CHAPTER-1

INTRODUCTION

1. INTRODUCTION

It is easiest to design new drugs when one has a full understanding of the disease process and reliable structural information about the target and its effectors. Unfortunately such a complete information is not often available. In the design of a new drug, structural information about the target molecule is frequently unavailable. Enzymes are the target of choice because they are generally smaller and not attached to other structures and thus often easier to isolate and characterize. Even when enzymes are selected as targets, there can be problems obtaining structural information. Sometimes it is difficult to isolate or produce sufficient quantities of the target enzyme to study it directly. All these are the obstacles in the drug design. To overcome these obstacles computer aided molecular modeling could be an answer since it can provide scientists with five major different types of information that is important for designing of a drug : (1) the three-dimensional structure of a molecule; (2) the chemical and physical characteristics of a molecule; (3) comparison of the structure of one molecule with different molecules; (4) visualization of complexes formed between molecules and (5) predictions about how related new molecules might look.

In this thesis, efforts have been made to design various novel enzyme inhibitors by using computer aided drug design techniques hence, in this chapter an overview of various computer aided drug designing techniques and the enzymes used in the study is provided.

1.1 Role of Computer Aided Drug Design in Drug Discovery

Human life is continuously threatened by ailments. Hence, drugs are always in great demand for the prevention and treatment of diseases. To meet the challenges of discovery of safe drugs, techniques/methods of drug discovery and development are in great demand. The process of drug discovery and development is an expensive and time-consuming affair. To introduce a chemical entity as a new drug in the market requires not less than 10-15 years of hard work and a huge amount of money. Hence, several new technologies have been developed and applied in drug research to cut short the research cycle and to reduce the expenses. Computer-aided drug design (CADD) is one of such evolutionary technologies [1].

Computer based drug design is a powerful, fast and reliable technique that improves the effectiveness of the process of drug development and thus reduces the cost [2]. Various computer simulation techniques can give a better insight into the action of the drugs and can highlight various properties that could definitely guide us in synthesizing right drug candidates. Revolutionary developments in computer technology provide the means to reduce greatly the number of new compounds that need to be synthesized and tested, thereby speeding up the process of drug discovery. Generally such type of computational work is done on workstations or high configuration computers. As we know, 'modeling' is the world of glamour which is attached with latest fashions and trends, "Molecular modeling" is in a way similar to this glamorous and thrilling profession. Here the ramp is the computer screen with beautiful graphical interface. The molecules perform a catwalk just in the same way as human models do. This catwalk is performed in order to attain the highest stable and energetically favorable structure "the desired conformation". The successful molecule or the conformation is thought to be the best structure hence, selected for interaction with the receptor. In other words, the selected candidate can be used for further development.

1.1.1 CADD and drug discovery

CADD is an exciting and diverse discipline wherein various aspects of applied and basic research merge and supplement each other. It is a unifying discipline focused on the prediction of chemical reactivity for non-synthesised virtual structures. The latest technological advances (QSAR/QSPR, structure-based design, combinatorial library design, cheminformatics and bioinformatics etc.), the growing number of chemical and biological databases and an explosion in currently available software tools, all provide a basis for the design of ligands and inhibitors with desired properties. Different terms applied to CADD, like computational drug design, computer-aided molecular design (CAMD), computer-aided molecular modeling (CAMM), rational drug design, *in silico* drug design and computer-aided rational drug design, all convey the same meaning [2].

Major objectives of CADD are lead identification, lead optimization and analysis. The three-dimensional structures of drug molecules and receptors with their conformational flexibility play important role in the process of drug designing. Molecular modeling, which comprises of variety of computer-based simulation techniques, can construct 3D models of chemical compounds and calculate various molecular properties,

mainly the energy of the system with respect to the atomic positions that highlight intra- and intermolecular interactions of the system. From this information one can design a molecule with desired properties. Thus, molecular modeling helps to simulate, explain and predict the 3D structures and physiochemical properties of biomolecules, which play a vital role in drug-interactions. Conformational energies are usually calculated using either quantum or molecular mechanics.

CADD tools have been developed initially for lead optimization (such as QSAR) and then later on extended for lead discovery (such as virtual screening). But in the post-genomic era, there has been a dramatic increase in information about small molecules and biomolecules. Thus, CADD tools now have been applied at almost every stage of drug research and development, changing greatly the strategy and the pipeline for drug discovery[1]. From its traditional application of lead discovery and optimization CADD has expanded in two directions [3]:

- Upstream for target identification and validation, and
- Downstream for preclinical study (ADMET predictions).

Successful drug design projects demand concerted efforts by a multidisciplinary team in producing new chemical entities for clinical use. A computational chemist can play a central role in the team in processing a vast amount of data produced by the other members of the research and development team, as that can facilitate decision making on the 'right' lead candidates for the 'right' target proteins [4]. Different techniques of computer-aided drug design are used depending on the available knowledge on the target structures and/or the ligands [5]. Based on the available information, either the ligand-based or the receptor-based molecular design methods can be applied. Ligand-based designing uses a known set of ligands but an unknown receptor site, whereas receptor-based designing starts with a known receptor, such as an enzyme or a protein binding site. Both of the approaches are actually very similar.

1.1.2 Ligand-based drug design

Ligand-based approach is used when the structure of the receptor site/enzyme is unknown, but a series of compounds have been identified that exert the activity of interest. In absence of the structure of the target receptor/protein, molecular modeling of known active ligands can be used to derive indirect information about the molecular

interactions at the protein binding site. To be used most effectively, structurally similar compounds with high activity, with no activity, and with a range of intermediate activities are required in this technique. Then, recognition site mapping attempts to identify a pharmacophore, which is nothing but the template derived from the structures of the compounds used in the study. Here, pharmacophore is a 3D space representation of the collection of common functional groups of active compounds, complementary to the geometry of the receptor site.

The pharmacophore can be generated by visual inspection or by statistical techniques such as 3D-qunatitative structure activity relationship (3D-QSAR), which maps the steric, charge, and hydrogen bonding interactions into a 3D grid for each known ligand. These maps are then compared to find features that the active compounds have in common. The map of common features is then converted into a pharmacophore. The next step is to search database for identifying new ligands that may bind to the chosen receptor. The 3D-pharmacophore is used in conformationally flexible searches for ligands that match the spatial distribution of the known ligands. The results of the database search may be used directly or may be modified to produce candidates for further study. The new ligands are then assessed for their use. This assessment involves prediction of the binding constant or biological activity of the ligand. Activity predictions are usually based on QSAR extrapolation. Finally, the candidates which have been predicted to have a good biological activity are synthesized and tested in the laboratory.

1.1.2.1 3D-QSAR

Classical QSAR techniques could not be applied to all types of datasets due to lack of availability of physicochemical parameters of the molecules or the substituents or due to difficulty in estimating those values. Molecular properties based on the 3D structures of compounds may be useful in describing the ligand receptor interactions. Due to recent advancements in computer hardware and software technologies, one can analyze 3D structures of macromolecules also in addition to the structure of small molecules. Over the years, the QSAR methodology has evolved from the classical 2D-QSAR approaches of Hansch and Free-Wilson to the present 3D-QSAR techniques like Comparative Molecular Field Analysis (CoMFA) [6], Comparative Molecular Similarity Indices Ananlysis (CoMSIA) [7] and the multidimensional or the nD-QSAR techniques [8,9]. In 3D-QSAR approaches, quantitative models are constructed correlating the biological

activity of small molecules with their 3D parameters. These techniques can be applied to structurally diverse set of compounds where the classical QSAR is often not suitable. Further, favorable and unfavorable molecular properties can be visualized in three dimensions. The 3D-QSAR approaches are based on the ligand structures [10]. Ligand based 3D-QSAR consists of determination of bioactive conformations of molecules, alignment of molecules, computation of the molecular properties, correlation of these properties with activity and visualization of regions favorable or unfavorable for the activity. The two most common 3D-QSAR techniques utilized in drug design are CoMFA [6] and CoMSIA [7].

