Published in final edited form as:
Mol Cell Endocrinol. 2011 July 4; 340(2): 120–126. doi:10.1016/j.mce.2010.09.012.

Aromatase, Estrone Sulfatase, and 17β-Hydroxysteroid Dehydrogenase: Structure-Function Studies and Inhibitor Development

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Abstract

Aromatase, estrone sulfatase, and 17β-hydroxysteroid dehydrogenase type 1 are involved in the key steps of 17β-estradiol biosynthesis. Structure-function studies of aromatase, estrone sulfatase and 17β-hydroxysteroid dehydrogenase type 1 are important to evaluate the molecular basis of the interaction between these enzymes and their inhibitors. Selective and potent inhibitors of the three enzymes have been developed as antiproliferative agents in hormone-dependent breast carcinoma. New treatment strategies for hormone-dependent breast cancer are discussed.

Keywords

Aromatase; estrone sulfatase; 17β-hydroxysteroid dehydrogenase; crystal structure; inhibitor; breast cancer

1. Introduction

The great majority of breast cancers are hormone-dependent, and it is widely accepted that estrogen plays an important role in the genesis and evolution of breast tumors. It is well established that the concentration of 17β-estradiol (E2), the most biologically active estrogen in breast tumors, can be as much as ten-fold higher than that in plasma in postmenopausal women (van Landeghem et al. 1985). Human breast cancer tissues contain the essential enzymes aromatase, estrone sulfatase, and 17β-hydroxysteroid dehydrogenase (17β-HSD), involved in the key steps of E2 biosynthesis (Figure 1). Two principle pathways are implicated in the formation of E2 in breast cancer tissues: the ‘aromatase pathway’, which converts androgens (androstenedione and testosterone) into estrogens (estrone and E2), and the ‘sulfatase pathway’, which converts estrone sulfate into estrone by estrone sulfatase. Another important step is the conversion of the functionally less active estrone to the biologically potent E2 by the action of 17β-HSDs. It has been proposed that intracrine biosynthesis of estrogens by aromatase, estrone sulfatase, and 17β-HSDs in the breast accounts for most of the estrogens in postmenopausal women. Because E2 has a stimulatory effect on the proliferation of breast cancer cells, blocking its formation by the inhibition of these enzymes should be of paramount importance for the control of breast tumor growth.

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Selective and potent inhibitors of these enzymes have been developed and have shown promise as antiproliferative agents in hormone-dependent breast carcinoma. For example, the third-generation aromatase inhibitors (AIs) (i.e., anastrozole, letrozole and exemestane) have been approved by the FDA for the treatment of hormone-dependent breast cancer in postmenopausal women. Dr. Mike Reed made important contributions in the demonstration of the functional importance of the three enzymes, as well as in the development of potent estrone sulfatase inhibitors and dual aromatase-sulfatase inhibitors to be potential drugs for estrogen-dependent breast cancer. To recognize Dr. Reed's contributions in these areas, his and other investigators' findings on structure-function studies of aromatase, estrone sulfatase, and 17β-HSDs and inhibitors of these enzymes are reviewed in this paper.

