CHAPTER-2

REVIEW OF LITERATURE

2. REVIEW OF LITERATURE

2.1 Molecular Modeling Studies of TACE and Its Inhibitors

High resolution crystal structures of proteins are the most common source of structural information for drug design. Molecular modeling techniques are useful for proteins that range in size from a few amino acids to 998 kDa [237]. Till date several crystal structures for TACE, co-crystallized with different inhibitors are available in the PDB databank. 1BKC, 1ZXC, 2A8H, 2DDF, 2FV5, 2OIO, 3B92 are some of the examples of TACE crystal structures co-crystallized with different inhibitors [238].

Some of the studies have also been reported on the homology modeling of TACE. Wasserman *et al.* reported a homology model structure of TACE based on the crystal structure of the related snake venom protein atrolysin. In this report, comparison of the developed model was carried out with crystal structures of MMPs and suggested a uniquely shaped S1' pocket that might be exploited for selectivity. A novel γ-lactam scaffold was used to explore the activity profile of P1' side chain, resulting in highly selective compounds consistent with the proposed hypothesis [239].

New γ-lactam TACE inhibitors were designed from known MMP inhibitors by Duan *et al.* in 2002. A homology model of TACE was built and examined to identify the S1' site as the key area for TACE selectivity over MMPs. Rational exploration of the P1'-S1' interactions resulted in the discovery of the 3,5-disubstituted benzylether as a TACE selective P1' group. Further optimization led to the discovery of IK-682 as a selective and orally bioavailable TACE inhibitor [240].

X-ray crystal structure of TACE-IK-682 was reported in 2006 by Niu *et al.* which clearly revealed that the compound IK-682 is embedded in the catalytic site of the enzyme. The hydroxamic acid moiety of IK-682 interacts with the zinc atom present in the active site of TACE and coordinates with the carboxylate of Glu406 and the carbonyl oxygen of Gly349. The carbonyl group of pyrrolidinone ring is responsible for formation of a hydrogen bond with the amide nitrogen of Leu348 and Gly349. The methyl group on the pyrrolidinone ring is in van der Waals contact with the carbonyl oxygen of Gly346. The phenyl group of IK-682 forms an aromatic stacking with the imidazole group of His405 [241].

Sheppeck *et al.* have reported the interaction study of various classes of non-hydroxamate TACE inhibitors and hydroxamate TACE inhibitors. In their report, it was suggested that hydroxamic acid forms two hydrogen bonds with Glu406 and Gly349 of TACE apart from a bidentate ligation of the hydroxyl and carbonyl oxygen to the zinc atom. The interaction of non-hydroxamate zinc binding groups with the TACE active site differ from those of the hydroxamate ones because they ligate Zn in monodentate fashion and hydrogen bond the same residues but in a fundamentally different way. In case of pyrimidinetriones, only two hydrogen bonds are observed, one with the NH of Leu348 and the carbonyl at the 4-position of pyrimidinetrione and the other one with the acid of Glu406 and the C_2 carbonyl of the pyrimidinetrione. The hydantoins bind to the catalytic site of TACE in monodentate fashion [242].

Sheppeck *et al.* have discovered selective and potent inhibitors of TACE that replace the common hydroxamate zinc binding group with a hydantoin, triazolone or imidazolone heterocycles. The potency and binding orientation of these inhibitors is discussed and they are modeled into the X-ray crystal structure of TACE [243].

MMPs and TACE assume independent, parallel or opposite pathological roles in cancer, arthritis and several other diseases. For therapeutic purposes, selective inhibition of individual MMPs and TACE is required in most cases due to their distinct roles in diseases and the need to preserve their activities in normal states. To achieve this goal, Lukacova *et al.* compared force-field interaction energies of five ubiquitous inhibitor atoms with flexible binding sites of 24 known human MMPs and TACE. The results indicated that MMPs 1-3, 10, 11, 13, 16, and 17 have at least one subsite very similar to TACE. S3 Subsite is the best target for development of specific TACE inhibitors. Specific binding to TACE as compared to most MMPs, is promoted by placing a negatively charged ligand either at the bottom of S2 subsite or at the entrance of S1' subsite, or as a part of S3' subsite, that is close to catalytic zinc. Numerous other clues consistent with available experimental data are provided for design of selective inhibitors [244].

