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Pharmacophore modeling strategies for the development of novel nonsteroidal inhibitors of human aromatase (CYP19)

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ABSTRACT

The present study utilizes for the first time the structural information of aromatase, an important pharmacological target in anti-breast cancer therapy, to extract the pharmacophoric features important for interactions between the enzyme and its substrate, androstenedione. A ligand-based pharmacophore model developed from the most comprehensive list of nonsteroidal aromatase inhibitors (AIs) is described and explained, as well. This study demonstrates that the ligand-based pharmacophore model contributes to efficacy while the structure-based model contributes to specificity. It is also shown that a 'merged' model (i.e., a merged structure-based and ligand-based model) can successfully identify known AIs and differentiate between active and inactive inhibitors. Therefore, this model can be effectively used to identify the next generation of highly specific and less toxic aromatase inhibitors for breast cancer treatment.

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The present study utilizes for the first time the structural information of aromatase, an important pharmacological target in anti-breast cancer therapy, to extract the pharmacophoric features important for interactions between the enzyme and its substrate, androstenedione. A ligand-based pharmacophore model developed from the most comprehensive list of nonsteroidal aromatase inhibitors (AIs) is described and explained, as well. This study demonstrates that the ligand-based pharmacophore model contributes to efficacy while the structure-based model contributes to specificity. It is also shown that a 'merged' model (i.e., a merged structure-based and ligand-based model) can successfully identify known AIs and differentiate between active and inactive inhibitors. Therefore, this model can be effectively used to identify the next generation of highly specific and less toxic aromatase inhibitors for breast cancer treatment.

Excluding cancers of the skin, breast cancer is the most frequently diagnosed cancer in women[1] and ranks second as a cause of tumor-related death after lung cancer. Currently, it is predicted that one in eight American women will develop invasive breast cancer some time during her life. Approximately two-thirds of breast cancer tumors are hormone-dependent and require estrogens to grow.[2] One approach in treating hormone-dependent cancer involves interfering with endogenous hormone production. Aromatase, also known as estrogen synthase, has always been considered the most promising target for the endocrine treatment of breast cancer[3] because, through inhibition of the aromatase enzyme, estrogen production is decreased, and tumor growth is stopped or reduced.

Aromatase is a multienzymatic complex that is mostly expressed in the ovaries of premenopausal women, in the placenta of pregnant women, and, additionally, in peripheral adipose tissue, breast tissue, and the brain.[4] The enzyme is overexpressed in or near breast cancer tissue and is responsible for local estrogen production and proliferation of breast cancer tumors.[5,6] It is located in the endoplasmic reticulum of cells and is composed of a cytochrome P450 heme protein (CYP19), which carries out the aromatization reaction, and a NADPH-cytochrome P450 reductase, a flavoprotein required for the electron transfer from NADPH to the cytochrome P450 enzyme.[7,8] Aromatase catalyzes the synthesis of estrogens via the aromatization of the A ring of androgen precursors, namely androstenedione and testosterone.

Considerable research efforts over the past decades have been devoted to the study of this enzyme and to the development of potent and selective agents able to interfere with its action. Several classes of steroidal and nonsteroidal aromatase inhibitors (AIs) have been developed,[2,7,9–20] and, on the basis of their inhibition mechanism and chemical origin, these molecules are divided into two classes: steroidal (type I) and nonsteroidal (type II).[2,6]

Steroidal AIs are derivatives or analogues of the preferred androgenic substrates and inhibit aromatase irreversibly. They can be further divided into competitive inhibitors and mechanism-based inhibitors. Competitive inhibitors bind non-covalently to aromatase in a manner similar to that of the natural substrate

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and block it from being enzymatically modified by aromatase. Mechanism-based inhibitors, also known as suicide inhibitors, bind to the enzyme and are converted into a reactive intermediate that covalently binds the enzyme and permanently inactivates it. This process often destabilizes the enzyme and also increases its rate of degradation by the intracellular proteosome.[6,21]

Nonsteroidal inhibitors, on the other hand, bind entirely non-covalently and contain a heteroatom that coordinates with the iron atom of the heme group to block the active site and reversibly inhibit the enzyme.[2,6] These types of AIs are further divided into categories based on the order in which they were discovered or synthesized: first-, second-, and third-generation AIs. Currently, the third-generation of triazole-derived AIs are approved as front-line therapy for early and even advanced cases of breast cancer in postmenopausal women.[21,22]

Nevertheless, for both steroidal and nonsteroidal AIs, important side effects—ranging from mild to severe, short-term to long-term—have been suggested or reported. For example, steroidal AIs often give way to androgenic side effects where other related systems are disturbed due to lack of inhibitor specificity.[23] Also, prolonged estrogen deprivation can lead to bone loss, osteoporosis, reproductive problems, or even other types of cancers.[21,24] Undoubtedly, for some women, the benefits of existent AIs outweigh the associated side effects. However, for many women, quality-of-life issues are serious enough to cause them to discontinue their use of the prescribed AIs. Therefore, more selective and less toxic CYP19 inhibitors are needed, especially since mutations in intratumoral aromatase, which can cause changes in its stability, efficiency, or sensitivity to different classes of AIs, can vary from patient to patient.[6]

Until fairly recently, no structural information on the enzyme was available, except several homology models[25–29] that proved to be valuable for understanding the binding determinants of several classes of inhibitors.[15,18,22,29–32] However, the low sequence identity between members of different P450 families has limited the success of these models in structure-based virtual screening. The recent determination of the crystal structure (X-ray) of aromatase complexed with androstenedione [PDB code: 3EQM][33] reveals the molecular basis for the enzyme's androgenic specificity and unique catalytic mechanism.

