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Hydrophobic Interactions Improve Selectivity to ERα for Benzothiophene SERMs

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Abstract

The discovery, pharmacology, and biophysical characterization of an 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 ERbeta (K_i=35 nM). In rodent models of estrogen action, BTPα blocks the effects of estrogen in the uterus but mimics the effects 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 supports 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 alpha receptor sub-type.

Keywords

Estrogen receptor alpha; estrogen receptor beta; 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, i.e, they can mimic the effects of estrogen in some tissues but block estrogen in other tissues.[1,2,3] All actions of estrogen and SERMs were thought to be mediated by a single 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. In order 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 towards 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.

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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.

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 (Ki = 0.25 nM) with significantly diminished affinity for ERβ (Ki = 35 nM) resulting in 140-fold selectivity for the alpha sub-type. 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*hr/mL, Cmax = 209.5 ng/mL, 10 mg/kg, p.o, data not shown). Based on this data, we evaluated BTPα in rodent models of estrogen-dependency in order 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, BTPα prevents bone loss in a dose-dependent manner after 5 weeks of oral administration (see Figure 1B). Taken together, this data indicates 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. This data supports ERα being an important regulator of bone metabolism.

In efforts to understand the molecular basis of the ER sub-type selectivity, we crystallized BTPα into the ligand binding domain (LBD) of ERα and compared it to the known protein co-crystal of **1**. As shown in Figure 2, the 6-OH 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 Ki's of 0.25 nM and 0.38 nM, respectively. In order to better understand the role that the *n*-butyl group of BTPα plays in regulating selectivity, we attempted to obtain protein co-crystals of this compound bound to the LBD of ERβ but were unsuccessful. In reviewing the known protein co-crystal structures of ligands bound in ERα and ERβ, we were surprised to find that despite the abundance of co-crystal structures of SERMs bound in ERα, there are only two example of a SERM bound in both receptors. This is for raloxifene, **1**[8] (PDB ID's: 1QKN for ERβ ; 1ERR for ERα) and 4-hydroxytamoxifen, **3** (PDB ID's: 2FSZ for ERβ; 2ERT for ERα). Since both ligands have only marginal affinity-based selectivity (see Chart 1), comparing these structures reveals little about the molecular determinants of receptor sub-type affinity. In order to understand why BTPα is selective for ERα we used hydrogen/deuterium exchange (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. In order to probe the use of HDX for quantifying such interactions, we evaluated the origins of receptor sub-type 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 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, 508-525 (numbering for ERα isoform 1). In ERβ BTPα stabilizes peptides 273-281, 295-309, 346-362and 462-476. The data from the HDX analysis was overlaid onto the static co-crystal structures of ERα LBD/BTPα (Figure 3A) as well as ERβ LBD (Figure 3B), the latter using the known co-crystal 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 which is 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, i.e., the peptide fragments containing Glu(353) and the Arg(394), are protected to exchange by 18% and 13%. This data is 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 co-crystal 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 angstroms), Met421 (3.3 angstrom), and His524 (3.4 angstroms). In ERβ, HDX shows no protection to exchange in this region, i.e., peptide $[363\text{-}375H]^{2+}$ (DRDEGKCVEGILE) is not protected (see Figure 4). This is likely due the branching on Ile373 (Met421 in alpha), one of the only two residues that are different between alpha and beta, that disrupts the van der Waal interactions that are observed in the BTPα/ERα complex. Thus, we propose that the receptor sub-type affinity-based differences between ERα and ERβ for BTPα results from favorable interactions between the *n*-butyl group on BTPα and the lipophilic amino acid residues on helix 7 in ERα, interactions which 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 affinity to the ERα receptor sub-type. This data provides the first direct biostructural evidence for the molecular basis of ERα subtype selectivity for benzothiophene SERM ligands.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References

1. Jordan VC. Antiestrogens and selective estrogen receptor modulators as multifunctional medicines.
   1. Receptor interactions. J Med Chem. 2003; 46:883–908. [PubMed: 12620065]

2. Jordan VC. Antiestrogens and Selective Estrogen Receptor Modulators as Multifunctional Medicines. 2. Clinical Considerations and New Agents. J Med Chem. 2003; 46:1081–1111. [PubMed: 12646017]

3. Dodge JA, Richardson TI. Novel Estrogen Receptor Modulators (SERMs). Annual Reports in Medicinal Chemistry. 2007; 42:147–160.