The crystal structural data of TACE, MMP-1, MMP-2, MMP-3 and MMP-9 were obtained from PDB database and the physico-chemical properties of their catalytic domains like conformations, molecular surface hydrophobicity and electrostatic potentials were analyzed and compared by Insight II molecular modeling software by

Zhao *et al.* In this study, it was found that the conformations and molecular surface hydrophobicity of catalytic domains of TACE and MMPs were not very different, but the molecular surface electrostatic potentials of the catalytic domains of TACE and MMPs had obvious differences [245].

Very recently Bahia *et al.* reported the ligand-based as well as structure-based molecular modeling and virtual screening of selective TACE inhibitors. In ligand based molecular modeling, two statistically reliable pharmacophore models HypoA1 (consisting of two hydrogen bond acceptor and three hydrophobic groups) and HypoB1 (consisting of one hydrogen bond donor, one ring (aromatic) and three hydrophobic groups were generated using a training set of 22 molecules. Virtual screening was performed with both models using in-house database of 1.2 million molecules. In structure-based molecular modeling, docking analysis was performed to explore important interactions between the ligands and the protein [246,247]. The same authors have applied a computational strategy based on molecular docking studies, followed by CoMFA and CoMSIA analyses on anthranilic acid based derivatives (ANT) as TACE inhibitors. This study was carried out by Bahia *et al.* with the aim to elucidate the atomic details of the TACE-ANT interactions and also to identify the most important features impacting TACE inhibitory activity of ANTs. The CoMSIA model has been reported to be more predictive than CoMFA model [248].

Feng *et al.* investigated the molecular basis of specificity for the interaction of peptidomimetic inhibitors with TACE. Four novel peptidomimetic TACE inhibitors were designed and synthesized by introducing a substituted sulfur group and a hydrophobic group to a novel matrix metalloprotease (MMP) inhibitor. Simulation studies were performed by this group involving the interactions of the inhibitors with the active site of TACE which was retrieved from the Brookhaven Protein Database (PDB). Four inhibitors showed significant *in vitro* activity in cytotoxicity tests, which corresponded to the molecular docking results [249].

Yu *et al.* have reported inhibitors of TACE which were designed using hydantoin as a zinc binding moiety. Crystal structures of the inhibitors bound to TACE revealed monodentate coordination of the hydantoin to the zinc. SAR of the inhibitors, molecular modeling and X-ray crystallographic studies have been described in this report. As per the claim of the authors, this was the first reported X-ray structure of TACE with zinc binding hydantoin ligand [250].

2.2 Molecular Modeling Studies of Neuraminidase, Inhibitors and Related Studies

Since the crystal structure of A/H1N1 is not available till date, majority of the computational work reported in the literature is regarding development of homology modelled structure of A/H1N1. Very few *in silico* studies have been reported on the swine influenza A/H1N1 and its inhibitors. Modeling studies done with neuraminidase and its inhibitors are important to get a clue about the structural features required for binding to this enzyme, hence are also reviewed below.

Le *et al.* have reported a molecular modeling of neuraminidase of swine influenza, Spanish H1N1 and avian H5N1 flu N1 bound to Tamiflu and Relenza. In this study a homology model of the swine influenza A/H1N1 type-I neuraminidase has been reported which was built using the pathogenic avian H5N1 type-I neuraminidase as a basis, due to the higher sequence identity between A/H1N1 and H5N1 (91.47%) compared to spanish H1N1 (88.37%) neuraminidase. All-atom molecular dynamics (MD) simulations of all three neuraminidases have been claimed to be performed, either as apo-structures or with commercial antiviral drugs Tamiflu or Relenza separately bound to the enzyme. The simulations helped for the identification of both conserved and unique drug-protein interactions across all of the three proteins. Specifically, the conserved network of hydrogen bonds stabilizing the drugs inhibitors in the sialic acid binding site of the simulated neuraminidases were analyzed, providing an insight into how disruption due to mutations may lead to increased drug resistance. In addition to that, a possible mechanism through which the residue 294 mutation acquires drug resistance is proposed by mapping the mutation site onto an electrostatic pathway which could play a role in controlling drug access to the binding pocket of neuraminidase [251].