1.1.2.1.1 CoMFA

CoMFA is the first approach dealing with electrostatic and steric interactions of molecules with their environment, taking into account the 3D shape of the molecules. In CoMFA, non-covalent intermolecular interactions producing biological properties of ligands can be appropriately described by steric and electrostatic fields [6,11] i.e. differences in the degree of biological activity of the molecules are co-related to local changes in the molecular field intensities. As per the CoMFA methodology, the molecules of the data set need to be aligned geometrically. Different alignment rules can be applied on the molecules of the data set under study. The aligned molecules are then placed into cubic lattice one by one, and interaction energies between the molecule under study and a defined probe atom (usually a proton or an sp^3 hybridized carbon atom with a positive charge) are calculated for each lattice point. Each lattice point defines a position in space relative to the molecule. The steric van der Waals interactions are derived from the Lennard-Jones potentials as per **Equation 1** and the electrostatic forces, utilize a simple Coulombic interaction term as per **Equation 2**. These potentials contribute only to the

$$E_{vdW} = \sum \left(\frac{A_{ij}}{r_{ij}^{12}} - \frac{A_{ij}}{r_{ij}^{6}}\right) \ldots\ldots\ldots\ldots\ldots (1)$$

Where, A_{ij} = repulsive term coefficient; B_{ij} = attractive term coefficient; r_{ij} = distance between the atoms i and j.

$$E_{elect} = \left(\frac{q_1 q_2}{\varepsilon r}\right) \ldots\ldots\ldots\ldots\ldots\ldots (2)$$

Where q_1 and q_2 = atomic charges of interacting atoms; ε = dielectric constant; r = interatomic distance.

enthalpic term of the free energy of binding, although it may be important to take into account the entropic effects (e.g. hydrophobicity) as well [12,13].

As per Cramer III *et al.* [11] the number of the resulting field energy values greatly exceed the number of the compounds, a multivariate statistical technique called partial least squares (PLS) [14,15] has to be utilized to evaluate the relationships between the biological activities i.e. Y variables and the calculated interaction energies i.e. X variables. In the iterative procedure of PLS, new components (latent variables) are extracted so that each time, the degree of commonality between the X and Y variables is maximized. Usually a maximum of five to six components is enough to generate a realistic model. The optimum number of components is traditionally determined by cross-validation, a technique that assesses the ability of a QSAR model to predict the biological data. In this technique, one or more compounds are left out form the model and their biological activities are predicted on the basis of the model derived with the remaining compounds. This procedure is repeated until each compound has been predicted once [11]. The resulting statistical values are q^2 (square of the cross-validated correlation coefficient, **Equation 3**) and S_{PRESS} (standard deviation of the error of predictions, **Equation 4**).

$$q^2 = 1 - \frac{PRESS}{\sum(Y - Y_{mean})^2} = 1 - \frac{\sum(Y - Y_{pred})^2}{\sum(Y - Y_{mean})^2} \quad \ldots\ldots\ldots\ldots\ldots\ldots (3)$$

Where, PRESS = the sum of the squared error of predictions; Y = experimental value; Y_{pred} = predicted value; Y_{mean} = mean value of the experimentally observed activities

$$S_{PRESS} = \sqrt{\frac{PRESS}{N - c - 1}} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (4)$$

Where, N = number of compounds; c = number of PLS components

The value of q^2 can range from 1.0 (perfect predictions) through zero to even negative values. The risk of chance correlation has been considered to be negligible with a $q^2 > 0.3$ [16,17]. However, robust and predictive models should have a q^2 of at least 0.4-0.5 [12,16]. The number of components used in the final model is recommended to be taken as that in which the S_{PRESS} value is smaller or it no longer decreases significantly [18].

This helps to avoid adding noise to the model. In fact, it has been shown that an excessive amount of noise (i.e. irrelevant grid points that do not contribute to explaining the biological activity) can be detrimental to the PLS prediction. Therefore, different variable selection techniques have been developed to eliminate irrelevant X-variables. The 'minimum-sigma' filter eliminates those grid points that express lower variance in the field values than the user-defined cut-off value (e.g., 2 kcal/mol). More advanced variable selection approaches include, for example, the GOLPE procedure [19] and the smart region definition method [20].

The conventional r^2 (square of the correlation coefficient) of a non-cross-validated final model shows how well this particular model fits the input data. In an analogous way with q^2 and S_{PRESS}, r^2 and the corresponding standard deviation can be calculated by replacing Y_{pred} with Y_{cal} (activity value calculated by the model) in **Equation 3** and **4**. The predictive r^2 (r^2_{pred}) is obtained if the model is used to predict the activities of a set of compounds not included in the model [11].

The beauty of the CoMFA QSAR equation is that it can be represented usefully as a 3D coefficient contour map in which the colored polyhedra denote the 3D location of the steric and electrostatic fields that significantly contribute to the model [6,11]. Two different contour levels are displayed for each CoMFA field, positive and negative. A positive steric contour shows a region that increases the activity of a molecule if occupied; whereas a negative contour reveals a sterically/electrostatically unfavorable region. A main advantage of CoMFA is that it allows you to make favorable modifications to the molecule under study and predict the activity. Thus, contour maps guide the modeller in the right direction to modify the molecule structurally to get higher activity.

1.1.2.1.2 CoMSIA

CoMSIA can be considered as an alternative approach to CoMFA to perform 3D-QSAR. In CoMSIA methodology, instead of field descriptors based on Lennard-Jones- and Coulomb-type potentials, molecular descriptors based on similarity indices of the aligned molecules are computed. The energy potentials used in CoMFA are very steep near the van der Waals surface of the molecules and they produce singularities at the atomic centers. In order to avoid too large energy values, arbitrary cut-off values have to be defined and evaluation of the potentials are restricted to regions outside the molecules.

To overcome such problem, CoMSIA utilizes a Gaussian-type function for the distance dependence between the probe and the atoms of the dataset molecules (**Equation 5**). Therefore, no cut-off limits are needed and the similarity indices can be calculated at all grid points inside and outside the molecules. This makes CoMSIA relatively insensitive to changes in grid spacing or orientation of the aligned molecules with respect to the lattice [21]. In CoMSIA technique, hydrophobic as well as H-bond donor and acceptor pro-

$$A_F, k^{q(j)} = -\sum_{i=1}^{n} W_{probe,k} W_{ik} e^{-\alpha r_{iq}^2} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (5)$$

Where, A_F = similarity index, k = physicochemical property, q = grid point, j = dataset molecule, $W_{probe,k}$ = probe properties (e.g., charge, radius), W_{ik} = actual value of k for atom i, α = attenuation factor, r_{iq} = distance between atom i and probe atom at q.

perties are included in addition to the steric and electrostatic similarity fields [21,22]. Statistical evaluation of the similarity indices is done by PLS in the same way as in CoMFA.

1.1.2.1.3 Evaluation of the predictive ability of the model: Validation of 3D QSAR models

It is of utmost importance to evaluate the predictive capacity or validity of the model before using the model for the interpretation and prediction of biological activity of the test compounds. Generally, validation of a method is done to establish the reliability and relevance of the method for a particular purpose. Reliability refers to the reproducibility of results, the relevance is related to the scientific use and practical usefulness, and the purpose refers to the intended application. Validation of a QSAR model is the process by which the predictive ability of the model is assessed for practical purposes. Validation means how accurately the model represents the reality, from the perspective of the intended application of the model.

