Pergamon

Bioorganic & Medicinal Chemistry Letters 11 (2001) 1939–1942

BIOORGANIC & MEDICINAL CHEMISTRY LETTERS

2-Amino-4,6-diarylpyridines as Novel Ligands for the Estrogen Receptor

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Received 29 March 2001; accepted 8 May 2001

Abstract—We have prepared a novel series of 2-amino-4,6-diarylpyridines that function as ligands of estrogen receptor alpha (ERα) and estrogen receptor beta (ERβ). These compounds bind to both ERα and ERβ with a modest selectivity for the alpha subtype. The most potent of these analogues, compound **19**, has a $K_i = 20$ nM at ERα. These molecules represent a novel template for designing potentially useful ligands for the estrogen receptor. © 2001 Elsevier Science Ltd. All rights reserved.

Estrogen is an effective treatment for both menopausal symptoms and the prevention and management of postmenopausal osteoporosis. Despite the beneficial effects of estrogen, there is evidence to suggest an increase in reproductive tissue cancer,[1] which leads to both a restriction of widespread use and long-term compliance issues. Thus, many pharmaceutical companies have engaged in the development of agents that can maintain the benefits of estrogen while avoiding the risks. The development of selective estrogen receptor modulators (SERMs) such as raloxifene and tamoxifen (Fig. 1) that have tissue-selective agonist or antagonist effects are agents that hold the promise of a safer alternative to estrogen.[2,3]

Estrogens exert their biological effects via the estrogen receptor (ER), a protein that functions as a ligand-modulated gene transcription factor. The recent discovery of a second ER subtype, termed ERβ, has increased the level of complexity of estrogen signaling.[4] The two receptor subtypes (ERα and ERβ) show significant sequence homology in their DNA and ligand binding domains; however, they exhibit differences in their tissue distribution patterns, ligand selectivity, and transcriptional properties.[5] Additionally, studies with receptor subtype-specific knockout mice suggest that the two ER subtypes have distinct biological roles.[6]

A detailed picture of both the binding requirements and the mode of action of steroidal and nonsteroidal ER ligands has been developed through the use of molecular modeling and X-ray crystallographic analysis of agonist- and antagonist-bound ERα and ERβ.[7–10] The ER binds a wide range of steroidal and nonsteroidal ligands with moderate to high affinity, with a minimal requirement of at least one phenol as the basic pharmacophore. The structural diversity of nonsteroidal estrogens having affinity for the ER is remarkable, with a number of chemically distinct templates being reported as ER ligands.[2,3] As part of our ongoing effort in the identification of novel ligands for nuclear receptors, we

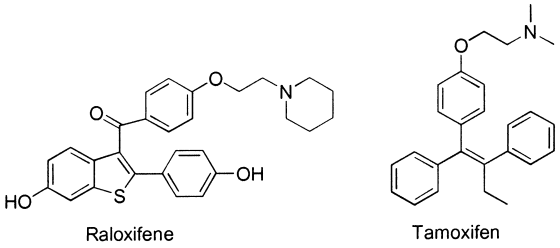

Figure 1. Representative ER ligands.

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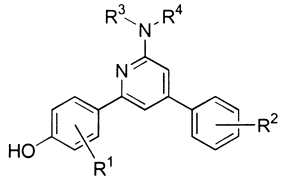
PII: S0960-894X(01)00321-3

sought to develop structurally novel templates that are readily amenable to parallel synthesis. Herein we report the synthesis and biological activity of a novel series of 2-amino-4,6-diarylpyridine ER ligands.

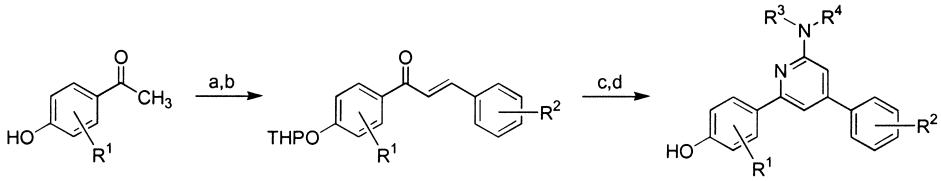
The preparation of compounds **1**–**19** (Table 1) is outlined in Scheme 1. The various substituted 4-hydroxy acetophenones were protected as their corresponding THP acetals using sulfuric acid adsorbed on silica gel as catalyst,[11] which allowed isolation of the product in high yield and purity by simple filtration of the catalyst and removal of solvent. Formation of the chalcone intermediates was best carried out by treating a 1 M ethanol solution containing equimolar amounts of the desired aromatic aldehyde and acetophenone with 0.25 equiv of finely ground solid NaOH.[12] Other methods of chalcone formation were less reliable and gave lower yields. Formation of the 2-aminopyridine nucleus was achieved using methodology previously described by Katritzky.[13] Treatment of the chalcone derivatives with an equimolar amount of 2-(benzotriazol-1-yl)acetonitrile and an excess (20 equiv) of the desired secondary amine in refluxing ethanol afforded the desired 2-amino-4,6-diarylpyridines in modest yields. It is worthwhile to note that while this reaction worked reasonably well with simple secondary amines, we were unable to isolate any of the desired 2-aminopyridine cyclization products when anilines or primary amines were employed as nucleophiles. While the failure of anilines to engage in this cyclization may be attributable to their poor nucleophilic character, the lack of success with primary amines remains puzzling. In addition, secondary amines containing a free hydroxyl group also failed to afford any desired product. Deprotection of the THP acetals was achieved by treatment with 80% aqueous acetic acid at 60 °C. The entire reaction sequence was readily

