Theoretical Studies of Anti-cancer Drug Tamoxifen and Estrogen Receptor Alpha (ERα)

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To my family
Abstract

For decades tamoxifen (TAM) has been widely used for treatment of breast cancer by mediating mainly the estrogen receptor α (ERα) signaling pathways, whereby it suppresses estrogen stimulated cancer cell growth. The clinical response of TAM has been linked to cytochrome P450 2D6 (CYP2D6), which is the main isoform responsible for the conversion of TAM to the active metabolites 4-hydroxyTAM (OHT) and endoxifen. Numerous clinical studies have thus attempted to assess the effects of CYP2D6 genetic variants on patients treated by TAM. However, the studies have resulted in contradictory conclusions. This thesis focuses on computational investigations of TAM and its main target ERα. The results obtained describe how the ligands contact with the ERα ligand binding domain (LBD), and provide possible mechanisms responsible for the CYP2D6 activating in TAM treatment. In addition, the CYP-mediated biotransformation of TAM-like compounds is investigated. All studies in this thesis aim to a step towards developing improved therapeutic agents for breast cancer treatment. In paper I, molecular dynamics simulations of ligand-LBD complexes have been performed. The results indicate that although OHT is a high affinity metabolite, it may have more undesired estrogen-like properties than the parent drug TAM, as a consequence of the additional 4-hydroxy group. In papers II and V, quantum mechanics calculations have been performed to study how the ligands are bound to ERα LBD. It is found that different conformational isomers of TAM-like ligands are discriminated by the LBD. The interactions between ligands and His524-Leu525 in the LBD are correlated with the transcriptional activity of estrogen agonist compounds. In papers III and IV, different CYP-mediated biotransformations of TAM and derivatives are studied. Based on the results from the computations, we suggest two modified compounds which are highly possible to be activated by other CYP isoforms besides CYP2D6, thereby avoiding CYP2D6 genetic polymorphism. Overall, the results generally agree with the hitherto available experimental results. Further experimental studies are needed to verify the proposed principles of ligands signaling through ERα, and to test the suggested CYP-mediated reactions and the bioactivity of the modified compounds.
List of papers

Following papers are included in this thesis:

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**Paper IV**  Gao, L.; Tu, Y.; Agren, H. and Eriksson, L. A. *Modification of the anti-cancer drug tamoxifen to avoid CYP2D6 polymorphism. In manuscript.*

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All the papers are the results of teamwork. I am responsible for the computations and a large part of the writing in all papers.
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Abbreviations

AI Aromatase inhibitor
Cpd I Compound I
CYP Cytochrome P450
DES Diethylstilbestrol
DFT Density functional theory
E₂ Estradiol
ER Estrogen receptor
GAFF The general amber force field
GEN Genistein
HSD Hydroxysteroid dehydrogenase
LBD Ligand binding domain
MTA 4-methyl-tamoxifen
MTO 4-methyl-toremifene
MD Molecular dynamics
NDT N-desmethyl-tamoxifen
OHT 4-hydroxy-tamoxifen
PDB Protein Data Bank
PME Particle mesh Ewald
RBA Relative binding affinity
RMSD Root-mean-square deviation
SERM Selective estrogen receptor modulators
TAM Tamoxifen, (Z)-2-[4-(1,2-diphenyl-1-butenyl)phenoxy]-N,N-dimethylethanamine
TOR Toremifene
ZPE Zero-point vibrational energy
W Way-169916
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CHAPTER 1. INTRODUCTION

The main focus of this work is the anti-cancer drug Tamoxifen (TAM), which is widely used to treat breast cancer, and for which the primary target is the estrogen receptor α (ERα). This chapter gives a brief introduction of TAM therapy for breast cancer and the related mechanistic pictures of small compounds signaling through ERα. TAM is a prodrug, and its biotransformation paths are introduced with particular focus on the \textit{in vivo} activation of the parent compound.

1.1 Breast Cancer

Breast cancer is the most prevalent cancer in women, especially in the western countries. As an example, in the United States, it is expected that 39,920 breast cancer patients will die during 2012, and at the same time the number of new cases among women is expected to be 290,170. Due to the earlier diagnosis and progress in treatment, deaths from breast cancer have decreased in the last decades, with the current 5-year survival rate of 90% compared to that of 63% in the early 1960s.\footnote{1}

The discovery of the link between breast cancer and estrogen has made a remarkable contribution to improve the cancer treatment and reduce the mortality rate. Approximately 70% of breast cancers are estrogen dependent, expressing estrogen receptors (ERs). The ER positive breast cancer cells exert an estrogen promoted proliferation through ER-regulated gene transcription. If the growth stimulated by estrogen can be blocked, then the breast cancer may be controlled. For example, TAM is a commonly used drug to treat breast cancer by directly blocking the actions of endogenous estrogen.

1.2 TAM: Chemical Structures

TAM (ICI 46,474) was first synthesized in the 1960s by the pharmaceutical company ICI (now known as AstraZeneca). It is an old drug with the brand name of Nolvadex, which has been used as first-line therapy for breast cancer since the 1970s, and is until now still widely used. It was initially marketed to treat breast cancer in 1973, and approved for breast cancer treatment and prevention by the Food and Drug Administration in 1977 and 2007, respectively.
**Scheme 1.1** The atropisomers of trans- and cis-TAM: trans- and cis-TAM in ‘counterclockwise’ propellers; trans'- and cis'-TAM in ‘clockwise’ propellers.

The chemical structures of TAM are seen in Scheme 1.1. The trans-isomer ((Z)-2-[4-(1,2-diphenyl-1-butenyl)phenoxy]-N,N-dimethylethanamine) but not the cis form is used for breast cancer treatment. The core structure of TAM is a triarylethylene, which has been found to exist in a molecular propeller.\(^2\) It is a highly conjugated system as the ethylene substituted by three aromatic rings. To stay in a low energy conformation, conjugation effects require the three rings to be coplanar in the ethylene plane, but this leads to increased steric repulsion of the rings. The aryl rings are thus forced to twist away from the ethylene plane to compromise between conjugation and steric repulsion, thereby the system stays in the low energy conformations, i.e., the ‘clockwise’ and ‘counterclockwise’ propellers (Figure 1.1).\(^3,4\) The two propellers differ in helicity, as the rings twist in opposite directions forming atropisomers. The interconversion between the atropisomers has been studied experimentally for more crowded triarylvinyl propellers, in which the rotational barriers were found to be in the range of 15-20 kcal/mol for the correlated rotations about the C(Ar)―C(sp\(^2\)) bonds.\(^3,5-7\) The thermal equilibrium between the ‘clockwise’ and ‘counterclockwise’ TAM propellers was too fast to be measured experimentally even at -75 °C.\(^8\) Therefore, the atropisomeric propellers of TAM are expected to have equivalent populations under physiological conditions. In such circumstance, the isolation of either TAM isomer is not possible, and the bioactivity of each
isomer is not clear. The pharmacological profiles of TAM atropisomers are thus compensatively studied by computational approaches in Paper II.

1.3 Estrogen

The endogenous estrogens in women are steroid hormones, which are biosynthesized from cholesterol via multiple enzymatic steps (Scheme 1.2). Aromatase is one of the most important enzymes catalyzing the biotransformation to eventually produce estrogens, of which 17β-estradiol (E2) is the most potent female hormone. In premenopausal women, estrogens are produced primarily in the ovaries. Oppositely, the ovaries almost cease to secrete estrogen in postmenopausal women and the serum concentration of estrogen thus decreases dramatically. Possible consequences of the lack of estrogen in postmenopausal women are frequently reported, including postmenopause symptoms, increased risks of osteoporosis, coronary heart disease and Alzheimer's disease. 9-13 On the other hand, a cumulative exposure to estrogen does encourage development of female reproductive cancers. Such examples include breast cancer and uterus cancer, which are found associated with hormone replacement therapy, early menarche and late menopause. 14 The contribution of estrogens in various physiological and pathological pathways highly depends on their binding to estrogen receptors and activating transcription of estrogen responsive genes. 15-18

Scheme 1.2 The major biosynthetic paths of endogenous estrogens.

1.4 Estrogen Receptor (ER)

ERs belong to the large superfamily of nuclear receptors and reside in the nuclei of estrogen target cells. They are expressed in two subtypes: ERα19 and ERβ. 20 The focus of the current work is the ERα isoform, which is primarily expressed in breast and uterine tissues. ERα is found intimately associated with breast cancer initiation and progression, and is hence very
important for breast cancer prevention and treatment. Breast cancer cells possess much higher levels of ERα than normal breast epithelial cells.\textsuperscript{21,22} The increase of ERα expression in benign breast epithelium has been linked to an increased risk of breast cancer.\textsuperscript{23,24}

ERs are composed of five function domains (Figure 1.1), referred to as the N-terminal A/B domain, the DNA binding domain (C), the hinge domain (D), the ligand binding domain (LBD, E), and the C-terminal F domain. As ERα and ERβ are encoded by homologous genes, their sequences are significantly similar at the amino acid level, in particular the DNA binding domain and the LBD, with identity of 97\% and 56\%, respectively. The DNA binding domain binds to specific estrogen response elements, and the resulting gene transcription is initiated through a ligand-independent or -dependent manner mediated by transcriptional activation domain 1 or 2 (AF-1 or AF-2), respectively.\textsuperscript{25}

![Figure 1.1 The human estrogen receptor α and β (hERα and hERβ).](image)

The ligand-dependent manner of the gene transcription through AF-2 makes it possible to interfere with the expression of estrogen responsive genes by small molecule ligands, which have the potential of activating or repressing ER signaling in estrogen-related diseases. Great efforts have been made in related studies, and a branch thereof is structural biology studies of ER ligands and the LBD. Since the crystallographic structures of the ligand-ERα LBD complexes were initially determined in the late 1990s, about 100 LBD structures of ERs have been deposited in the RCSB Protein Data Bank (PDB). However, the other domains, except the DNA binding domain, have not yet been solved, and the complete structure of the five ER domains as a whole is still lacking.

Of particular interest is that the LBD shows binding properties of trapping a variety of exogenous compounds besides the endogenous steroid hormones. As a result, a wide range of compounds have been cocrystallized with the wild type or mutated LBD, and have shown to induce different conformational changes of the receptor. The crystallographic structures of the ligand-ERα LBD complexes are generally classified as agonist or antagonist conformations based on the positions of the C-terminal Helix 12 (H12, Figure 1.2), which displays a high degree of dynamic flexibility. H12 closes the binding site in the agonist conformation (Figure 1.2A), which enables co-activator binding and the dimerization process of the ligand-ERα
complex to form a transcription unit that triggers gene expression or cell proliferation. This closed/agonist conformation is thus asserted to be an active conformation, and is frequently found in the cases of LBD in complex with E₂ and agonist ligands. Conversely, antagonists have bulky tails and bind to the same site of the LBD as agonists do, but force H12 to project towards the open/antagonist conformation (Figure 1.2B), whereby they occupy part of the co-activator binding groove. The co-activator binding is blocked with the open/antagonist-LBD, which slows or stops ER-mediated transcription.²⁶,²⁷ The open/antagonist-LBD is a relatively inactive conformation compared to the closed/agonist-LBD, but it is not completely inactive. The contradictory bioactivities have been the key issues in the studies of estrogen antagonists, and more detailed information is given later in this summary.

Figure 1.2 The closed/agonist E₂-ERα LBD complex (A, 1GWR²⁶) and the open/antagonist TAM-ERα LBD complex (B, 3ERT²⁶), H12 in cyan and coactivator in blue.