Cross-validation has been used to evaluate the predictive power of the CoMFA/CoMSIA PLS models. In the 'leave-one-out' (LOO) procedure, each molecule is separately left out from the model and its biological activity is predicted on the basis of the data from the molecules existing in the model. Unfortunately, a high degree of redundancy in the chemical structures of the model compounds the result in the form of providing too optimistic q^2 values [23]. Additionally, it has been shown that there is no real

relationship between q^2 and the external predictivity of a model [24-26]. This phenomenon in which internally predictive models result in low external predictivity and vice versa has been called the 'Kubinyi paradox' [27-29]. High q^2 value is a necessary but an insufficient condition for endowing high predictive power, and therefore, one should not blindly rely on (LOO) q^2 [26]. Cross-validation in random groups (Leave-some-out, LSO) may produce more reliable q^2 values. In particular, a more stringent leave-half-out (LHO) cross-validation has been reported to give a good estimate of model predictivity [30]. A random-group cross-validation procedure should be repeated several times as the difference in distribution of the compounds in the groups during each PLS analysis affects the q^2 value. For establishing a more reliable QSAR model, Golbraikh *et al.* recommended the use of an external test set [26]. However, a counter argument is given that it is better to use properly conducted cross-validation than to waste valuable information by holding back compounds for a test set while validating a typical QSAR model with small or modest sample size [31].

Robustness of PLS model can be assessed by using response (Y) randomization. In response randomization, the biological activities are scrambled among the training set molecules and novel PLS models are generated with the scrambled data. If the new models give q^2 values comparable to the original model, there is a strong possibility that the original model is fitting only the noise. Therefore, a loss of statistical significance in cross-validation after response scrambling is considered to be a necessary condition for the robustness of a model. However, it is not a sufficient condition particularly in case of redundant datasets [17]. Progressive scrambling method has been developed to address the overly optimistic cross-validation or response randomization results for redundant datasets [32,17]. In this approach, small random perturbations are introduced into a dataset in such a way as to allow the underlying covariance structure of the data to remain undisturbed. This causes the nominal predictivity of unstable models to fall off rapidly, whereas robust models are relatively stable. The deprecated predictivity (Q_s^{*2}) and standard error of prediction ($SDEP_s^*$), the two characteristic statistical values are produced by a progressive scrambling analysis. By correcting for the effect of introduced perturbation, one obtains the adjusted statistical values Q_0^{*2} and $SDEP_0^*$ that correspond to the q^2 and S_{PRESS} respectively of cross-validation. The instantaneous slope of the predictivity with respect to the degree of perturbation ($dq^2/dr^2_{yy'}$), the third characteristic statistics from progressive scrambling depicts the model sensitivity to perturbation at a critical threshold level of perturbation (s, typically 0.85). The slope value is reported to be

a reliable indicator of model complexity and thus, it helps to avoid over fitting due to too many PLS components. In general, a particular number of PLS components yielding a dq^2/dr^2_{yy} slope near unity should be optimal [32].

1.1.2.2 Pharmacophore modeling

Pharmacophore modeling is considered to be an important tool in drug discovery process because of considerable development of pharmacophore technology. A pharmacophore can be defined as an "ensemble" of steric and electrostatic features that is necessary to ensure optimal molecular interactions with a specific biological target structure and to trigger (or to block) its biological response". It can also be defined as "the spatial arrangement of key chemical features recognized by a receptor" [33]. Such a model is derived by superimposing a set of active molecules and identifying their common chemical features that contribute to the binding interactions at the target protein.

Pharmacophore modeling can also be useful if shape constraints describing the steric boundaries of the binding site can be inferred from the dataset [29]. For example, a region occupied by inactive molecules but not by their active analoges can be defined as sterically forbidden. A pharmacophore model can be used to explain the SAR of a series of ligands, to guide the molecular alignment process in 3D-QSAR and to search for novel structures with the same pharmacophore; hence the model is useful in lead discovery and optimization [29, 34].

Alignment of pharmacophore features is usually more straightforward for rigid molecules than for flexible molecules whose bioactive conformations are often unknown. Hence, conformational analysis of flexible structures is an important part of the pharmacophore mapping. Different theoretical approaches such as systematic search, Monte Carlo random search or molecular dynamics (MD) can be used to examine the energetically accessible conformations of a molecule [12]. A bioactive conformer is considered to be one of the low-energy conformations of a molecule, however, not necessarily the one with the lowest energy [35,36]. A study on 150 crystallized ligand-protein complexes demonstrated that 60% of the drug-like ligands did not bind in a global minimum conformation and at least 10% of the ligands had their binding conformation energies over 9 kcal/mol above the global minimum [37].

While developing a pharmacophore model, it is of utmost importance to use correct structures as well as the use of consistent and mechanistically homogenous biological data collected following the same protocol for all of the compounds having a similar binding mode [29]. It is also important to remember that usually multiple pharmacophore alignments are possible for a dataset. The quality of a pharmacophore model can only be measured by the model's success in prospective application to drug design i.e. how well it can facilitate lead optimization and the accuracy in selection of active compounds to be synthesized, or how many active hit compounds can be found in virtual screening with a query based on the model [29].

Using different alignment protocols and the manner of generating conformations, a number of programs like PHASE [38], DISCO [39,40], Catalyst [41,42], GASP [43] etc. are available for pharmacophore modelling studies. PHASE [38] is an overlapping tool that can be used for pharmacophore modelling, 3D-QSAR and 3D database search. PHASE allows pharmacophore model generation using a single reference structure by automated protocol that utilizes a set of actives for an extensive search of common features. The biological activity data of the compounds can be used to develop 3D-QSAR models using pharmacophore as a mode of alignment.

1.1.3 Structure-based drug design

The three-dimensional structure of a protein provides important information for understanding its biochemical function and interaction properties at molecular level [44]. In addition to the pharmacophore-based strategies, site-directed mutagenesis on the binding site can give important information on the interactions between the binding of amino acids and the ligand. Nevertheless, none of the indirect approaches can compete with the actual insight obtained from experimentally determined target-protein structures or ligand-protein complexes. Structure based approach applies when a reliable model of the receptor/enzyme site is available. With the availability of the receptor/enzyme site, it is possible to use docking techniques to design ligands that interact favorably at the site.

The process of the structure-based drug design is an iterative one and often proceeds through multiple cycles before an optimized lead goes into phase I clinical trials [44]. The first step is to determine the structure of the binding site using standard analysis from X-ray diffraction, NMR studies, homology modeling or calculations involving molecular orbital or molecular mechanics and molecular dynamics techniques.

X-ray crystallography and NMR spectroscopy are commonly used to derive detailed 3D protein structures. The number of the solved protein structures in the Protein Data Bank (PDB) [45] lags far behind the number of determined protein sequences [46,47] because X-ray structure determination is often a complicated and tedious task and the size of protein sets a limit for NMR experiments. Thus, computational approaches such as *ab initio* methods [48,49], threading (fold recognition) [50,51] or comparative (homology) modelling [52,53] are usually applied for generating 3D models of proteins. Since we do not yet know general rules for folding of proteins, *ab initio* methods, which predict the structure directly from the sequence, are usually able to produce models with only a low resolution. Additionally, these methods can only be applied to protein sequences of limited size (less than 100 residues). On the other hand, comparative modelling, which utilizes homologous protein structures as templates, perform better in these aspects and its applicability will no doubt increase in future as the number of novel solved protein structures increases [54].

The process of structure based drug design involves the following steps:

- Using computer algorithms, compounds or fragments of compounds from a database are docked into a selected region of the structure. These compounds are scored and ranked based on their steric and electrostatic interactions with the target site and the best compounds are tested with biochemical assays.
- In the second cycle, structure determination of the complex of the target and the lead (that lead molecule which has shown micromolar inhibition in the *in vitro* studies from the first cycle) reveals sites on the compound that can be optimized to increase potency.
- Additional cycles include synthesis of the optimized compounds, usually showing marked improvement in binding affinity and often specificity for the target followed by repetition of the above second and third steps.

