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Differential estrogen receptor subtype modulators: Assessment of estrogen receptor subtype-binding selectivity and transcription-regulating properties of new cycloalkyl pyrazoles

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ABSTRACT

Several new cycloalkyl-fused diaryl pyrazoles were synthesized and their binding affinity for the estrogen receptor (ER) subtypes, ERα and ERβ, and subtype-specific agonist/antagonist properties were determined. Cyclopentane- and cyclohexane-fused pyrazoles with *p*-hydroxyphenyl rings at positions 1 and 3 displayed modest ERβ-binding selectivity and variable agonism through ERα, while behaving as full estrogen antagonists through ERβ in estrogen-responsive element (ERE)-dependent gene expression assays. By contrast, the 2,3-diphenolic derivatives were non-selective and considerably less effective ERβ antagonists compared to 1,3-diphenolic ones. The cyclohexane-fused 1,3-diphenolic pyrazole **8**, in particular, behaved as full ERα agonist/ERβ antagonist in these assays. Molecular modelling revealed the structural determinants possibly accounting for the differential regulation of transcription through the two ERs exhibited by **8**. The data also shows that the ER subtype-binding selectivity and agonist/antagonist efficacy of the 1,3-diphenolic pyrazoles is influenced by the cycloalkyl ring fused to the pyrazole core. Using **8** we show that, though the mutant androgen receptor (AR) of LNCaP cells is required for estrogen as well as androgen stimulation of cell growth, estrogen responsiveness of the cells depends on ERβ and AR but not on ERα.

1. Introduction

Estrogens affect the physiology of many tissues and organs mostly *via* regulating the transcription of estrogen target genes through two estrogen receptor (ER) subtypes, ERα and ERβ [1,2]. ER regulate transcription by binding as homo- or hetero-dimers to DNA sequences known as estrogen-responsive elements (EREs) or by acting as co-regulators of other transcription factors in the promoters of estrogen target genes. Alternatively, they affect gene expression by interacting with various signaling proteins such as growth factor receptors [3]. Both ER subtypes are known to possess very similar modular structure, comprising distinct functional domains for ligand binding, DNA binding and regulation of transcription, and yet exhibit different ligand-binding and/or transcription-regulating properties. The X-ray structures of the ligand-binding domains of ERα and ERβ with 17β-estradiol (estradiol), raloxifene, 4-hydroxy-tamoxifen, diethylstilbestrol or genistein, revealed the molecular determinants of ER subtype-specific ligand binding and regulation of transcription ([1,2] and references therein). While the affinity of ligand binding to either form of ER primarily reflects the presence of two hydroxyl groups with an O–O distance close to that between the 3- and 17β-hydroxyl groups of estradiol, the ability of raloxifene and 4-hydroxy-tamoxifen to display tissue-specific ER antagonist activity depends on their lengthy side chains, which can stabilize receptor conformations capable of recruiting transcriptional co-repressors rather than co-activators depending on the relative levels of expression of these co-regulators in a particular cell or tissue [1,2,4]. Ligands lacking lengthy extensions but possessing core features (e.g. hydrophobic bulkiness) that allow them to take advantage of the subtle differences in the amino acid residues lining the ligand-binding cavity of ERα and ERβ, may display differential regulation of transcription through these receptors [5–7]. ER ligands displaying subtype-selective binding and/or potency of transcriptional regulation are tools to study the function of one subtype in the presence of the other [6,8,9].

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Many estrogen target tissues, including breast, prostate and uterus, comprise cells expressing both ER subtypes [1,2]. Uterine cells are known to express ERβ as well as ERα and experiments with knock-out mice have indicated that ERβ may function as negative regulator of ERα in the uterus [10]. Treatment of normal female rats with subtype-specific agonists corroborated these findings and showed, in addition, that modulation of ERα activity by ERβ depends on the ligand used and the uterine response measured [11]. Ishikawa endometrial adenocarcinoma cells express both ER subtypes and respond to estrogen by inducing expression of hundreds of genes, including placental-like2 alkaline phosphatase [12]. The ensuing increase in alkaline phosphatase (AlkP) activity is ER-dependent and is considered to be a marker of estrogenic activity [12,13]. The AlkP response of Ishikawa cells is often used as a means to assess the ERα agonist or antagonist character of new ligands [13,14]. However, whether this response may also reflect ERβ modulation of this character is usually not addressed.

ERα and ERβ are also known to be key regulators of mammary epithelial cell proliferation, differentiation and apoptosis [15,16]. Nonetheless, in most cases transformation of mammary ductal epithelial cells causes them to lose expression of ERβ while maintaining expression of ERα [17]. For instance MCF-7 breast adenocarcinoma cells express hardly any ERβ and their estrogen-dependent growth is mediated by ERα [18,19]. In contrast, human prostate epithelial cells express ERβ and although their level of receptor expression is known to drop considerably during prostate cancer progression, it is recovered in metastatic lesions [20]. Previous research on ERβ knock-out animals indicated that the receptor acts as a negative regulator of proliferation and survival of prostate epithelial cells [21]. In tissue culture, prostate epithelial cancer cells often display androgen-dependent growth predominantly mediated by androgen receptor (AR). Nonetheless, conversion of dihydro-testosterone (DHT) to 5α-androstane-3β,17β-diol, an estrogen of considerable selectivity for ERβ, reportedly allows this receptor to negatively regulate the proliferative and metastatic potential of prostate cancer cells [22]. However, others have shown that ERβ may cooperate with a mutant form of AR to positively regulate proliferation of the androgen-responsive LNCaP prostate cancer cells in an estrogen as well as androgen-dependent manner [23,24].

Development of ER subtype-selective agonists or antagonists is expected to improve our understanding of whether and how the two ER subtypes may affect endocrine cancer cell growth and response to treatment [11]. It has been reported that 1,3-diphenolic pyrazoles may exhibit very different affinities and preferences of binding to ERα and ERβ, depending on the alkyl substitutions at positions 4 and 5 [25]. In the course of our ongoing investigation on the role of the substitution pattern in determining the ER-modulating activity of various pyrazoles [26], we were interested to investigate the effect of incorporation of a cycloalkyl moiety on their ER subtype-binding selectivity and transcription-regulating properties. Recent reports have addressed the utilization of α,α-disubstituted β,γ-unsaturated cyclohexane systems in the total synthesis of bioactive natural products [27]. In addition, various α-substituted cyclopentanones have been used as precursors for the construction of benzopyran derivatives that displayed potent and selective agonistic activity for ERβ [28]. We therefore used cyclohexanone and cyclopentanone moieties as substrates for the construction of several mono- and diphenolic pyrazoles with a cycloalkyl system fused at positions C4,5 of the pyrazole core. Furthermore we determined their ER subtype-binding selectivity using purified preparations of human ERα and ERβ and their agonist/antagonist properties using reporter cell lines stably transfected with an ERE-dependent luciferase gene. We found that the cyclohexane-fused 1,3-diphenolic pyrazole **8** displayed, in addition to a modest ERβ-binding selectivity, full antagonism of estrogen signaling through ERβ and full estrogen agonism through ERα. We therefore used pyrazole **8** as a means to study the role of ERα and ERβ in the estrogen responsiveness of Ishikawa and LNCaP cells.

2. Materials and methods

2.1. Chemicals, cell lines and tissue culture media

LNCaP (human prostate adenocarcinoma) cells were from ATCC, whereas Ishikawa (human endometrial adenocarcinoma) cells were from ECACC. All cells were maintained as recommended by the supplier. If not stated otherwise, fine chemicals and culture media were from Sigma and sera were from Gibco (Invitrogen). ICI 182,780 (fulvestrant, faslodex®), ICI 176,334 (bicalutamide, casodex®) and THC, the *R*,*R*-enantiomer of tetrahydrochrysene [(*R*,*R*)-5,11-diethyl-5,6,11,12-tetrahydro-2,8-chrysenediol], were from Tocris bioscience.