Wang *et al.* reported the findings from homology modeling and molecular simulations of 2009 A(H1N1) neuraminidase complexed with zanamivir, oseltamivir, and several herb extracts with potential activities. The docked results of herb extracts HR1039 and HR1040 suggested that they are potential potent inhibitors of neuraminidase. Also, the binding modes of HR1039/HR1040 have been reported and claimed to be different from those of oseltmivir and zanamivir, and could be effective in treating oseltamivir-resistant influenza virus strains [252].

To understand at the molecular level, how new H1N1 virus can be inhibited by the current anti-influenza drugs, homology modeling and MD simulations have been applied on the H1N1 neuraminidase complexed with oseltamivir, and the M2-channel with adamantanes bound by Rungrotmongkol *et al.* The H1N1 virus was predicted to be susceptible to oseltamivir, with all important interactions with the binding residues being well conserved. In contrast, adamantanes are not predicted to be able to inhibit the M2 function and have completely lost their binding with the M2 residues. The reason for this has been reported by Rungrotmongkol *et al.* mainly due to the fact that the M2 transmembrane of the new H1N1 strain contains the S31N mutation which is known to confer resistance to adamantanes [253].

To predict the susceptibility of the probable 2009 influenza A (H1N1-2009) mutant strains to oseltamivir, MD/LIE approach was applied by Rungrotmongkol *et al.* to oseltamivir complexed with the most frequent drug-resistant strains of neuraminidase subtypes N1 and N2: two mutations on the framework residues (N294S and H274Y) and the two others on the direct-binding residues (E119V and R292K) of oseltamivir. This study suggested that the H1N1-2009 influenza with R292K substitution conferred a high level of oseltamivir resistance, while the other mutants revealed moderate resistance levels [254].

The finding reported by Wang *et al.* provided useful insights for modifying the existing drugs, such as oseltamivir and zanamivir, not only to make them more effective against H1N1 virus but also effective against H5N1 virus. The neuraminidase (NA) of influenza virus is the target of anti-flu drugs oseltamivir and zanamivir. Clinical practices showed that oseltamivir was effective to treat the 2009-H1N1 influenza but failed for the 2006-H5N1 avian influenza. To perform an in-depth analysis on such a drug-resistance problem, the 2009-H1N1-NA structure was developed by Wang *et al.* To compare it with the crystal 2006-H5N1-NA structure as well as the 1918 influenza virus H1N1-NA structure, the multiple sequential and structural alignments were performed. It has been revealed that the hydrophobic residue Try347 in H5N1-NA does not match with the hydrophilic carboxyl group of oseltamivir unlike the case of H1N1-NA. This could be the reason as mentioned by the Wang *et al.* why H5N1 avian influenza virus was drug-resistant to oseltamivir [255].

Durrant *et al.* have used computer program AutoGrow to generate novel virtual neuraminidase inhibitors. Protein dynamics were also incorporated into the computer-aided drug-design process. Several potential inhibitors have been identified that are predicted to bind to neuraminidase better than currently approved drugs [256].

In search for new antiviral drug leads from nature, the seed extract of Alpinia katsumadai has been phytochemically investigated by Grienke *et al.* Out of the six isolated constituents, four diarylheptanoids showed *in vitro* NA inhibitory activities in low micromolar ranges against human influenza virus A/PR/8/34 a subtype of H1N1. The most promising constituent, katsumadain A ($IC_{50} = 1.05 \pm 0.42\ \mu M$), also inhibited the NA of four H1N1 swine influenza viruses, with IC_{50} values between 0.9 and 1.64 μM. Extensive molecular dynamics (MD) simulations were performed to study the putative binding mechanism of the T-shaped diarylheptanoids in the flexible loop region of NA. Docking results showed well-established interactions between the protein and the core of this novel NA-inhibiting natural scaffold, excellent surface complementarity to the simulated binding pocket and with experimentally derived SAR data [257].

Surflex-Dock is employed to investigate interactions between neuraminidase inhibitors (NIs) and neuraminidase (NA), which illustrated that carboxyl, amino (guanidino), amide, hydroxy groups are crucial. Hydrogen bonds and hydrophobic interactions impacted the activities of NIs. There was a strong correlation between binding affinity and pIC_{50}, with $r = 0.813$. The authors have developed three-dimensional holographic vector of atomic interaction field analysis (HoVAIFA) as a new method of 3D-QSAR to understand chemical-biological interactions. Electrostatic, steric and hydrophobic interactions affected activities of NIs as per this study. HoVAIFA and docking results matched each others, which illustrated that HoVAIFA is an effective methodology for characterization of complex interactions of drug molecules [258].