1.1.3.1 Homology modeling

Homology modeling is also called comparative modeling. Homology modeling techniques have been developed to build three-dimensional models of a protein (target) from its amino-acid sequence on the basis of an alignment with a similar protein with known structure (template). In general homology modeling consists of four main steps:

- Identifying evolutionarily related proteins with experimentally solved structures that can be used as template(s) for modeling the target protein of interest.
- Mapping the corresponding residues of the target sequence and template structure(s) by means of sequence alignment methods and manual adjustments.
- Building a three-dimensional model of the target protein or a fragment of it on the basis of the alignment.
- Evaluating the quality of the resulting model. This procedure can be repeated until a satisfactory model is obtained [54].

The starting point in the process of homology modeling is to search for known structures of proteins (template) that are related (homologous) to the unknown protein (target). Potential structural templates are identified using a search for homologous proteins in a library of experimentally determined protein structures. From the resulting list of possible candidate structures, a template structure is chosen on the basis of its suitability according to various criteria such as the level of similarity between the sequences of the query and the template, the experimental quality of the solved structures, the presence of ligands or cofactors and so on. Ideally, a large segment of the query sequence should be covered by a single high-quality template, although in many cases, the available template structures will correspond to only one or more distinct structural domains of the protein. Sequence comparison tools such as BLAST and PSI-BLAST [55] are used to find related sequence and identify structurally conserved regions (SCRs) in the template and the target proteins. If multiple related structures are available, then a template(s) with the highest quality (e.g., resolution) and highest sequence similarity to the target protein is chosen for the modelling project.

The final modelling alignment of the target and the template(s) can be performed with ClustalW [56]. Aligning correctly the target sequence with the template is a crucial step and one of the primary sources of errors in the whole modeling procedure. If the target-template sequence identity is less than 40%, the alignment accuracy can be improved by considering the information obtained from the template structure, multiple sequence alignment of many related sequences, or secondary structure prediction of the target sequence [54,57]. The gaps in the SCRs should be avoided. Coordinates of the SCRs are then transferred from the template structure to the target protein. Coordinates of the identical amino acids are taken as such but in case of diverse residues, only the backbone

coordinates are used. The missing amino acid side chains are usually chosen from a rotamer library. Structurally variable regions (SVRs, usually loops) can be modelled either by retrieving the fitting peptide segments from a protein database or by generating the loop structure *de Novo* (from scratch). Finally, the model usually needs to be refined by energy minimization and/or MD techniques to remove steric strains and repulsive van der Waals contacts and to find energetically more favored conformations of the protein [12].

Internal validation of a model can be done by assessing the stereochemical quality of the protein by using programs like PROCHECK [58] and WHATCHECK [59], which help to identify amino-acid conformations deviating from expected values for structural features such as bond lengths and angles. Consistency with experimental data (e.g. ligand binding and site-directed mutagenesis) is a good measure for external validation/evaluation. In case of a reasonable sequence similarity, comparative modelling can result in models comparable to medium resolution experimental structures, although the loops and side-chains may contain large errors [57].

Each of the four steps mentioned above in homology modeling requires specialized software as well as access to up-to-date protein sequence and structure databases. SWISS-MODEL [60,61] and PRIME [62] are examples of the softwares which are used for homology modeling. Comparative models have been reported to be useful, for example, in identifying active sites [58,63], indicating important amino acids for mutagenesis studies [64], expanding different binding modes of diverse ligands [65] or in virtual screening and lead optimization [66, 67].

1.1.3.2 Molecular dynamics

Molecular dynamics (MD) simulations can be applied for conformational sampling of small molecules as well as proteins. In cases of protein models or crystal structures, MD is frequently used in the model refinement. Main aim of the MD simulation is to report the time-dependent movement of a molecule. Scientists have demonstrated that if we are able to calculate the relative positions of the protein atoms at small intervals of time, we can then predict the behavior of the atoms over a longer time scale. The principal tool used for such calculations is MD simulations that was first introduced in the late 1950s by Alder and Wainwright [68,69]. In the 1960s Rahman carried

out the first realistic MD simulation studies with liquid argon and liquid water [70,71] and later in 1976, the first protein simulation was performed by McCammon *et al.* [72,73]. Since then researchers have used MD to investigate the structural, dynamic and thermodynamic properties of biological molecules, including such items as the characterization of protein folding kinetics and pathways, protein structure refinement and protein–protein interactions [74].

Newton's law of motion is integrated at successive time steps (e.g., every 2 femtoseconds, fs) and the coordinates and velocities of the particles in the system are recorded at each step. The resulting trajectory can be analyzed for the time-dependent properties of the system. To mimic the natural environment of a protein, the simulated system can also include explicit water molecules, counter ions, and even a lipid bilayer when simulating membrane proteins [75,76]. Extensive simulations i.e. lasting for nanoseconds are performed to study the dynamic motions related to ligand binding or the formation of important hydrogen bond network at the active site [77-79].

Simulated annealing is a modification of MD simulations at high temperature. An elevated temperature is used to overcome energy barrier between different molecular conformations. In simulated annealing, the molecule is first heated to high temperature and then slowly cooled down until the movement practically ceases (near 0 K). A low-energy conformation is then saved and used as an input into the next annealing cycle. Repeating the cycle many times and collecting the low energy structures results in a set of low energy conformations [80].

1.1.3.3 Molecular docking

Molecular Docking is the process in which two molecules fit together in 3D space. It has been widely used in drug design to suggest binding modes of ligands at their target proteins. The docking process consists of sampling the coordinate space of the binding site and scoring each possible ligand pose, which is then taken as the predicted binding mode for that compound.

There are a large number of docking programs available for use in virtual screening and they differ in the sampling algorithms used, the handling of ligand and protein flexibility, the scoring functions they employ and the cpu time required to dock a molecule to a given target. If in the process of docking, both ligand and the protein are

treated as rigid bodies [81], the conformational flexibility of ligands can be taken into account by creating an ensemble of conformers and docking each one of them separately into the target site. Alternatively, the conformational flexibility of ligands can be explored during the docking process. There are various examples of semi-flexible docking approaches like incremental growth methods [82], genetic algorithms (e.g., GOLD [83]), Tabu search (e.g., PRO_LEADS [84], and combined Monte Carlo and simulated annealing methods (e.g., Dock Vision [85]). Molecular docking exercise carried out by keeping the target protein rigid is sometimes an inaccurate approximation, since the binding of a ligand can induce large conformational changes in a receptor binding site [86, 87]. One possibility to consider the protein plasticity is to use an ensemble of protein conformers, for example, obtained from NMR studies or molecular dynamics simulations. The FlexE program combines structural information from superimposed target protein conformers and treats the dissimilar protein regions as distinct alternatives [88].

If a pose has been generated for a compound in the binding site, it needs to be scored to rank the quality of the pose with respect to other poses of the compound. There is a wide choice of scoring functions available, and they can be categorized as being physical-based (force-field), empirical or knowledge-based. Scoring functions estimate the free energy of binding of a ligand in a protein-ligand complex. They are used to optimize the placement of the ligands during the docking process and after docking they are applied to rank the resulting ligand poses with respect to the other poses and ligands [12, 89]. There are wide choice of scoring functions available that are grouped into force-field based, empirical and knowledge-based functions [90]. It is a well-known fact that these fast scoring methods do not perform so accurately as the time-consuming free energy perturbation technique [91]. Using a combination of one or more scoring functions (i.e., consensus scoring) has been reported to improve the results [92-94].

In the structure-based virtual screening, docking and scoring is considered to be an integral part of the process. Molecular docking to target protein structures has also been used successfully as an automated alignment method in creating 3D-QSAR models. Docking studies using both, protein crystal structures [95,96] as well as comparative models [97-99] have resulted in statistically significant and predictive QSAR models. In the absence of a precise pharmacophore, automated docking can be a useful tool for generating an alignment that is not biased by subjectivity. Some of the docking programs which are used in drug designing are FlexX [82,100] GOLD [101], GLIDE [102,103] and DOCK [104].