4. Kuiper GGJM, Enmark E, Pelto Huikko M, Nilsson S, Gustafsson JA. Cloning of a novel estrogen receptor expressed in rat prostate and ovary. Proc Natl Acad Sci U S A. 1996; 93:5925–5930. [PubMed: 8650195]

5. Norman BH, Dodge JA, Richardson TI, Borromeo PS, Lugar CW, Jones SA, Chen K, Wang Y, Durst GL, Barr RJ, Montrose-Rafizadeh C, Osborne HE, Amos RA, Guo S, Boodhoo A, Krishnan V. Benzopyrans Are Selective Estrogen Receptor β Agonists with Novel Activity in Models of Benign Prostatic Hyperplasia. J Med Chem. 2006; 49:6155–6157.10.1021/jm060491j [PubMed: 17034120]

6. Grese TA, Cho S, Finley DR, Godfrey AG, Jones CD, Lugar CW, Martin MJ, Matsumoto K, Pennington LD, Winter MA, Adrian MD, Cole HW, Magee DE, Phillips DL, Rowley ER, Short LL, Glasebrook AL, Bryant HU. Structure–Activity Relationships of Selective Estrogen Receptor Modulators: Modifications to the 2-Arylbenzothiophene Core of Raloxifene. J Med Chem. 1997; 40:146–167.10.1021/jm9606352 [PubMed: 9003514]

7. Brzozowski AM, Pike ACW, Dauter Z, Hubbard RE, Bonn T, Engstrom O, Ohman L, Greene GL, Gustafsson JA, Carlquist M. Molecular basis of agonism and antagonism in the oestrogen receptor. Nature. 1997; 389:753–758. [PubMed: 9338790]

8. Pike ACW, Brzozowski AM, Hubbard RE, Bonn T, Thorsell A-G, Engstrom O, Ljunggren J, Gustaffson J-A, Carlquist M. Structure of the ligand-binding domain of oestrogen receptor beta in the presence of a partial agonist and a full antagonist. Embo J. 1999; 18:4608–4618. [PubMed: 10469641]

9. Dai SY, Chalmers MJ, Bruning J, Bramlett KS, Osborne HE, Montrose-Rafizadeh C, Barr RJ, Wang Y, Wang M, Burris TP, Dodge JA, Griffin PR. Prediction of tissue-specificity of selective estrogen receptor modulators by using a single biochemical method. Proc Natl Acad Sci USA. 2008; 105:7171. [PubMed: 18474858]

10. Dai SY, Burris TP, Dodge JA, Montrose-Rafizadeh C, Wang Y, Chalmers MJ, Griffin PR. Unique ligand binding patterns between estrogen receptor alpha and beta revealed by hydrogen-deuterium exchange. Biochemistry. 2009; 48:9668. [PubMed: 19739677]

11. West GM, Chien EYT, Katritch V, Gatchalian J, Chalmers MJ, Stevens RC, Griffin PR. Ligand-Dependent Perturbation of the Conformational Ensemble for the GPCR b2 Adrenergic Receptor Revealed by HDX. Structure. 2011; 19:1–9.10.1016/j.str.2011.08.001 [PubMed: 21220108]

12. Chalmers MJ, Busby SA, Pascal BD, West GM, Griffin PR. Differential hydrogen/deuterium exchange mass spectrometry analysis of protein-ligand interactions. Expert Rev Proteomics. 2011; 8:43–59. [PubMed: 21329427]

