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Hydrophobic Interactions Improve Selectivity to ERα for Benzothiophene SERMs

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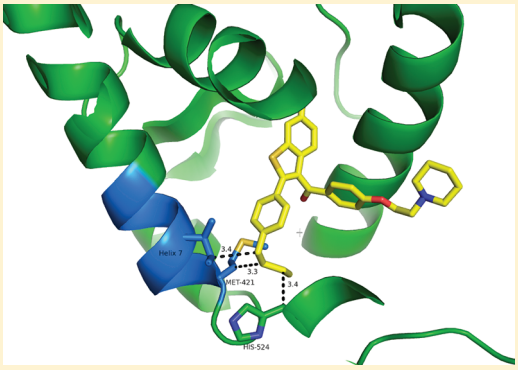
Supporting Information

ABSTRACT: The discovery, pharmacology, and biophysical characterization of an estrogen receptor α (ERα) selective benzothiophene (BTPα) is described. BTPα (**4**) is a high-affinity ligand with 140-fold greater selectivity for ERα (K_i = 0.25 nM) over estrogen receptor β (ERβ) (K_i = 35 nM). In rodent models of estrogen action, BTPα blocks the effects of estrogen in the uterus but mimics the effects of estrogen on bone. The basis of ERα selectivity for BTPα was evaluated by using protein crystallography and hydrogen/deuterium exchange (HDX) mass spectrometry. HDX data support that the *n*-butyl chain of BTPα stabilizes helix 7 in ERα relative to that of ERβ, which we propose leads to an enhancement of affinity to the α-receptor subtype.

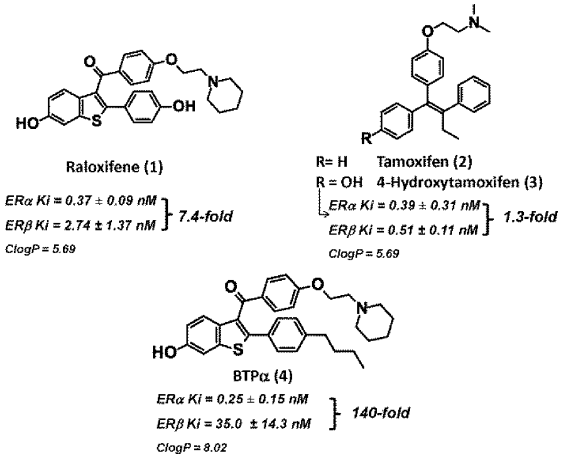


KEYWORDS: *estrogen receptor α, estrogen receptor β, SERM, hydrogen/deuterium exchange*

The steroid hormone estrogen mediates a number of biological processes that range from reproductive health to bone maintenance. Selective estrogen receptor modulators (SERMs) such as raloxifene (**1**) and tamoxifen (**2**) represent a class of therapeutic agents that demonstrate tissue selective pharmacology; that is, they can mimic the effects of estrogen in some tissues but block estrogen in other tissues.[1−3] All actions of estrogen and SERMs were thought to be mediated by a single estrogen receptor (ER) until 1996 when a second isoform, termed ERβ, was discovered.[4] The identification of ERβ has added further complexity to the molecular origin of tissue selectivity for SERMs. To better probe the biological roles of ERα and ERβ, we sought to identify highly selective ligands for each subtype to use as chemical tools. We have previously reported such efforts toward selective ERβ agonists.[5] Herein, we describe the discovery and pharmacology of a highly selective ERα SERM. The molecular basis of selectivity for ERα over ERβ is revealed by hydrogen/deuterium exchange (HDX) mass spectrometry.