1.1.3.4 Structure-based virtual screening

In the past decade virtual screening (VS) has become a promising tool for discovering active (leading) compounds, and has been integrated into the drug discovery process in most of the pharmaceutical companies [105]. One of the most powerful forms of computational screening is made possible when the structure of the biological target, such as a protein, enzyme or receptor structure is known. Researchers have demonstrated the efficiency of virtual screening, which has been shown to enrich the hit rate (defined as the number of compounds that bind to the target at a particular concentration divided by the total number of compounds experimentally tested) by hundred to thousand-fold over random screening (e.g. high throughput screening). Aiming at speeding up the drug discovery process researchers use computational methods to evaluate virtual libraries (databases) against virtual receptors (targets) in the *in silico* laboratory [106].

VS is aimed for searching large scale hypothetical databases of chemical structures or virtual libraries by using computational analysis and for selecting a limited number of candidate molecules that are likely to be active against a chosen biological receptor [107]. It is a complementary alternative to the experimental high throughput screening (HTS) approach [108]. The ligand-based VS methodologies utilize, for example, exact match or substructure search and similarity or pharmacophoric searching [109] whereas structure-based VS employs molecular docking and scoring. Pharmacophoric search queries can also be constructed on the basis of the structural features of the target binding site of known ligands which are first docked at this specific binding site. Since 1990s, docking based VS has become a more popular tool for discovering active compounds because it directly reflects the ligand-receptor binding process. Apart from the accuracy of the scoring functions in docking, the quality and the conformation of the target protein also affect the results of the screening process. It has been shown that ligand-bound i.e. holo crystal structures yield meaningful enrichment of known ligands from a database containing mostly decoy molecules [110]. However, apo (without ligand) and modelled structures in some cases may also perform better than the holo structures. Successful results from many groups suggest that in VS of GPCR ligands, it is beneficial to utilize information derived from known ligands or mutational studies when constructing the 3D models. It is suggested that a combined ligand- and structure-based VS approach may be more efficient than either of the methods alone [76,111-117]. There are some reports that emphasize the use of available target structure information when building pharmacophoric queries for database searches [118]. Although the 3D structures of targets

may be known, pharmacophore based VS is still used as a complementary approach to docking-based VS for pre-processing of the databases (libraries) of small molecules to remove compounds not possessing features known to be essential for binding or for post-filtering of the compounds selected by docking approaches [119].

Ligand-based virtual screening results tend to find structures that are closely related to known active products. In ligand-based VS, databases of chemical structures are searched to find compounds that are similar to known actives (similarity searching) or possess a pharmacophore or substructure in common with a known active (pharmacophore substructure searching). Similarity substructure searching may be carried out with reference to the 2D- or 3D-structure of various compounds to be screened. Ligand based screening methods are based on the principle of similarity, i.e. similar compounds are assumed to produce similar effects. In this case, if one or more active chemical entities are known, it is possible to search a database for more potent similar molecules [120]. The top ranked compounds, called hits, obtained as output of database screening, are submitted for further filtering to reduce the number of compounds to be chosen for biological testing. The Lipinski's 'rule of five' [121] has frequently been used as a crude criterion for drug-like properties. As per this rule a compound that has a molecular weight more than 500, calculated log P more than 5, the number of H-bond donors more than 5, and the number of H-bond acceptors more than 10 is likely to be poorly absorbed and will be unlikely to permeate through cell membranes. Other filtering criteria can include requirements for chemical diversity, geometric complementarity of the protein-ligand complexes, absence of reactive functional groups, ease of synthesis and patentability [122-124].

1.1.2.5 *de Novo* designing

The basic aim of both of the techniques HTS and VS is to find a "needle in the haystack" by searching for hits in large compound collections. These collections overlap to a certain extent between different pharmaceutical companies, thereby leading to the detection of identical hits by companies that work on the same drug target. Application of *de Novo* designing minimizes the risk of identical hit retrieval as it does not rely directly on existing compound collections. Hence, *de Novo* design may provide a competitive advantage. Computational *de Novo* design may be regarded as a complementary technique to HTS and VS [125]. *de Novo* design aims at generating novel molecular structures that exhibit desired pharmacological activity and match the binding pattern of a particular

biological target. This binding pattern can be defined by either the 3D target structure (structure-based) or at least one known reference ligand (ligand-based) [125].

Majority of the current computer-based molecular design approaches are structure-based that rely on the availability of the three-dimensional target structure. Structure-based designing cannot be practiced when a high-resolution structure of a biological target is unavailable. This applies to many membrane-bound receptors including a large group of G-protein coupled receptors [126]. The goal of structure-based drug design is to build novel molecular structures or ligands that bind to the receptors of these proteins. Computational methods have been used in the designing of novel ligands. A scoring function primarily considers information related to the binding affinity of candidate compounds to a particular biological target (primary target constraints). Primary target constraints may be derived from the 3D receptor structure (structure-based design) or from known ligands (ligand-based design). Scoring functions that are employed in structure-based *de Novo* designing are analogous to scoring functions as used in docking experiments. They can be subdivided into explicit force-field approaches, empirical scoring functions and knowledge-based scoring functions. All of them aim for the estimation of the free energy of binding. Softwares for structure-based *de Novo* drug design can be divided into three categories:

- Database search techniques e.g. Hook/MCSS [127] which exhaustively search potential binding pockets and docks functional groups into these sites. The functional groups are then linked with templates from a database.

- Atom-based methods that build structures using one atom at a time e.g. LEGEND [128], are atom-based structure generation programs which build structures by adding one atom at a time. The atom by atom building proceeds as follows. The first atom is placed in a specified distance from a potential hydrogen bonding atom of the receptor. The subsequent atoms are placed by randomly choosing an atom on the existing ligand, and then placing the new atom at a random point on the circle of all possible dihedral angles with fixed bond lengths and angles. This new atom is assigned an atom type and a state of hybridization. If the new atom occupies a forbidden position on the grid then it is rejected. This procedure terminates when the structure reaches the user defined size.

- Fragment joining techniques that build structures from a library of common molecular fragments. The Allegro [129] software starts with a pre-computed grid map that classifies the binding zones within the active site. A root atom is chosen and new atoms and fragments are grown from this. Growth points are determined and one is randomly selected to form a connection with another randomly chosen atom or functional group. The new atom or group is placed and a complementary score evaluated from the binding zone classification. A Monte Carlo (MC) sampling criteria is used to decide whether this atom or group is to be accepted. If accepted, new growth points are determined and structure generation is repeated from any one of the potential growth points of the molecule. If the distance and bond angle of the growing structure are appropriate, the MC procedure can also spontaneously perform ring closure. This is also called an undirected random search. SPROUT [130] constructs structures using a fragment joining technique. It carries out five main functions: (i) locates binding pockets in a receptor, (ii) identifies potential interaction sites, (iii) docks molecular fragments to target sites, (iv) generates novel chemical structures by incremental construction from templates and (v) scores, sorts and clusters the solutions for an efficient means of evaluating the results.

Ligand-based *de Novo* designing complements structure-based approaches by working independently from the 3D target structure [131]. One or more ligands that bind to the biological target of interest are a prerequisite for ligand-based *de Novo* design. Ligand-based designing derives primary target constraints from one or more known ligands. Structure-based design unavoidably operates in 3D space and has to deal with the complexity of molecular conformations and conformer sampling. Ligand-based scoring functions on the other hand, may or may not take into account the 3D information of known ligands. Ligand-based scoring functions quantify the similarity between a candidate compound and one or more known reference ligands. Pharmacophore models, QSAR equations and pair-wise chemical similarity have been employed to assess the potential binding affinity of candidate compounds in *de Novo* design [132,133]. LeapFrog, a *de Novo* ligand design tool interfaced with Sybyl 7.1 was used successfully by Kumar *et al.* to design new ligands using the CoMFA results. In this study LeapFrog makes use of the CoMFA contours for the generation of a hypothetical cavity and then design new ligands based on the hypothetical cavity features [134]. It should be kept in mind that automatically

designed structures cannot be expected to represent lead structures or even binding molecules. Rather, *de Novo* design should be regarded as an "idea generator"

1.2 Enzymes as Targets for Discovery of New Drugs

The abnormal functioning of the biochemical pathways may lead to a diseased state and there are many conditions in which biochemical pathways could become abnormal. Therefore, knowledge of the molecular basis of the disease is important in order to select a target [136]. Current drug therapy rests on about 218 biological targets which are classified into three main categories i.e. enzymes, receptors and nucleic acids (DNA, RNA, ribosomes). There are approximately 6000 drugs currently in the market for these drug targets [137]. Thirty percent of the currently available drugs in the market act by inhibition of some enzyme function.