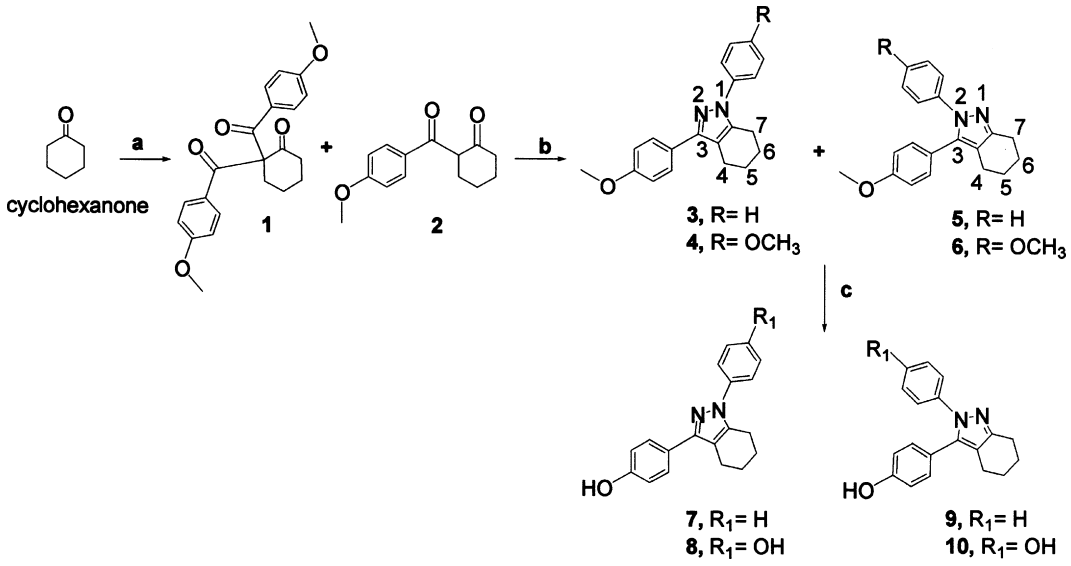

Fig. 1. Reagents and conditions used for the synthesis of cyclohexane-fused diaryl pyrazoles **7**–**10**. (a) LiHMDS, $CH_3OC_6H_4COCl$, THF; (b) $RC_6H_4NHNH_2{\cdot}HCl$, DMF/THF 3:1, 120 °C; (c) BBr_3, CH_2Cl_2, −78 °C.

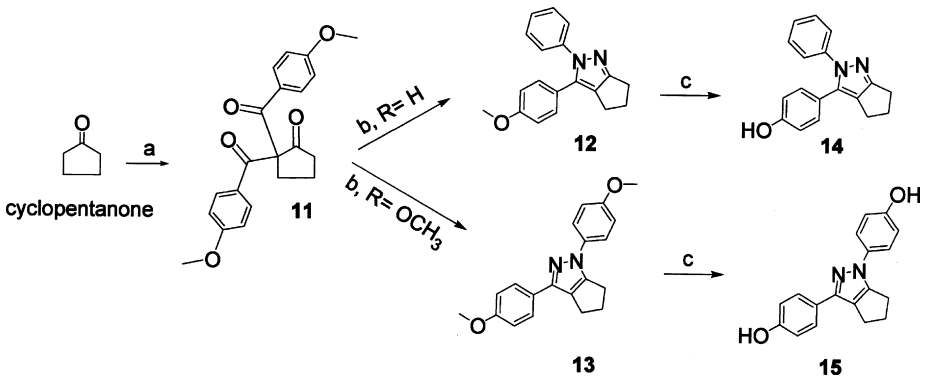

Fig. 2. Reagents and conditions used for the synthesis of cyclopentane-fused diaryl pyrazoles **14** and **15**. (a) LiHMDS, $CH_3OC_6H_4COCl$, THF; (b) $RC_6H_4NHNH_2$·HCl, DMF/THF 3:1, 120 °C; (c) BBr_3, CH_2Cl_2, −78 °C.

2.2. Synthesis and structural elucidation of the cycloalkyl pyrazole derivatives

The synthesis of the cyclohexane-fused diaryl pyrazoles **7**–**10** (Fig. 1), the cyclopentane-fused diaryl pyrazoles **14** and **15** (Fig. 2) and elucidation of their structures with NMR spectroscopy are described in detail in Supplementary material.

2.3. Assessment of binding affinities of the pyrazoles for ERα and ERβ

The binding affinities of the pyrazoles relative to that of estradiol (relative binding affinity, RBA) for isolated ERα and ERβ (RBAα and RBAβ) were assessed using a Beacon 2000 Fluorescence Polarization Reader (Invitrogen) as previously described [29]. Briefly, we determined the concentrations of estradiol, THC, **7**–**10**, **14** and **15**, that inhibited the binding of the fluorescent estrogen ES2 (Invitrogen) to the isolated recombinant human ERα or ERβ (Invitrogen) by 50% (IC_{50}), and used them to derive the RBA values of Table 1 as described in the legend to the table.

2.4. Pyrazole effects on the expression of estrogen-regulated reporter genes

Pyrazole inductions of ERE-dependent gene expression through ERα and ERβ were assessed using MCF-7:D5L and HEK:ERβ cells, respectively. MCF-7:D5L cells, a clone of MCF-7 cells that is stably transfected with the estrogen-responsive plasmid pERE-gl-Luciferase and maintains wild-type levels of ERα, have been described [8]. HEK:ERβ cells, a clone of human embryonic kidney (HEK-293) cells that is stably transfected with the estrogen-responsive reporter plasmid pERE-tk-Luciferase and an expression plasmid coding the full-length human ERβ, have been also described [7]. Assessment of pyrazole regulation of luciferase expression in MCF-7:D5L and HEK:ERβ cells was carried out as already described [7,8]. Briefly, the cells were plated in 96-well microculture plates at a density of 10,000 cells per well in MEM devoid of phenol-red and supplemented with 5% DCC-FBS, i.e. FBS that was treated with 10% dextran-coated charcoal (DCC) to remove endogenous steroids as previously described [30]. 72 h after plating, the cells were exposed to increasing concentrations (0.03–10 μM) of the test compounds (stock solutions were prepared using tissue culture grade DMSO as vehicle) in the absence (vehicle to a final concentration ≤0.2%) or presence of 0.1 nM estradiol (1 nM estradiol in the case of HEK:ERβ cells) or 10 μM fulvestrant for 16 h, and the induction of luciferase was assessed using the Steady-Glo Luciferase Assay System (Promega). Appropriate controls for full agonism (cells exposed only to 0.1 or 1 nM estradiol), full antagonism (cells exposed to 10 μM fulvestrant as well as estradiol) and non-agonism/antagonism (cells exposed only to vehicle) served to classify the pyrazoles that significantly affected luciferase expression as full, partial or weak ER agonists or antagonists as described in the legend to Table 2.

Table 1
Relative ERα- and ERβ-binding affinity and selectivity of the pyrazoles.[a].

Compound	RBAα	RBAβ	β/α
Estradiol	100	100	100
THC[b]	25.1 ± 2.5	60.4 ± 11.8	2.41
7	1.21 ± 0.38	4.68 ± 0.89	3.87
8	0.56 ± 0.12	2.43 ± 0.81	4.34
9	0.63 ± 0.07	0.81 ± 0.04	1.29
10	1.17 ± 0.32	1.16 ± 0.33	0.99
14	0.58 ± 0.16	2.41 ± 0.82	4.16
15	0.35 ± 0.05	2.01 ± 0.58	5.74

[a] The RBA values (mean ± SEM of at least three independent experiments) of the pyrazoles for ERα (RBAα) and ERβ (RBAβ) were calculated by [RBA = (IC_{50} estradiol/IC_{50} pyrazole) × 100], where IC_{50} values are estradiol or pyrazole concentrations capable of inhibiting the binding of the fluorescent estrogen ES2 (1 nM) to ERα and ERβ by 50%. IC_{50} values of estradiol for ERα and ERβ were 3.02 ± 0.17 and 2.73 ± 0.35 nM, respectively. The RBAα and RBAβ of estradiol were set equal to 100. The ERβ-binding selectivity (β/α) is calculated by [β/α = (RBAβ/RBAα)].