The ERα selective benzothiophene **4** (Chart 1, BTPα) was discovered during the course of structure−activity studies on the benzothiophene nucleus.[6] This compound is a high-affinity ligand for ERα (K_i = 0.25 nM) with significantly diminished affinity for ERβ (K_i = 35 nM), resulting in 140-fold selectivity for the α subtype. In an assay of cell-based function, BTPα is an antagonist of estrogen action in MCF-7 breast cancer cells (IC_{50} = 33 nM). BTPα has good oral exposure after administration to rats (AUC 0−24 *h* = 2337 ng h/mL, C_{max} = 209.5 ng/mL, 10 mg/kg, po, data not shown). On the basis of these data, we evaluated BTPα in rodent models of estrogen dependency to determine tissue specificity. In young female rats, BTPα is a potent antagonist of estrogen-induced uterine hypertrophy as shown in Figure 1A. In older female rats that have been ovariectomized (OVX), BTPα prevents bone loss in a dose-dependent manner after 5 weeks of oral administration

Chart 1. Structures, ER Binding Affinities, Clog *P* Values (Chemaxon), and Receptor Subtype Selectivity for SERMs



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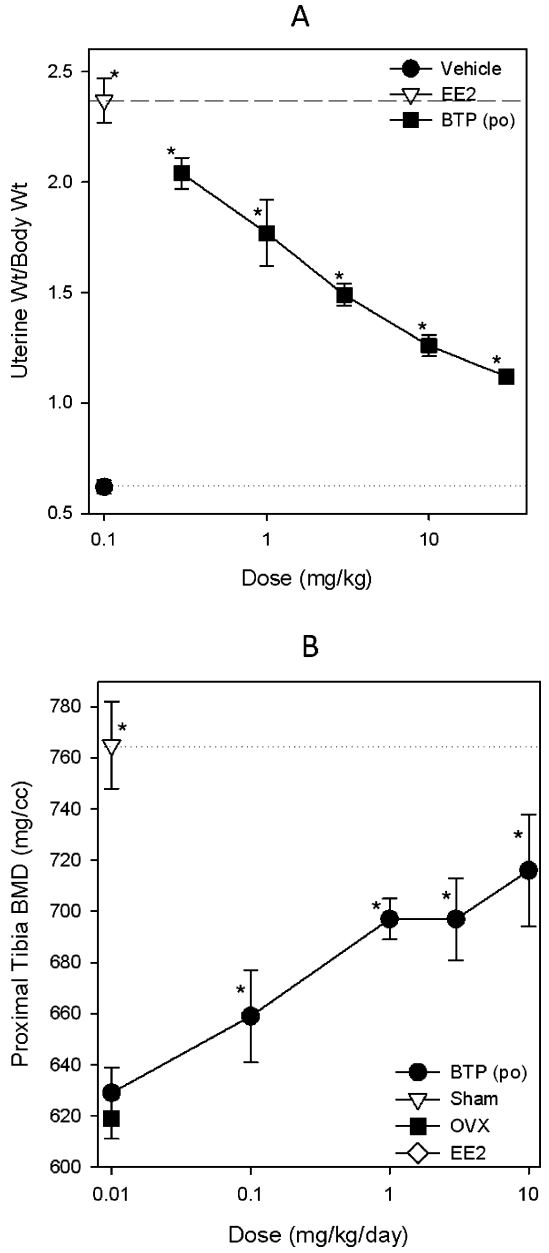

Figure 1. (A) Three week old Sprague–Dawley (SD) female rats were orally treated with estradiol (0.1 mg/kg) and 1.0, 0.1, and 0. 01 mg/kg SERM for 3 days, 6 rats per group. *Significant decrease from estradiol alone for each dose, $p < 0.05$. (B) Six month old SD were OVX and were orally treated with compound once daily or by intrapertaoneal (ip) injections starting 4 days postovariectomy. After 42 days of treatment, animals were sacrificed. Volumetric bone mineral density (vBMD) of the proximal metaphsis of excised tibea was measured using quantitative computed tomography. *Significant increase from OVX control, $p < 0.05$.