2. Aromatase

Aromatase is a cytochrome P450 (CYP450) and is the rate-limiting enzyme in estrogen biosynthesis. Through interaction with NADPH-cytochrome P450 reductase (CPR), aromatase catalyzes three steps of hydroxylation to convert androgen to estrogen. Significant efforts from a number of laboratories were made to study the mechanisms of aromatization (Akhtar et al. 1982; Brodie et al. 1969; Hackett et al. 2005; Hahn and Fishman 1984; Miyairi and Fishman 1985; Morand et al. 1975; Numazawa et al. 1994). To understand the structure-function relationship of aromatase, molecular characterization of purified aromatase (Hong et al. 2007; Kagawa et al. 2004; Yoshida and Osawa 1991), site-directed mutagenesis (Auvray et al. 2002; Hong et al. 2008; Hong et al. 2007; Kadohama et al. 1993; Kao et al. 1996; Kao et al. 2001; Kao et al. 1998), and structural modeling analysis (Favia et al. 2006; Graham-Lorence et al. 1995; Hong et al. 2007; Laughton et al. 1993) have been carried out. The crystal structure of full-length aromatase in complex with androstenedione solved at 2.9 Å resolution marks a major milestone in structure determination of CYP450s (Ghosh et al. 2009), as this is the first crystal structure of full-length transmembrane CYP450, although the structure of the N-terminal transmembrane domain was not well defined (Figure 2A). The active-site cleft of the complex is relatively small (< 400 $Å^3$) when compared with other CYP450s, thus an androstenedione molecule fits snugly into this androgen-specific cleft (Figure 2B). This crystal structure confirms several key active site residues predicted from previous site-directed mutagenesis and structure modeling, including D309 and T310 (I helix), F134 (B-C loop), S478 (β-4 sheet), and V370-M374 (3′-flanking loop of the K helix) (Hong et al. 2007), and suggests additional active site residues F221, W224, M447, and S470.

Third-generation AIs (Figure 3) including two triazole derivatives, anastrozole (Arimidex) (Plourde et al. 1995) and letrozole (Femara) (Lipton et al. 1995), and one steroid analogue, exemestane (Aromasin) (Evans et al. 1992), are currently used clinically for the endocrine treatment of hormone-dependent breast cancer in postmenopausal patients (Bajetta et al. 2000;Plourde et al. 1995;Smith and Dowsett 2003). The x-ray crystal structure of aromatase in the presence of the substrate androstenedione helps us to understand better how the steroidal inhibitor exemestane interacts with the enzyme (Ghosh et al. 2009). However, the current information is not sufficient to explain the molecular basis of the mechanism-based inhibition by exemestane. Two nonsteroidal inhibitors, letrozole and anastrozole, which don't resemble the chemical structure of the androgen substrate, bind to aromatase with high affinity and specificity. Letrozole, the most potent aromatase inhibitor reported so far, has a Ki value of 0.1 to 1 nM (Choate and Resko 1996). Without a crystal structure of aromatase with bound letrozole, results from aromatase site-directed mutagenesis experiments (Kao et al. 1996;Kao et al. 2001) have been used to develop a model to explain how nonsteroidal inhibitors bind to aromatase (Hong et al. 2009b).

CPR is essential for aromatase to catalyze the formation of estrogen. Enzyme kinetic analysis from our laboratory demonstrates that during estrogen synthesis, CPR binds strongly to aromatase with a Km value of 0.5 nM (Hong et al. 2009a), which supports three simultaneous hydroxylation steps in converting androgen to estrogen. The site-directed mutagenesis experiments also provided evidence that the electrostatic interactions between residues N175/T177 of CPR and residue K108 of aromatase play an important role in their association (Hong et al. 2009a). Our findings suggest that the site-specific interaction between aromatase and CPR is critical for effective estrogen synthesis, while CPR attaches aromatase at regions away from the active site where androgen is converted to estrogen, and where AIs bind.

In addition to synthetic AIs, a number of phytochemicals and environmental chemicals act as competitive inhibitors of aromatase (Adams and Chen 2009; Brueggemeier et al. 2001; Hong and Chen 2006). Most of these chemicals inhibit aromatase with Ki values in the micromolar range. The binding nature of these chemicals has been reported by our laboratory using site-directed mutagenesis and computer modeling analysis (Hong et al. 2008; Kao et al. 1998).