Table 1. In vitro profile of ER ligands **1**–**19**



Compd	Structure[a]				Binding[b]	
	R^1	R^2	R^3	R^4	ERα K_i (nM)	ERβ K_i (nM)
1	H	H	$-CH_3$	$-(CH_2)_2$-Ph	490 ± 10 (2)	1050 ± 250 (2)
2	H	*p*-OH	$-CH_3$	$-(CH_2)_2$-Ph	> 3000 (2)	> 3000 (2)
3	H	*o*-CH_3	$-CH_3$	$-(CH_2)_2$-Ph	650 ± 50 (2)	1100 ± 300 (2)
4	*m*-CH_3	H	$-CH_3$	$-(CH_2)_2$-Ph	160 ± 10 (4)	680 ± 190 (2)
5	H	H	$-CH_3$	$-(CH_2)$-CH-$(CH_3)_2$	620 ± 60 (2)	1260 ± 400 (4)
6	*m*-CH_3	H	$-CH_3$	$-(CH_2)$-CH-$(CH_3)_2$	210 ± 50 (2)	660 ± 100 (2)
7	*m*-CH_3	H	$-CH_3$	$-(CH_2)_3$-CH_3	660 ± 85 (2)	790 ± 10 (2)
8	*m*-CH_3	H	$-(CH_2)_4-$	IA[c]	IA	
9	*m*-CH_3	H	$-CH_3$	$-CH_2$-Ph	380 ± 10 (4)	1120 ± 300 (2)
10	*m*-CH_3	H	$-CH_3$	$-CH_2$-(1-naphthyl)	450 ± 20 (2)	1410 ± 300 (2)
11	*m*-CH_3	H	$-CH_3$	$-(CH_2)_2$-N-$(CH_3)_2$	IA	IA
12	*m*-CH_3	H	$-CH_3$	$-(CH_2)_2$-(*o*-pyridyl)	1260 ± 50 (2)	> 3000 (2)
13	H	H	$-(CH_2)_2CH_3$	$-CH_2$-(*o*-pyridyl)	180 ± 20 (4)	710 ± 320 (6)
14	*m*-CH_3	H	$-(CH_2)_2CH_3$	$-CH_2$-(*o*-pyridyl)	70 ± 10 (4)	330 ± 120 (6)
15	*o*-CH_3	H	$-(CH_2)_2CH_3$	$-CH_2$-(*o*-pyridyl)	IA	IA
16	*m*-CH_3	H	$-CH_2CH_3$	$-CH_2$-(*p*-pyridyl)	830 ± 140 (4)	1000 ± 320 (6)
17	*m*-CH_3	H	$-CH_2$Ph	$-(CH_2)_2$-Ph	130 ± 10 (2)	250 ± 10 (2)
18	*m*-CH_3	H	$-(CH_2)_2OCH_2CH_3$	$-(CH_2)_2OCH_2CH_3$	2000 ± 30 (2)	1170 ± 300 (2)
19	*m*-CH_3	H	$-(CH_2)_2CH(CH_2-Ph)-(CH_2)_2-$		20 ± 10 (4)	110 ± 10 (4)
Raloxifene		—	—	—	0.22 ± 0.11 (8)	10 ± 5 (8)
Estradiol		—	—	—	2.2 ± 1 (30)	3.5 ± 1 (30)

[a]See Table 1 figure.
[b]The values for K_i were obtained from least squares fit of the concentration–response curves according to the equation $-b = -b_0/1 + [L]/K_i$ where b_0 = the counts bound in the absence of test compound and b = counts bound in the presence of test compound at concentration [L] ± standard deviation (number of determinations).
[c]IA = inactive at 10^{-5} M.