(see Figure 1B). Taken together, these data indicate that BTPα is a SERM that blocks the effects of estrogen on uterine tissue and breast cells while mimicking the effects of estrogen on bone tissue. These data support ERα being an important regulator of bone metabolism.

In efforts to understand the molecular basis of the ER subtype selectivity, we crystallized BTPα into the ligand binding domain (LBD) of ERα and compared it to the known protein cocrystal of **1**. As shown in Figure 2, the 6-OH

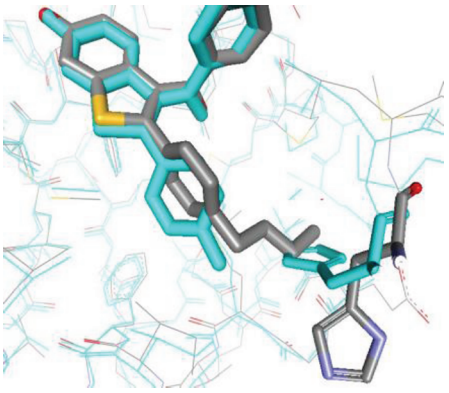

Figure 2. Protein crystal structures: Overlay of respective cocrystal structures of BTPα (gray) and **1** (teal) in ERα LBD.

group of BTPα anchors the benzothiophene in a manner similar to that seen with **1**.[7] In contrast, the *n*-butyl group at the 4′-position of BTPα significantly displaces histidine524, which in the case of **1** provides a hydrogen bond between the phenol and the protein. Given that the BTPα/ERα complex lacks this important hydrogen bond, we were intrigued that BTPα and **1** bind with the same high affinity to ERα with K_i' values of 0.25 and 0.38 nM, respectively. To better understand the role that the *n*-butyl group of BTPα plays in regulating selectivity, we attempted to obtain protein cocrystals of this compound bound to the LBD of ERβ but were unsuccessful. In reviewing the known protein cocrystal structures of ligands bound in ERα and ERβ, we were surprised to find that despite the abundance of cocrystal structures of SERMs bound in ERα, there are only two examples of a SERM bound in both receptors. This is for raloxifene, **1**[8] (PDB IDs: 1QKN for ERβ and 1ERR for ERα) and 4-hydroxytamoxifen, **3** (PDB IDs: 2FSZ for ERβ and 2ERT for ERα). Because both ligands have only marginal affinity-based selectivity (see Chart 1), comparing these structures reveals little about the molecular determinants of receptor subtype affinity. To understand why BTPα is selective for ERα, we used HDX mass spectrometry (MS) to determine the ligand-induced changes that BTPα confers on protein dynamics. HDX coupled with proteolysis and MS has evolved as a powerful biophysical method for characterizing the interactions of nuclear receptors.[9,10] In addition, this technology is highly versatile and has been successfully applied to other gene families including kinases and G-protein-coupled receptors.[11] Under HDX assay conditions, the local environment of backbone amide hydrogens in a protein can be probed by measuring their rates of exchange with deuterium by MS, the kinetics of which vary as a function of hydrogen bonding and, to a lesser degree, solvent accessibility.[12] While HDX has been used extensively to characterize proteins, this technology has received considerably less attention as a biostructural tool to study how small molecules impact protein dynamics. To probe the use of HDX for quantifying such interactions, we evaluated the origins of receptor subtype selectivity for BTPα.

HDX analysis was conducted with BTPα in the presence and absence of both ERα LBD and ERβ LBD. The exchange kinetics for the relevant regions of the LBD is summarized in the Supporting Information in Tables 1 and 2. The values represent the average difference in deuterium incorporation percentages for each of the on-exchange time points [percent deuterium (% D) vs log time]. For consistency, we have used ERα and ERβ isoform-1 numbering in the discussion of both