3. Estrone sulfatase

Estrone sulfatase, along with aromatase and 17β-HSD, is responsible for maintaining high levels of E2 in tumor cells. After estrone is synthesized by aromatase, it can be converted to estrone sulfate in the liver by the catalysis of estrogen sulfotransferase (Tseng et al. 1983). Through circulation, estrone sulfate can be then stored in tissues, including breast tumors. Estrone sulfatase catalyzes the hydrolysis of estrone sulfate to estrone, which is subsequently reduced to E2 by 17β-HSD. In breast tumor tissue, especially in postmenopausal women, the concentration of estrogen sulfate is several times higher than that found in the plasma or in the area of the breast considered as normal (Edery et al. 1981; Millington 1975; Pasqualini et al. 1996; van Landeghem et al. 1985). It is now widely recognized that this reservoir of estrogen sulfates provides an important source of estrogens in tumors via the action of estrone sulfatase. Previous studies have reported that, in human breast tumors, the sulfatase pathway predominates over the aromatase pathway (Gunnarsson et al. 2001; Vihko et al. 2006). Thus, estrone sulfatase has been considered to be an attractive target for therapeutic intervention.

The structure of estrone sulfatase, purified from the microsomal fraction of human placenta, was determined at 2.6 Å by x-ray crystallography (Figure 4A) (Hernandez-Guzman et al. 2003). The catalytic amino acid is created by post-translational modification of a highly conserved cysteine residue to hydroxylformylglycine (FG), followed by a covalent linkage to a sulfate moiety as a sulfate ester of FG (FGS) (Bond et al. 1997). The residue FGS75 is involved in stabilizing the calcium ion and the sulfate ester in the active site (Bond et al. 1997). The calcium ion is located at the center of the catalytic site near the residue FGS75 and is required for estrone sulfatase activity (Hernandez-Guzman et al. 2003). The posttranslational conversion of this cysteine residue was also well studied by several other groups, demonstrating that the conversion is required for generating catalytically active sulfatases (Dierks et al. 1997; Schmidt et al. 1995). The active site residues of estrone sulfatase were identified to be D35, D36, FGS75, R79, K134, H136, H290, D342, Q343, and K368 (Figure 4B).

Several classes of estrone sulfatase inhibitors (Figure 5) have been reported, mainly by Dr. M. Reed and his colleagues in the past 10 years. Estrone-3-sulfamate is the first potent estrone sulfatase inhibitor that causes a time- and concentration-dependent irreversible inactivation of the enzyme (Howarth et al. 1994;Purohit et al. 1995). Sulfamoyloxy-

substituted 2-phenylindoles have been developed as antiestrogen-based inhibitors of estrone sulfatase (Golob et al. 2002). Compound STX64 (also known as 667 Coumate and BN83495), which has an IC50 value of 8 nM in placental microsomes (Foster et al. 2008), has become the first estrone sulfatase inhibitor to enter clinical trail for postmenopausal patients with advanced hormone-dependent breast cancer and has shown encouraging results (Stanway et al. 2007).

Since aromatase is needed for the synthesis of estrogen that is then converted to estrogen sulfate by estrogen sulfotransferase, hormone-dependent breast cancer may be more effectively treated by dual inhibition of aromatase and steroid sulfatase. A new design strategy was explored that involves introducing the aromatase inhibitory pharmacophore into a template that has been designed primarily for sulfatase inhibition (Woo et al. 2010). A series of compounds that can inhibit both aromatase and sulfatase has been developed based on the structure of estrone 3-sulfamate, a typical estrone sulfatase inhibitor (Numazawa et al. 2006). In addition, a series of dual aromatase-sulfatase inhibitors that are sulfamate derivatives of nonsteroidal AIs, including letrozole and anastrozole, has been successfully developed (Woo et al. 2007; Woo et al. 2008; Woo et al. 2003). The design of these dual aromatase-sulfatase inhibitors shares a common strategy; that is, to engender the sulfatase inhibitory pharmacophore into an established aromatase inhibitor with minimal structural change incurred to the original scaffold in order to retain and maximize aromatase inhibition.