[b] THC, the *R,R*-enantiomer of tetrahydrochrysene, is a full ERβ antagonist that reportedly functions as partial agonist/partial antagonist through ERα [34–36].

2.5. Pyrazole effects on the alkaline phosphatase expression of Ishikawa cells

Pyrazole effects on the alkaline phosphatase (AlkP) expression of Ishikawa cells were assessed using 96-well microculture plates plated with 12,000 cells per well in phenol-red-free DMEM supplemented with 5% DCC-FBS. 24 h after plating, the cells were exposed to the test compounds in the absence or presence of 0.1 nM estradiol or 10 μM fulvestrant for 72 h, and alkaline phosphatase activity was assessed as previously described [13]. Controls were as described above (Section 2.4). The pyrazoles that induced AlkP expression were classified as full, partial or weak ER agonists or antagonists as described in the legend to Table 2.

2.6. Pyrazole effects on the growth of LNCaP cells

Pyrazole effects on the growth of LNCaP cells were assessed as already described [31], with minor modifications. Briefly, the cells were plated in 96-flat-bottomed-well microplates at a density of 8000 cells per well in phenol-red-free RPMI 1640 supplemented with 5% DCC-FBS. After plating, the cells were exposed to serial dilutions of the test compounds or 10 μM fulvestrant or bicalutamide in the absence (vehicle to a final concentration ≤0.3%) or

Table 2
Biological responses to the pyrazoles as compared to those of estradiol and fulvestrant.

Compound	Luciferase expression (MCF-7:D5L cells)		Luciferase expression (HEK:ERβ cells)		Alkaline phosphatase expression (Ishikawa cells)	
	Agonism (% of 0.1 nM E2)[a]	Antagonism (% of 10 μM fulv)[b]	Agonism (% of 1 nM E2)[a]	Antagonism (% of 10 μM fulv)[b]	Agonism (% of 0.1 nM E2)[a]	Antagonism (% of 10 μM fulv)[b]
THC	Weak (26)	Partial (64)	Non-significant	Full (97)	nd	nd
7	Partial (35)	Partial (47)	Non-significant	Full (74)	Partial (66)	Weak (27)
8	Full (70)	Non-significant	Non-significant	Full (80)	Partial (51)	Partial (34)
9	Partial (38)	Non-significant	Non-significant	Partial (53)	Full (73)	Non-significant
10	Partial (64)	Non-significant	Partial (37)	Partial (52)	Partial (34)	Partial (47)
14	Partial (56)	Non-significant	Partial (47)	Partial (49)	Full (107)	Non-significant
15	Non-significant	Partial (45)	Non-significant	Full (95)	Partial (38)	Weak (31)

Statistically significant agonist or antagonist effects were classified as full, partial, or weak depending on whether they were, respectively, >66–100, >33–66 and ≤33% of the effect of E2 or fulvestrant. nd: not determined.

[a] The agonist effect of the pyrazoles (10 μM) and THC (1 μM), expressed as % of that of estradiol (E2), was calculated by [(expression in the presence of pyrazole − expression in vehicle) × 100/(expression in E2 − expression in vehicle)].

[b] Antagonism of the agonist effect of E2 by the pyrazoles (at 10 μM) and THC (1 μM), expressed as % of fulvestrant (fulv), was calculated by [(expression in E2 − expression in pyrazole as well as E2) × 100/(expression in E2 − expression in fulvestrant as well as E2)].

presence of 10 nM estradiol and/or 10 nM DHT, and after incubation for 4 days, the relative numbers of viable cells were determined using MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] conversion to coloured formazan. Cells exposed only to estradiol and/or DHT, fulvestrant or bicalutamide served as controls. Similar results were obtained using the sulforhodamine B (SRB) assay as described by Takahashi et al. [24].

2.7. Molecular modelling

Calculations were performed using Macromodel 9.0 (Schroedinger). The ligand-binding domains of ERα and ERβ in the agonist (PDB entries 1ERE and 1X78, respectively) or the antagonist conformation (PDB entries 3ERT and 1L2J, respectively) were chosen as starting structures for docking calculations. On each complex the crystallographic ligand was replaced by compound **8**. Partial charges of compound **8** were calculated using the AM1 algorithm as implemented in Mopac7. Docking calculations were performed using 1000 step search of the mixed Monte Carlo/Low Mode (MC/LMOD) search algorithm with a ratio of 0.5 and AMBER* force field, as already described [4,8]. A distance-dependent dielectric "constant" of 4*r* was used. All residues within 6.0 Å from the ligand were allowed to move freely while the remaining residues were treated as "frozen atoms". After each successful run the complex was minimized using the TNCG algorithm (rmsG < 0.01 kJ/mol Å). Unique conformations were stored only if they were within the lowest 50 kJ/mol. Each global minimum of the docking run was re-minimized for only 1 cycle using the continuum solvent model GB/SA and the interaction energy of the complex (sum of electrostatic and van der Waals interaction energies) was calculated using the EMBRACE module as implemented on Macromodel 9.0.

2.8. Statistics

The statistical significance of the differences observed was determined using one-way ANOVA with a Tukey Post Hoc test for multiple comparisons and the SPSS 10.0 statistical package. Differences were considered significant for values of $p < 0.05$.

3. Results and discussion

3.1. Synthesis of the cycloalkyl-fused diaryl pyrazoles

The synthesis of cycloalkyl-fused diaryl pyrazoles was achieved by α-acylation of the corresponding cycloketones followed by condensation with the appropriate hydrazine derivative. Specifically, the commercially available cyclohexanone was acylated (*via* its Li enolate) to yield a 3:1 mixture of **1** and **2** (Fig. 1). Analytical samples of the latter compounds were obtained by semi-preparative HPLC. Then, a crude mixture of **1** and **2** was then double condensed with phenylhydrazine or 4-methoxyphenylhydrazine to predominantly produce **5** and **6**, and a small amount of their regioisomers, **3** and **4** (with 8 and 16% yields, respectively). Demethylation of compounds **3**–**6** furnished the cyclohexane-fused diaryl pyrazoles **7**–**10**. A different route to the synthesis of compound **8** has been described [25]. Similarly, the α-acylation of cyclopentanone provided the triketone **11** (Fig. 2), which by treatment with phenylhydrazine or 4–methoxyphenylhydrazine furnished **12** and **13**, respectively, as the exclusive products. Subsequent deprotection provided the cyclopentane-fused diaryl pyrazoles **14** and **15** in high yields.

3.2. ER subtype-binding affinity and selectivity of the pyrazole derivatives

ERα and ERβ are known to bind with high affinity steroidal and non-steroidal compounds that possess two hydroxyl groups with an O–O distance similar or even higher than estradiol (10.8 Å) such as the potency-selective ERβ agonist genistein (12.1 Å) and the full ERβ antagonist THC (12.1 Å) that reportedly behaves as partial agonist/partial antagonist through ERα [6,32–36]. The O–O distance of the OH pair of compounds **8**, **10** and **15** was estimated equal to 12.2, 7.7 and 12.3 Å, respectively. Table 1 shows that the RBAα and RBAβ values of all the pyrazoles are rather modest (<5%). As described in detail below (Section 3.4), docking calculations showed that, e.g. the 1,3-diphenolic pyrazole **8** fits inside the ERα binding pocket with the cyclohexane ring located in a position very similar to that of the B hydrophobic ring of estradiol [32], where favourable van der Waals interactions with Met388, Leu391 and Leu428 stabilize the complex (see Fig. 4A). However, in this orientation the phenolic ring of **8** is displaced compared to the A ring of estradiol, forming weaker hydrogen bonds with Arg394, Glu353 and the adjacent water molecule (Supplementary material, Fig. 1A). This could cause the pyrazoles to display RBA values substantially lower than estradiol.