Androgens, the preferred substrates of aromatase, are believed to enter the enzyme's catalytic cleft and active site through an access channel open to the outside.[33] The catalytic cleft of the aromatase enzyme encompasses a volume of approximately 400 $Å^3$, which is considerably smaller than the volume of about 530 $Å^3$ of the binding pockets in CYP3A4 and CYP2D6, the two CYP450 enzymes with highest sequence identities to human aromatase.[33] Due in part to this smaller volume and also to the unique locations of the catalytically important residues, the catalytic cleft of aromatase is very specific to its androgenic substrates.

Since there has been no recent report on developing inhibitors using the newly published aromatase structure [PDB code: 3EQM],[33] the present study provides a hypothetical picture of the primary structural and chemical features responsible for activity and is expected to provide useful knowledge for developing the next generation of inhibitors targeted to human aromatase.

Using a comprehensive database of nonsteroidal AIs and structural data on aromatase, two pharmacophore models—one ligand-based (LB) and the other structure-based (SB), respectively,—were developed. The LB pharmacophore model was generated using Molecular Operating Environment (MOE)[34] while the SB pharmacophore model was generated on LigandScout.[35,36] These two models were then merged using MOE to generate a 'Merged' Model that combined the different strengths of each original model.

Over the past several years, numerous potential inhibitors of human aromatase have been tested for biological efficacy. To create the LB pharmacophore model, a database of 56 active nonsteroidal aromatase inhibitors, presented in the literature as having been tested in human placental microsome assays, plus nine inactive nonsteroidal compounds, was compiled. Homologous half maximal inhibitory concentration (IC_{50}) values for each were also noted. This collection of 65 total nonsteroidal compounds was divided into three sets: a Training Set (Table 1) to create the model and two Test Sets—one Active (Table 2) and one Inactive (Table 3)—to validate it.

The Training Set was comprised of 20 of the most active yet structurally diverse nonsteroidal AIs. The Active Test Set differs from the Training Set in that it contains 36 AIs that are slightly less potent, yet just as structurally diverse. The Inactive Test Set contains nine potential inhibitors that have been determined to be inactive against aromatase. Molecules in the Training Set and in the two Test Sets were created using ChemSketch[37] and were compiled into databases using the Molecule Builder function in MOE.[34]

The LB pharmacophore model, generated with MOE, was derived from the Training Set of 20 of the most potent yet structurally diverse nonsteroidal AIs known. This was done using the PCHD scheme in the Pharmacophore Elucidation function in MOE,[36] defining H-bond acceptors and donors features, as well as putative points from hydrogen bond donors and acceptors that are projected in the approximate direction of the hydrogen bond.[36] Different conformations of the molecules of the Training Set were taken into consideration using the Conformation Import function during the Pharmacophore Elucidation.[36]

The resulting LB Model identified four pharmacophore features: two hydrophobic/aromatic (**Hyd|Aro**), one hydrogen-bond acceptor (**Acc**), and one hydrogen-bond acceptor projection (**Acc2**). The 3D and 2D representations of the LB Model are shown in Figures 1 and 2, respectively. In Figure 1, the solid spheres represent the four pharmacophore features identified by the LB Model. Figure 2 shows the dimensions of the model and the distances between the pharmacophoric features. Featuring coverage of 20 out of 20 of the best structurally diverse AIs, the LB Model was assigned an overlap score of 12.7354 out of a maximum of 20 and an accuracy of 100%.

Originally, attempts at generating the ligand-based (LB) pharmacophore model were made based on sets of AIs with activity levels that are lower and more comparable to biologically relevant potencies (data not shown). However, several problems with this method soon became apparent. Since, in general, less potent AIs tend to differ considerably in chemical features and structural characteristics, lower-potency ligand-based (LB) models based on these AIs could be made to encompass only a maximum of only 11 or 12 lower-potency AIs. With some lower-potency Training Sets, only a two-point LB model could be generated, if at all. On the other hand, the final LB model based on the most potent AIs allowed for more than 20 compounds to align well enough for at least four-point pharmacophore generation. It was also observed that, if fewer than 20 of the most potent compounds were used, a five-point model could be developed, as well (data not shown).

A three-dimensional (3D) pharmacophore model (i.e., structure-based pharmacophore model) of the CYP19 binding pocket was created with LigandScout[35,36] using the X-ray structure deposited in the Protein Data Bank (PDB) [PDB code: 3EQM].[33] The model was based on interactions that define aromatase inhibition, such as hydrophobic interactions, hydrogen bonding, and electrostatic interactions.[35,55,56] Features identified by the LigandScout software are those that take into consideration chemical functionality but not strict structural topology or definite functional groups. As a result, completely new potential pharmacons can be identified through database screening. Moreover, to increase selectivity, the LigandScout model includes spatial information regarding areas inaccessible to any potential ligand, thus reflecting possible steric

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