4. 17β-HSDs

17β-HSDs are a group of enzymes that catalyze dehydrogenation of 17-hydroxysteroids in steroidogenesis. Most studies on the 17β-HSDs have primarily assessed type 1. 17β-HSD1 is an important enzyme for E2 production because it can use estrone as a substrate generated by both aromatase and sulfatase pathways, and it principally reduces the 17β-keto group of estrone to 17β-hydroxyl group using NADPH as a cofactor (Aka et al. 2010). This enzyme has been demonstrated to be involved in maintaining high E2 levels in breast tumors of postmenopausal women (Miyoshi et al. 2001; Poutanen et al. 1995). In certain breast cancer patients, intratumoral E2 was produced by the 17β-HSD1-mediated reduction of estrone produced by aromatase (Sasano et al. 2008; Sasano et al. 2006). Higher expression of 17β-HSD1 mRNA has been demonstrated in almost 50% of breast cancer tissues (Vihko et al. 2004). The expression and activity of 17β-HSD1 are significantly higher in breast cancer than in normal breast tissue (Pasqualini 2004), and it has been suggested that this higher expression could explain the elevated E2 concentration in breast tumors (Vermeulen et al. 1986). A high level of 17β-HSD1 correlates with an increased risk of developing a late relapse of breast cancer in ER-positive breast cancer patients (Day et al. 2006; Vihko and Apter 1989). In addition, 17β-HSD2 and 17β-HSD7 have been suggested to play a secondary role in the balance between estrone and E2 (Dunbier et al. 2010; Haynes et al. 2010; Lonning et al. 2009).

The first diffraction-quality crystal of 17β-HSD1 was obtained in the early 1990s (Zhu et al. 1994). A dozen different complex structures have been solved to date (Azzi et al. 1996; Breton et al. 1996; Gangloff et al. 2003; Lin et al. 2000; Lin et al. 1996; Persson et al. 2003; Puranen et al. 1997; Qiu et al. 2002; Sawicki et al. 1999; Shi and Lin 2004). The crystal structures of 17β-HSD1 complexed with testosterone or E2 are shown in Figure 6A. Both testosterone and E2 bind in the narrow hydrophobic tunnel of 17β-HSD1 with a high degree of complementarity (Figure 6B). However, testosterone is bound in an alternative orientation to 17β-HSD1 compared with E2. The residue L149 plays an important role in the discrimination between C19 androgen and C18 estrogen (Gangloff et al. 2003).

When testosterone is positioned in the same binding mode as E2, a steric clash between the 19-methyl group and the residue L149 is observed. This steric hindrance may compel testosterone to bind in a reverse binding mode (Gangloff et al. 2003), which results in a much lower binding affinity of C19 androgen when compared to C18 estrogen (Labrie et al. 2000).

Inhibitors of 17β-HSDs constitute a growing interest in biomedical research, and new compounds have been developed in recent years (Brozic et al. 2008; Day et al. 2008; Laplante et al. 2008; Poirier 2003; Poirier et al. 2005). To develop new compounds capable of inhibiting 17β-HSD1, a series of structure-function studies were conducted in several laboratories focusing on the understanding of steroid binding to the enzyme (Azzi et al. 1996; Gangloff et al. 2003; Mazumdar et al. 2009; Miyoshi et al. 2001; Poutanen et al. 1995; Qiu et al. 2007; Shi and Lin 2004). A hybrid inhibitor having both the substrate and cofactor parts, EM-1745 (Figure 7), competed with the substrate as well as the cofactor cores (Qiu et al. 2002). The E2-adenosine hybrid compound EM-1745 revealed key interactions with two different enzyme-binding sites, namely the substrate- and the cofactor-binding sites. In addition, the IC50 value for a new inhibitor E2B was determined to be 42 nM in T47D cells (Mazumdar et al. 2009). A kinetic study demonstrated that E2B inhibits the conversion of estrone to E2, with a Ki of 0.9 ± 0.15 nM (Mazumdar et al. 2009). Such strong inhibition is in agreement with the effective interaction of E2B with the enzyme, suggesting its potential as a lead compound for breast cancer therapy.