In addition, Table 1 shows that, while the RBAα of all the pyrazoles and the RBAβ of **9** and **10** are around 0.5–1, the RBAβ values of **7**, **8**, **14** and **15** are between 2 and 5, resulting in ∼5-fold preference for ERβ. Evidently, in 1,3-diaryl pyrazoles, the RBAβ decreases by substituting the cyclopentane for the cyclohexane ring, while the opposite is true for the 2,3-diaryl pyrazoles. In fact, both cyclopentane-fused 1,3-diaryl and 2,3-diaryl pyrazoles display preference for ERβ, while this is so for cyclohexane-fused 1,3-diaryl but not 2,3-diaryl pyrazoles, indicating that the bulky cyclohexane ring may suffer more steric inhibition from binding pocket residues in the 2,3- than the 1,3-diaryl pyrazoles. As shown

below (Section 3.3), the ERβ-binding selectivity of the 1,3-diaryl pyrazoles **7**, **8** and **15** is accompanied by full antagonism through ERβ, while this is not the case with **14**. In fact, **9**, **10** and **14** display only partial antagonism through ERβ. Molecular modelling of **7**-, **8**- and **15**-ERβ antagonist complexes revealed the molecular basis of their ERβ-binding selectivity (see Fig. 4B). The 2,3-diaryl pyrazoles **9**, **10** and **14** can assume a somewhat different conformation, where interactions of the cyclopentane ring and the 2-aryl moiety of **14** with Ile373 and Leu476, respectively, appear to stabilize a structure of higher energy compared to **7**, **8** and **15** (Supplementary material, Fig. 1B); and where the more bulky cyclohexane ring exhibits steric interactions that could account for the lower RBAβ of **9** and **10** compared to **14**.

Notably, the RBAα and RBAβ of **8** are identical and approx. 50-fold higher, respectively, than previously reported [25]. For comparison, we also determined the RBA values of THC. In accordance with previous reports [34,35], we found that THC displays a modest selectivity for ERβ. The previously reported RBA of **8** and THC were obtained using a competitive radiometric assay carried out with tritiated estradiol and purified preparations of the ligand-binding domains of ERα (residues 304–554) and ERβ (256–505) expressed in *Escherichia coli* [25,34] or (only for THC) baculovirus-expressed full length human ERα and ERβ from the same commercial source we also used [35]. On the basis of the THC data the radiometric assay and our fluorescence polarization assay for determining RBA could be considered as being grossly equivalent. In light of this the 50-fold lower RBAβ of **8** previously reported could be the result of the heterologous expression causing ERβ to misfold in a manner affecting the binding of **8** but not THC.

3.3. ER subtype-dependent pyrazole effects on ERE-dependent gene expression

First, we assessed the ability of the pyrazoles to induce ERE-dependent gene expression through ERα in MCF-7:D5L cells. These cells, like the wild-type MCF-7 cells, express ERβ to a much lower level than ERα ([19] and data not shown). As already reported [8,29], we consistently observed maximal (4.5-fold) induction of luciferase expression at ≥0.1 nM estradiol (Fig. 3A). Treatment of estrogen-free MCF-7:D5L cells with **7**–**10** or **14** resulted in significant induction of luciferase expression, while **15** was totally ineffective up to 10 μM (Fig. 3A; $^{\#}p < 0.05$). Importantly, inductions were ER-dependent, since they were fully inhibited in the presence of the ER destabilizer fulvestrant (Fig. 3A). Induction efficacies at 10 μM ranked in the order: **8** ≈ **10** ≥ **14** > **7** ≈ **9** and potencies were in the micromolar range, with **8** displaying an EC_{50} = 3.8 μM (Fig. 3C). Compared to estradiol (full agonist), the pyrazoles that significantly induced luciferase expression at 10 μM were classified as full (**8**) or partial (**7**, **9**, **10** and **14**) ERα agonists (Table 2, column 2). Treatment of MCF-7:D5L cells growing in the presence of 0.1 nM estradiol with the pyrazoles resulted in significant suppression of hormonal induction of luciferase expression at 10 μM **7** or **15** (Fig. 3A; $^{*}p < 0.05$). Compared to fulvestrant (full antagonist), **7** and **15** behaved as partial ERα antagonists (Table 2, column 3). However, even 10 μM **8**–**10** and **14** failed to significantly suppress induction of luciferase expression at 0.1 nM estradiol and were totally ineffective in this respect at 1 nM hormone (not shown). Considering that the ER-binding affinities and selectivity of **8** and **15** are similar, their difference in inducing luciferase expression in MCF-7:D5L cells is striking and probably reflects the involvement of