It is worthwhile to note that the ligand activated transcription is completely silent for the unbound LBD. However, the crystallization of an unbound ERα LBD (without any ligand) is hampered by the conformational flexibility of the LBD. A large part of the LBD is formed by rigid helixes as seen in Figure 1.2, while extreme flexibility is mainly due to the variation of H12. The apo conformation (Figure 1.3) of a crystallized ERα LBD (1a52²⁹) is suggested to be a representative of the unbound LBD, in which the Helix 12 is solvent exposed and extended away from the domain, and is thus very flexible. The reorientation of H12 occurs in the capture of a ligand, resulting in the corresponding closed/agonist- or open/antagonist-LBD depending on the ligand molecules.
1.5 ERα Ligands

Beside steroid hormones, numerous exogenous compounds are able to bind to ERα due to the versatile binding properties of the LBD, albeit these ligands are structurally very different, and result in anti-estrogen and/or estrogen like properties. According to the differences in functional activities, ERα ligands are generally classified into four orders: full agonist, partial agonist, partial antagonist and pure antagonist, with examples in Scheme 1.3.

Full and partial agonists stabilize ERα LBD in the closed/agonist conformation (Figure 1.4A). E2 is known as the most potent female hormone in women, and is frequently used as a standard full agonist in experimental studies. Diethylstilbestrol (DES) is a synthetic full agonist, which has been used in pregnant women and blamed for many adverse effects on the offspring. The bioactivity profiles of partial agonists are much more complicated due to the mixed agonist/antagonist properties. The soy phytoestrogen genistein (GEN) is a partial agonist, and the consumption of soy food has been suggested to reduce the risk of developing breast cancer. However, GEN has also been found to stimulate breast cancer cell growth in some assays. Way-169916 (W) is an even weaker partial agonist, and has broad anti-inflammatory activity instead of the conventional agonistic effects of transcriptional activation. Besides the reduced capacity in transcriptional activation, partial agonists were experimentally found more difficult to be cocrystallized with the LBD than full agonists. With the aid of mutational techniques for success in the cocrystallization, the crystal structures of partial agonists in complex with LBD have the same closed/agonist conformation as that of full agonists. Little is known about how the ligands affect the allosteric conformational changes in H12, and subsequently affect the transcriptional or regular bioactivities. We thus discuss what may elicit such differences between full and partial agonists in Paper V.
In contrast to full and partial agonists, partial antagonists stabilize ERα LBD in the open/antagonist conformation (Figure 1.4B). Well known such examples are raloxifene and TAM, with 4-hydroxyTAM (OHT) as one of its active metabolites. The compounds in this order exert tissue-specific effects of both anti-estrogen and estrogen like activities, and are thus classified as selective estrogen receptor modulator (SERM). SERMs act like estrogens in some tissues, whereas they act like anti-estrogens in others. As mentioned earlier, estrogen actions are blamed for breast cancer; meanwhile, lack of estrogen has been linked to the progressive changes in postmenopausal women. Therefore, compounds with ideal SERM profiles have attracted considerable attention in drug development. We will see examples of such attempts later in this chapter. Generally, ligand molecules of partial antagonist display no apparent differences in stabilizing the open/antagonist-LBD conformation. Despite this fact, subtle changes in ligand structures have, however, been found to result in significant
differences in bioactivities (agonism and/or antagonism). The means by which the compounds share similar crystal structures of the ligand-LBD complexes, but differ in their ER signaling are poorly understood. We here discuss in Paper I the possible mechanism which may govern the agonist and antagonist properties of SERMs.

Pure antagonists competitively bind to ERα as partial antagonists do, but unlike SERMs, they exert anti-estrogen actions in all tissues. The mechanism of pure antagonists has been described as downregulation of ER levels and inhibition of dimerization, and hence the ligand-ERα complex does not work as a transcription initiation unit. The pure antagonist fulvestrant is used in breast cancer patients who have failed in TAM therapy. The ligands in this order are not a focus of the current study.

Figure 1.4 Superposed crystallographic structures of ligands in complex with ERα LBD: A. full and partial agonists, 1G50 in red (E2), 3ERD in gray (DES), 1X7R in blue (GEN), and 2QZO in green (W). B. partial antagonists, OHT-LBD (3ERT in red, 2JF9.A in gray, and 2BJ4.A in yellow) and raloxifene-LBD (1ERR.A in blue, and 2JFA.A in green). Reproduced with permission of the copyright owner.

1.6 Endocrine therapy

Endocrine therapy is also called hormone therapy. In this treatment hormones are usually added, blocked or removed. In the case of breast cancer, the ER-positive cancer cell proliferation is stimulated by estrogens, and hence hormone therapy for breast cancer is in fact anti-hormone treatment. This is an approach through which the tumor growth is suppressed by preventing ERα from binding the endogenous estrogens, which the tumor cells require to grow.

Different strategies are used in endocrine therapy. Surgical removal of ovaries (oophorectomy), which decreases the levels of circulating estrogens in premenopausal women,
was applied to breast cancer patients even before the discovery of ERα and corresponding estrogen responsiveness in the cancer cells. As simpler and safer alternatives to surgical approaches, such as oophorectomy, adrenalectomy, and hypophysectomy, drugs are commonly used to block the estrogen stimulated cancer cell proliferation. Based on different mechanisms, these drugs can either work as estrogen antagonism to block estrogen binding to ERα, or directly block the estrogen biosynthetic process. TAM treatment represents the most commonly used approach by competitively binding to ERα, thereby TAM functions as estrogen antagonist in breast tissue. For the other approach, aromatase inhibitors (AIs) are usually applied to inhibit the aromatase enzyme which catalyzes the essential conversion to produce estrogens.

1.6.1 TAM for breast cancer treatment

TAM is the first clinically used SERM for breast cancer treatment. The SERM profiles, the mixed estrogen agonist and antagonist properties depending on the specific tissue, and related effects of TAM\(^4^8\) are briefly summarized below:

- **Anti-estrogenic properties in breast tissue:** protect against tumor relapse in breast cancer treatment, and reduce the incidence of breast cancer as a preventive drug.

- **Estrogenic properties in the cardiovascular system:** decrease heart disease and reduce serum concentration of cholesterol.

- **Estrogenic properties in bone:** increase the bone density in postmenopausal women and thus reduce the fracture rate.

- **Estrogenic properties in uterine tissue:** increase the risk of endometrial cancer and increase endometrial thickness.

- **Menopausal symptoms** are most frequently reported adverse effects of TAM, such as hot flashes and atrophic vaginitis, which may be caused by deprivation of estrogen due to the antagonist effects of TAM. Other adverse effects include slightly increased risks of cataract and thromboembolic.

Overall, TAM significantly reduces the risk of cancer recurrence and death\(^4^9\) with beneficial effects on bone and the cardiovascular system, and has no serious side effects except the slightly increased incidence of endometrial cancer. Compared to other cancer therapies, such as radiation or chemotherapy, TAM therapy specifically targets the ERα signaling pathways, which does not damage normal cells and tissues, and thus causes relatively fewer and milder side effects. The drug is well tolerated by breast cancer patients with benefits that are much larger than its adverse effects.
1.6.2 Aromatase inhibitor

Anastrozole, exemestane and letrozole are commonly used AIs for breast cancer treatment, which block the biosynthesis of endogenous estrogens by inhibiting the aromatase enzyme and thus significantly decrease the levels of circulating estrogen. The mechanism of this approach is based on estrogen deprivation all over the body, in some sense close to that of pure antagonists, and is not discussed further in this thesis.

1.7 TAM: in vivo Metabolism

TAM undergoes in vivo Phase I and Phase II reactions and the processes can vary significantly among individuals due to patients carrying different genotypes of the metabolic enzymes, resulting in dramatic differences in plasma levels of TAM and its metabolites among patients. Phase I reactions of TAM mainly consist of oxidations catalyzed by cytochrome P450 (CYP) enzymes, which produce both active and toxic metabolites.50-52 Phase II reactions are also known as conjugation reactions, which usually combine polar functional groups with the Phase I products, and thus facilitate drug elimination. The conjugation of TAM and its metabolites is most commonly catalyzed by sulfotransferase (SULT) and UDP-glucuronosyltransferase (UGT). Genetic variation in patients occurs with the enzymes participating in Phase I or Phase II reactions, and affects the plasma levels of TAM and its metabolites. Hence, the influences of enzyme genotypes in clinical response of TAM therapy have been the focus of studies on TAM treatment for decades. We will see a more detailed introduction in the following section.

1.7.1 The major TAM metabolic pathways

TAM undergoes extensive hepatic oxidations, i.e. the CYP-mediated Phase I reactions (Scheme 1.4). The sequential biotransformation mainly constitutes CYP-mediated N-demethylation, 4-hydroxylation, and α-hydroxylation, yielding the active or toxic metabolites.50-52 The primary metabolites, including N-didesmethyl-TAM (NDT), α-hydroxy-TAM, and OHT, are generated via direct oxidation of the parent drug TAM. The metabolites may undergo further oxidations into secondary metabolites, including N-didesmethyl-TAM, endoxifen, and α-hydroxy-NDT. A variety of CYP isoforms are involved in the TAM biotransformation, including CYP1A2, CYP1B1, CYP2B6, CYP2C9, CYP2C19, CYP2D6, CYP3A4, CYP3A5 and CYP3A4, of which CYP3A4/5 and CYP2D6 play prominent roles. CYP3A4 is the most abundant isoform in adult humans. It does not have a clinically significant polymorphism, and mainly catalyzes N-demethylation and α-hydroxylation. With CYP3A5 also contributing to the N-demethylation, N-desmethyl-TAM (NDT) is the most prevalent in vivo metabolite. CYP2D6 is responsible for 4-hydroxylation, hence it is of particular importance in the metabolic activation of the prodrug. The CYP2D6 isoform is
functionally important but quantitatively minor in human liver CYPs, with the activity frequently affected by the genetic polymorphism or other inhibitive drugs. CYP1B1-catalyzed cis-trans isomerization of OHT represents a quantitatively minor route, which attracts research interest because it is associated with resistance to TAM therapy. More detailed introductions are given in the following sections.

**Scheme 1.4** The important metabolic paths of TAM and the major CYP isoforms mediating the biotransformation.\(^{50,51,53,54}\)

1.7.2 **CYP-mediated α-hydroxylation and DNA adducts**

Both TAM and NDT undergo α-hydroxylation catalyzed mainly by CYP3A4, which constitutes approximately 1/3 of the total hepatic CYPs of adults. The α-hydroxyl metabolites constitute a considerable part of the total metabolites, and have been found to irreversibly bind to DNA. As a result, TAM is considered to be a genotoxic carcinogen in rats, as TAM-induced tumors have been found in rat liver.\(^{55}\) The β-chlorinated TAM, toremifene (TOR), undergoes CYP-mediated α-hydroxylation as well, however, it does not form DNA adducts.
The postulated mechanism of forming DNA adducts is related to the Phase II conjugation of the α-hydroxyl group. The allylic carbocation electrophile subsequently formed is capable of binding DNA, illustrated in Scheme 1.5.53,54 TOR was developed with the purpose to improve the safety of TAM with regard to forming DNA adducts.56,57 Experimentally, DNA adducts were not clearly found in patients treated by TAM or TOR.58,59 TOR was proven no better than TAM, as it showed equivalent potency to TAM in suppressing breast cancer and in encouraging the development of endometrial cancer.56 The incidence of endometrial cancer was thus proven to be a consequence of TAM or TOR actions via the ER signaling pathway rather than genotoxicity of DNA adducts.