5. Discussion

While aromatase, estrone sulfatase, and 17βHSD1 all use sex steroids as substrates, with the information we have so far it is difficult to compare the similarity among the substrate binding sites of these three enzymes. This is not unexpected, as they are different types of enzymes with distinct catalytic mechanisms. The dissimilarity in the active site regions of these enzymes allows the development of specific inhibitors for each enzyme. As reviewed in this paper, a majority of the inhibitors of were developed through structure-activity studies and biochemical analysis. The three-dimensional structures of these enzymes have not been shown to be useful in drug design, but they provide valuable information to help understand the catalytic mechanisms and molecular basis of inhibitor binding. The x-ray structures are certainly valuable to verify the importance of key amino acid residues in the active sites and in the binding pockets for substrates and inhibitors.

Among the inhibitors of aromatase, estrone sulfatase, and 17β-HSDs, AIs are the best-developed, and clinically effective for hormone-dependent breast cancer. Letrozole, anastrozole, and exemestane have shown to be superior in terms of relapse-free survival and recurrence in the endocrine treatment of hormone-dependent breast cancer in postmenopausal patients. Although these three inhibitors are efficacious drugs for hormone-dependent breast cancer, there are concerns for acquired resistance and side effects associated with estrogen deprivation. It is essential to consider new therapeutics to reduce side effects and overcome resistance to current AIs. Inhibitors that target estrone sulfatase or 17β-HSDs may not be very effective for monotherapy because aromatase is the only enzyme that catalyzes the synthesis of estrogen from androgen. Although estrone is less active than E2, it is still an active estrogen. However, there will be a benefit for combination therapies using inhibitors of estrone sulfatase or 17β-HSDs with an AI, which could lower the dose of the AI, thus reduce side effects created by the AI, and delay the development of resistance to either drug. Considering that the expression levels of estrone sulfatase and 17β-HSD1 are significantly higher in breast tumor than in normal breast tissue, the use of inhibitors of these two enzymes together with AIs may also enhance tumor-selective reduction of E2 levels. New treatment strategies combining inhibitors of estrone sulfatase or 17β-HSDs with

AI could also extend the period of time of endocrine therapy, thereby disease progression and the need for chemotherapy may be significantly delayed. Preclinical, translational and clinical studies to evaluate such treatment options are critically needed.

Dr. Mike Reed demonstrated his vision in the endocrine therapy of breast cancer by the design and characterization of dual aromatase-sulfatase inhibitors. These inhibitors could be the new generation of drugs to treat hormone-dependent breast cancer. His contributions in hormonal carcinogenesis and endocrine therapy will be greatly missed by all of us.

Abbreviations

AI	aromatase inhibitor
CPR	NADPH-cytochrome P450 reductase
CYP450	cytochrome P450
E2	17β-estradiol
17β-HSD	17β-hydroxysteroid dehydrogenase
17β-HSD1	17β-hydroxysteroid dehydrogenase type 1
ER	estrogen receptor
FG	hydroxylformylglycine

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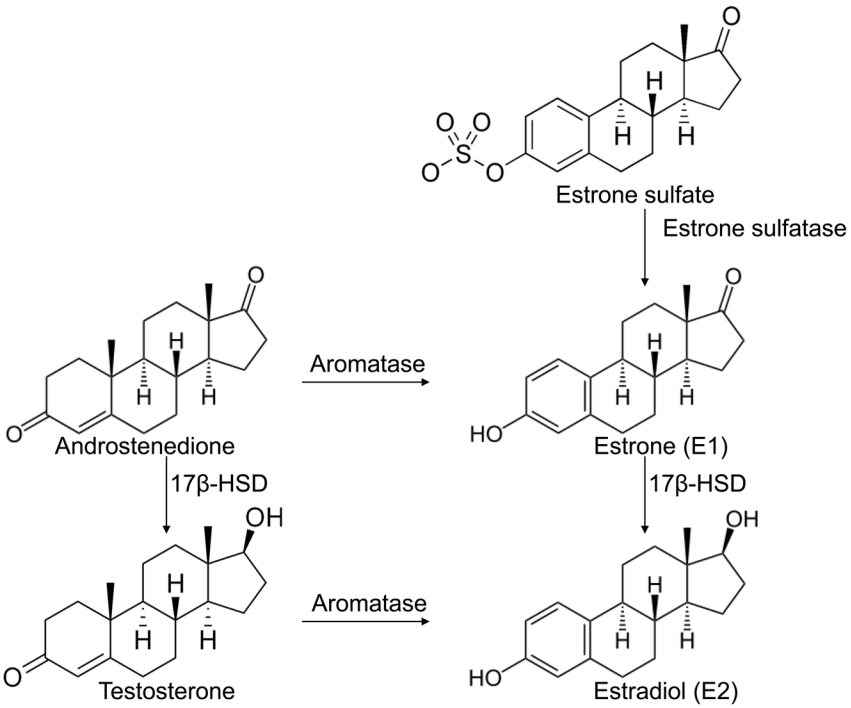


