangeable* s, 1H, OH), 11.14 (*exchangeable* s, 1H, OH). ^{13}C NMR (acetone-d_6) δ (ppm): 115.55, 116.38, 118.12 (d, J = 22.8 Hz), 118.28, 126.78 (d, J = 3.7 Hz), 132.20 (d, J = 6.4 Hz), 132.35, 132.61, 133.92, 145.17 (d, J = 12.8 Hz), 150.36, 151.73 (d, J = 238.6 Hz), 159.06. MS *m*/*z* 281 (M^+, 100), 263 ($M^+ - H_2O$, 64). Mp: 125–127 °C. HPLC, t_R 10.7 min.

7.2. Biological methods

7.2.1. General

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 PerkinElmer, Inc. (Waltham, MA) and had 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 [34,35]. 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.

7.2.2. 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 [21,22]. 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) [22]. 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.

7.2.3. Cell culture and transient transfections

Human endometrial cancer (HEC-1) cells were maintained in culture as described [30]. 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, 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 [30].

7.3. Docking methods

The crystal structure of ERα (pdb code 2I0J) [36] and ERβ (pdb code 2I0G) [36] was taken from the Protein Data Bank. After adding hydrogen atoms the two proteins complexed with their reference inhibitor were minimized using Amber 9 software [37] and parm03 force field at 300 K. 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 counter ions 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 [38] and were minimized by means of Macromodel [38] 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 [25]; Autodock Tools 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. In order 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. 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 500,000 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 [39].

7.3.1. QM/MM calculations

Geometry optimization was performed by means of quantum mechanical calculations based on the Gaussian 09 software [27]. Prior to QM/MM the ERβ–**2d** complex was energy-minimized using AMBER 11 and the parm96 force field [40]. The complex was placed into a rectangular parallelepiped water box; an explicit solvent model for water, TIP3P, was used, and the complex was solvated with a 10 Å water cap. Sodium ions were added as counter ions to neutralize the system. Two steps of minimization were then carried out. In the first stage, we kept the complex 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 20,000 steps of steepest descent followed by conjugate gradient until a convergence of 0.05 kcal/(mol Å) was attained. All the α carbons of the protein were blocked with a harmonic force constant of 10 kcal/(mol Å^2). The minimized structure was used as starting structure for the QM/MM calculations. The QM region was described by the B3LYP chemical model with a 6-31G++** basis set and contained the ligand, the structural water molecule and the side chains of T299, E305 and R346. The B3LYP model is a combination of the Becke three-parameter hybrid functional [41] with the Lee–Yang–Parr correlation functional (which also includes density gradient terms) [42]. On the MM region, the parm96 force field was applied using no constraints.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ejmech.2011.03.030.

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