**Scheme 1.5 α-hydroxy and DNA adducts.**53,54

![Scheme 1.5 α-hydroxy and DNA adducts](image)

1.7.3 CYP2D6-mediated activation of TAM: 4-hydroxylation

Both TAM and NDT undergo 4-hydroxylation predominantly catalyzed by CYP2D6.50,51 Being the opposite of the CYP3A4 distribution, CYP2D6 is present in less than 5% of total CYPs in the liver. However, the importance of CYP2D6 in metabolizing TAM does not parallel its hepatic abundance. CYP2D6 catalyzes the 4-hydroxylation of TAM and NDT to produce the active metabolites OHT and endoxifen, respectively (Scheme 1.6). The additional 4-hydroxyl group forms hydrogen bonds with the side chains of Glu353 and Arg394 in ERα (Figure 1.5). OHT binds to ERα with affinity equivalent to that of E2, and so does endoxifen. OHT and endoxifen are thus much more potent than the TAM parent drug, which binds to ERα with fairly weak affinity.60-62 The conversion of TAM to 4-hydroxy metabolites results in the metabolic activation of the prodrug, thereby it is intuitively seen to be positive for breast cancer treatment. In contrast, the subsequent sulphate conjugation mediated by
sulphotransferase (SULT) 1A1 results in rapid elimination of the active 4-hydroxyl metabolites.\textsuperscript{63,64}

**Scheme 1.6** Important metabolites of TAM and the major isoforms responsible for the biotransformation\textsuperscript{50,51} and clearance of active 4-hydroxyl metabolites.\textsuperscript{63,64} Reproduced with permission of the copyright owner.\textsuperscript{47}

![Scheme 1.6](image)

**Figure 1.5** H-bond interactions between OHT and protein residues in ERα LBD (3ERT). Reproduced with permission of the copyright owner.\textsuperscript{47}
1.7.4 CYP2D6: the debate over TAM therapy

The key step, 4-hydroxylation, in the bioactivation of TAM is predominantly, though not exclusively, catalyzed by CYP2D6. CYP2D6 is not an inducible isoform, with individual activity in patients under the control of genetic variants. A variety of genotypes of CYP2D6 have been found in humans. The genetic polymorphism of CYP2D6 is particularly problematic, because most allelic variants of CYP2D6 show diminished or absent catalytic activity compared to the wild type CYP2D6*1. CYP2D6*4 is the most common polymorphism in Caucasian women, encoding inactive CYP2D6. CYP2D6*10 is frequently carried by Asian women with decreased CYP2D6 activity. The polymorphic variants affect the plasma concentrations of the active metabolites through the genetically determined CYP2D6 activities, and hence intimately affect the clinical response to TAM treatment. The conjugation of the active 4-hydroxyl metabolites mediated by SULT1A1 may also affect the outcome. Therefore, an intuitive implication in individualized therapy for breast cancer is that patients carrying null CYP2D6 alleles and high-activity SULT1A1*1 allele might respond poorly to TAM treatment, whereupon they would be best treated by other approaches.

To establish a more personalized treatment, the link between CYP2D6 genotypes and the clinical outcome of TAM treatment has been extensively investigated in breast cancer patients. Patients carrying null CYP2D6 alleles were frequently reported to have an increased risk of cancer recurrence than women with two-wide type CYP2D6*1 alleles.65-70 These findings were intuitively right, but conflicting with other studies, which reported that patients carrying one or two null CYP2D6*4 alleles benefited more than those with two functional CYP2D6*1 alleles.71,72 It was also reported that CYP2D6 activity had no effect on breast cancer recurrence in other studies,73,74 and even in two recent large studies.75,76 Another counterintuitive clinical result was that patients with low SULT1A activity had higher risk of death than those with high SULT1A1 activity.77 The effects of higher levels of active metabolites remained elusive, meanwhile markedly lower intracellular levels of the prodrug TAM were found in resistant tumors.78

1.7.5 CYP-mediated cis-trans isomerization of OHT

Many patients suffer from the recurrences in TAM therapy due to acquired TAM resistance. In TAM-resistant tumors, the relatively high ratio of cis:trans-OHT was clinically observed besides the observation of a lower cellular concentration of TAM.78,79 Cis-OHT was found to be isomerized directly from the active metabolite trans-OHT, with much weaker anti-estrogen activity compared to the trans form.80 In contrast to the facile cis-trans isomerization of OHT, the isomeric interconversion was not observed for the parent compound TAM (Scheme 1.7). The isomerization of OHT was proven to be catalyzed by human liver microsomes in the presence of NADPH, which served as a typical cofactor for CYP-mediated reactions.81
more detailed study by recombinant individual CYP isoforms found that CYP1B1 was the major isoform catalyzing this isomerization, with CYP2B6 and CYP2C19 also involved. The cis-trans isomerization of OHT elicits no changes in the redox (reduction-oxidation) state between the cis and trans isomers, however, a CYP-mediated redox process is argued by the requirement for NADPH. Less clear is the mechanistic picture of this CYP-mediated cis-trans isomerization, which is a rarely reported CYP reaction. A postulated isomerization process is thus discussed in Paper III.

**Scheme 1.7** The cis-trans isomerization.

![Scheme 1.7](image)

### 1.7.6 CYP reactions

As seen in the TAM metabolism, the CYP superfamily consists of a great variety of CYP enzyme forms (isoforms). These enzymes are particularly versatile and perform diverse types of transformations, such as N-dealkylation, hydroxylation, epoxidation, and desaturation. The CYP-mediated reactions are of vital importance in drug metabolism as well as in other biotransformations. The activation and detoxification of a drug may depend on the activity of the related CYP isoforms, which controls the rate and the amount of a compound that is metabolized. In contrast, the activity of CYP isoforms can be affected by many drugs, either decreased by directly inhibiting the activity of a specific isoform or increased by inducing the biosynthesis of an inducible isoform.
Most CYP isoforms have a heme group (Figure 1.6) tethering to a cysteine side chain in the active site. The prosthetic heme group most commonly contains an iron atom in the center of a large porphyrin ring, and has been the essential focus of theoretical studies of CYP reactions for decades.\textsuperscript{85-90} A generally accepted catalytic pathway of CYP reactions is illustrated in Scheme 1.8. The heme group is activated by dioxygen in the presence of substrate molecules. The resulting ferrous-dioxygen complex may evolve through the ferric-hydroperoxide, and finally be activated to the high-valent iron-oxo intermediate, Compound I (Cpd I), which is well asserted as the driving force in the efficient CYP oxidations. The chemical nature of the highly active Cpd I is electrophilic, and hence the reactions are initiated from either electrophilic attack of the \( \pi \) system or abstraction of an H atom from the substrate. Some generally consensus pathways are illustrated in Scheme 1.9.\textsuperscript{83,85,86,91-97}

**Scheme 1.8** An overall equation and a representative pathway of CYP-mediated hydroxylation of hydrocarbon C—H bond.

\[
\text{RH} + \text{O}_2 + \text{NADPH} + \text{H}^+ \xrightarrow{\text{CYP}} \text{ROH} + \text{NADP}^+ + \text{H}_2\text{O}
\]
Scheme 1.9 The CYP catalyzed reaction paths of hydroxylation and dealkylation.

While the explicit heme-containing oxidation steps are intriguing, they are, however, not a focus of the current studies. The CYP-mediated Phase I metabolism of TAM is investigated based on the most well justified initial step, the H atom abstraction from the individual reactive sites leading to different metabolites. Different CYP reactions are thus compared at the substrate levels with low computational cost.

1.8 Comparisons of Selected SERMs

An ideal SERM should fulfill certain criteria, such as (1) having antagonist actions in breast tissues with a potency high enough to suppress the estrogen-stimulated cancer cell growth, (2) having estrogenic effects on bone to protect against bone loss and fracture, on the cardiovascular system to protect against coronary heart disease, on the nervous system to improve cognitive function and protect against Alzheimer’s, (3) having less undesired agonist properties in the uterus and being safe in the long run. Great effort has been made for such attempts with many SERMs under development or abandoned, and some of which are briefly compared herein (Figure 1.7 and Scheme 1.10). New structures are proposed in Paper IV.
1.8.1 Geometries of SERMs in complex with ERα LBD

We performed geometric comparison of five structurally different SERMs in complex with ERα LBD (Figure 1.7). Lasofoxifene can be viewed as a fixed ring analogue to OHT, and for which the isomerization to the cis-isomer is prohibited. GW5638 is a triphenylethylene derivative, and is thus structurally analogous to TAM. The lack of the high affinity 4-hydroxy group does not affect the binding mode of GW5638 to LBD. It binds to the receptor in the same site and orientation as OHT does. For this reason, OHT-LBD can represent the anti-cancer drug TAM and its major metabolites bound to the receptor. The binding of raloxifene and the benzoxathiin derivative is also in a similar manner, albeit they are structurally very different from TAM-like compounds. The positioning of SERMs in the binding site is compared in Figure 1.13(3), showing identical binding behavior of the B-rings, slight deviations of the A-rings, and most flexible orientations of the C-rings. The comparison indicates that ERα ligands require the proper orientations of A- and B-rings, which have proven by experiments as affecting the relative binding affinity (RBA) to ERα.8,98

The ligand-induced allosteric changes lead to identical structures of these SERM-LBD complexes except for GW5638, as the superimposed backbones are shown in Figure 1.13(2). The GW5638-LBD complex (1R5K, Figure 1.13(2), in blue) shows a distinct H12 conformation, of which H12 gets closer to the binding pocket than it does in others. This may be due to the shorter acrylate side chain of GW5638 compared to the bulky tails of other SERMs, whereby the allosteric changes of the LBD are again proven to depend on the ligand molecules.

Of particular interest is the comparison between raloxifene and TAM. The piperidinoethoxy side chain of raloxifene stabilizes H12 exactly in the same geometry as the dimethylaminoethoxy tail of TAM does. The positioning of H12 affects the recruitment of co-regulators and subsequently up/down regulates the target gene transcription. Therefore, similar physiological functions of raloxifene and TAM have been found experimentally. They both have estrogenic properties in bone showing similar effects on maintaining bone density, and have anti-estrogenic properties in breast cells showing equivalent potency in preventing breast cancer. In addition, raloxifene also displays cross-resistance in TAM-resistant breast cancer.99-101 However, raloxifene is less estrogenic than TAM in uterine tissues,102 in which TAM works as an agonist and encourages development of endometrial cancer.103,104 Raloxifene is thus a safer preventive drug than TAM in health women, and is approved for the prevention and treatment of osteoporosis with beneficial side effect of preventing breast cancer. The SERM profiles of raloxifene have been rigorously studied and analyzed, and finally ascribed to the charge neutralization between the basic amine of raloxifene and the negatively charged residue Asp351 in the LBD.105,106 The distance between the basic amine (\(\text{N}_{\text{lig}}\)) and Asp351 (O\(^{\delta351}\)) has been found to be ~1 Å shorter of raloxifene-LBD (1ERR) compared to that of OHT-LBD (3ERT, Figure 1.5), which is thus assumed to be the
mechanism explaining why raloxifene is less estrogenic than TAM. However, the mechanism does not account for the agonist or antagonist properties, as shown by the distances measured from other crystal structures in Table 1.1. TAM and raloxifene have equivalent charge-neutralizing capacity seen in the crystal structures except for 3ERT. A comparison of the static structures is not sufficient to decipher the SERM profiles, and further discussions are presented in Paper I.

![Figure 1.7 (1)](image)

**Figure 1.7 (1).** SERMs used in the superposition comparison; (2). Superposed backbones of ERα LBD cocrystallized with different SERMs: 1ERR-raloxifene (black)\(^27\), 1R5K-GW5638 (blue)\(^{107}\), 2OUZ-lasofoxifene (green)\(^{108}\), 3ERT-OHT (red)\(^{26}\), 1SJ0-benzoxathiin derivative (yellow)\(^{109}\); (3). The ligands in the superposed backbones. Reproduced with permission of the copyright owner.\(^{110}\)
Table 1.1 Distances (Å) between Asp351 and the N atom of the tertiary amine of OHT and Raloxifene in complex with ERα LBD.