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the HDX and the X-ray data. The peptides showed differential HDX protection in a region-specific manner for each receptor. For ERα, the amino acid regions that are most protected to exchange (>20%) in the presence of BTPα include 311−319, 320−327, 349−367, 391−402, 403−410, 422−428, and 508−525 (numbering for ERα isoform 1) . In ERβ, BTPα stabilizes peptides 273−281, 295−309, 346−362, and 462−476. The data from the HDX analysis were overlaid onto the static cocrystal structures of ERα LBD/BTPα (Figure 3A) as well as

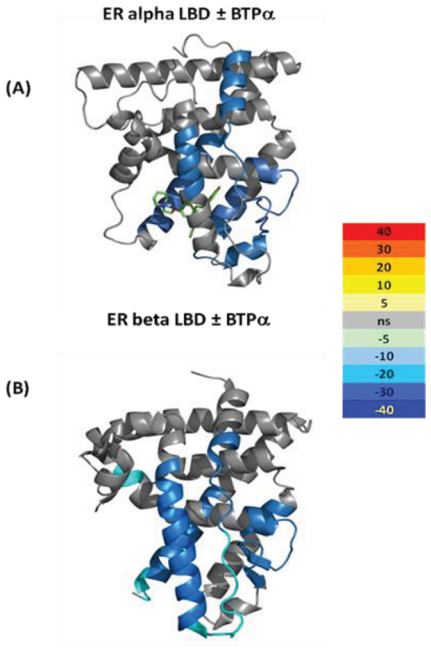

Figure 3. Average differential HDX profile of BTPα (**4**) overlaid onto ERα (A) and ERβ LBD (B) crystal structures. The color legend shows the differential HDX between Apo ERα or ERβ LBD and the BTPα bound ones.

ERβ LBD (Figure 3B), the latter using the known cocrystal of **1** in ERβ (PDB ID: 1QKN) as a template. When comparing the differences between the two HDX fingerprints, helix 3 is similarly stabilized in both receptors, data that are consistent with other SERMs.[6,7] In this helix, the amino acid residues that anchor the phenol at the 6-position of the benzothiophene of BTPα, that is, the peptide fragments containing Glu(353) and the Arg(394), are protected to exchange by 18 and 13%. These data are supported by the Gly353−Arg394 interactions observed in the static atomic structure shown in Figure 2. Likewise for ERβ, the analogous fragments containing Glu(305) and Arg(346) are protected to exchange.

The largest HDX differences between ERα or ERβ and BTPα are observed in helix 7 where the lower end of the helix is protected to exchange in ERα (residues MVEIFDM) but not ERβ (residues DRDEGKCVEGILE). The deuterium build-up curves for each of these peptides are shown in Figure 4. This stabilization is supported by the cocrystal structure of BTPα in ERαLBD for which favorable van der Waal interactions (see Figure 5) are observed between the methylene groups in the *n*-butyl alkyl chain and Ile424 (3.4 Å), Met421 (3.3 Å), and His524 (3.4 Å). In ERβ, HDX shows no protection to exchange in this region, that is, peptide $[363-375H]^{2+}$ (DRDEGKCVEGILE) is not protected (see Figure 4). This is likely due the branching on Ile373 (Met421 in α), one of the only two