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

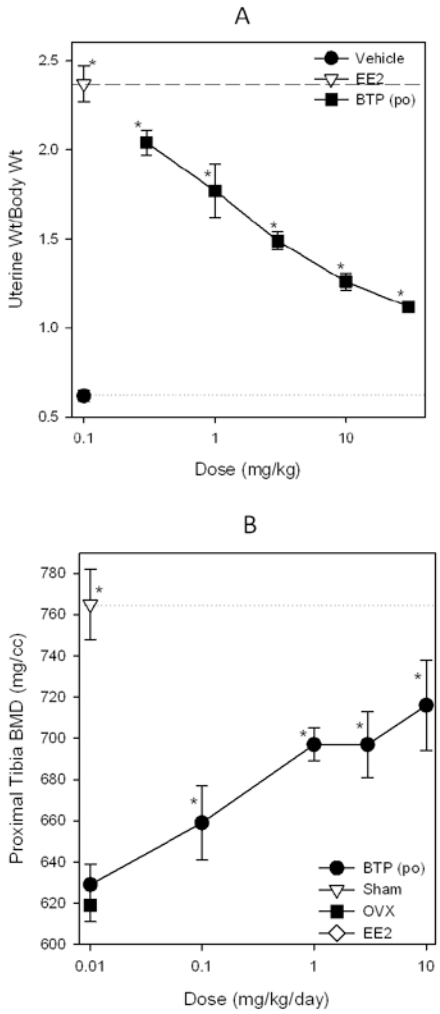

Figure 1.

(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 ovariectomized and were orally treated with compound once daily or by intrapertaoneal (IP) injections starting 4 days post-ovariectomy. 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, $p < 0.05$.

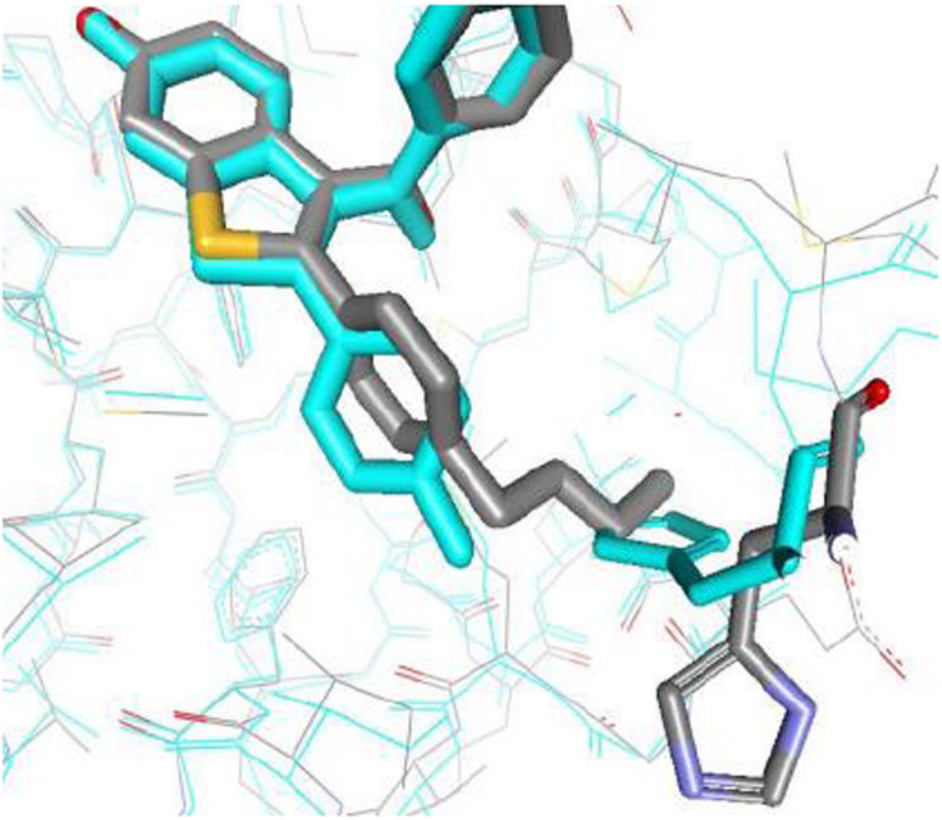

Figure 2.
Protein crystal structures: Overlay of respective co-crystal structures of BTPα (grey) and **1** (teal) in ERα LBD.