<table>
<thead>
<tr>
<th>OHT</th>
<th>Raloxifene</th>
</tr>
</thead>
<tbody>
<tr>
<td>3ERT^{25}</td>
<td>2JF9(A. B. C.)^{35}</td>
</tr>
<tr>
<td>3.82</td>
<td>2.74</td>
</tr>
</tbody>
</table>

1.8.2 SERMs with indication of breast cancer

The area of SERMs for the treatment of breast cancer has been the focus of research due to problems found with TAM, such as the genetic polymorphism of CYP2D6, acquired resistance, and the undesired uterine stimulation. In order to avoid CYP-mediated activation of prodrug, many SERMs (Figure 1.7 and Scheme 1.10) have a hydroxyl group on the B-rings to achieve high affinity to ERα. Hence, the Phase II conjugation of these compounds is much faster than that of TAM, resulting in more rapid clearance and much shorter \textit{in vivo} lifetime.{^{111,112}} As a result, droloxifene,^{113-115} miproxifene^{116}, raloxifene^{117} and arrozoxifene^{118} were all proven inferior to TAM in breast cancer treatment. It suggests that the therapeutic agents in breast cancer are required to be long-acting. In contrast, idoxifene and nafoxidine are metabolically more stable, and they were stopped from further development due to the side effect of uterine prolapse^{119} and liver toxicity,^{120} respectively. Unfortunately, none of the new developed SERMs has proven to be superior to TAM in treating breast cancer. The studies of the SERMs launched or rejected provide a wealth of information for the pursuit of ideal SERMs.

Scheme 1.10 Structures of selected SERMs.
1.9 Aims of the Study

The overall aim of this project was to elucidate the possible explanations for the CYP2D6 debate in the TAM-treated breast cancer patients, in order to provide new information that might be useful to develop novel and improved therapeutic agents.

Paper I — To investigate what governs the estrogenic or anti-estrogenic SERM profiles of compounds which stabilize ER$\alpha$ LBD in the canonical open/antagonist conformation.

Paper II — To explore how the TAM-like ligands contact ER$\alpha$ LBD. We aimed to gain a better knowledge on the three-dimensional geometry of the ER$\alpha$ ligand binding pocket.

Paper III — To study the mechanism of the CYP-catalyzed cis-trans isomerization of OHT, which is a rarely reported CYP-reaction. Understanding the reaction mechanism might be a step forward to avoiding the cis-trans isomerization problem, which is related to increased risk of cancer recurrence.

Paper IV — To provide more insight into the comprehensive CYP-mediated metabolism of TAM. We aimed to create new SERMs to avoid being activated by CYP2D6.

Paper V — To study the agonists binding to ER$\alpha$ LBD, and to understand the signaling differences of small ligands which stabilize the LBD in the canonical closed/agonist conformation.
CHAPTER 2. COMPUTATIONAL METHODS

All studies presented in this thesis were carried out by computational approaches to investigate the issues introduced in Chapter 1. Quantum Mechanics (QM) calculations were performed in Papers II-V to investigate the properties of small systems, which contain one to several molecules in the studies. Of these computations, energies are generated from the distribution of electrons, which are explicitly modeled. In contrast, electrons are treated implicitly in Molecular Mechanics (MM) calculations, which are widely used to model larger systems closer to real objects, such as proteins. As the systems consist of many nuclei and several times more electrons, atoms are treated as a whole using “classical” hard spheres. MM calculations are thus based on “classical” mechanics mainly of Newton’s second law, and have been performed in Papers I, II, IV, and V.

The computational methods and programs used in the current studies are briefly introduced in the following section.

2.1 Quantum Mechanics Approaches

In QM, Schrödinger equation\(^{121}\) is used to describe the quantum states of physical systems. Equation 2.1 is the time-independent Schrödinger equation,

\[
\hat{H}\psi = E\psi
\]  

(2.1)

where the Hamiltonian operator \(\hat{H}\) acts on the wavefunction \(\psi\), yielding the total energy \(E\) of the system multiplied by \(\psi\).

\[
\hat{H} = \hat{T} + \hat{V}
\]  

(2.2)

\[
\hat{H} = \hat{T}_N + \hat{T}_e + V_{NN} + V_{Ne} + V_{ee}
\]  

(2.3)

The Hamiltonian \(\hat{H}\) is the total energy operator as a combination of kinetic \(\hat{T}\) and potential \(\hat{V}\) energy operators (Equation 2.2), consisting of the kinetic energies of nuclei \(\hat{T}_N\), the kinetic energies of electrons \(\hat{T}_e\), and three terms of potential energies, \(V_{NN}\), \(V_{Ne}\), and \(V_{ee}\), describing the interactions of nuclei and electrons in the system (Equation 2.3).

By solving the Schrödinger equation, the eigenfunction \(\psi\) and eigenvalue \(E\) are found, from which various properties of the system are derived. However, the Schrödinger equation cannot be exactly solved for many-electron systems, which are usually the systems of interest. Hartree-Fock (HF) method is a primary approach to solve the Schrödinger equation (2.1) in large systems. A series of approximations has been applied in the HF method, such as (1) the Born-Oppenheimer approximation: splitting of the movements of nuclei and electrons based
on the fact that nuclei move much slower than electrons; (2) the relativistic effects are not considered; (3) electrons are assumed to be uncorrelated and move in the mean field independently of each other, and the eigenfunction of a many-electron system is thus described by a single Slater determinant; (4) the LCAO-MO approximation: the wavefunction of each molecular orbital (MO) can be constructed by a linear combination of atomic orbitals (LCAO), expressed in terms of a finite number of basis functions.

HF methods are referred to as wavefunction-based \textit{ab initio} approaches. Based on certain approximations, the Schrödinger equation is solved iteratively using the self-consistent field (SCF) approach. The iteration process starts from an initial guess of the wavefunction, which is refined in each energy minimization step until convergence is reached. As guaranteed by the variational principle, the obtained HF energy of a given system is an upper bound to the energy of the exact wavefunction. The HF energies of many-electron systems are always overestimated since electrons are assumed to be uncorrelated, which is apparently an over approximation accounting for a major portion of energy errors in HF calculations. Of the system being described, the energy gap between the HF energy $E_{HF}$ and the exact nonrelativistic energy $E_{\text{exact}}$ gives rise to the definition of correlation energy $E_{\text{corr}}$ (Equation 2.4), which is always negative.

$$E_{\text{corr}} = E_{\text{exact}} - E_{\text{HF}}$$  \hspace{1cm} (2.4)

The correlation energy is relatively small and usually less than 1% of the total energy of the system. However, the chemical properties of interest, such as reaction barriers, are practically described by the relative energies, i.e., the energy changes between different states. Not as in the absolute total energy, the effects of correlation energies are not negligible in the relative energy.

In addition to the primitive HF methods, many computational methods have been developed, among which Density Functional Theory (DFT) methods have evolved as widely used approaches. Different from HF theory, DFT is based on the electron density $\rho$ instead of the explicit wavefunction $\Psi$. The energy of a given system $E[\rho]$ is thus described by a functional of the electron density $\rho$. Therefore, in an $N$-electron system, only three spatial variables are required in DFT, whereas in wavefunction-based methods $(3+1)N$ variables are required to describe the spatial and spin wavefuntions. DFT computations are hence much faster than wavefunction-based methods, and are even more efficient in systems consisting of more electrons.

DFT in principal is an accurate method, and in practice, the computations are most commonly performed using the Kohn-Sham approach, in which the electron density is generated by non-interacting electrons to model the interacting electrons of a real system. The Kohn-Sham equation is solved iteratively in a similar manner to HF calculations. The DFT energy $E[\rho]$ is expressed in terms of the kinetic energy $T[\rho]$ of the non-interacting electrons, the attraction
energy $E_{Ne}[\rho]$ between nuclei and electrons, the Coulomb energy among electrons $J[\rho]$, and the exchange-correlation energy $E_{XC}[\rho]$ representing the interactions not included in the other terms (Equation 2.5).

$$E[\rho] = T[\rho] + E_{Ne}[\rho] + J[\rho] + E_{XC}[\rho]$$  \hspace{1cm} (2.5)

The main focus in DFT methods is the challenge of describing the exchange-correlation energy, in which approximation has to be made. The accuracy of DFT functionals highly depends on the exchange-correlation functional, which is usually decomposed into an exchange functional and a correlation functional (Equation 2.6).

$$E_{XC}[\rho] = E_X[\rho] + E_C[\rho]$$  \hspace{1cm} (2.6)

Hence, efforts have been made to improve the exchange-correlation functional, and various types of exchange-correlation functionals have been developed. The approximations used include the local density approximation (LDA), generalized gradient approximation (GGA), and meta-GGA. LDA depends only on the local density at a given coordinate, and in general does not yield sufficient accuracy. As an improvement, GGA takes the gradient of the local density into account. One such example is B88\textsuperscript{122} which is a frequently used exchange functional, and usually in conjunction with the GGA correlation functional LYP developed by Lee, Yang and Parr\textsuperscript{123}. A further development is meta-GGA, which includes the Laplacian (the second derivative) of the electron density as the additional ingredients. Besides these DFT methods, exchange energy could be extracted by the HF method, in which exchange energy is fully accounted for and considered as exact exchange. Hybrid exchange-correlation functionals are thus developed by incorporating a portion (usually not 100%) of HF exchange with DFT exchange and correlation. Such examples are B3LYP\textsuperscript{123-125} a hybrid GGA; and M06-2X\textsuperscript{126,127} a hybrid meta-GGA. The hybrid functionals represent a great improvement in DFT functionals and provide satisfactory accuracy in many situations.

All QM calculations in this thesis were performed using the Gaussian09\textsuperscript{128} program packages. In Paper II, the geometry of TAM was optimized using a range of DFT functionals with the 6-31+G(d,p) basis set. The pure GGA functional BLYP\textsuperscript{122,123} and the hybrid GGA functional B3LYP\textsuperscript{123-125} gave rise to the optimized structures in best agreement with the X-ray geometry, and were thus used to investigate the flip-flop process of the TAM propeller and derivatives. The more extended 6-311++G(2d,2p) basis set was applied in the computations of the rotational barriers about the C(Ar)—C(sp\textsuperscript{2}) single bond. Frequency calculations were performed at the same level of theory to analyse the obtained structures to be local minima or transition states. The solvent effects were considered using the SMD continuum solvation model\textsuperscript{129} with single-point calculations. Water ($\varepsilon = 78.36$) was used as a solvent to model the physiological conditions.