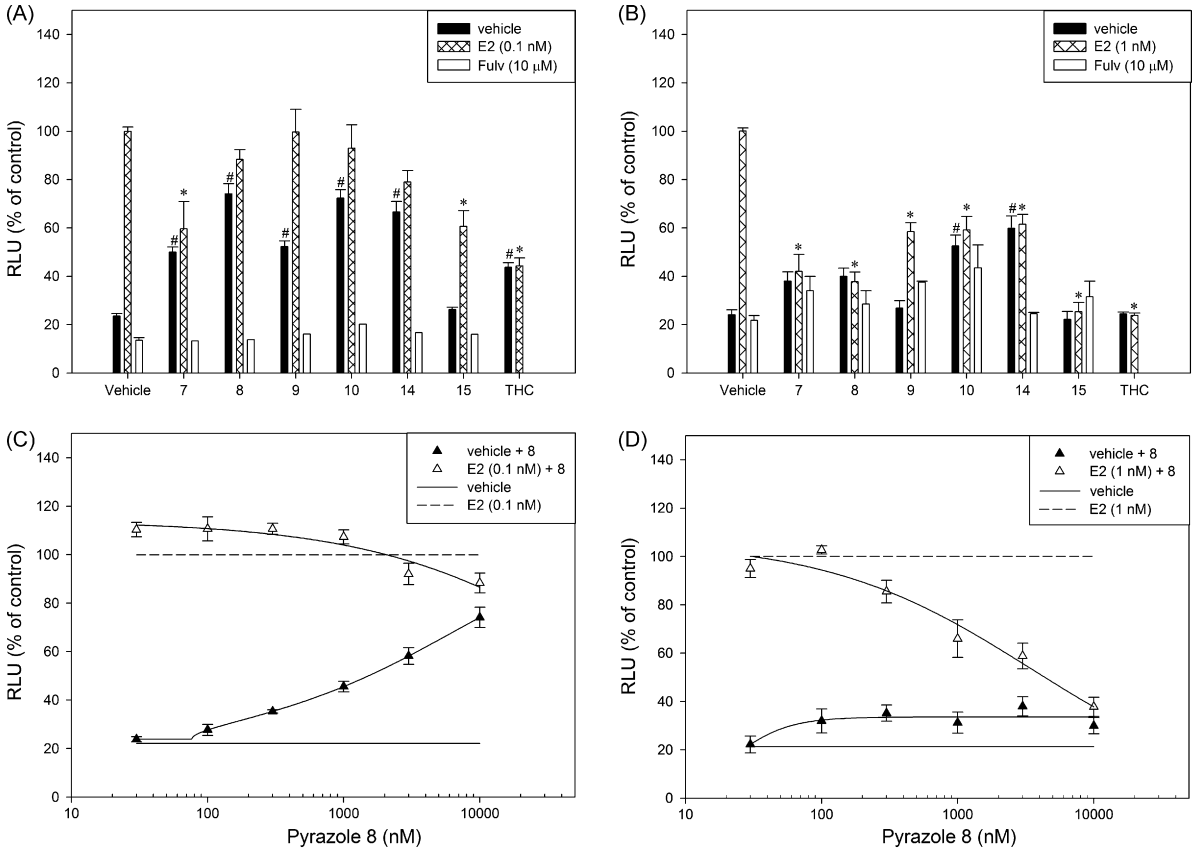

Fig. 3. Luciferase expression of (A) MCF-7:D5L cells and (B) HEK:ERβ cells exposed to pyrazoles **7**–**15** (10 μM) or THC (1 μM) in the presence of vehicle (black bars), estradiol (stripped bars) or fulvestrant (white bars). Effects of increasing concentrations of pyrazole **8** in the expression of luciferase in (C) MCF-7:D5L cells and (D) HEK:ERβ cells in the absence or presence of estradiol. Expression in the presence of estradiol alone was set equal to 100 (control). Values (% of control) are mean ± SEM ($n \geq 3$); $^{*}p < 0.05$ *vs* control; $^{\#}p < 0.05$ *vs* vehicle. E2: estradiol. Fulv: fulvestrant. RLU: relative light units.

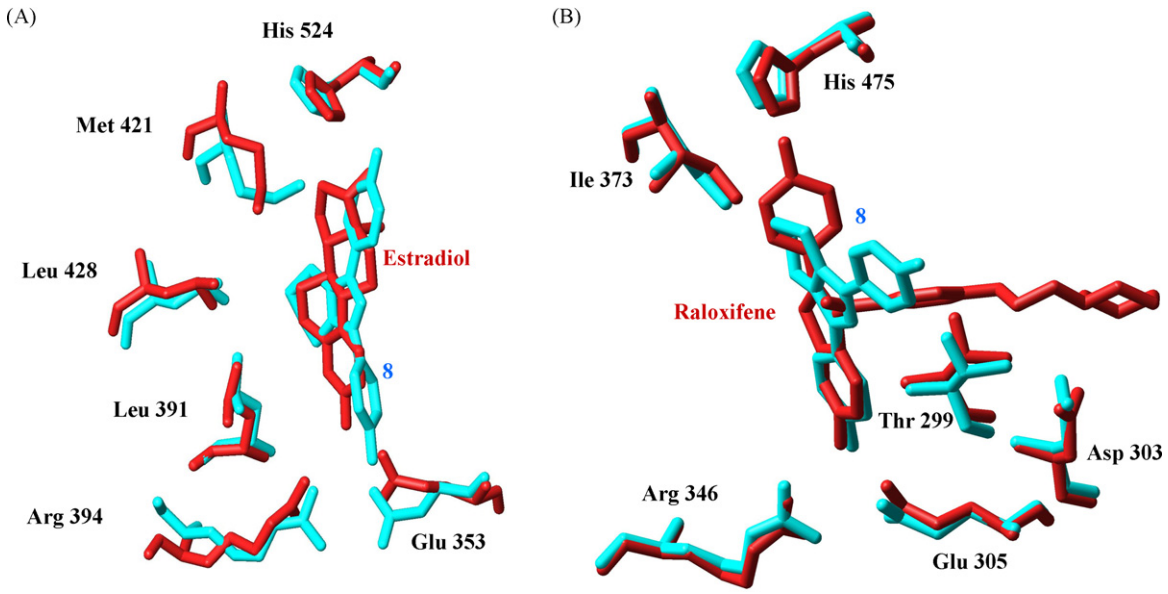

Fig. 4. Crystal structure of (A) estradiol-ERα and (B) raloxifene-ERβ complex (red) superimposed to the global minimum structure of **8** on ERα and ERβ, respectively, (blue), as obtained using docking calculations. Only the most important amino acid residues of the ER-binding cavity are shown.

the more bulky cyclohexyl moiety in improving the agonist character of the former compared to the later pyrazole in ERE-dependent gene transcription. For comparison, we also determined THC regulation of the expression of the ERE-dependent luciferase reporter that is embedded in MCF-7:D5L cells in the presence and absence of estradiol. Fig. 3A shows that THC displayed weak agonist activity through ERα in this reporter system.

Next, we assessed the ability of the pyrazoles to induce ERβ-dependent gene expression in HEK:ERβ cells. In accordance with previous findings [7], we observed maximal induction of luciferase expression at ≥1 nM estradiol (Fig. 3B). Treatment of estrogen-free HEK:ERβ cells with the pyrazoles resulted in significant induction of luciferase expression only with **10** or **14** (Fig. 3B; $^{\#}p < 0.05$). Compared to estradiol, **10** and **14** behaved as partial ERβ agonists (Table 2, column 4). The induction of luciferase was ERβ-dependent, since it was fully inhibited by fulvestrant (Fig. 3B). Treatment of HEK:ERβ cells growing in the presence of 1 nM estradiol with the pyrazoles resulted in significant suppression of luciferase expression (Fig. 3B; $^{*}p < 0.05$). Compared to fulvestrant, the pyrazoles behaved as partial (**9**, **10** and **14**) or full (**7**, **8** and **15**) ERβ antagonists at 10 μM (Table 2, column 5). Unlike **7** and **15**, however, pyrazole **8** did not antagonize ERα significantly (Table 2, column 3). Thus, it appears that only **8** can maintain full induction of ERE-dependent gene expression through ERα and at the same time fully suppress the effect of estradiol through ERβ with an IC_{50} = 1.9 μM (Fig. 3D). We also determined the effect of THC on the expression of the ERE-dependent luciferase reporter that is embedded in HEK:ERβ cells. THC behaved as full antagonist through ERβ in this reporter system (Fig. 3B), in accordance with previous findings with a range of ERE-dependent enhancers commonly found in estrogen-regulated promoters [36].

3.4. Molecular modelling

Docking calculations of **8** bound to the ligand-binding domains (LBDs) of ERα and ERβ in the agonist or the antagonist conformation were performed and interaction energies were calculated as the sum of electrostatic and van der Waals energy terms in order to get an insight into the mode of binding of this pyrazole to the two ER subtypes. The interaction energy of **8**-ERα complex was found to be lower for the agonist rather than antagonist LBD conformation, in line with **8** behaving as ERα agonist in ERE-dependent gene transcription. Fig. 4A depicts the calculated global minimum energy structure of **8**-ERα superimposed to the reported estradiol-ERα crystal structure [32]. The two phenol OH groups form hydrogen bonds, one with Arg394 and Glu353 and the other with His524 in the distal part of the ligand-binding cavity (Fig. 4A). Notably, the side chain of Met421 of ERα is reoriented from the initial conformation in the crystal structure in order to interact with the proximal aromatic ring of **8**. This interaction is not possible in the ERβ complex, where Ile373 substitutes for Met421. Similar orientation and hydrogen bond formation were observed in the **8**-ERβ agonist complex, although in this case the ligand is rotated by 180° around the N2-C6 axis (not shown). However, the interaction energy of the **8**-ERβ complex in the agonist LBD conformation was found to be higher (by 2.4 kcal/mol) than that in the antagonist conformation, in line with **8** behaving as ERβ antagonist. Fig. 4B depicts the calculated global minimum energy structure of **8**-ERβ antagonist complex superimposed to the reported raloxifene-ERβ crystal structure [33]. It appears that the ligand is now oriented towards the channel occupied by the side chain of raloxifene. This structure seems to be stabilized by the formation of a hydrogen bond between Thr299 and the phenolic OH group of **8**. Again, a similar structure was obtained for the ERα antagonist conformation (not shown), but as already said, it was energetically less favoured than the **8**-ERα agonist structure of Fig. 4A. *Ab initio* quantum chemical calculations show that an attractive interaction exists between –SCH3 and aromatic ring moieties, with the energy stabilization calculated to be up to 2.2 kcal/mol [37]. Thus, agonism is favoured in **8**-ERα because, in this case, the ligand is able to interact with the –SCH3 group of Met421. This interaction could rationalize the shift of the equilibrium **8**-ERα agonist ↔ **8**-ERα antagonist towards the agonist conformation.