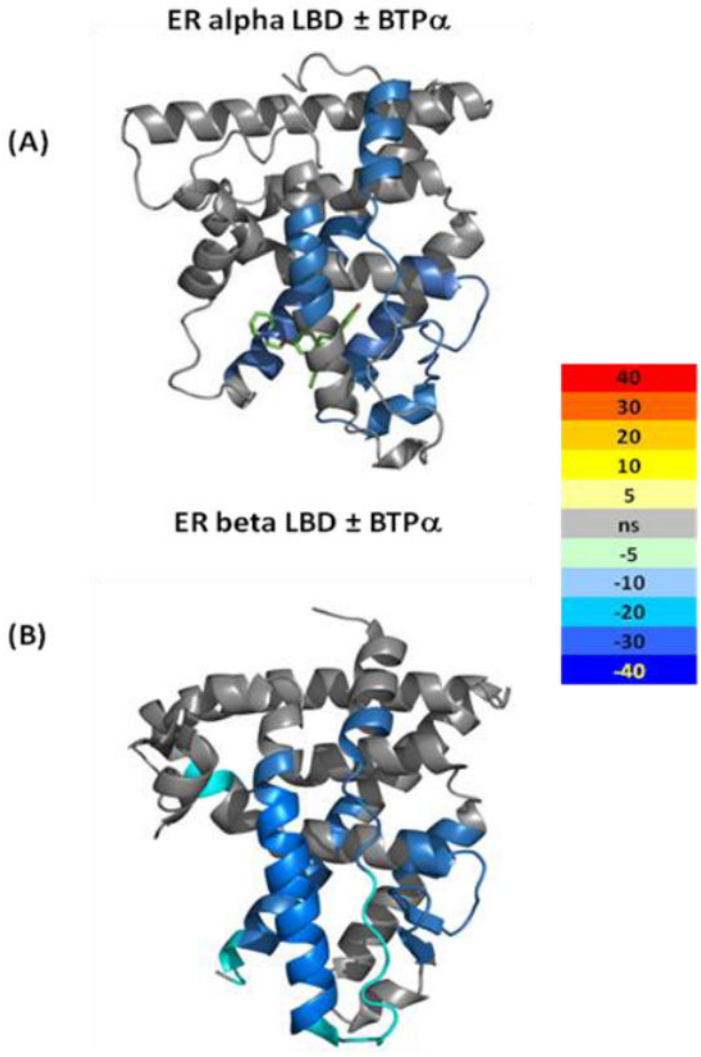

Figure 3.
Average differential HDX profile of BTPα (**4**) overlaid onto ERα (3A) and ERβ LBD (3B) crystal structures:. The color legend shows the differential HDX between Apo ERα or ERβ LBD and the BTPα bound ones.