Scheme 1. Reagents and conditions: (a) dihydropyran, $H_2SO_4{\cdot}SiO_2$ (cat), CH_2Cl_2, rt, 15 min, 85–95%; (b) ArCHO, NaOH, EtOH, rt, 16 h, 75–90%; (c) 2-(benzotriazol-1-yl)acetonitrile, R^3R^4NH, EtOH, reflux, 24–48 h, 20–60%; (d) 80% AcOH, 60 °C, 18 h, 50–65%.

adapted to a solution-phase, parallel-synthesis approach. Parallel chromatographic purification on silica gel was done after chalcone formation and then again after deprotection to afford the final target compounds. Compound identity was established both by mass spectrometry and ^{1}H NMR. Compound purity was assessed by HPLC analysis, with all reported compounds displaying >90% purity (data not shown).

Compounds were tested for their ability to bind to ERα and ERβ via a scintillation proximity assay (SPA) using a bacterial lysate containing overexpressed GST-hERα or GST-hERβ ligand binding domain. Yttrium silicate SPA beads were suspended in assay buffer (10 mM K_2HPO_4, 10 mM KH_2PO_4, 2 mM EDTA, 50 mM NaCl, 1 mM DTT, 2 mM CHAPS, 10% glycerol) and dispensed at 0.5 mg/well. Lysates containing GST-hERα or GST-hERβ were diluted in assay buffer and added to plates to give a final concentration of ~0.15–0.2 μg protein with a final assay volume of 100 μL. Test compounds were dissolved in DMSO, serially diluted in assay buffer and added to the wells in 10 μL aliquots. 1 nM [^{3}H]17β-estradiol was then added and the plates were shaken for 2 h before radioactivity was counted.

The de novo design of this template originated by comparison of this scaffold to the known pharmacophore and structural requirements for binding to the ER, coupled with the assumption that a range of analogues could be readily produced in parallel fashion using the chemistry established by Katritzky.[13] As part of the template design process, we docked representative compounds from this series into the published crystal structure of ERα bound with raloxifene[8] using an in-house molecular modeling package.[14] Figure 2 illustrates the overlay of raloxifene and compound **19** obtained from this docking procedure. Table 1 summarizes the binding affinity of compounds **1**–**19** from this series to ERα and ERβ and serves as a brief summary of the structure–activity relationships within the series. Compounds within this set displayed a modest (2- to 5-fold) selectivity for binding to ERα versus ERβ. Only a limited set of substituents on the two phenyl rings were evaluated in this initial compound set, with R^1 and R^2 being limited to –H, –CH_3, and –OH. Placement of the hydroxyl group in the *para* position of the 6-phenyl ring was important for activity, as all analogues with a *meta*-phenol were inactive at both ER subtypes (data not shown). A methyl group *meta* to the phenol (R^1 = *m*-CH_3) increased the affinity modestly for both ER subtypes versus the corresponding unsubstituted phenol (compare entries **1** vs **4**, **5** vs **6** in Table 1). This increased affinity may be due to the methyl group's occupation of a hydrophobic pocket in both ERα and ERβ corresponding to the 6-position of the B-ring in estradiol and, as evident in Figure 2, the sulfur in the thiophene ring of raloxifene. Placement of this methyl group *ortho* to the phenol led to a drastic loss of binding affinity at both receptor subtypes (cf. **14** vs **15**, Table 1). Methyl substitution at the *ortho* position of the 4-phenyl ring did not provide any substantial change in affinity over the unsubstituted analogues (Table 1, **1** vs **3**). However, addition of a second phenol moiety either in the *para* (**2**) or *meta* position (data not shown) of the 4-phenyl ring led to a significant loss of receptor binding affinity. This result might seem surprising since addition of a second phenolic group tends to increase ER binding affinity in many nonsteroidal estrogens. However, our modeling (Fig. 2) suggests that the 4-phenyl and 6-phenyl groups in our template span a greater distance compared with the corresponding aryl groups of raloxifene. Thus, placing substituents at the *meta* and *para* positions of the 4-phenyl ring may not be tolerated sterically within the binding pocket.