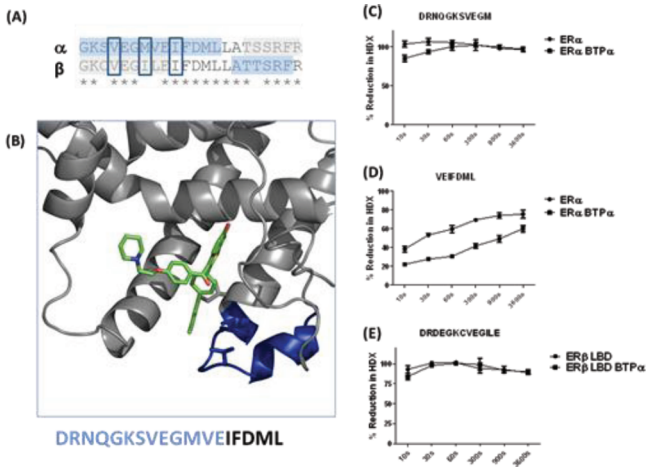

Figure 4. BTPα stabilizes helix 7 within ERα LBD but not ERβ LBD. (A) Sequence alignment of ERα and ERβ. Regions of the sequence protected to exchange upon compound binding are colored blue, and those colored gray exhibited no perturbation in HDX upon binding. Regions with no color were not covered in the HDX MS experiment. Blue boxes show ERα residues Ile424, Met421, and Val418 involved in van der Waal interactions with BTPα. (B) BTPα is shown in the LBD of ERα (H3 removed for clarity). Regions shaded blue represent those residues protected to HDX in ERα but not ERβ (sequence shown at the bottom of the figure). (C and D) Percent deuterium vs time plots showing protection to exchange in ERα upon binding of BTPα. (E) Minimal protection to HDX was observed for the corresponding sequence of ERβ upon binding of BTPα.

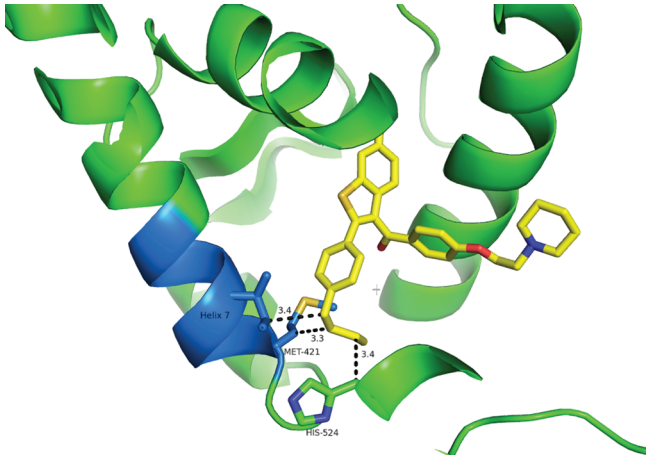

Figure 5. Van der Waal interactions of BTPα with ERα. The colored region shows HDX stabilization.

residues that are different between α and β, that disrupts the van der Waal interactions that are observed in the BTPα/ERα complex. Thus, we propose that the receptor subtype affinity-based differences between ERα and ERβ for BTPα result from favorable interactions between the *n*-butyl group on BTPα and the lipophilic amino acid residues on helix 7 in ERα, interactions that are not compatible in ERβ.

In summary, we have identified BTPα, a synthetic benzothiophene SERM that is highly selective for ERα over ERβ. In rodents, BTPα is an antagonist of estrogen action on uterine tissue and an agonist on bone. The basis of ERα selectivity for BTPα was evaluated by using HDX in which the *n*-butyl chain of BTPα was observed to stabilize helix 7 in ERα relative to that of ERβ, presumably enhancing the relative

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affinity to the ERα receptor subtype. These data provide the first direct biostructural evidence for the molecular basis of ERα subtype selectivity for benzothiophene SERM ligands.

■ ASSOCIATED CONTENT

Supporting Information

Detailed experimental procedures for biological assays, HDX, and protein crystallography. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

M.J.C. designed and interpreted HDX experiments and contributed to the writing of the manuscript. Y.W. designed and interpreted protein crystallography experiments. S.N. performed HDX experiments. M.S. and H.U.B. designed and interpreted rodent experiments. C.M.-R. designed and interpreted binding and cell experiments. P.R.G. designed and interpreted HDX experiments and contributed to the writing of the manuscript. J.A.D. designed and interpreted experiments and contributed to the writing of the manuscript.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

HDX, hydrogen/deuterium exchange mass spectrometry; ER, estrogen receptor; SERM, selective estrogen receptor modulator; LBD, ligand binding domain; MS, mass spectrometry; OVX, ovariectomized; SD, Sprague–Dawley; PDB, protein data bank

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