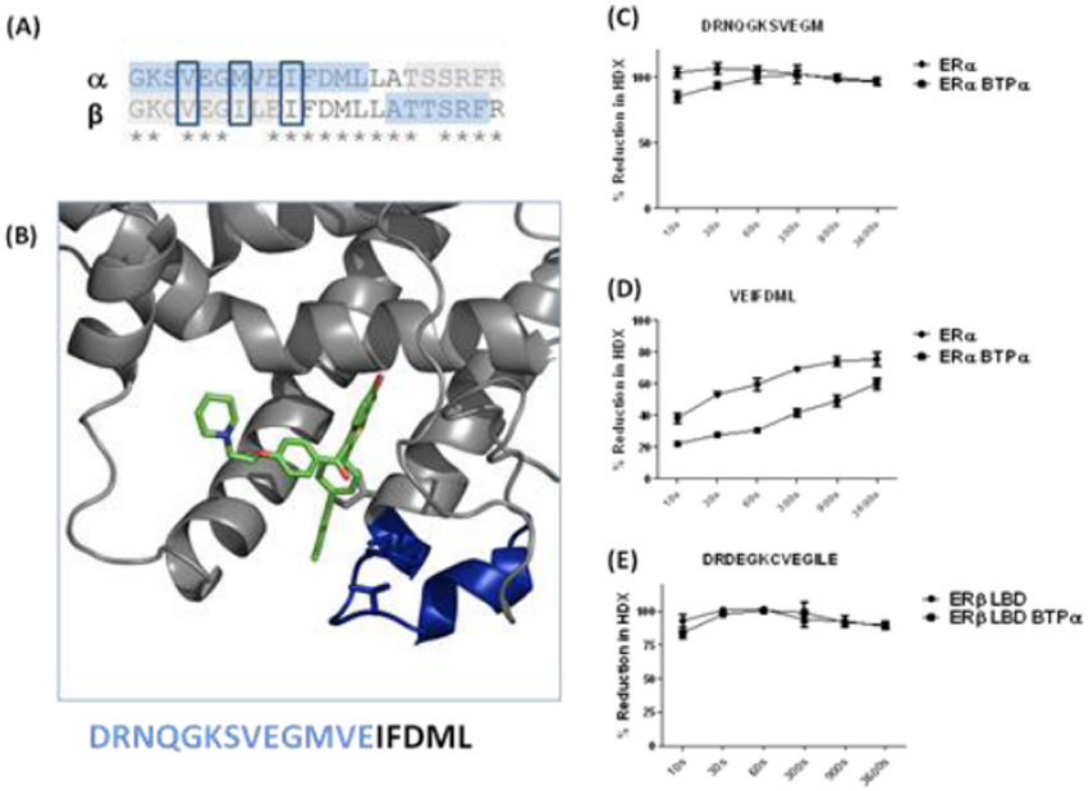

Figure 4.

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, those colored grey 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.