In all the calculated structures, the bulky cyclohexane ring together with the overall molecular scaffold (e.g. long molecule exhibiting 12.2 Å distance between the two phenol OH groups) lead to steric interactions with binding pocket residues. This is particularly evident in the agonist conformation where docking calculations showed that in several energy-minimum-structures the cyclohexane moiety exhibits steric interactions with residue

side chains, pushing the ligand towards displacing His524/His475 (Fig. 4A and data not shown), while also favouring the agonism of **8** compared to **15** through ERα. Notably, it has been reported that productive hydrogen bonding with the ligand holds His524/His475 in a position that allows initiation of a network of hydrogen bonds that involves Glu419/Glu371, extends to the C-terminal of helix 11, and eventually stabilizes helix 12 in the agonist orientation [38]. Displacement of His524/His475 and disruption of the network of polar and non-polar interactions that stabilizes the productive conformation of helix 12 is known to compromise ER agonism and, as already reported to be the case with THC, to affect ERβ more readily than ERα [6]. Thus, the steric interactions of the cyclohexane moiety may also account for the agonism of pyrazole **8** preferentially through ERα.

3.5. Effects of pyrazoles on the AlkP expression of Ishikawa cells

We tested the ability of the pyrazoles to induce the expression of AlkP in Ishikawa cells. As previously reported [13,29], treatment of estrogen-free Ishikawa cells with ≥0.1 nM estradiol resulted in maximal (~4-fold) induction of AlkP expression (Fig. 5). Similarly, treatment with the pyrazoles resulted in significant inductions of AlkP expression (Fig. 5; $^{\#}p < 0.05$). Compared to estradiol, the pyrazoles behaved as partial (**7**, **8**, **10** and **15**) or full (**9** and **14**) agonists at 10 μM (Table 2, column 6). The induction of AlkP was ER-dependent, since it was fully inhibited by fulvestrant (Fig. 5). Treatment of Ishikawa cells growing in the presence of 0.1 nM estradiol with the pyrazoles resulted in non-significant (**9** and **14**), weak (**7** and **15**) or partial (**8** and **10**) suppressions of AlkP expression at 10 μM pyrazole (Fig. 5; $^{*}p < 0.05$; Table 2, column 7). Since **8** and **10** were found above not to antagonize ERα-dependent luciferase expression in MCF-7:D5L cells significantly (Fig. 3A), the inference is that cell and/or promoter-specific mechanisms account for the difference between the two systems as regards suppression of estrogen-dependent gene expression by these pyrazoles. In line with this, it has been reported that levels of ERα and ERβ are comparable in Ishikawa cells [12]; and that while ERβ is a negative regulator of ERα function in uterine epithelial cells [10], it positively regulates serum prolactin levels and PCNA protein expression in luminal epithelial cells in mice [11]. It is therefore possible that estrogen induction of AlkP in Ishikawa cells is mediated by ERβ as well as ERα, causing **8** to behave as partial rather than full agonist in this system. Furthermore, the relative level of expression

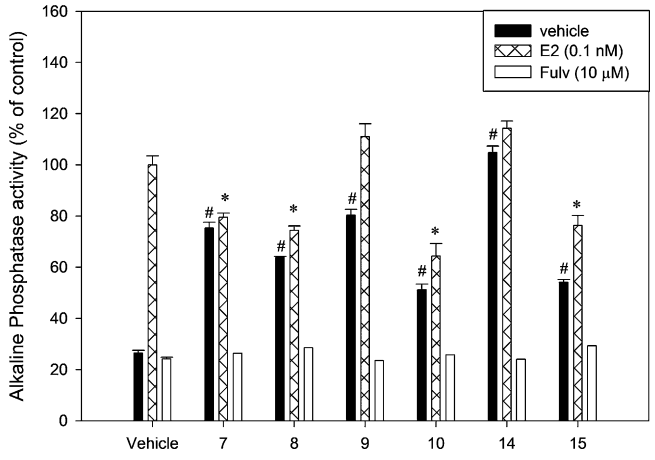

Fig. 5. Alkaline phosphatase expression of Ishikawa cells exposed to pyrazoles **7**–**15** (10 μM) in the presence of vehicle (black bars), estradiol (stripped bars) or fulvestrant (white bars). Expression in the presence of estradiol alone was set equal to 100 (control). Values (% of control) are mean ± SEM ($n \geq 3$); $^{*}p < 0.05$ *vs* control; $^{\#}p < 0.05$ *vs* vehicle.

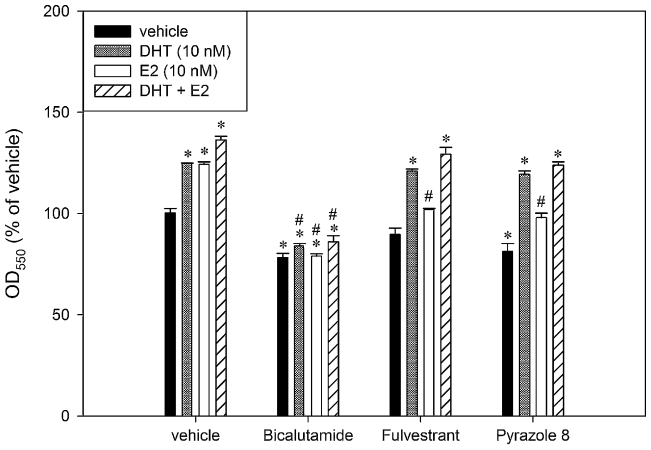

Fig. 6. Effects of pyrazole **8** (10 μM), fulvestrant (10 μM) and bicalutamide (10 μM) on the growth of LNCaP cells in growth medium supplemented with vehicle (black bars), DHT (dotted bars), estradiol (white bars) or a combination of DHT and estradiol (stripped bars), as measured using the MTT assay. OD at 550 nm in the presence of vehicle is set equal to 100. Values (% of vehicle) are mean ± SEM ($n \geq 3$); $^{*}p < 0.05$ *vs* vehicle; $^{\#}p < 0.05$ *vs* the respective hormone(s) in the presence of vehicle.

of co-activators and co-repressors may also impact on the agonist/antagonist character of **8** through the AlkP promoter. Indeed, cell- and promoter-specific differences have been shown to account for the higher agonism of 4-hydroxy-tamoxifen in uterine as compared to breast cancer cells [39].

3.6. Effects of pyrazole 8 on the growth of LNCaP cells

We finally looked for the contribution of ERα and ERβ in the proliferation of LNCaP cells, known to express both forms of ER as well as a mutant AR [24,40]. Estrogens as well as androgens have been reported to promote the growth of these cells but the mechanism of estrogen action and the ER subtype(s) involved in mediating their responsiveness to estrogen remains elusive [23]. Suppression of ERβ expression using siRNA indicated that both estrogen and androgen stimulation of growth of LNCaP cells depends on ERβ and that both fulvestrant and the AR antagonist bicalutamide could abrogate stimulation of cell growth by either estrogen or androgen [23]. On the other hand, fulvestrant and bicalutamide reportedly could only block ER- and AR-mediated gene expression, respectively [23]. In accordance with previous findings [23], we observed that LNCaP cell growth was significantly stimulated by 10 nM estradiol, 10 nM 5α-dihydro-testosterone (DHT) or a combination of the two (Fig. 6; $^{*}p < 0.05$); and that bicalutamide repressed cell response to either hormone as well as to their combination, suggesting that both estrogen and androgen stimulation of growth of LNCaP cells depends on AR. However, fulvestrant suppressed the effect of estradiol, but failed to impact on the DHT effect, suggesting that androgen stimulation of our LNCaP cells is independent of both ERα and ERβ. This is consistent with recent findings showing that fulvestrant is able to block estrogen-mediated but not androgen-mediated expression of genes jointly regulated by ER and AR in LNCaP cells [24]. Interestingly, the effects of **8** on cell growth closely resembled those of fulvestrant (Fig. 6). Taken together the data of Fig. 6 suggests that estrogen stimulation of growth of LNCaP cells depends on ERβ and AR but not on ERα.