Figure 1.
The last steps of E2 biosynthesis by aromatase, estrone sulfatase, and 17β-HSD.

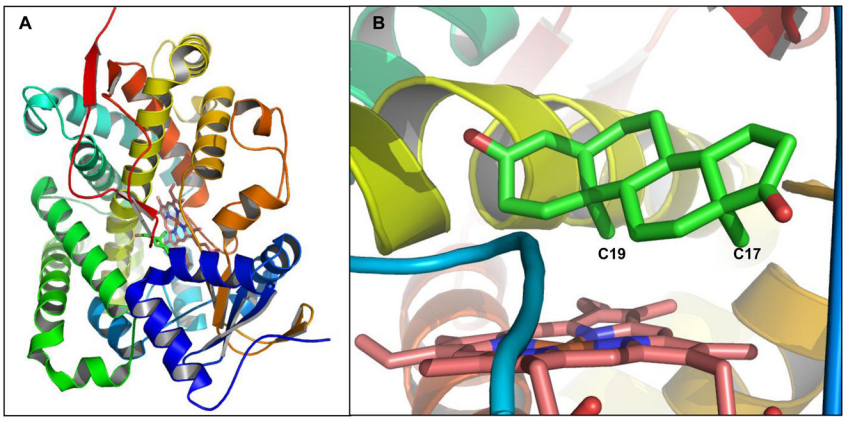
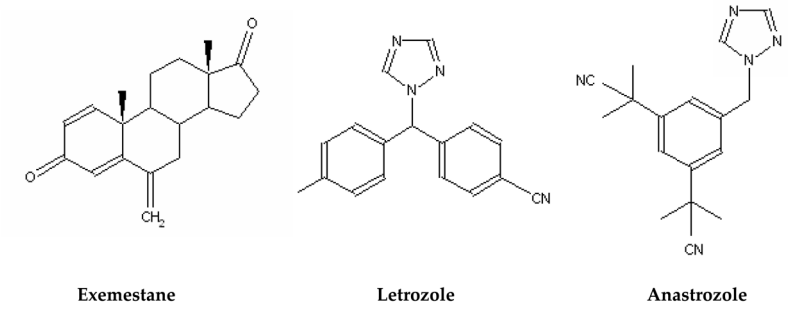


Figure 2.

A. The crystal structure of human placental aromatase cytochrome P450 complexed with androstenedione (PDB# 3EQM) (Ghosh et al. 2009). B. The active site of human aromatase. The substrate androstenedione is shown in green.



Exemestane **Letrozole** **Anastrozole**

Figure 3.
Structures of aromatase inhibitors exemestane, Letrozole, and anastrozole.

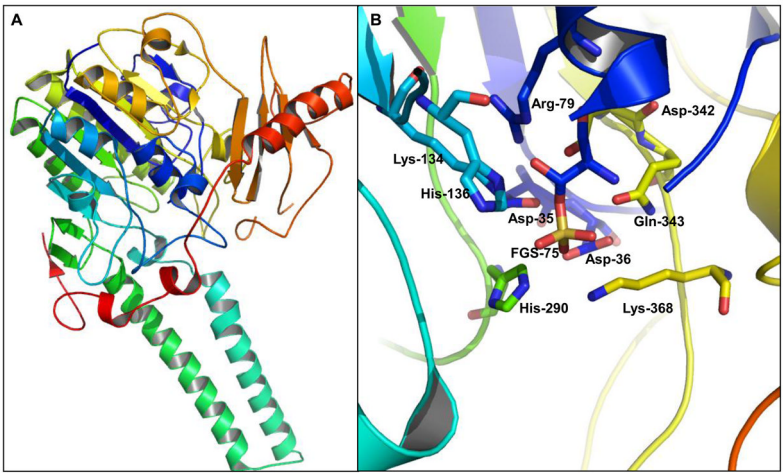


Figure 4.

