Toward Selective ERβ Agonists for Central Nervous System Disorders: Synthesis and Characterization of Aryl Benzthiophenes

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Received October 12, 2001

Abstract: In an effort to identify selective ligands for the estrogen receptor subtype ERβ, a series of aryl benzthiophenes was synthesized. In a radioligand binding assay and reporter gene assays in HeLa and SH-SY5Y cells, compounds were characterized as ERβ-selective agonists. By targeting ERβ in the brain, these compounds could lead to drugs able to separate the beneficial effects of estrogens on mood, learning, and memory from side effects such as the stimulation of endometrial and breast cancer.

For the past decade, the physiological effects of estrogens were attributed to a single receptor of the ligand activated transcription factor family, now known as ERα. The recent discovery of a second estrogen receptor ERβ[1,2] has fueled an intensive effort to elucidate differences in function and to evaluate the pharmacological potential of selective ligands.[3,4]

Both estrogen receptors bind 17β-estradiol with high affinity and bind to classical estrogen response elements in a similar fashion. However, there are differences in tissue distribution and transcriptional activity.

The distribution pattern of both receptors in the central nervous system (CNS) and periphery is intriguing. While ERα and ERβ coexpress in some brain areas, ERβ is the dominant receptor in the hippocampus, in nuclei of the basal forebrain, and in the neocortex.[5,6] On the other hand, there is little or no expression of ERβ in reproductive tissues such as the uterus.[7] In support of these results, the uterus of ERα knock-out mice is insensitive to 17β-estradiol.[8]

Taken together, this distribution pattern suggests that a selective ERβ agonist could emulate the beneficial effects of estrogens on mood, learning, and memory, while being free of the unwanted side effects associated with estrogen replacement therapy, most notably the stimulation of endometrial (uterus) and breast cancer.[3]

Human ERα and ERβ show a 60% homology in their ligand binding domains (LBD).[1,9] However, the crystal structures of liganded ERα[10] and ERβ[11] LBDs show little variation in the direct vicinity of the ligand (Figure 1). While this helps to explain the high affinity of 17β-estradiol to both ERα and ERβ, it also highlights why attempts to find selective ligands have met with limited success to date.

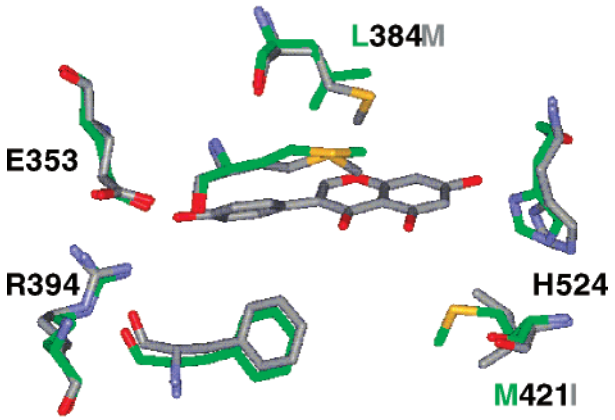

Figure 1. Superposition of experimental X-ray structures (PDB entries: 1ere, 1qkm) of the ligand binding domains of ERα (green C atoms) and ERβ with liganded genistein (gray C atoms). For clarity, only some selected residues of the ligand binding sites are shown. Polar interactions are formed by residues E353, R394, and H524. The only residues different in the ligand binding sites of ERα and ERβ are L384 and M421 in ERα and M336 and I373 in ERβ, respectively.

Although compounds with some selectivity for ERα have been reported in the literature,[12] the isoflavonoid genistein remains the only agonist with appreciable, although modest, selectivity for ERβ reported to date.[13] However, genistein and other isoflavonoid compounds have been shown to induce DNA strand breaks and chromosomal aberations.[14]

This genotoxicity has been linked to inhibition of topoisomerase II and to a common structural element of bioflavonoids comprising the benzpyran ring system.[15] No genotoxic activity has been reported for the antiestrogen raloxifene, which is built on a structurally similar motif, namely a benzthiophene core. We therefore decided to explore the potential of aryl benzthiophenes as lead structures for selective ERβ agonists. Related structures such as aryl indoles and aryl benzofuranes have been evaluated as estrogen receptor antagonists.[16] Here we report our findings in a series of aryl benzthiophenes.

Chemistry. Aryl benzthiophene compounds were synthesized via a Suzuki coupling reaction of benzthiophene boronic acids with aryl bromides.[17] The boronic acid precursors were easily accessible by treatment of benzthiophenes[18] with *n*-BuLi followed by a quench with triisopropylborate and cleavage of the resulting boronic ester with HCl (Scheme 1).