In Paper III, IV, and V, the hybrid meta-GGA functional M06-2X\textsuperscript{126,127} was used, as it is highly parameterized for main group atoms and recommended for studies of weak interactions,
such as hydrogen binding and dispersive interactions. The 6-31+G(d,p) basis set was used for most calculations. In Paper III and IV, the systems of single molecules were optimized both in vacuo and in nonpolar medium ($\varepsilon = 4.24$) mimicking the protein environment of CYP enzymes. Corresponding harmonic vibrational frequency calculations were performed to extract zero-point vibrational energies (ZPE) and thermal corrections to the Gibbs free energies. In paper V, the systems containing one or more molecules were optimized in vacuo, and corresponding harmonic vibrational frequencies were calculated in vacuo and in solvents ($\varepsilon = 78.36$ and $\varepsilon = 4.24$). The ZPE-corrected binding energies between ligands and part of the ligand binding region were calculated by Equation 2.7:

$$\Delta \Delta E_{\text{binding}} = \Delta E_{\text{total}} - \Delta E_{\text{ligand}} - \Delta E_{\text{residues}}$$ (2.7)

### 2.2 Molecular Mechanics Approaches

The requirement of computational studies of systems with thousands to millions of atoms has evoked the development of MM methods. In MM, the system is constructed with classical spheres representing the atoms, whereby only the motion of nuclei is modeled and the electrons are not explicitly considered as in QM methods. The MM calculations are thus simplified to handle large systems, of which, Newton’s second law (Equation 2.8) is the main principle of modeling a system evolving with time. It gives rise to the concept of Molecular Dynamics (MD) simulation of motions of atoms and molecules.

$$F = ma$$ (2.8)

where $F$ is the force acting on a body with mass $m$, yielding an acceleration $a$. The propagation of the system is calculated from Equation 2.9.

$$\frac{d^2 x_i}{dt^2} = \frac{F_x}{m_i}$$ (2.9)

The computation accuracy thus highly depends on the force $F$ generated from the force field which is used to describe the potential energy of the system. The potential energy defined by a force field usually contains contributions of covalent-bonded interactions and noncovalent-bonded interactions (Equation 2.10),

$$V = V_{\text{bonded}} + V_{\text{non-bonded}}$$ (2.10)

in terms of (Equation 2.11 and 2.12):

$$V_{\text{bonded}} = V_{\text{bond}} + V_{\text{angle}} + V_{\text{torsion}}$$ (2.11)

$$V_{\text{non-bonded}} = V_{\text{vdW}} + V_{\text{elec}}$$ (2.12)
Chapter 2. Computational methods

The covalent-bonded interactions are generally described by Equations 2.13–2.15:

\[ V_{\text{bond}} = \frac{1}{2} k_b (l_i - l_{i,0})^2 \]  
\[ (2.13) \]

\[ V_{\text{angle}} = \frac{1}{2} k_\theta (\theta_i - \theta_{i,0})^2 \]  
\[ (2.14) \]

\[ V_{\text{torsion}} = \frac{V_n}{2} \left[ 1 + \cos(n\omega - \gamma) \right] \]  
\[ (2.15) \]

Another term of improper torsion is described in similar ways.

The noncovalent-bonded interactions are decomposed into van der Waals interactions and electrostatic interactions, modeled by the Lenard-Jones potential (Equations 2.16) and Coulomb’s law (Equations 2.17), respectively:

\[ V_{LJ} = 4\varepsilon_{ij} \left[ \left( \frac{\sigma_{ij}}{r_{ij}} \right)^{12} - \left( \frac{\sigma_{ij}}{r_{ij}} \right)^{6} \right] \]  
\[ (2.16) \]

\[ V_{\text{elec}} = \frac{q_i q_j}{4\pi \varepsilon_0 r_{ij}} \]  
\[ (2.17) \]

MM methods used in the current studies:

Molecular Operating Environment (MOE) program\(^{130}\) was used to visualize and analyze the crystal structures. Comparisons were made of the cocrystallized ligand-LBD structures through structural alignments with the root-mean-square deviation (RMSD) calculated from \( \text{C}^\alpha \) atoms.

YASARA structure program\(^{131}\) was used to perform MD simulations of the ligand-LBD complexes. All starting geometries were constructed from crystal structures, and in some cases the ligands in the crystal structures were manually modified. Unrestrained all-atom MD simulations were conducted using a predefined macro (md_run) within the YASARA package. In all simulations, the ligand-LBD complexes were solvated with TIP3P\(^{132}\) water molecules in a periodic box, which extended 10 Å outside the protein. The AM1-BCC model\(^{133}\) was used to calculate partial atomic charges of each ligand. The systems were then neutralized by adding counter ions,\(^{134}\) \( \text{Na}^+ \) or \( \text{Cl}^- \), and additional ions were added to give the NaCl concentration of 0.9 % to mimic the physiological solution. Long-range Coulomb interactions were included using particle-mesh Ewald (PME) summation with a cut-off of 7.86 Å. All runs were carried out at 298 K using the AMBER03\(^{135}\) force field for proteins and the general amber force field\(^{136}\) (GAFF) for ligands. The systems evolved with multiple time steps, 1.25 fs for intramolecular forces and 2.5 fs for intermolecular forces.
The dynamic behaviours of the ligands in contacting with the LBD were analyzed from the MD trajectories. Geometry changes were directly monitored from the atomic coordinates in each trajectory. The conformational drift of the structures was compared according to RMSD values of Ca atoms throughout the simulation time. The RMSDs were calculated with regard to the initial conformation by Equations 2.18 (R is the vector linking n corresponding Ca-atom pairs in space):

$$\text{RMSD} = \sqrt{\frac{\sum_{i=1}^{n} R_i^2}{n}}$$  \hspace{1cm} (2.18)
CHAPTER 3. ANTAGONISTS AND THE ANTAGONIST-ERα LBD COMPLEXES

Paper I

3.1 More Stable, More Estrogenic—the SERM Profiles

As it is introduced in Chapter 1, the estrogen-related diseases have provoked a wide use of SERMs in both patients and healthy women, such as therapeutic and preventive agents for breast cancer. SERMs generally act as partial antagonists. The rationale behind the SERM actions is the fact that they stabilize ERα LBD in the open/antagonist conformation which is significantly less active compared to the closed/agonist conformation. The SERM profiles, i.e. how potent they work as agonists or antagonists, vary significantly by individual compounds. However, they are not distinguishable from the crystal structures of the SERM-LBD complexes.

The ERα-mediated estrogenic and antiestrogenic properties are of vital importance for the clinical applications of SERMs, and have been tested and compared for many compounds. The results indicated the crucial effects of the basic side chain on the biological activity in uterine tissue, as seen in dihydrobenzoxathiin analogous (Scheme 3.1) compounds 15, 16, 18 and 19 (Table 3.1, numbering from ref [43]). Significant differences in the SERM profiles have been found for compounds 15 vs 16 and 18 vs 19, which have subtle changes in the ligand structures as they differ only in the orientation of one or two methyl substituents.

Scheme 3.1 Dihydrobenzoxathiin.

Fortunately, the crystal structures were solved for these ligands bound to ERα LBD, which made it possible to study the SERM profiles by modeling techniques. The overall structures are compared by structure alignments (Table 3.1 and Figure 3.1). A highly conserved open/antagonist conformation is found for the compounds in complex with the LBD, with
fairly slight deviations computed from Cα atoms of the LBD. The charge-neutralizing capacity, the once believed mechanism for the SERM profiles, are again compared by the distances measured between Oδ351 and Nlig. of the crystal structures. The distances of 2.6~2.8 Å are found in all SERM-LBD complexes except 3ERT, showing no apparent differences of these ligands in shielding the charge of Asp351. Hence, it cannot be the rationale behind the different SERM profiles of compounds 15 vs 16, 18 vs 19, or raloxifen vs TAM.

**Table 3.1** Biodata of SERMs and geometry comparisons of the cocrystallized structures. Reproduced with permission of the copyright owner.47

<table>
<thead>
<tr>
<th>SERM</th>
<th>IC₅₀ (nM)</th>
<th>Uterine activity</th>
<th>PDB</th>
<th>Oδ₃₅¹-Nlig.</th>
<th>RMSD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>hERαa</td>
<td>MCF-7b</td>
<td>%Ant. %Ag.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>E₂</td>
<td>1.3</td>
<td>—</td>
<td>100</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>OHT</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>3ERT²⁶</td>
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</tr>
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<td></td>
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</tr>
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<td>Comp. 15</td>
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<td>103</td>
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<tr>
<td>Comp. 16</td>
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<td>1XP9</td>
</tr>
<tr>
<td>Comp. 19</td>
<td>1.7</td>
<td>0.5</td>
<td>18</td>
<td>71</td>
<td>1XPC</td>
</tr>
</tbody>
</table>

*In the ERα ligand binding assays used tritiated estradiol. **The in vitro MCF-7 breast cancer cell proliferation assays were performed in the presence of low levels of estradiol. † The %Ant. (%antagonism) and %Ag. (%agonism of estradiol control as 100% agonism) were compared in the uterine weight assay with compounds dosed orally.43*
Compounds 16 and 19 were proven much more estrogenic than compounds 15 and 18. More detailed comparisons of the cocrystal structures are seen in Figure 3.1. All of the four ligands form hydrogen bonds with the side chains of Glu353, Arg394 and His524 in the LBD in the same manner. The ligands differing in contacting the receptor are seen only for the positioning of the methyl substituents. The methyl groups of 16 and 19, no matter whether they are substituted at the pyrrolidine rings or the linkers, approach the L11-12 loop region more closely than 15 and 18 do. Although no strong interactions are found between the methyl groups and the LBD, the loop region is sterically stabilized by the methyl substituents of 16 and 19 better than that of 15 and 18. As seen in Figure 3.2, the conformational drift is compared for 18 vs 19 in complex with the LBD through the 20 ns simulation time. The RMSDs, monitored for the overall structure and for the region from L11-12 to the C-termini,
show a higher conformational stability of 19 in complex with the LBD than in the case of 18. In comparison with the bioactivities in Table 3.1, we here propose that the partial agonist properties of SERMs are in line with the conformational stability of the SERM-LBD complex, i.e., more stable, more estrogenic. It might indicate that easier or faster allosteric changes of the LBD are induced by 19 than by 18, and the differences in the folding processes are independent of the binding affinity to ERα. As far as the SERM-LBD complex folds into the canonical open/antagonist conformation, the ERα is activated to some extent with the potency depending on the specific tissues. It may partly explain why all the SERMs have more or less estrogen-like activities.

![Figure 3.2 RMSDs calculated from the MD trajectories for A) Cα atoms of the LBD (Res 307-551), B) Cα atoms of L11-12 to C-terminals (Res 527-551). C) Distances between Asp351 (Oδ351) and the tertiary amine of ligand (N18). Reproduced with permission of the copyright owner.47](image-url)
3.2 The SERM Profiles of TAM vs OHT

TAM is highly successful in the treatment of ER-positive breast cancer, with antiestrogenic properties in breast tissue. The 4-hydroxy metabolites, OHT and endoxifen, have \(~100\) fold higher affinity to ER\(\alpha\) than TAM itself, and are thus simply assumed to be more active antagonists in breast cancer cells. As CYP2D6 is the primary isoform responsible for producing the 4-hydroxy metabolites, patients who have null alleles for inactive CYP2D6 resulting in limited amounts of OHT and endoxifen, have been believed to benefit less from TAM treatment than patients who have wide type \(CYP2D6\) alleles. However, this intuitively right concept has been greatly challenged through many clinical studies, in particular the very recent large studies which suggest that CYP2D6 activity does not predict clinical response to TAM therapy.\(^{75,76}\) The effects of higher-affinity metabolites on TAM treatment have remained elusive. However, the decreased concentration of the parent drug TAM has been related to the failure in TAM treatment despite the fact that it binds to ER\(\alpha\) with fairly low affinity.\(^{78}\)

OHT and endoxifen have been assumed to work as potent anti-estrogens in breast cancer cells, but the truth lies in very complex actions, because they have both estrogen agonist and antagonist properties, as seen in compounds 15, 16, 18 and 19 (Table 3.1). However, there is as yet no report of experimental comparisons on the SERM profiles between TAM and OHT, since the experiments are not easy to carry out due to TAM is \textit{in vivo} metabolized to OHT, etc. We thus performed \textit{in silico} comparisons of the SERM profiles based on the principle of more stable, more estrogenic. As seen in Figure 3.3A, a higher conformational drift is found for TAM than OHT in complex with the LBD. The difference in dynamic stability between TAM- and OHT-LBD complexes is a result of the additional 4-hydroxyl group, which stabilizes the inter-helix interactions (Figure 1.5) through hydrogen bond interactions as seen in Figure 3.3B. Constant distances between \(\text{C}_\delta^{353}\) (on Helix 3) and \(\text{C}_\zeta^{394}\) (on Helix 6) are found for OHT but not TAM in the modeled systems. The simulation results show that TAM-LBD complex is less dynamically stable than OHT-LBD. TAM may thus have more antagonist properties than OHT does. Hence, the conversion of TAM into OHT mediated by CYP2D6 has complex effects on TAM treatment. The 4-hydroxyl group increases the affinity to ER\(\alpha\), and also results in more estrogen-like properties. The mixed agonist-antagonist properties of OHT are necessary to be testified experimentally. Conclusive experimental studies are still lacking to clarify the clinical effects of CYP2D6 activity on TAM therapy.