4. Conclusions

It has been reported that appropriately substituted phenolic pyrazoles may differ considerably in respect to ER subtype-binding selectivity and transcription-regulating potency: the 1,3,5-triphenolic-4-propyl pyrazole (PPT) behaves as selective

ERα agonist, while the 1,3-diphenolic-4-methyl pyrazole with a protracted side chain at C5 (MPP), behaves as selective ERα antagonist [5,36]. In addition, it has been reported that THC, the *R,R*-enantiomer of tetrahydrochrysene, is a full antagonist through ERβ and a partial agonist through ERα; and that it displays approx. 50% of the efficacy of estradiol (or less in our ERα reporter system) in inducing the expression of a variety of estrogen-regulated genes [36]. However, full ERβ antagonists that also behave as full ERα agonists are lacking. The present report extends the repertoire of pyrazole-based ER modulators by showing that (i) the 1,3-diphenolic-4,5-cyclohexyl pyrazole **8** is a full ERα agonist/ERβ antagonist, (ii) the ERα agonism and ERβ antagonism of **8** could result from two different binding orientations, providing for ligand interactions with Met421 in ERα and Thr299 in ERβ, respectively, and (iii) the ERα agonism of 1,3-diphenolic-4,5-cycloalkyl pyrazoles depends on the type of cycloalkyl ring fused to the pyrazole core, possibly allowing for the development of differential ER subtype modulators with better binding and transcriptional characteristics. Using **8** as a means to fully block ERβ while maintaining ERα activity, we obtained data indicating that the estrogen-dependent growth of LNCaP cells depends on ERβ and AR but not on ERα.

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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.jsbmb.2009.09.006.