A. The crystal structure of human estrone sulfatase (PDB# 1P49) (Hernandez-Guzman et al. 2003). B. The active site of estrone sulfatase. The substrate binding site is located at the pocket underneath the residues D36, FGS75, H290, and K368

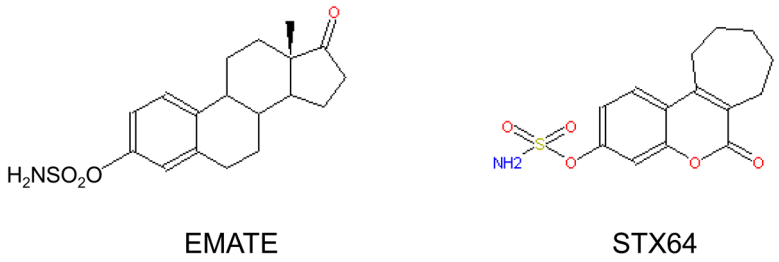


Figure 5.
Structures of estrone sulfatase inhibitors estrone-3-sulfamate (EMATE) and STX64.

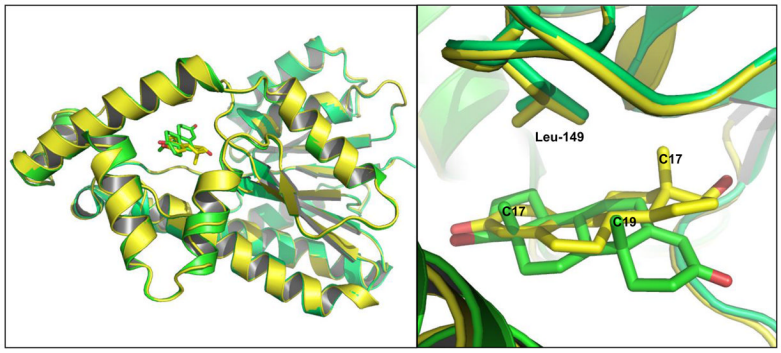


Figure 6.
A. The crystal structures of human 17β-HSD1 complexed with testosterone (PDB# 1JTV, green), or estradiol (PDB# 1IOL, yellow)(Azzi et al. 1996). B. The substrate binding site of 17β-HSD1: testosterone (green), estradiol (yellow).

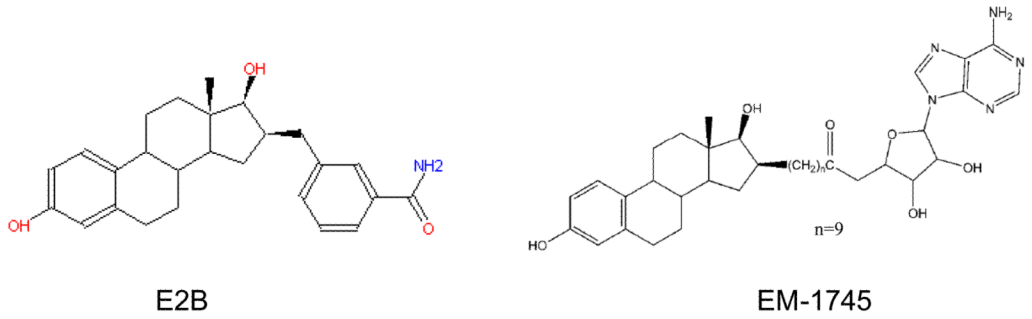


Figure 7.
Structures of 17β-HSD1 inhibitors E2B and EM-1745.