The quantitative structure-activity relationship (QSAR) of 30 acylthiourea analogs was studied by Jing *et al.* by using 3D-HoVAIF. The descriptors obtained were processed by stepwise multiple regression (SMR) and a partial least-squares (PLS) regression model was built. The model showed favorable stability and good prediction capability [259].

Abu *et al.* have reported pharmacophore modeling, QSAR, and shape-complemented *in silico* screening for influenza neuraminidase inhibitors. In this study the pharmacophoric space of influenza NA was explored using three sets of diverse inhibitors. Genetic algorithm and multiple linear regression analysis were employed to select optimal combinations of pharmacophoric models and 2D descriptors capable of yielding self-consistent and predictive QSARs for 181 training compounds. The optimal QSAR equations were validated using 43 external test compounds with r^2_{PRESS} values ranging from 0.488 to 0.591. Interestingly, five orthogonal pharmacophores emerged in the optimal QSAR equations suggesting the existence of several distinct ligand/NA binding modes within the NA binding pocket. The reported pharmacophores were complemented with tight shape constraints and employed as three-dimensional (3D) search queries for the National Cancer Institute (NCI) list of compounds. Several hits were reported having potent inhibitory activities against NA. The highest ranking hit demonstrated an *in vitro* IC_{50} value of 1.8 μM. Docking studies supported the binding modes as suggested by the pharmacophore/QSAR analysis [260].

Li *et al.* have designed derivatives of Tamiflu through a fragment-based approach combining multiple computational methods. Over 1000 FDA-approved drugs were computationally screened targeting at the open conformation of N1 with the GOLD program in combination with the X-Score scoring function. Some chemical fragments on the top-scored hits, which were able to fit into the cavity, were transplanted onto the core structure of Tamiflu to produce a total of 30 new molecules. Then, binding of these designed molecules to N1 was evaluated by docking. The promising ones were further subjected to molecular dynamics simulations of 3 ns long, and their free energies for binding were computed by using the MM-PB/SA method. Some of the designed molecules were predicted to have comparable or even better binding affinities than that of Tamiflu [261].

D'Souza *et al.* have synthesized some new derivatives of oseltamivir by modifying the amino group with glycyl, acetyl, benzyl and prolyl moieties. The interactions of these derivatives with neuraminidase have been probed by molecular modeling techniques. Results indicated that the glycyl derivative of oseltamivir had more profound effects on the membrane compared to other derivatives and seemed to be the most promising derivative for further pharmacological evaluation as a neuraminidase inhibitor [262].

2.2 Molecular Modeling Studies of Aromatase Enzyme and its Inhibitors

Understanding the binding of ligands in the active site of a membrane-bound protein is difficult in the absence of a crystal structure. When these proteins are the enzymes involved in drug metabolism, it leaves little option but to use site-directed mutagenesis and *in vitro* studies to provide critical information relating to determinants of binding affinity. Pharmacophore models and three-dimensional quantitative structure-activity relationships have been used either alone or in combination with protein homology model to provide this information for cytochrome P450s. At present, application of pharmacophore model, 3D QSAR and homology modeling techniques have been directed to the major enzymes but this may escalate in future as more *in vitro* data are generated for other P450s. The review published by De Groot *et al.* outlines the methodologies and models as well as future prospects for applying these technologies to P450s in the hope that future drugs will be selected with increased metabolic stability and fewer incidences of undesirable drug-drug interactions [263].

Karkola *et al.* reported a homology model of aromatase using the first crystallized mammalian cytochrome enzyme, rabbit 21-progesterone hydroxylase 2C5 as a template structure. This model was validated with exhaustive molecular dynamics simulation with and without the natural substrate androstenedione. Enzyme-substrate complex showed very good stability and only two of the residues were in disallowed regions in a Ramachandran plot as per the authors' claim [264].

A three-dimensional (3-D) structure of human aromatase was modeled on the basis of the crystal structure of rabbit CYP2C5, the first solved X-ray structure of an eukaryotic cytochrome P450 and was evaluated by docking *S*-fadrozole and the steroidal competitive inhibitor (19*R*)-10-thiiranylestr-4-ene-3,17-dione, into the enzyme active site by Loge *et al.* [265].