Every aspect of molecular biology requires the chemistry of bond formation and bond breaking at different stages. Majority of these biochemical reactions are spontaneous reactions, but left to themselves they would proceed too slowly to support life. The catalytic power of enzymes enhances the rates of these reactions to make them useful for sustaining life. The genome of the cell controls these chemical reactions by regulating the expression of different proteins (including enzymes). Overexpression or underexpression of these proteins may lead to certain pathophysiological or pathological conditions. When abnormally high enzymatic activity underlies a diseased state, it can be treated by inhibiting the enzyme.

In other instances specific enzymes may be required for pathogenic organisms or cancerous cells to live and grow, thereby causing diseases. Inhibition of such enzymes would prevent the growth of the enzymes or cells and hence reverses the disease [136]. Enzymes are considered to be the most promising targets out of all of the protein targets (receptors, carrier proteins, enzymes etc.) because of the following reasons:

- Purification of enzymes is generally a much simpler task than receptor isolation and purification [138]. This however is changing fast due to introduction of recombinant DNA technology in the field of drug development.
- A homogeneous enzyme preparation can be easily obtained for preliminary *in vitro* screening purposes.

- Knowledge of the mechanism of action of an enzyme can be used in the design of various inhibitors like transition state analogs [139], multisubstrate, slow tight–binding and mechanism-based enzyme inactivators.
- Effective receptor antagonists often may not bear structural resemblance with the agonists whereas, enzyme inhibitors are generally very similar in molecular structure to the substrates or products of the target enzyme. Consequently, lead compounds are readily obtainable for enzyme targets [136].

About half of the top 20 drugs sold worldwide are enzyme inhibitors. Inhibitors of a single or a group of enzymes have great potential as drugs by selectively blocking certain metabolic pathways, decreasing the concentration of enzymatic products or increasing the concentration of enzymatic substrates [140].

1.2.1 Tumor necrosis factor alfa converting enzyme (TACE) as an attractive target for the treatment of Rheumatoid arthritis (RA)

Tremendous attention has been paid towards the autoimmune diseases by pharmaceutical industries due to the remarkable progress made in the understanding of the immune and inflammatory processes. RA is one of the most common autoimmune inflammatory conditions affecting approximately 1% of the world's population [142]. The pathogenesis of RA is characterized by an inflamed synovium (lining the joint cavity), degradation of articular cartilage and erosion of subchondral bone. Although the cause of RA is unknown, the presentation of an arthritogenic self-antigen to a genetically susceptible individual is believed to trigger the activation of autoimmunological pathways that lead to RA [142]. Conventional treatment of RA includes the following three categories of drugs:

- Non-steroidal anti-inflammatory drugs (NSAIDs): These drugs act by blocking cyclooxygenase, an enzyme responsible for synthesis of prostaglandins. NSAIDs do not treat the underlying causes of the disease, but treat only the symptoms like pain and inflammation associated with the disease. Apart from causing gastric irritation, this class of drugs also causes renal and cardiac disturbances [143].

- Corticoids: These steroidal agents have been shown to halt the process of joint destruction in arthritis patients [144]. This class of drugs acts by lowering the activity of white blood cells and inhibits the production of prostaglandins. When used

for a long time these drugs become less effective and also cause major side effects like osteoporosis, bone cell death, onset of diabetes, artery hardening etc.

- Disease modifying anti-rheumatic drugs (DMARDs): Although a number of DMARDs are used to treat RA, it has been shown that only methotrexate, sulfasalazine, aurothioglucose and leflunomide significantly reduce the rate of joint destruction [145] by suppressing the immune response. There are major toxic issues associated with the use of DMARDs [146].

Non-pharmacological methods [147] such as physiotherapy, occupational therapy and electrotherapy are becoming popular these days as there is no reliable pharmacological treatment available for the cure of RA. These non-pharmacological methods not only play a vital role in acute flares and in chronic stage but are also helpful in relieving symptoms and protecting joints from further damage. Patients may benefit from surgical procedures such as tenosynovectomy, tendon repair and joint replacements in severe conditions.

1.2.1.1 Cytokines and RA

Cytokines are critical in the induction and resolution of inflammatory responses. There are numerous so called 'pro-inflammatory cytokines' but considerably fewer 'anti-inflammatory cytokines', known so far. The classic 'pro-inflammatory cytokines' include Inerleukin (IL)-1, 6 and 12, Granulocyte and monocyte colony stimulating factor (GM-CSF), tumor necrosis factor-α (TNF-α) and interferon-γ (IFN-γ), whereas the 'anti-inflammatory cytokines' include transforming growth factor-β (TGF-β) and IL-101 [148].

Abundant laboratory and clinical evidences suggest that pro-inflammatory cytokines play a major role in the pathogenesis of RA [149]. Several studies have indicated that cytokines, especially TNF-α, are present in high concentrations in serum, synovial fluid, synovial fluid cells and synovial tissue specimens of patients with RA [150]. Although the precise mechanism of bone and cartilage destruction in RA has not been completely understood, but the cytokines especially IL-1 and TNF-α, play important roles in the process. These cytokines are abundant in inflamed joints and promote the influx of inflammatory neutrophils and monocytes into the joints, stimulate the cells in the inflamed synovium to produce proteolytic enzymes, including collagenase and stromelysin that can degrade tissue and cause systemic manifestations such as malaise and

fatigue. Thus, although the initial cause of RA remains unknown, the maintenance and propagation of the disease appear to be related to immunologically mediated inflammatory processes. Hence, interfering with key steps in the inflammatory process would be expected to provide symptomatic relief and to slow down the disease progression.

The earliest change observed in the joints of arthritis patients is swelling and congestion of the synovial membrane and the underlying connective tissues which get infiltrated with lymphocytes (especially CD4 T-cells), plasma cells and macrophages. In the initial cell-mediated response macrophages engulf and process antigens and present them to T-lymphocytes. The processed antigens are recognized by the Major Histocompatibility Complex (MHC) proteins on the lymphocyte surface, resulting in T-cell activation and production of cytokines and cytotoxins that ultimately result in joint damage.

Activated β-lymphocytes produce plasma cells from antibodies, in combination with their complements, resulting in accumulation of polymorphonuclear leukocytes (PMNs). PMNs release cytotoxins, free oxygen radicals and hydroxyl radicals that cause cellular damage to synovium and bone. During active phases of the disease, effusion of synovial fluid takes place into joint space and hypertrophy of synovial membrane occurs resulting into formation of lymphoid follicles, resembling an immunologically active lymph node. Inflammatory granulation tissue known as pannus, spreads over and under the articular cartilage, which is progressively eroded and destroyed. Later, fibrous or bony ankylosis may occur [151].