This sequence allowed the preparation of a variety of substituted aryl benzthiophene compounds (**1**−**13**, Table 1) and is easily amenable to fast parallel synthesis.

Biology. To screen compounds for their affinity and selectivity for ERβ vs ERα we set up an in vitro ligand binding assay[19] on recombinant human ERα and ERβ using [^{3}H]17β-estradiol as radioligand. [^{3}H]17β-estradiol bound in a stereoselective, saturable, and protein dependent manner, apparently to a single site of ERα and ERβ, respectively.

Compounds were also evaluated for their ability to activate an estrogen response element (ERE) in a

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10.1021/jm015577l CCC: $22.00 © 2002 American Chemical Society
Published on Web 02/26/2002

Scheme 1. Synthesis of Benzthiophene Compounds

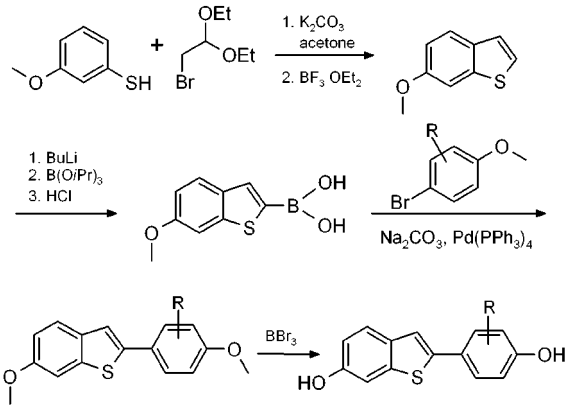
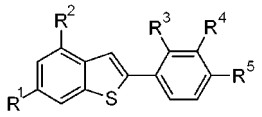


Table 1. Benzthiophene Compounds **1**−**13**



compd	R^1	R^2	R^3	R^4	R^5
1	OH	H	H	H	H
2	OH	H	H	H	CH_3
3	OH	H	H	H	OH
4	OH	H	H	F	OH
5	OH	H	H	CH_3	OH
6	OH	H	H	CH_2CH_3	OH
7	OH	H	H	$CH_2CH_2CH_3$	OH
8	OH	H	H	OH	OH
9	OH	H	H	OH	H
10	OH	H	H	$C(O)CH_3$	OH
11	OH	H	CH_3	H	OH
12[18]	OH	OH	H	F	OH
13[18]	OH	OH	H	CH_3	OH

Table 2. Estrogen Receptor Binding Affinities ([^{3}H] Binding) and Reporter Gene Activation in HeLa Cells (ERE) of Reference Compounds and **1**−**13**

	[^{3}H] binding[a]		ERE[a]	
compd	EC_{50} ERβ [nM]	EC_{50} ERα [nM]	EC_{50} ERβ [nM]	EC_{50} ERα [nM]
17β-estradiol	24	24	0.07	0.02
17α-estradiol	1230	570	4.5	1.5
diethylstilbestrol	9	12	0.02	0.06
estrone	1135	480	2.1	0.7
genistein	**200**	**3920**	**4.1**	**48**
raloxifene	260	22	na	na
1	4310	3710	166	209
2	>10000	>10000	174	178
3	**114**	**1410**	**17**	**50**
4	**115**	**1030**	**32**	**138**
5	1830	3650	302	148
6	4710	7440	257	191
7	3940	>10000	186	162
8	**249**	**4980**	525	363
9	**740**	**2210**	245	269
10	>10000	>10000	>1000	>1000
11	**129**	**341**	**15**	**48**
12	7470	>10000	>1000	1000
13	>10000	>10000	>1000	>1000

[a] Values are means of 3−5 experiments, and standard deviation was typically 15% of mean or less (na = no agonist activity).

luciferase reporter gene assay[20] (Table 2). We used HeLa cells stably transfected with the human ERα or ERβ (full length) receptor and subsequently with a luciferase reporter gene bearing an estrogen response element driven by a β-globulin minimal promoter.

Table 3. Reporter Gene Activation in Neuroblastoma Cells (ERβ)

compd	neuroblastoma cells EC_{50} ERβ [nM][a]
17β-estradiol	0.10
genistein	**1.7**
1	69
2	120
3	**10**
4	**13**
7	257
11	**16**

[a] Values are means of 3−5 experiments, and standard deviation was typically 15% of mean or less.

The examination of a number of reference compounds indicated that the pharmacology of both ERα and ERβ in these assays was in line with what would be expected from estrogen receptors.

The endogenous ligand 17β-estradiol showed equally high affinity and activity on both estrogen receptors while genistein displayed some selectivity for ERβ. As expected, the tissue-selective antagonist/partial agonist raloxifene showed no agonist activity on either receptor in the functional assay.