Murthy *et al.* reported a study which sheds new light on the role of acidic residues present in the active site cavity of human aromatase. Eight acidic residues (E129, D222, E245, E302, D309, E379, D380 and D476) lining the cavity were identified and studied using comparative modeling, docking, molecular dynamics as well as statistical techniques. The structural environment of these acidic residues was studied to assess the stability of the corresponding carboxylate anions. Results in this study indicated that the environment of the residues E245, E302 and D222 was most suitable for carboxylate ion formation in the uncomplexed form. However, the stability of D309, D222 and D476 anions was seen to increase on complexation to steroidal substrates. The interaction between D309 and T310, which assists proton transfer, was found to be formed following androgen/nor-androgen complexation. The residue D309 was found to be clamped in the presence of substrate which was not observed in the case of other residues although they exhibited changes in properties following substrate binding. The inhibition of aromatase activity by 4-hydroxy androstenedione (formestane) is attributed to a critical hydrogen bond formation between the hydroxy moiety and T310/D309 as well as the large distance from D476. As per the authors' claim these results corroborated well with site directed mutagenesis studies [266].

Schuster *et al.* have reported a ligand-based, selective pharmacophore model for aromatase enzyme. The HipHop and HypoRefine algorithms implemented in the Catalyst software package were employed to create both common feature and quantitative models. The common feature model for aromatase reported in this includes two ring aromatic features in its core and two hydrogen bond acceptors at the ends. The models were used as database search queries to identify active compounds from the NCI database [267].

Nagar *et al.* have reported pharmacophore mapping studies for falvone derivatives [268], isoflavone [269], benzofuran [270], diarylalkylimidazole and diarylalkyltriazole derivatives[271] and benzcyclohexane/pentane derivatives [272] for aromatase inhibition.

Muftuoglu *et al.* have used the structural information of aromatase to extract the pharmacophoric features important for interactions between the enzyme and its substrate, androstenedione. In this study a ligand-based pharmacophore model developed from the most comprehensive list of nonsteroidal aromatase inhibitors (AIs) is described and explained. As per the claim by the authors 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 mixed structure-based and ligand-based model) can successfully identify known AIs and differentiate between active and inactive inhibitors [273].

Aromatase inhibition is usually achieved with steroids structurally related to the substrate or alternatively, with non-steroidal azole compounds. Substituted androstenedione derivatives with Δ^1, Δ^6 and $\Delta^{1,6}$ unsaturations and 6-alkyl/phenyl aliphatic substitutions, are among the most potent steroidalal aromatase inhibitiors known to date. Neves *et al.* have combined the common pharmacophoric and shape features of these molecules into a new pharmacophore model useful for virtual screening of large compound databases. Small subsets of the best fitting anti-aromatase candidates were extracted from the NCI database and experimentally tested in an *in vitro* assay with human placental aromatase and new potent aromatase inhibitors were identified [274].

The five-dimensional quantitative structure-activity relationships (5D-QSAR) of a series of androstenedione analogs developed as aromatase inhibitors were studied using the Raptor program by Oliveira *et al.* The best model showed contributions of the hydrophobic, hydrogen-bond-donating and hydrogen-bond-accepting fields to the activity [275].

Synthesis, biological evaluation and molecular modeling of a series of novel Dual Aromatase-Sulfatase Inhibitors (DASIs) were reported by Jackson *et al.* It was postulated that dual inhibition of the aromatase and steroid sulfatase enzymes, both responsible for the biosynthesis of oestrogens, would be beneficial in the treatment of hormone-dependent breast cancer. The compounds based upon aromatase inhibitor anastrozole template was modified to include a phenol sulfamate ester motif, the pharmacophore for potent irreversible steroid sulfatase inhibition while maintaining the haem ligating triazole moiety crucial for enzyme inhibition. *In vitro*, the lead compound showed a high degree of potency against aromatase (IC_{50} = 3.5 nM), comparable with that of anastrozole (IC_{50} = 1.5 nM) whereas, only moderate activity against steroid sulfatase was found. Most active compound was modelled into the active site of a homology model of human aromatase and the X-ray crystal structure of steroid sulfatase [276].

The design, synthesis, and biological evaluation of a series of new aromatase inhibitors bearing an imidazole or triazole ring linked to a fluorene, indenodiazine, or coumarin scaffold were reported by Leonetti *et al.* The modeling of the aromatase inhibition data by Comparative Molecular Field Analysis (CoMFA/GOLPE 3D-QSAR approach) led to the development of a PLS model with good fitting and predictive powers. The relationship between aromatase inhibition and the steric and electrostatic fields generated by the examined azole inhibitors enabled a clear understanding of the nature and spatial location of the main interactions modulating the aromatase inhibitory potency[277].