In addition, the salt bridges between \(\text{N}^{188}\) and the negatively charged Asp351 are found not retained in solvent in all the simulations (Figures 3.2 C and 3.3 C). The side chain of Asp351 is more likely to be solvent exposed, facilitating the interaction with other domains of ER\(\alpha\) or coregulators, and thus contributes to the overall folding of the ER\(\alpha\) transcription unit. It has proven by experiments that mutations of Asp351 into noncharged residues (Ala, Val and Gly) result in reduced agonism of SERMs.\(^{144-146}\)
Chapter 3. Antagonists and the antagonist-ERα LBD complexes

Figure 3.3 A) RMSDs of Cα atoms of the LBD (Res 306-551), calculated from the MD trajectories. B) Distances between Glu353 (Cδ$_{353}$) and Arg394 (Cζ$_{394}$). C) Distances between Asp351 (Oδ$_{351}$) and the tertiary amine of ligand (N$_{lig}^\text{N+}$). Reproduced with permission of the copyright owner.47

**Paper II**

**3.3 Flip-Flop: Rotation about the C(Ar)—C(sp$^2$) Bond**

Chemical chirality profoundly affects drug development since enantiomers have been frequently reported as differing in efficacy and even dangerously in toxicity. Chiral drugs are thus highly recommended to be developed into pure eutomers, i.e. the more active enantiomers. Differing from the classical enantiomers with chiral centers, atropisomers are generated by torsion thereby they display thermal equilibration at a certain temperature depending on the barrier of the hindered rotation. The chirality of TAM has been largely
overlooked due to the fast interconversion between the atropisomeric propellers (clockwise and counterclockwise triarylvinyl propellers) through the rotation about the \( \text{C(Ar)}-\text{C(sp}^2) \) bonds (Scheme 3.2).

The spontaneous racemization of the TAM propellers is too fast to be investigated by dynamic NMR methods,\(^8\) whereas computational methods are applied in the current studies. The possible pathways of the helicity reversal, through the simultaneous rotations of the three rings, are illustrated in Scheme 3.2, with corresponding barriers summarized in Table 3.2. The rotation of the rings is referred to as ‘flip’ or ‘nonflip’ according to the pass through the plane perpendicular or coplanar to the vinyl plane, respectively. The lowest activation energies of the helical inversion of TAM-like compounds are in the range of 2–4 kcal/mol through the three-ring flip pathway. Besides this threshold pathway, the middle ring noflip pathway has the barrier of \( \sim 5 \) kcal/mol, which could be an alternative route for the helicity reversal.

Scheme 3.2 Idealized depictions of the competing flip-flop mechanisms. The transition states are illustrated in the middle. The ‘flip’ rings via the perpendicular plane are denoted as \( \text{flip} \). Reproduced with permission of the copyright owner.\(^{110}\)

\[\begin{align*}
\alpha &= 2-1-7-8 \\
\beta &= 2'-1'-7-8 \\
\gamma &= 2''-1''-8-7 \\
\delta &= 7-8-9-10
\end{align*}\]
Table 3.2 Enantiomerization free energies of TAM and OHT in gas phase and in aqueous solution at 298 K, calculated at B3LYP and BLYP levels in conjunction with the 6-311++G(2d,2p) basis set.\textsuperscript{a}

Reproduced with permission of the copyright owner.\textsuperscript{110}

<table>
<thead>
<tr>
<th></th>
<th>$\Delta G_{\text{gas}}^{298}$</th>
<th>$\Delta \Delta G_{\text{gas}}^{298}$</th>
<th>$\Delta G_{\text{aq}}^{298}$</th>
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</tr>
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</tr>
<tr>
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<td></td>
<td>TS$_{\text{Three-ring flip}}$</td>
<td>-1213.171214</td>
<td>3.42</td>
<td>-1213.273524</td>
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</table>

\textsuperscript{a} Absolute energies in a.u., and relative energies in kcal/mol.

As seen in Table 3.2, the threshold barriers are too low to allow for the resolution of TAM atropisomers, resulting in that TAM appears as an achiral compound. Atropisomers were defined by Oki in 1983, as the isomers interconverted slowly enough to be resolved, with a half life not less than 1000 seconds.\textsuperscript{147} However, recognizing the existence of atropisomers of TAM and its metabolites is of great importance, because these atropisomers are discriminated by the drug target, ER$\alpha$. As seen in Figure 3.4 and Table 3.3, only the clockwise propellers of...
the ligands are found in the cocrystallized structures, while the counterclockwise conformation is found in crystalline TAM. The counterclockwise propeller is not tolerable by the ligand binding pocket of ERα, as tested by MD simulations with the detailed results seen in Paper II. Therefore, the counterclockwise isomers of TAM and its metabolites do not bind to ERα directly, but rapidly flip forward to the clockwise isomers to cater for the ligand binding pocket of ERα. Although the fast interconversion between the atropisomers of TAM-like compounds makes no distinguishable atropisomeric conformations in the solvent, the clockwise atropisomers are the pharmacologically functional isomers, which work as agonists or antagonists in TAM treatment through ERα signaling pathways.

**Figure 3.4** Superposed crystal structures of OHT in complex with ERα LBD solved by three independent research groups, 3ERT (red)²⁶, 2JF9.A (blue)⁴⁵, and 2BJ4.A (green)⁴⁶. Reproduced with permission of the copyright owner.¹¹⁰

**Table 3.3** Torsional angles measured from the crystal structures of crystalline TAM and OHT/GW5638 cocrystallized with ERα LBD. Reproduced with permission of the copyright owner.¹¹⁰
3.4 CYP-Catalyzed Rotation about the C(sp²)=C(sp²) Bond

\textit{Trans}-TAM is prescribed to treat ER-positive breast cancer as an estrogen antagonist in breast tissue, whereas \textit{cis}-TAM is an estrogen agonist. \textit{Cis}-4-hydroxyTAM (\textit{cis}-OHT) was, besides \textit{trans}-OHT, found in patients who took TAM drug in the \textit{trans}-form. \textit{Cis}-OHT was a direct product from \textit{trans}-OHT mainly due to the CYP-mediated \textit{cis}-\textit{trans} isomerization of OHT. The analogous isomerization process was, however, not observed with TAM itself. Of these isomers, the relative binding affinities to ER (which was 100\% of estradiol) were reported as 0.3 vs 2.5\% for \textit{cis}- vs \textit{trans}-TAM, and 2.8 vs 310\% for \textit{cis}- vs \textit{trans}-OHT.\textsuperscript{80} The \textit{trans}-to-\textit{cis} isomerization of OHT results relatively high \textit{cis}:\textit{trans}-OHT ratio \textit{in vivo}, which has been associated with an increased risk of tumor relapse possibly owning to the less potent \textit{cis}-OHT in suppressing tumor growth.\textsuperscript{78,79}

The CYP-catalyzed interconversion between \textit{cis}- and \textit{trans}-OHT implies a redox process by the attendance of NADPH as cofactor, thereby the oxidation steps are most likely to be initiated from the additional 4-OH group, since the isomerization is forbidden between \textit{cis}- and \textit{trans}-TAM. Hence, the 4-OH substituent is assumed to undergo an initial H-atom abstraction, which has been regarded as a typical initiation step of many CYP reactions, resulting in a radical intermediate. This putative radical intermediate is also possible to be further oxidized into a cationic intermediate by the heme-oxygen complex. In both cases, the C(sp²)=C(sp²) bond is disturbed due to the oxidation of the 4-hydroxy group in OHT or endoxifen, and the subsequent rotation process is allowed to occur via either the radical or the cationic intermediate (Scheme 3.3A).

Hence, as the key step of the isomerization process, rotations about the disturbed alkene bond were studied via the radical (Scheme 3.3B) or cationic intermediates (Scheme 3.3C). The rotations via the radical intermediates have similar barriers (15–18 kcal/mol) for \textit{cis}→\textit{trans} and \textit{trans}→\textit{cis} isomerizations. Alternatively, the barriers are much lower via the cationic intermediates, estimated at 8–11 vs 2–5 kcal/mol for \textit{cis}→\textit{trans} vs \textit{trans}→\textit{cis} isomerization. The rotational barriers indicate a faster conversion of \textit{trans}→\textit{cis} than \textit{cis}→\textit{trans} via the cationic pathway. The experimental studies were performed by Williams et al. using human CYP’s. Pure \textit{cis}- or \textit{trans}-OHT were incubated with human liver microsomes in the presence of NADPH cofactor, and the conversion rates were 51–64\% and 22–27\% for \textit{trans}→\textit{cis} and \textit{cis}→\textit{trans} reactions, respectively.\textsuperscript{81} The rotational energy differences between the \textit{trans}→\textit{cis} and \textit{cis}→\textit{trans} processes are seen only in the cationic pathways, mainly due to the \textit{cis}-d\textsuperscript{+} isomers are energetically favored than the \textit{trans}-d\textsuperscript{+} isomers (Figure 3.5), and are in agreement with the order of the experimental conversion rates. The optimized geometries of \textit{cis}/\textit{trans}cation intermediates and the detailed isomerization potentials are depicted in Figure 3.5.
**Scheme 3.3** Possible radical and cationic intermediates for the trans-to-cis isomerization of OHT/endoxifen catalyzed by CYP. The cis-to-trans reaction may proceed by analogous mechanisms, via the cis-c• or cis-d• intermediates. Reproduced with permission of the copyright owner.148

Hitherto, the cationic pathways haven’t verified experimentally. Comparisons are made between CYP-mediated isomerization and desaturation, which appear as distinctly different reactions, but derive from the same phenolic hydroxyl group (Scheme 3.4). Instead of isomerization, 4-hydroxyacetanilide and raloxifen undergo CYP-catalyzed dehydrogenation, in which process CYP enzymes abstract two hydrogen atoms from the substrate. However, the second abstractable proton is absent in cis- or trans-OHT, thereby the stable dehydrogenation product is not yielded by the CYP oxidation steps, and a radical or cationic intermediate is instead formed, resulting in cis-trans isomerization. We propose that
analogous CYP-catalyzed oxidation steps of phenolic compounds evolve into isomerization or desaturation reactions, largely depending on structures and properties of the substrates. The ionization potentials and deprotonation energies are thus computed for cis/trans-OHT, 4-hydroxyacetanilide and raloxifen, of which the results are in a similar range (Table 3.4). The redox processes mediated by CYP enzymes are thus assumed to be accessible for all the three substrates under physiological conditions, albeit the computations have not been addressed in the CYP active site.