The ligand binding site of both estrogen receptors is characterized by hydrogen bond acceptors at the far ends of the cavity (Figure 1), and the structural differences between ERα and ERβ in the ligand binding site (L384M, M421I) are located in between. Residues in the R2, R3, and the R4 position of aryl benzthiophenes are most likely to interact with these amino acids, and we decided to focus structural variation on these positions. As a result, the series of aryl benzthiophenes **1**−**13** outlines a SAR for ERβ selectivity (Table 2, columns 2 and 3). The unsubstituted phenol benzthiophene **3** displays some selectivity for ERβ, and a fluorine substituent at R4 is tolerated (**4**). However, the ERβ selectivity of genistein is not surpassed with these compounds. Increasing the size of the substituent at R4 (**5**−**7**, **10**) results in a gradual loss of activity. Position R3 seems sterically less restricted, at least a methyl group is tolerated (**11**). On the benzthiophene core, introduction of a hydroxy substituent in R2 completely deletes affinity.

The selectivities seen in the binding assay could largely be confirmed in a reporter gene assay in HeLa cells (Table 2, columns 4 and 5).

As a pleiothropic hormone, the effects of 17β-estradiol in different tissues depend among other factors on the specific promoter architecture and co-activator recruitment in a target cell. Different ligand activation of ERα and ERβ on different response elements[21] as well as differential co-activator recruitment[22] of these two receptors have been demonstrated.

Therefore, having determined ERβ selectivity of several aryl benzthiophenes in the cervical adenocarcinoma cell line HeLa, we wanted to test whether these compounds would retain agonist activity in a neuronal cell. To this end, the human neuroblastoma cell line SH-SY5Y was simultaneously transfected with the human ERβ (full length) receptor and the luciferase reporter gene bearing an estrogen response element driven by a β-globulin minimal promoter.[23]

As shown in Table 3, in the human neuroblastoma cell line SH-SY5Y all compounds retained agonism with

an EC_{50} comparable to what was previously observed in HeLa cells.

In summary, we have synthesized and tested a series of aryl benzthiophene derivatives and have found agonists with selectivity for ERβ. These findings could lead to drugs that, by targeting ERβ in the brain, are able to separate the beneficial effects of estrogens on mood, learning, and memory from unwanted side effects such as the stimulation of endometrial and breast cancer.

Acknowledgment. We thank W. Huber, J. Zadrobilek, I. Bobirnac, P. Martin, and N. Reymann for expert technical assistance.

Supporting Information Available: Analytical and spectral characterization data of key compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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(19) [^{3}H] binding assay: The radioreceptor assay was performed by using 96-well microtiterplates (Picoplates, Packard) in volumes of 0.2 mL of incubation buffer (50 mM Tris, pH 7.4). The incubation mixture contained 5 nM ERα or 6 nM ERβ long form receptors, 8 nM [^{3}H]17β-estradiol ($\approx$180 000 total counts), the compound to be tested, and 0.25 mg/well SPA-beads. After incubation at room temperature for 240 min, the reaction was terminated by centrifugation at room temperature (10 min at 1000*g*). The radioactivity was counted at least 3 h after completion of the experiment in a Packard Topcount scintillation counter. Nonspecific binding was defined as the remaining radioactivity in the presence of 10 μM nonradioactive 17β-estradiol (Sigma). Assays were performed in triplicate.

(20) Reporter gene assay in HeLa cells: Cells were obtained from P. Balaguer, Montpellier: Balaguer, P.; François, F.; Comunale, F.; Fenet, H.; Boussioux, A.-M.; Pons, M.; Nicolas, J.-C.; Casellas, C. Reporter cell lines to study the estrogenic effects of xenoestrogens. *Sci. Total Environ.* **1999**, *233*, 47−56. Cells were seeded at a density of 75 000 cells/well in 96-well microtitre plates. After 5 h, 17β-estradiol, test compounds, or vehicle (DMSO 0.1% final concentration) were added and left in contact with the cells for 20 h. After aspiration of the medium, the cells were subjected to the luciferase assay using the LucLite reagent kit (Packard). Luminescence activity was counted for 6 s in a TopCount (Packard). Cells were maintained in a medium deprived of phenol red and containing charcoal stripped serum to keep steroid levels as low as possible. Compounds were tested in duplicate over the concentration range of 1 pM−1 μM, in parallel with 17β-estradiol (0.1 pM−0.1 μM). Concentration−response curves were fitted to the nonlinear logistic function of the Microcal Origin software package to yield EC_{50} values. The E_{max} parameter was constrained to the value obtained for 17β-estradiol in the same experiment.

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(23) Reporter gene assay in SH-SY5Y cells: the assay was performed essentially as described in ref 20.

JM015577L