Five quantitative spectroscopic data-activity relationships (QSDAR) models for 50 steroidal inhibitors binding to aromatase enzyme have been developed by Berger *et al.* based on simulated ^{13}C NMR data. Three of the models were based on comparative spectral analysis (CoSA), and the other two models were based on comparative structurally assigned spectral analysis (CoSASA). A CoSA QSDAR model based on five principal components had an explained variance (r^2) of 0.78 and a leave-one-out (LOO) cross-validated variance (q^2) of 0.71. A CoSASA model that used the assigned ^{13}C NMR chemical shifts from a steroidal backbone at five selected positions gave an r^2 of 0.75 and a q^2 of 0.66. The ^{13}C NMR chemical shifts from atoms in the steroid template positions 9, 6, 3, and 7 each had correlations greater than 0.6 with the relative binding activity to the aromatase enzyme. The QSDAR models developed by Berger *et al.* provided a rapid and simple way to model the steroid inhibitor activity in relation to the aromatase enzyme [278].

A series of 6-ester and 6-ether substituted androst-4-ene-3,17-diones (androstenediones) and their 1,4-diene analogs as well as 6-substituted 4,6-diene and 1,4,6-triene steroids were synthesized by Numazawa *et al.* as aromatase inhibitors to gain insight into the structure-activity relationship between various substituents and inhibitory activity. All of the synthesized inhibitors blocked aromatase in a competitive manner. In the series of 4-ene and 1,4-diene steroids, the 6α-substituted steroids had higher affinity for the enzyme than the corresponding 6β-isomers. In the 1,4-diene series, 6β-substituted steroids caused a time-dependent inactivation of aromatase, whereas their 6α-isomers essentially did not. The ether substituted 1,4,6-trienes inactivated the enzyme in a time-dependent manner; in contrast, their 4,6-diene analogs did not. The substrate androstenedione blocked the inactivation, but no significant effect of *L*-cysteine was observed. Based on molecular modeling with the PM3 method, along with the present inhibition and inactivation results, the authors concluded that both, the steric effects of the 6-substituents as well as the electronic effects of the C-6 oxygen functions, play critical role in the binding of inhibitors to the active site of aromatase [279].

An approach to compare quantitatively a ligand-based (CoMFA) model and an enzyme active site model was investigated by Cavalli *et al.* The active site of the cytochrome P450 human aromatase was constructed by homology modeling techniques and two structurally different non-steroidal aromatase inhibitors were docked into it. A

CoMFA model was then developed on a related series of non-steroidal inhibitors by correlating their inhibitory activity versus eleven steric descriptors [i.e. C (sp^3)-ligand steric interaction energies]. The resulting 3D-QSAR coefficients (11) and the steric field values of the aromatase active site calculated at the same points of the CoMFA lattice [i.e. eleven C(sp3)-protein steric interaction energies] were compared pair-wise [280]. Oprea *et al.* have also reported the 3D-QSAR of steroid aromatase inhibitors by using CoMFA analysis [30].

Molecular basis of the inhibition of human aromatase by flavone and isoflavone phytoestrogens have been studies by Kao *et al.* by using a site-directed mutagenesis study. Flavone and isoflavone phytoestrogens are plant chemicals that are known to be competitive inhibitors of cytochrome P450 aromatase with respect to the androgen substrate. In this study, the inhibition profiles of four flavones [chrysin (5,7-dihydroxyflavone); 7,8-dihydroxyflavone; baicalein (5,6,7-trihydroxyflavone) and galangin (3,5,7-trihydroxyflavone)], two isoflavones [genistein (4',5,7-trihydroxyisoflavone) and biochanin A (5,7-dihydroxy-4'-methoxyisoflavone)], one flavanone [naringenin (4',5,7-trihydroxyflavanone)] and one naphthoflavone (α-naphthoflavone) on the wild-type and six human aromatase mutants (I133Y, P308F, D309A, T310S, I395F and I474Y) were determined. In combination with computer modeling, the binding characteristics and the structure requirements for flavone and isoflavone phytoestrogens to inhibit human aromatase were obtained. These compounds were found to bind to the active site of aromatase in an orientation in which rings A and C mimic rings D and C of the androgen substrate, respectively. This study provided a molecular basis to explain why isoflavones are significantly poorer inhibitors of aromatase than flavones [281].