Figure 3.5 Computed energy profiles for the cis-trans isomerization of OHT via the cationic intermediate. The DFT energies ($\varepsilon = 1$ and $\varepsilon = 4.24$) are obtained at the M06-2X/6-31+G(d,p) level of theory. Reproduced with permission of the copyright owner.\textsuperscript{148}

Table 3.4 Ionization potentials and deprotonation energies ($\Delta\Delta G$, in kcal/mol) of the oxidation processes shown in scheme 3.4.\textsuperscript{a} Reproduced with permission of the copyright owner.\textsuperscript{148}

<table>
<thead>
<tr>
<th>Reactant</th>
<th>Products\textsuperscript{b}</th>
<th>\textsuperscript{trans-OHT}</th>
<th>\textsuperscript{cis-OHT}</th>
<th>\textsuperscript{4-hydroxyacetanilide}</th>
<th>\textsuperscript{Raloxifene}\textsuperscript{d}</th>
</tr>
</thead>
<tbody>
<tr>
<td>a</td>
<td>$\text{b}^{+\ast} + e^-$</td>
<td>158.6(132.8)</td>
<td>158.7(132.7)</td>
<td>175.1(138.5)</td>
<td>158.6 (133.3)</td>
</tr>
<tr>
<td>b$^{+\ast}$</td>
<td>$e^- + H^+$</td>
<td>237.8(263.1)</td>
<td>236.5(262.0)</td>
<td>219.5(254.5)</td>
<td>236.0 (261.1)</td>
</tr>
<tr>
<td>c$^-$</td>
<td>$d^+ + e^-$</td>
<td>164.1(134.0)</td>
<td>161.6(132.2)</td>
<td>174.0(134.6)</td>
<td>155.6 (127.9)</td>
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<tr>
<td>d$^+$</td>
<td>$e^- + H^+$</td>
<td>—$^c$</td>
<td>—$^c$</td>
<td>208.8(249.9)</td>
<td>234.0 (262.6)</td>
</tr>
</tbody>
</table>

\textsuperscript{a} All values are relative to the free energies of the corresponding reaction intermediates. \textsuperscript{b} Energies of free $H^+/e^-$ are not included on product side. \textsuperscript{c} The final dehydrogenation product $e$, does not exist for trans- or cis-OHT. \textsuperscript{d} Geometry optimizations and frequency calculations were performed at the M06-2X/6-31G(d,p) level of theory.
**Scheme 3.4** Possible mechanism for CYP-catalyzed (1) trans-cis isomerization of OHT and (2) dehydrogenation of 4-hydroxyacetanilide to iminoquinone and (3) dehydrogenation of raloxifene to diquinone methide. Reproduced with permission of the copyright owner.

An early study suggested that the dehydrogenation of raloxifene initiated from the 6-hydroxy group catalyzed by CYP3A4.

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**Paper IV**

### 3.5 Modified SERMs

A significant portion (30–50%) of patients eventually developed resistance to TAM treatment, and suffered from tumor recurrences. Besides the elevation of the cis:trans-OHT ratio, another validated clinical fact in TAM-resistant tumors is the reduced cellular concentration of TAM. This indicates that therapeutic agents are required to be long-acting, as suggested by other studies aimed for better SERMs for the treatment of breast cancer. As a result, TAM remains one of the best choices for endocrine treatment of breast cancer. The CYP-mediated in vivo biotransformation is intimately involved in TAM response. Although the effects of CYP2D6 gene variants on TAM therapy are as yet not clear, the genetic polymorphism does have potential effects on the clinic responses of drugs. Hence, a new developed drug had better avoid being specifically metabolized by CYP2D6. We here propose two modified
structures, i.e., MTA and MTO (Scheme 3.5), of which both the cis-trans isomerization and the CYP2D6 polymorphism are most likely avoided according to the computational comparisons of the CYP-mediated biotransformation.

**Scheme 3.5** Structures and reaction sites labeling of TAM and derivatives.

TAM-like compounds undergo several types of CYP-mediated oxidations, involving N-demethylation, 4-hydroxylation, α-hydroxylation, and cis-trans isomerization. The CYP reactions are initiated by electrophilic-type attack due to the electron deficiency of the high-valent iron-oxo complex (Por^+Fe(IV)O), Cpd I, which is an important intermediate in the CYP catalytic cycle. Different reactivity patterns have been found, among which the initial abstraction of a H atom has been frequently suggested.83,85,92 Although computational studies of CYP reactions have been performed extensively, the mechanistic studies of CYP-mediated TAM metabolism are seldom reported, partly due to that the compound is relatively large. In order to gain a better understanding of the biotransformation of TAM-like compounds, whereby they evolve into different products by CYP reactions, the initial H-abstraction is assumed to be the mechanistic choice for all the reactions studied herein. The reaction energies of the H-abstractions are computed for the distinctively different CYP reactions (Scheme 3.5), and results are shown in Tables 3.5.
Table 3.5 Total energies and reaction Gibbs free energies\(^a\) in gas phase (\(\varepsilon = 1\)) and in solvent (\(\varepsilon = 4.24\)) of the initial H-abstraction to yield the possible CYP-catalyzed oxidations of TAM, MTA, MTO, NDT and OHT (\(\Delta G\), in a.u. and \(\Delta \Delta G\), in kcal/mol).

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<th>Systems</th>
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<td>MTO’(α) + H’(α)</td>
<td>-1636.035476</td>
<td>83.5</td>
<td>-1636.043857</td>
<td>83.7</td>
</tr>
<tr>
<td>NDT</td>
<td>-1098.049051</td>
<td>0.0</td>
<td>-1098.056855</td>
<td>0.0</td>
</tr>
<tr>
<td>NDT’(4) + H’(4)</td>
<td>-1097.884082</td>
<td>103.5</td>
<td>-1097.891836</td>
<td>103.6</td>
</tr>
<tr>
<td>NDT’(α′) + H’(α′)</td>
<td>-1097.914145</td>
<td>84.7</td>
<td>-1097.919474</td>
<td>84.7</td>
</tr>
<tr>
<td>NDT’(α) + H’(α)</td>
<td>-1097.931567</td>
<td>73.7</td>
<td>-1097.932882</td>
<td>73.8</td>
</tr>
<tr>
<td>OHT</td>
<td>-1212.509640</td>
<td>0.0</td>
<td>-1212.519888</td>
<td>0.0</td>
</tr>
<tr>
<td>OHT’(4α) + H’(4α)</td>
<td>-1212.374731</td>
<td>84.7</td>
<td>-1212.385290</td>
<td>84.5</td>
</tr>
<tr>
<td>OHT’(α′) + H’(α′)</td>
<td>-1212.385204</td>
<td>78.1</td>
<td>-1212.395876</td>
<td>77.8</td>
</tr>
</tbody>
</table>

\(\Delta G\) and \(\Delta \Delta G\) are in a.u. and kcal/mol, respectively.

\(\Delta G_{\text{gas}}^{298.15}\) and \(\Delta \Delta G_{\text{gas}}^{298.15}\) are the total energies and reaction Gibbs free energies in gas phase.

\(\Delta G_{\text{sol}}^{298.15}\) and \(\Delta \Delta G_{\text{sol}}^{298.15}\) are the total energies and reaction Gibbs free energies in solvent.

\(\varepsilon\) is the solvent polarity.

\(a\) Each value corresponds to the formation of a specified metabolite, and energy of the free H• is estimated at the same level of theory and thus included in the total energies.
Although the reaction energies do not account for activation energies or reaction rates, comparisons can be made between different reactions regarding to how endothermic the H-abstraction processes are. The H-abstraction energies decrease in the order of 4(4′)-hydroxylation (~103 kcal/mol) > β-hydroxylation (~93 kcal/mol) > N-demethylation ~ 4α-hydroxylation (~84 kcal/mol) > cis-trans isomerization (~78 kcal/mol) > α-hydroxylation (~74 kcal/mol). The most endothermic process is the aromatic 4(4′)-H-abstraction yielding the CYP-mediated aromatic hydroxylation. This hydroxylation has been proposed through a more efficient reactivity pattern, which initiates from a direct attack of the π electrons forming the Fe-O-Ph intermediate. The β-H-abstraction energy is 10 kcal/mol lower than that of the 4(4′)-H-abstraction, however, the β-hydroxyl product of TAM hasn’t been found experimentally. Therefore, the β-H-abstraction is not a rational reactivity step, and neither is the 4(4′)-H-abstraction. For N-demethylation, 4α-hydroxylation, cis-trans isomerization, and α-hydroxylation, the energies of the initial H-abstraction are in the range of 74–84 kcal/mol, which are 20–30 kcal/mol lower than that of aromatic H-abstraction. These H-abstractions with relatively low reaction energies are thus assumed to be accessible, as analogous H-abstractions have been frequently proposed in other studies.

In the experimental studies of TAM biotransformation, CYP2D6, CYP3A4/5, and CYP3A4 were found to be the main isoforms responsible for 4-hydroxylation, N-demethylation, and α-hydroxylation, respectively. The members of CYP3A are able to catalyze N-demethylation and α-hydroxylation, but not 4-hydroxylation. CYP2D6 is hence the main isoform for 4-hydroxylation, which eventually produces the high affinity metabolites OHT and endoxifen. The alkane 4α-hydroxylation of the modified structures, MTA and MTO, are highly possible to be catalyzed by CYP3A through the initial H-abstraction step, which has relatively low reaction energy. In this process, the CYP2D6 polymorphism is circumvented by the CYP3A mediated activation of the modified compounds. The subsequent 4-hydroxymethyl products are different from the 4-hydroxy products of TAM, without the phenolic para-hydroxy group, and are not likely to undergo the cis-trans isomerization. More experimental studies are required to testify the proposed metabolic paths of MTA and MTO, and to examine the anticancer efficacy and other beneficial or adverse side effects, because of which most SERMs have failed in the clinical trials.

The SERM profiles and related ER-dependent side effects are difficult to estimate through computational approaches. An ER-independent side effect of genotoxicity is, however, possible to be investigated by quantum mechanics calculations. TAM genotoxicity is found in rats, whereby it is caused by the DNA adducts forming from the α-hydroxy metabolites of TAM (Scheme 1.5). The formation of DNA adducts has been proposed to occur through the allylic carbocation intermediate, of which a high formation enthalpy results in low stability and thus leads to low possibility to form DNA adducts. This physicochemical property has been calculated, and the results are shown in Table 3.6. Regarding to formation of DNA adducts, the computed potentials decrease in the order of OHT > MTA > TAM ~
MTO. The modified structures MTO and MTA are thus expected to have no increased genotoxicity by forming DNA adducts.

Table 3.6. Comparison of the stability of reactive carbocation intermediates by calculations in gas phase (in kcal/mol).

<table>
<thead>
<tr>
<th>Compound</th>
<th>$\Delta\Delta H^a$</th>
<th>Relative Difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>TOR</td>
<td>248.0</td>
<td>4.4</td>
</tr>
<tr>
<td>MTO</td>
<td>247.8</td>
<td>4.2</td>
</tr>
<tr>
<td>TAM</td>
<td>243.6</td>
<td>0.0</td>
</tr>
<tr>
<td>MTA</td>
<td>242.1</td>
<td>-1.5</td>
</tr>
<tr>
<td>OHT</td>
<td>240.7</td>
<td>-2.9</td>
</tr>
</tbody>
</table>

$^a \Delta \Delta H = \Delta H(\text{carbocation intermediate}) + \Delta H(\text{H}^-) - \Delta H(\text{intact compound})$
CHAPTER 4. AGONISTS AND THE AGONIST-ERα LBD COMPLEXES

Paper V

As shown in Figure 1.4A, full and partial agonists in complex with ERα LBD resemble each other in the canonic closed/agonist conformation. Comparisons are made between full and partial agonists, including the well known natural and synthetic full estrogens E2 and DES, the partial agonists of phytoestrogen GEN and another synthetic compound W, with structures seen in Schemes 4.1, and 4.2. One of the experimentally found differences is that partial agonists are more difficult to cocrystallize with the LBD, since they may stabilize the LBD in mixed closed/agonist and open/antagonist conformations. Therefore, mutation techniques were applied to aid the cocrystallization of partial agonists with the LBD, by facilitating the folding process of H12 into the closed/agonist conformation.40,41

Full and partial agonists differ mainly in the ligand-dependent transcription activity, i.e., the conventional agonist action of proliferative effects, which decreases in the order of E2 ~ DES > GEN > W (not detectable) for the ligands studied herein.37-39 Molecular modeling techniques were used in this study to investigate how the full and partial agonists communicate ERα LBD. The cocrystal structures are compared among E2–LBD (1G50), DES–LBD (3ERD), GEN–LBD (1X7R), and W–LBD (2QZO). Figure 4.1 represents the main interactions of agonists binding to the receptor displayed by the E2–LBD cocrystal structure.