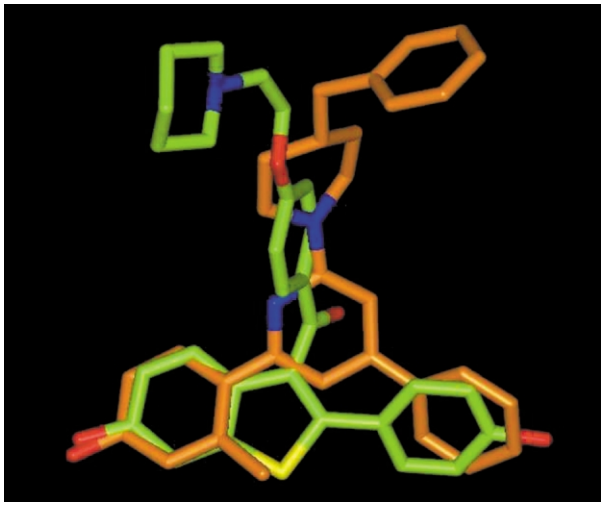


Figure 2. Overlay of raloxifene (in green) and compound **19** (in orange) based on the docking of compound **19** into the crystal structure of raloxifene bound to ERα.

Investigation into the substituents on the 2-amino group revealed that sterically large, nonpolar groups were necessary to achieve good receptor affinity. The affinity of these compounds may be due to their interactions with residues along a large hydrophobic tunnel present in the ligand-occupied antagonist conformations of ERα and ERβ.[9] Either alkyl or aralkyl groups at R^3 and R^4 provided compounds with submicromolar affinity. Increasing chain length and bulk at either R^3 (Table 1, cf. entries **12** vs **14**, **4** vs **17**) or R^4 (Table 1, cf. entries **6** vs **7**, **4** vs **9**) led to modest increases in receptor binding affinity. Even the 1-naphthyl derivative **10** showed submicromolar affinity at ERα. Small cyclic hydrocarbons such as the pyrrolidine derivative **8** were inactive. Placement of polar groups within R^3 and R^4 was detrimental to binding affinity. For example, compound **11**, which contains a basic nitrogen in this region, lost all affinity for the ER, and compound **18**, containing two alkyl ethers, displayed only weak affinity for both ER subtypes. Replacement of the phenyl group in R^4 with pyridyl also led to a loss in receptor affinity (Table 1, cf. entries **4** vs **12** and **9** vs **16**). Interestingly, this potency loss can be recovered by increasing the steric bulk of the R^3 substituent, as compounds **13** and **14** are among the most potent compounds tested. Conformationally constraining the phenethyl moiety in R^4 into a 4-benzyl piperazine system afforded compound **19**, which is the most potent analogue within this series at both receptor subtypes, with a K_i = 20 nM at ERα and a K_i = 110 nM at ERβ.

In order to assess the functional profile of these novel ER ligands we tested the transcriptional activity of compound **4**, as a representative of this series, in a transient transfection assay utilizing a human breast carcinoma cell line (T47D). T47D cells were transfected with expression vectors containing full length hERα or hERβ, β-galactosidase, and an estrogen-responsive reporter gene construct consisting of two copies of an estrogen receptor response element, the estrogen-responsive HSV tk promoter, and a SPAP reporter gene. Alkaline phosphatase activity was corrected for transfection efficiency using β-galactosidase activity as an internal standard. Drug dilutions were prepared in phenol red-free DMEM/F-12 with 15 mM HEPES buffer supplemented with 10% charcoal-stripped, delipidated calf serum. Drug-treated cells were incubated for 24 h, after which the medium was sampled and assayed for β-galactosidase and SPAP activity. Compound **4** profiled as a functional antagonist at ERβ, fully suppressing estradiol-stimulated transcriptional activity with an $IC_{50} = 160$ nM ($n = 2$). Compound **4** was a weak partial agonist at ERα, with a maximal stimulation of 16% relative to estradiol and an $EC_{50} = 30$ nM ($n = 2$). The partial agonist nature of compound **4** at ERα was confirmed by demonstrating that **4** was able to antagonize the effects of 17-β estradiol to 65% of its maximal efficacy, with an $IC_{50} = 30$ nM ($n = 2$). Compound **4** was roughly 5-fold more potent in this assay than in the binding assay, which is within reasonable agreement. The modest selectivity for the ERα subtype observed in this cell-based functional assay ($\beta/\alpha = 5.3$) is also in good agreement with the subtype selectivity observed in the binding assay ($\beta/\alpha = 4.3$).

In summary, utilizing solution-phase, parallel-synthesis techniques we have synthesized a series of 2-amino-4,6-diarylpyridines that are novel ligands for ERα and ERβ. These compounds show a modest preference for binding to the ERα subtype, with the most potent compound in this series (**19**) having a $K_i = 20$ nM at ERα. Compound **4** from this series profiled as a full antagonist at ERβ and a weak partial agonist at ERα in a cell-based functional assay. These ligands may have utility in the treatment of various diseases associated with estrogen loss.

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