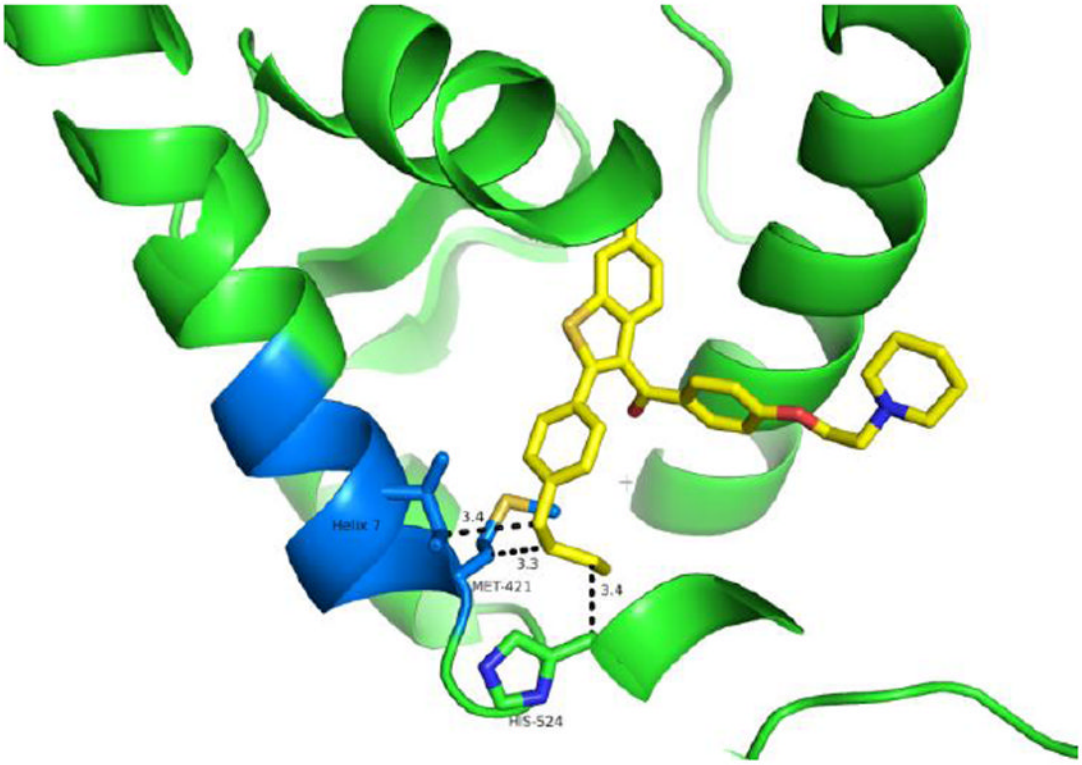

Figure 5.
Van der waal interactions of BTPα with ERα. Colored region show HDX stabilization.

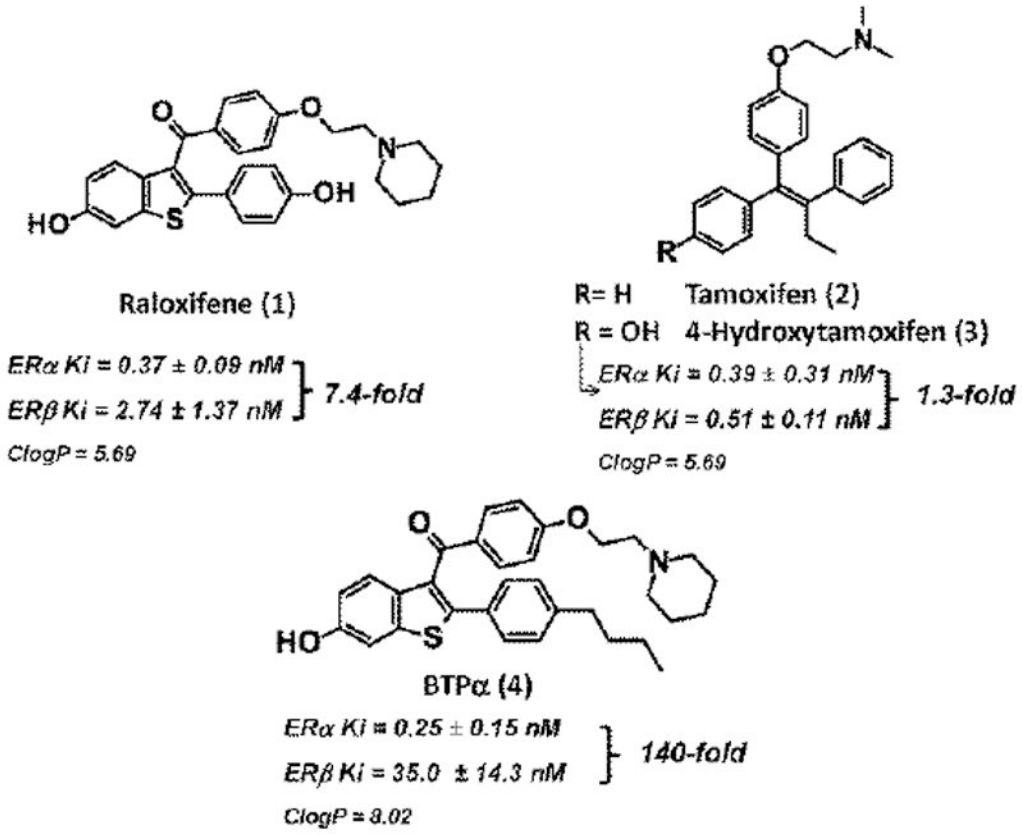

Chart 1.
Structures, ER binding affinities, ClogP's (Chemaxon), and receptor subtype selectivity for SERMs