Figure 4.1 E2 in complex with the agonist-ERα LBD, which represents in general how agonists form contacts with the receptor; PDB code 1G50.44 Reproduced with permission of the copyright owner.150
The interactions of highly consensus are found for the phenolic hydroxyl group of each ligand bound to the side chains of Glu353 and Arg394. In the other end of the ligands, they communicate with His524-Leu525 in different manners, which are expected to be responsible for different bioactivities. As it was found experimentally, His524 and Leu525 were intimately involved in ligand-induced transcriptional response of ERα agonists, as mutations in either of them to alanine resulted in large reductions in binding affinity and subsequent ligand-induced transcriptional activation.151,152 The interactions have thus been studied in terms of ligands bound to His524 only (Scheme 4.1) and together with Leu525 (Scheme 4.2).

**Scheme 4.1** Side chain of histidine and the structures of systems modeled for the ligand…His524…Glu419 H-bonding network. Asterisks indicate the centers which are frozen in the geometry optimization steps. Reproduced with permission of the copyright owner.150
Scheme 4.2 Structures of systems modeled for ligands bound to His524-Leu525 in ERα LBD. Asterisks indicate the centers which are frozen in the geometry optimization steps. Reproduced with permission of the copyright owner.150

The connections between the ligands and His524 highly depend on the state of the imidazole ring of His524 (Scheme 4.1), which is not explicitly shown in the crystal structures. The δ-tautomer (Nδ—H) does not have a H atom at the Nε position to form the H-bond connection between His524 and the carbonyl group of the Glu419 backbone (Nε—H⋯O=C), which is a conserved interaction seen in the crystal structures, and this tautomer is thus excluded. His524 functions either as H-bond acceptor in the ε-tautomer (Nε—H) or H-bond donor in the protonated form (Schemes 4.1 and 4.2). The strength of the H-binding connection depends on how potent the ligand functions as H-bond donor or acceptor in the presence of different...
Chapter 4. Agonists and the agonist-ERα LBD complexes

states of His524. DFT calculations have been performed to investigate the properties of ligands related to H-bond formation, including deprotonation energies and proton affinities. The results are shown in Table 4.1, along with binding energies to His524 and to the extended system, in which Leu525 is involved. The strength of H-binding to His524 is in line with the decrease in deprotonation energy or increase in proton affinity of ligand corresponding to the two states of His524. In the models of protonated His524, partial agonists have relatively low binding energies to the region of His524-Leu525 and are thus not able to retain a stable $O^{\text{Hig}...\cdot H-N_{\epsilon}}$(His524)$N_{\epsilon}^\delta$ network displayed by MD simulations, with the details presented in Paper V. It may be a possible explanation of that partial agonists are expected to bind the LBD in both agonist and antagonist conformations.

Table 4.1 ZPE-corrected binding energies of ligands bound to His524 (Scheme 4.1), and the binding energies in parentheses of ligands bound to His524-Leu525 (Scheme 4.2), and the deprotonation energies and proton affinities of the corresponding hydroxyl groups bound to His524. (in kcal/mol) Reproduced with permission of the copyright owner.150

<table>
<thead>
<tr>
<th></th>
<th>$\epsilon = 1$</th>
<th>$\epsilon = 4.24$</th>
<th>$\epsilon = 78.36$</th>
<th>$\epsilon = 1$</th>
<th>$\epsilon = 4.24$</th>
<th>$\epsilon = 78.36$</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\epsilon$-tautomer $O^{\text{Hig}}\cdots H-N_{\epsilon}$(His524) (---Leu525)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E2</td>
<td>6.63(13.53)</td>
<td>5.35(11.47)</td>
<td>4.64(10.32)</td>
<td>365.9</td>
<td>326.2</td>
<td>312.2</td>
</tr>
<tr>
<td>DES</td>
<td>13.32(16.38)</td>
<td>11.46(13.77)</td>
<td>10.42(12.69)</td>
<td>341.2</td>
<td>305.9</td>
<td>293.9</td>
</tr>
<tr>
<td>GEN</td>
<td>14.71(16.77)</td>
<td>12.60(14.05)</td>
<td>11.46(12.26)</td>
<td>327.0</td>
<td>294.8</td>
<td>284.6</td>
</tr>
<tr>
<td>Protonated $O^{\text{Hig}}\cdots H-N_{\epsilon}$(His524) (---Leu525)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E2</td>
<td>17.58(18.92)</td>
<td>9.50(10.66)</td>
<td>6.83(7.90)</td>
<td>193.4</td>
<td>230.7</td>
<td>242.5</td>
</tr>
<tr>
<td>DES</td>
<td>13.95(16.66)</td>
<td>7.46(10.61)</td>
<td>5.57(8.72)</td>
<td>180.2</td>
<td>218.9</td>
<td>230.8</td>
</tr>
<tr>
<td>GEN</td>
<td>11.34(12.06)</td>
<td>5.48(5.82)</td>
<td>3.82(2.79)</td>
<td>172.6</td>
<td>211.8</td>
<td>224.1</td>
</tr>
<tr>
<td>W</td>
<td>7.82(7.55)</td>
<td>-0.30(2.68)</td>
<td>0.01(1.33)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
CONCLUSIONS

SERMs have been more and more widely used as multifunctional drugs acting as ERα signaling modulators. A very successful example is TAM, which has been used to treat breast cancer for decades. Computational studies in this research have led to new information on how ligands act on ERα, and have provided better understanding of the CYP-mediated biotransformation of TAM. The studies aimed for new SERMs with optimized in vivo metabolism. The main results thus obtained are:

— The SERM profiles of partial antagonists are related to the conformational flexibility of the SERM-ERα LBD complex. The principle of more stable, more estrogenic is used to interpret between MD simulation and experimental results.

— Base on this principle, a plausible explanation thus obtained for the CYP2D6 debate is that the CYP2D6-mediated activation of TAM has contradictive effects on TAM treatment: the positive side is that OHT has much higher binding affinity to ERα than the prodrug TAM does, while the negative side is that OHT may provide more estrogenic properties.

— Rotation about the C(Ar)—C(sp²) single bond of TAM is a lower energy cost process, resulting in spontaneous helicity reversion of the molecular propeller. Only the clockwise propellers fit the ERα binding pocket.

— Rotation about the C(sp²)=C(sp²) double bond of OHT (cis-trans isomerization), a rarely reported CYP reaction, evolves from a conventional CYP-mediated redox process analogous to the CYP-catalyzed dehydrogenation. The evolution towards distinctly different reactions is mainly a result of the chemical properties of the substrate compounds.

— TAM and TOR are modified by adding a methyl substituent to the para-position. Activation of the resulting structures is highly possible to be a CYP3A-mediated aliphatic hydroxylation. The modified structures are likely to be used in a wider range than TAM, as both the CYP2D6 genetic polymorphism and the undesired cis-trans isomerization are possibly avoided.

— The full agonists E2 and DES display sufficiently strong interactions with His524-Leu525 so as to stabilize the agonist conformation of the LBD, whereas the partial agonist GEN does not, which explains the different transcriptional activities of full and partial agonists.

The complete mechanism of SERM actions is still quite a mystery. Much more research is required for deciphering small ligands signaling via ERα pathways in order to develop idea SERMs. Computational methods are powerful tools to analyze chemical properties of compounds, reaction mechanisms, et al., and can assist development of new drugs in addition to experimental methods.
REFERENCES

1 American cancer society. cancer facts and figures 2012
4 Kuramochi H. Conformational studies and electronic structures of tamoxifen and toremifene and their allylic carbocations proposed as reactive intermediates leading to DNA adduct formation. Journal of Medicinal Chemistry 1996; 39:2877-2886
5 Biali SE, Rappoport Z. Stable Simple Enols .3. Static and Dynamic Nmr Behavior of Crowded Triarylethenols and Related-Compounds - 3-Ring Flip as the Threshold Mechanism for Enantiomerization of Crowded Triarylvinyl Propellers. Journal of the American Chemical Society 1984; 106:477-496
12 Riggs BL, Khosla S, Melton LJ. Sex steroids and the construction and conservation of the adult skeleton. Endocrine Reviews 2002; 23:279-302
13 Sherwin BB. Estrogen and cognitive aging in women. Trends in Pharmacological Sciences 2002; 23:527-534


References


76 Rae JM, Drury S, Hayes DF, et al. CYP2D6 and UGT2B7 Genotype and Risk of Recurrence in Tamoxifen-Treated Breast Cancer Patients. Journal of the National Cancer Institute 2012; 104:452-460
78 Osborne CK, Coronado E, Allred DC, et al. Acquired Tamoxifen Resistance - Correlation with Reduced Breast-Tumor Levels of Tamoxifen and Isomerization of Trans-4-Hydroxytamoxifen. Journal of the National Cancer Institute 1991; 83:1477-1482
84 Guengerich FP. Common and uncommon cytochrome P450 reactions related to metabolism and chemical toxicity. Chemical Research in Toxicology 2001; 14:611-650
References

93 de Visser SP, Shaik S. A proton-shuttle mechanism mediated by the porphyrin in benzene hydroxylation by cytochrome P450 enzymes. Journal of the American Chemical Society 2003; 125:7413-7424
95 Groves JT. Key Elements of the Chemistry of Cytochrome-P-450 - the Oxygen Rebound Mechanism. Journal of Chemical Education 1985; 62:928-931
102 Sato M, Rippy MK, Bryant HU. Raloxifene, tamoxifen, nafoxidine, or estrogen effects on reproductive and nonreproductive tissues in ovariecotomized rats. Faseb Journal 1996; 10:905-912
108 Vajdos FF, Hoth LR, Geoghegan KF, et al. The 2.0 A crystal structure of the ERalpha ligand-binding domain complexed with lasofoxifene. Protein Science 2007; 16:897-905
116 Nomura Y, Nakajima M, Tominaga T, et al. [Late phase II study of TAT-59 (miproxifene phosphate) in advanced or recurrent breast cancer patients (a double-blind comparative study with tamoxifen citrate)]. Gan To Kagaku Ryoho 1998; 25:1045-1063
121 Schrödinger E. An undulatory theory of the mechanics of atoms and molecules. Physical review 1926; 28:1049
126 Zhao Y, Truhlar DG. The M06 suite of density functionals for main group thermochemistry, thermochemical kinetics, noncovalent interactions, excited states, and transition elements: two new functionals and systematic testing of four M06-class functionals and 12 other functionals. Theoretical Chemistry Accounts 2008; 120:215-241
127 Zhao Y, Truhlar DG. Density functionals with broad applicability in chemistry. Accounts of Chemical Research 2008; 41:157-167
130 Molecular Operating Environment (MOE). Chemical Computing Group, Montreal, Canada


144 MacGregor Schafer J, Liu H, Bentrem DJ, et al. Allosteric silencing of activating function 1 in the 4-hydroxytamoxifen estrogen receptor complex is induced by substituting glycine for aspartate at amino acid 351. Cancer Research 2000; 60:5097-5105


147 Oki M. Recent Advances in Atropisomerism. Topics in Stereochemistry 1983; 14:1-81