References

[1] N. Heldring, A. Pike, S. Andersson, J. Matthews, G. Cheng, J. Hartman, M. Tujague, A. Strom, E. Treuter, M. Warner, J.-A. Gustafsson, Estrogen receptors: how do they signal and what are their targets, Physiol. Rev. 87 (3) (2007) 905–931.
[2] K. Dahlman-Wright, V. Cavailles, S.A. Fuqua, V.C. Jordan, J.A. Katzenellenbogen, K.S. Korach, A. Maggi, M. Muramatsu, M.G. Parker, J.-A. Gustafsson, International union of pharmacology. LXIV. Estrogen receptors, Pharmacol. Rev. 58 (4) (2006) 773–781.
[3] E.R. Levin, R.J. Pietras, Estrogen receptors outside the nucleus in breast cancer, Breast Cancer Res. Treat. 108 (3) (2008) 351–361.
[4] G. Lambrinidis, M. Halabalaki, E. Katsanou, A.-L. Skaltsounis, M. Alexis, E. Mikros, The estrogen receptor and polyphenols: molecular simulation studies of their interactions, a review, Environ. Chem. Lett. 4 (3) (2006) 159–174.
[5] S.R. Stauffer, C.J. Coletta, R. Tedesco, G. Nishiguchi, K. Carlson, J. Sun, B.S. Katzenellenbogen, J.A. Katzenellenbogen, Pyrazole ligands: structure-affinity/activity relationships and estrogen receptor-alpha-selective agonists, J. Med. Chem. 43 (26) (2000) 4934–4947.
[6] A.K. Shiau, D. Barstad, J.T. Radek, M.J. Meyers, K.W. Nettles, B.S. Katzenellenbogen, J.A. Katzenellenbogen, D.A. Agard, G.L. Greene, Structural characterization of a subtype-selective ligand reveals a novel mode of estrogen receptor antagonism, Nat. Struct. Mol. Biol. 9 (5) (2002) 359–364.
[7] G. Skretas, A.K. Meligova, C. Villalonga-Barber, D.J. Mitsiou, M.N. Alexis, M. Micha-Screttas, B.R. Steele, C.G. Screttas, D.W. Wood, Engineered chimeric enzymes as tools for drug discovery: generating reliable bacterial screens for the detection, discovery, and assessment of estrogen receptor modulators, J. Am. Chem. Soc. 129 (27) (2007) 8443–8457.
[8] N. Fokialakis, G. Lambrinidis, D.J. Mitsiou, N. Aligiannis, S. Mitakou, A.-L. Skaltsounis, H. Pratsinis, E. Mikros, M.N. Alexis, A new class of phytoestrogens: evaluation of the estrogenic activity of deoxybenzoins, Chem. Biol. 11 (3) (2004) 397–406.
[9] E.C. Chang, T.H. Charn, S.-H. Park, W.G. Helferich, B. Komm, J.A. Katzenellenbogen, B.S. Katzenellenbogen, Estrogen receptors {alpha} and {beta} as determinants of gene expression: influence of ligand, dose, and chromatin binding, Mol. Endocrinol. 22 (5) (2008) 1032–1043.
[10] Z. Weihua, S. Saji, S. Makinen, G. Cheng, E.V. Jensen, M. Warner, J.A. Gustafsson, Estrogen receptor (ER) beta, a modulator of ERalpha in the uterus, Proc. Natl. Acad. Sci. U.S.A. 97 (11) (2000) 5936–5941.
[11] J. Frasor, D.H. Barnett, J.M. Danes, R. Hess, A.F. Parlow, B.S. Katzenellenbogen, Response-specific and ligand dose-dependent modulation of estrogen receptor (ER) alpha activity by ERbeta in the uterus, Endocrinology 144 (7) (2003) 3159–3166.
[12] S.M. Johnson, M. Maleki-Dizaji, J.A. Styles, I.N. White, Ishikawa cells exhibit differential gene expression profiles in response to oestradiol or 4-hydroxytamoxifen, Endocr. Relat. Cancer 14 (2) (2007) 337–350.
[13] K.M. Kasiotis, C. Mendorou, S.A. Haroutounian, M.N. Alexis, High affinity 17alpha-substituted estradiol derivatives: synthesis and evaluation of estrogen receptor agonist activity, Steroids 71 (3) (2006) 249–255.
[14] W.J. Hoekstra, H.S. Patel, X. Liang, J.B. Blanc, D.O. Heyer, T.M. Willson, M.A. Iannone, S.H. Kadwell, L.A. Miller, K.H. Pearce, C.A. Simmons, J. Shearin, Discovery of novel quinoline-based estrogen receptor ligands using peptide interaction profiling, J. Med. Chem. 48 (6) (2005) 2243–2247.
[15] C. Forster, S. Makela, A. Warri, S. Kietz, D. Becker, K. Hultenby, M. Warner, J.A. Gustafsson, Involvement of estrogen receptor beta in terminal differentiation of mammary gland epithelium, Proc. Natl. Acad. Sci. U.S.A. 99 (24) (2002) 15578–15583.
[16] S.O. Mueller, J.A. Clark, P.H. Myers, K.S. Korach, Mammary gland development in adult mice requires epithelial and stromal estrogen receptor alpha, Endocrinology 143 (6) (2002) 2357–2365.
[17] P. Roger, M.E. Sahla, S. Makela, J.A. Gustafsson, P. Baldet, H. Rochefort, Decreased expression of estrogen receptor beta protein in proliferative preinvasive mammary tumors, Cancer Res. 61 (6) (2001) 2537–2541.
[18] G. Lazennec, J.L. Alcorn, B.S. Katzenellenbogen, Adenovirus-mediated delivery of a dominant negative estrogen receptor gene abrogates estrogen-stimulated gene expression and breast cancer cell proliferation, Mol. Endocrinol. 13 (6) (1999) 969–980.
[19] E.C. Chang, J. Frasor, B. Komm, B.S. Katzenellenbogen, Impact of estrogen receptor beta on gene networks regulated by estrogen receptor alpha in breast cancer cells, Endocrinology 147 (10) (2006) 4831–4842.
[20] I. Leav, K.M. Lau, J.Y. Adams, J.E. McNeal, M.E. Taplin, J. Wang, H. Singh, S.M. Ho, Comparative studies of the estrogen receptors beta and alpha and the androgen receptor in normal human prostate glands, dysplasia, and in primary and metastatic carcinoma, Am. J. Pathol. 159 (1) (2001) 79–92.
[21] O. Imamov, A. Morani, G.J. Shim, Y. Omoto, C. Thulin-Andersson, M. Warner, J.A. Gustafsson, Estrogen receptor beta regulates epithelial cellular differentiation in the mouse ventral prostate, Proc. Natl. Acad. Sci. U.S.A. 101 (25) (2004) 9375–9380.
[22] V. Guerini, D. Sau, E. Scaccianoce, P. Rusmini, P. Ciana, A. Maggi, P.G. Martini, B.S. Katzenellenbogen, L. Martini, M. Motta, A. Poletti, The androgen derivative 5alpha-androstane-3beta,17beta-diol inhibits prostate cancer cell migration through activation of the estrogen receptor beta subtype, Cancer Res. 65 (12) (2005) 5445–5453.
[23] M. Maggiolini, A.G. Recchia, A. Carpino, A. Vivacqua, G. Fasanella, V. Rago, V. Pezzi, P.A. Briand, D. Picard, S. Ando, Oestrogen receptor beta is required for androgen-stimulated proliferation of LNCaP prostate cancer cells, J. Mol. Endocrinol. 32 (3) (2004) 777–791.
[24] Y. Takahashi, S.N. Perkins, S.D. Hursting, T.T.Y. Wang, 17beta-estradiol differentially regulates androgen-responsive genes through estrogen receptor-beta- and extracellular-signal regulated kinase-dependent pathways in LNCaP human prostate cancer cells, Mol. Carcinog. 46 (2) (2007) 117–129.
[25] G.A. Nishiguchi, A.L. Rodriguez, J.A. Katzenellenbogen, Diaryl-dialkyl-substituted pyrazoles: regioselective synthesis and binding affinity for the estrogen receptor, Bioorg. Med. Chem. Lett. 12 (6) (2002) 947–950.
[26] K.M. Kasiotis, N. Fokialakis, S.A. Haroutounian, Synthesis of novel conformationally constrained pyrazolo[4,3-c]quinoline derivatives as potential ligands for the estrogen receptor, Synthesis 2006 (11) (2006) 1791–1802.
[27] H.-J. Liu, T.W. Ly, C.-L. Tai, J.-D. Wu, J.-K. Liang, J.-C. Guo, N.-W. Tseng, K.-S. Shia, A modified Robinson annulation process to [alpha],[alpha]-disubstituted-[beta],[gamma]-unsaturated cyclohexanone system. Application to the total synthesis of nanaimoal, Tetrahedron 59 (8) (2003) 1209–1226.
[28] B.H. Norman, J.A. Dodge, T.I. Richardson, P.S. Borromeo, C.W. Lugar, S.A. Jones, K. Chen, Y. Wang, G.L. Durst, R.J. Barr, C. Montrose-Rafizadeh, H.E. Osborne, R.M. Amos, S. Guo, A. Boodhoo, V. Krishnan, Benzopyrans are selective estrogen receptor beta agonists with novel activity in models of benign prostatic hyperplasia, J. Med. Chem. 49 (21) (2006) 6155–6157.
[29] M. Halabalaki, X. Alexi, N. Aligiannis, G. Lambrinidis, H. Pratsinis, I. Florentin, S. Mitakou, E. Mikros, A.-L. Skaltsounis, M.N. Alexis, Estrogenic activity of isoflavonoids from Onobrychis ebenoides, Planta Med. 72 (06) (2006) 488–493.
[30] A.D. Gritzapis, C.N. Baxevanis, I. Missitzis, E.S. Katsanou, M.N. Alexis, J. Yotis, M. Papamichail, Quantitative fluorescence cytometric measurement of estrogen and progesterone receptors: correlation with the hormone binding assay, Breast Cancer Res. Treat. 80 (1) (2003) 1–13.
[31] E.S. Katsanou, M. Halabalaki, N. Aligiannis, S. Mitakou, A.L. Skaltsounis, X. Alexi, H. Pratsinis, M.N. Alexis, Cytotoxic effects of 2-arylbenzofuran phytoestrogens on human cancer cells: modulation by adrenal and gonadal steroids, J. Steroid Biochem. Mol. Biol. 104 (3–5) (2007) 228–236.
[32] A.M. Brzozowski, A.C. Pike, Z. Dauter, R.E. Hubbard, T. Bonn, O. Engstrom, L. Ohman, G.L. Greene, J.A. Gustafsson, M. Carlquist, Molecular basis of agonism and antagonism in the oestrogen receptor, Nature 389 (6652) (1997) 753–758.
[33] A.C. Pike, A.M. Brzozowski, R.E. Hubbard, T. Bonn, A.G. Thorsell, O. Engstrom, J. Ljunggren, J.A. Gustafsson, M. Carlquist, Structure of the ligand-binding domain of oestrogen receptor beta in the presence of a partial agonist and a full antagonist, EMBO J. 18 (17) (1999) 4608–4618.
[34] J. Sun, M.J. Meyers, B.E. Fink, R. Rajendran, J.A. Katzenellenbogen, B.S. Katzenellenbogen, Novel ligands that function as selective estrogens or antiestrogens for estrogen receptor-alpha or estrogen receptor-beta, Endocrinology 140 (2) (1999) 800–804.

[35] M.J. Meyers, J. Sun, K.E. Carlson, B.S. Katzenellenbogen, J.A. Katzenellenbogen, Estrogen receptor subtype-selective ligands: asymmetric synthesis and biological evaluation of cis- and trans-5,11-dialkyl- 5,6,11, 12-tetrahydrochrysenes, J. Med. Chem. 42 (13) (1999) 2456–2468.
[36] W.R. Harrington, S. Sheng, D.H. Barnett, L.N. Petz, J.A. Katzenellenbogen, B.S. Katzenellenbogen, Activities of estrogen receptor alpha- and beta-selective ligands at diverse estrogen responsive gene sites mediating transactivation or transrepression, Mol. Cell Endocrinol. 206 (1–2) (2003) 13–22.
[37] E.S. Manas, Z.B. Xu, R.J. Unwalla, W.S. Somers, Understanding the selectivity of genistein for human estrogen receptor-beta using X-ray crystallography and computational methods, Structure 12 (12) (2004) 2197–2207.
[38] M. Gangloff, M. Ruff, S. Eiler, S. Duclaud, J.M. Wurtz, D. Moras, Crystal structure of a mutant hERalpha ligand-binding domain reveals key structural features for the mechanism of partial agonism, J. Biol. Chem. 276 (18) (2001) 15059–15065.
[39] Y. Shang, M. Brown, Molecular determinants for the tissue specificity of SERMs, Science 295 (5564) (2002) 2465–2468.
[40] D. He, C.N. Falany, Inhibition of SULT2B1b expression alters effects of 3beta-hydroxysteroids on cell proliferation and steroid hormone receptor expression in human LNCaP prostate cancer cells, Prostate 67 (12) (2007) 1318–1329.