Vasoactive substances (histamine, kinins, PGs) are released at the site of inflammation thereby increasing the blood flow and vascular permeation that cause edema, warmth and pain [146,152]. Around 30 cytokines are found to be present in the tissues and synovial fluid of active RA affected joints [153,154]. Many of these appear to mediate closely related functions, like induction of cartilage destruction, mediated by IL-1, TNF-α and LT-α. There is considerable evidence to suggest that in an inflammatory site there is a cytokine network/cascade, where the actions of certain cytokines are regulated by certain others. The rheumatoid synovium produces significant levels of IL-1α, IL-1β, TNF-α, IL-6, IL-8, GM-CSF and other pro-inflammatory cytokines [149]. Evidence for the cytokine network was obtained by the use of neutralizing antibodies for TNF-α, and IL-1 and its

receptor antagonist (IL-1Ra) in dissociated cultures of rheumatoid synovium. It was found that anti-TNF-α antibodies reduce the production of IL-1, IL-6, IL-8 and GM-CSF, whereas IL-1Ra reduces the production of IL-6, IL-8 and GM-CSF, but not of TNF-α. This experiment led to the notion of existence of a network of cytokines in rheumatoid joints with TNF-α at the apex followed by IL-1, IL-6, IL-8 and GM-CSF downstream [153-155].

1.2.1.2 Tumor necrosis factor-α

In 1975, TNF was defined as an endotoxin-induced serum factor which produced necrosis of tumors both in *in vitro* and *in vivo* experiments [156]. In 1984, Goeddel and colleagues successfully cloned and sequenced human TNF-α [157]. In the late 1980s, TNF-α was included in the class of cytokines because of its striking similarities in activities with IL-1 [157, 158].

The predominant source of TNF-α is cells of the monocyte/macrophage lineage, but other important sources are neutrophils, T cells, mast cells, epithelial cells, osteoblasts and dendritic cells. TNF-α synthesis is induced by biological, chemical and physical stimuli including virus, bacterial and parasitic products, ischemia, trauma and irradiation. Transcription of TNF-α gene is regulated in a complex manner and there are multiple transcription factors like NF-κB, AP-1, NFIL-6, and NFAT. The p38 MAPK signaling pathway also plays a critical role in the production of TNF-α [158-160].

TNF-α is produced in our body as pro-TNF-α. It is a type II membrane protein (i.e. the N-terminus contains the transmembrane sequence). Pro-TNF-α is biologically inactive and to secrete the active and soluble form TNF-α, a proteolytic cleavage of 26 KDa Pro-TNF-α is needed. The active form is a 17 KDa protein. This proteolytic cleavage is brought about by a zinc endopeptidase that is a member of ADAM (A Disintegrin And Metalloproteinase) [159,160]. This enzyme is known as TNF-α Converting Enzyme (TACE) which specifically cleaves the Ala76-Val77 bond of Pro-TNF and releases TNF-α, the active and soluble form.

The biological responses of TNF-α are mediated through two distinct receptors, namely TNF-R1 and TNF-R2. Both of them are transmembrane glycoproteins. Although their extracellular domains share structural and functional homology, their intracellular domains are distinct. TNF-R1 is constitutively expressed in most tissues while TNF-R2

is typically found in cells of immune system. It has been experimentally proved that under physiological conditions, signalling through TNF-R1 seems to be primarily responsible for pro-inflammatory properties of TNF-α [161]. Much information is not available about TNF-R2, but it has been speculated that it acts as ligand passer, at least in some cells [162].

It has been experimentally proved that TNF-α plays a pivotal role in the origin and progression of RA. TNF-α is responsible for causing a variety of inflammatory effects in inflamed joint [150,163,164]. Apart from arthritis, over-expression of TNF is implicated in a number of diseased states like Crohn's disease, multiple sclerosis, diabetes, ulcerative colitis and congestive heart failure [158,164,165].

Once the role of TNF-α was clearly elucidated in RA, efforts were made to develop inhibitors of TNF-α. Some protein-based drugs have been approved by USFDA for their use in the treatment of RA [166]. Etanercept is genetically engineered version of TNF-R2 that binds and inactivates TNF-α, pre-empting its role in RA. Infliximab (Mouse-human chimeric anti-human TNF-α antibody) and Adalimumab (Human anti-human TNF-α antibody) have also been approved for the treatment of RA [159,167]. These TNF-α blockers are effective for the treatment of RA and show significant, documentable improvement in symptoms, signs and laboratory parameters within 8-12 weeks of treatment [168,169].

The success of these biological agents proved that inhibition of TNF-α could result in effective control of RA. Despite the remarkable activity shown by these biological agents that neutralize TNF-α, these macromolecules have their own disadvantages [170] namely:

- Commercial scale up may be difficult and costly
- Purity may be difficult to achieve and pure product would be still more costly
- Conformational stability may vary in the environment of body fluids
- They may be excluded from some body compartments due to existence of some barriers in the body like blood-brain barrier
- They may lead to the development of neutralizing antibodies and
- Low patient compliance as parenteral route of administration only is possible.

Hence, scientists all over the world thought of developing small molecule orally bioavailable TNF inhibitors [167,170,171]. Most of the above listed disadvantages of macromolecules may be overcome by small molecule inhibitors of TNF.

Attempts to disable TNF functions have been targeted at several levels. TNF can be inhibited at three different stages:

- Synthesis: It has been shown that the biosynthesis of TNF-α is upregulated by activation of transcription factors like Nuclear Factor-κB, (NF-κB). The activation of cyclic adenosine monophosphate also upregulates the synthesis of TNF-α. Hence, NF-κB and cAMP inhibitors (especially PDE4 inhibitors) are being developed in order to block the effects of TNF-α.

- Processing: As pro-TNF is biologically inactive and needs to be cleaved proteolytically by TACE to the active form of TNF. Newton *et al.* demonstrated that inhibition of TACE by small molecular weight orally bioavailable drugs would be more effective than the biological agents in blocking downstream cytokine production [171].

- Inhibition of TNF-α effects: The biological agents fall under this category. These agents capture the active form of TNF-α from the blood stream and thus can be helpful in preventing diseases caused by the over-expression of TNF-α.

In vitro and *in vivo* studies have clearly shown that TACE inhibitors are more effective than biological macromolecules in eliminating TNF from biological fluids and hence in treating inflammatory disease [170]. Inhibition of TACE is an attractive target for medicinal chemists all over the world to develop new TACE inhibitors for the treatment of RA.

1.2.1.3 Structure of TACE

The structure of TACE reveals that it is part of ADAM family of proteins. This family falls within the metzincin superfamily which also includes metalloproteinases [172]. TACE was cloned by two research groups at the same time [173,174]. Like most ADAMs, TACE is a muti-domain, type-I transmembrane protein, which includes a zinc dependent catalytic domain and a disintegrin cysteine rich sequence, as shown in **Figure 1.1a and 1.1b.**

The pro-domain contains a cysteine that interacts with the active site of zinc of the catalytic domain and this interaction must be replaced for the generation of an active enzyme. Furin is the enzyme that is responsible for the generation of active form of TACE [175,176]. The role of disintegrin domain of TACE is not known but it may have an

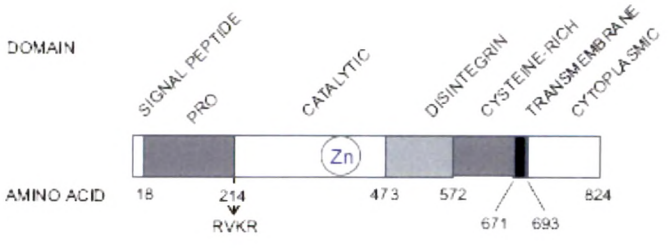

Figure 1.1a Schematic representation of structure of TACE

adhesion function since this domain in some other ADAMs has been shown to interact with integrins. Of the 31 ADAMs that have been identified till date, only ADAM-10 has highly significant sequence homology with TACE [177], however the catalytic domain of TACE shows substantial topological similarity to the catalytic domains of other ADAMs [176].