Series of 6α- and 6β-alkylandrosta-1,4-diene-3,17-diones were synthesized and evaluated as time-dependent inactivators of aromatase in human placental microsomes to gain insight into the structure-activity relationship of varying the 6-*n*-alkyl substituents (C-1 - C-7) to the time-dependent inactivation activity. All of the synthesized inhibitors were good to powerful competitive inhibitors of aromatase. These results along with molecular modeling with the PM3 method indicate that the length and the stereochemistry of a straight alkyl substituent at the C-6 position of androsta-1,4-diene-3,17-dione both play important roles in causing a time-dependent inactivation of aromatase. No significant correlation between affinity for the enzyme and the inactivation ability of the 6-alkylandrosta-1,4-diene-3,17-diones was observed [282].

The same authors have reported synthesis and biological evaluation of a series of 6α- and 6β-alkylsubstituted androst-4-en-17-ones and their 17β-reduced derivatives (alkyl: methyl, ethyl, *n*-propyl, *n*-pentyl, *n*-octyl) as aromatase inhibitors. Androst-4-en-17-ones having an oxygen function (hydroxy, acetoxy, or methoxy group) at C-6α and C-6β were also tested for their abilities to inhibit aromatase. All of the studied steroids inhibited human placental aromatase in a competitive manner. These results along with molecular modeling with the PM3 method suggested that compounds may produce a thermodynamically stable enzyme-inhibitor complex in the hydrophobic binding pocket with a limited accessible volume. A carbonyl group at C-17 of the 6-alkylandrost-4-enes is essential for the tight binding. Moreover, the binding pocket also tolerates a polar hydroxy group at the 6β-position rather than at the 6α-position [283].

The enantiomers of the potent nonsteroidal inhibitor of aromatase, fadrozole hydrochloride, have been separated and their absolute configuration determined by X-ray crystallography by Furet *et al.* On the basis of a molecular modeling comparison of the active enantiomer and one of the most potent steroidal inhibitors reported to date, (19*R*)-10-thiiranylestr-4-ene-3,17-dione, a model describing the relative binding modes of the azole-type and steroidal inhibitors of aromatase at the active site of the enzyme was proposed. Authors have suggested that the cyanophenyl moiety present in the most active azole inhibitors partially mimics the steroid backbone of the natural substrate for aromatase, androst-4-ene-3,17-dione [284]. Cepa *et al.* have reported newly synthesized A, D-modified steroids as irreversible aromatase inhibitors. The specific interactions of these compounds with aromatase active sites were analysed by computer aided molecular modeling studies [285].

Derivatives of 4-pyridylacetic acid are known to be inhibitors of the cytochrome P450 enzymes and lyases (17α-hydroxylase/C_{17-20}lyase), and are therefore of interest in the treatment of hormone dependent breast and prostate cancers. Laughton *et al.* reported the determination of the crystal structure of one such derivative, the 4-*t*-butylcyclohexyl ester, and molecular modeling studies on two related inhibitors, the cyclohexyl ester and its α-methyl derivative. These latter two compounds show a marked difference in their relative activities against aromatase and lyase. Two models are proposed for the interaction of these molecules with the target enzymes on the basis of their ability to adopt conformations that partially mimic steroid substrates. From these models an explanation could be advanced for the fact that when compared with the unmethylated analoge, the (racemic) α-methylated compound was seven times weaker as an inhibitor of aromatase but seven times stronger as an inhibitor of lyase. The model proposed for binding to aromatase placed the α-carbon of the ester group in the position occupied by C2 of steroid substrates. In contrast, the model proposed for binding to lyase placed this atom in the position occupied by C17 of steroid substrates. The introduction of steric bulk at C2 was known to be unfavorable for aromatase inhibition, while its introduction at C17 might lead to a better mimicry of the steroid D-ring improved lyase inhibiting activity [286].

Recently Ghosh *et al.* have reported the crystal structure of human placental aromatase, the only natural mammalian full-length P450 to be crystallized so far. This crystal structure will definitely speed up the modeling studies in the right direction for designing of novel potent aromatase inhibitors [222,287].