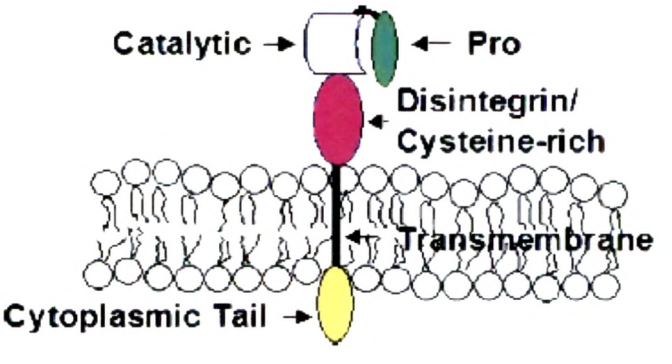

Figure 1.1b Structure of TACE

The X-ray structure of catalytic domain of TACE shows that the zinc environment and the placement of the major structural elements closely resemble those of snake venom members of the ADAM family. In addition, a number of loops protrude outward from the core, which could be involved in regulatory or substrate interactions. The binding pocket has an unusual 'tunnel' connecting the S1' and the S3' site (the side chains that accommodate the first and third residues of the substrate following the cleaved peptide bond) [178].

Analysis of the amino acid sequence of TACE indicates the presence of an EGF-like domain and a crambin-like domain, unique to TACE, within the cysteine-rich domain. The cysteine-rich domain is responsible for the substrate recognition and also for the process of TACE maturation [179]. The cytoplasmic tail of TACE contains potential sites for interaction with Src-homology-2 and Src-homology-3, and a potential tyrosine phosphorylation site as well as potential MAPK phosphorylation site [180].

The catalytic site of TACE (**Figure 1.2**) has oblate ellipsoid shape, notched at its flat side to give a relatively small active site cleft separating a small 'lower' subdomain from an upper main molecular body (**Figure 1.3**). Central to it is a highly twisted 5-stran-

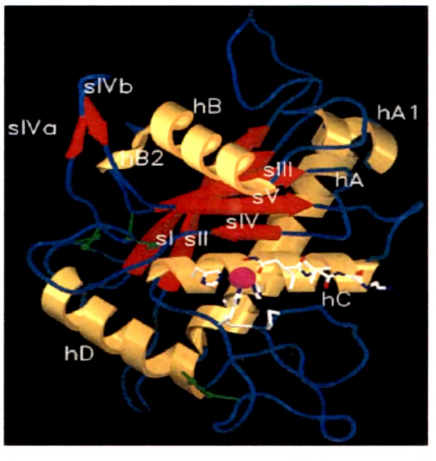

Figure 1.2 Ribbon diagram of TACE catalytic site [176]

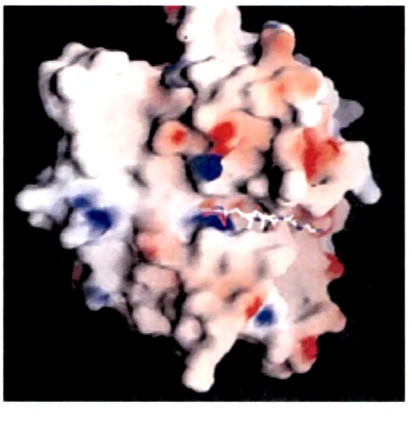

Figure 1.3 Solid Surface representations of the catalytic domain of TACE

ded β-pleated sheet (strands sI-sV, as shown in red color in **Figure 1.2**) flanked on its convex side by α-helices (hB and hB2, as shown in **Figure 1.2**) and on its concave side by helices hA and hC, as shown in **Figure 1.2**. β-Strands sII and sIII are linked by a large

'multiple-turn loop', a long 'intermediate' α-helix (hB) and an adjacent short α-helix (hB2), all arranged on 'top' of the β-sheet and thus fully shielding its central part from bulk water (**Figure 1.2**). The multiple-turn loop is bulged out at two sites, giving rise to a 'spur-like' and an 'acidic' protuberance (visible in **Figure 1.3** on top of the molecule). The sIII-sIV linker terminates in a short 'bulge' before entering the 'edge' strand sIV, the only antiparallel β-strand. The sIV-sV connecting segment is dissected into two large 'ear-like' surface loops, the first one nestling to the main molecular body (giving rise to the 'blue' surface, center left in **Figure 1.3**), and a long β-hairpin loop (sIVa–sIVb) projecting from the molecular surface (upper left in **Figure 1.2** and **1.3**). A bulged-out loop links sV with the 'active-site helix' hC, which is located in the center of the molecule and stops abruptly at the strictly conserved Gly412. At this point, the chain kinks down to build the lower sub-domain [176].

The last 61 residues of the catalytic domain forms three short and straight, perpendicularly arranged segments linked by two 'narrow' super twisted loops. From these it returns back to the surface where it kinks at Pro437 to form the outer wall of the S1'crevice. Then a wide loop precedes the C-terminal α-helix hD, after which the chain ends up on the molecular 'back' surface close to the N-terminus (**Figure 1.2**). The first of the two 'narrow' loops is disulfide-linked to the N-terminus of helix hD. The C-terminal

Figure 1.4 A closer view of solid surface representation of the active site of TACE

end of this helix is clamped to the 'ear-like' sIV-sV linker peptide through Cys365-Cys469. The spatially adjacent third disulfide bridge, Cys225-Cys333, connects the N-terminal parts of β-strands sI and sIII. The last defined residues, Arg473 and Ser474 are fixed via hydrogen bonds to the main molecular body.

The active site cleft of TACE (**Figure 1.4**) is relatively flat on left hand side but becomes notched towards the right. The catalytic zinc residing in its centre is penta-coordinated by the three imidazole rings of His405, His409 and His415. This zinc-imidazole ensemble is placed above the distal Met435. The deep S1' pocket (hydrophobic) invaginates immediately to the right of the catalytic zinc (**Figure 1.4**).

Further to the right through the polar entrance a second hydrophobic S3' pocket opens, which merges inside the molecule with the S1' site pocket bridged by the opposing side chains of Ala439 and Leu348. The dominant intermolecular interactions are made by the P1' *i*.butyl side chain of the peptide inhibitor and hydrophobic bottleneck to the S1' pocket, which accommodates three additional solvent molecules linked through a water channel to the S3' pocket. The P2' *t*.butyl side chain extends away from the enzyme, but nestles against the hydrophobic canopy formed by the bulge of the enzyme. The P1' to P3' segment Val77-Arg78-Ser79 of a bound Pro-TNF-α probably binds TACE in a similar fashion, with the *i*.propyl side chain of Val77 just fitting into hydrophobic S1' neck, Arg78 side chain anchoring to the carbonyl and the hydroxyl groups at the upper rim and the Ser79 hydroxyl interacting with the polar entrance to the S3' pocket. Thus the preferential Pro-TNF-α at Ala76 and Val77 by TACE can partly be explained by favorable interaction in the active site vicinity. Experimental facts suggest that the cleavage site is also determined by its arrangement relative to the base of the compact cone formed by the associated C-terminal segments of three Pro-TNF-α molecules [181,182].

The catalytic domain of TACE is similar to ADAM family members with some unique characteristics. Their close structural similarity is reflected by the superposition of the central β-sheet, the long α-helices and loosely arranged N-terminal and disulfide bridge Cys365-Cys469. The difference is that the chain in TACE is much longer, a more bulged out sV-hC connector [176].

1.2.1.4 Functions of TACE

The role of TACE in shedding TNF has been confirmed by *in vitro* experiments. Both, T-cells and monocytes derived from 'TACE $^{\Delta Zn/\Delta Zn}$ transgenic mice, are deficient in releasing TNF[173]. Apart from this, these mice were also shown to be deficient of Transmembrane Growth Factor-α (TGF-α). Hence, most 'TACE $^{\Delta Zn/\Delta Zn}$ mice show developmental defects in the embryos such as failure of eyelids to fuse and specific hair

and skin defects that closely resemble the defects characteristic for TGF-α deficiency. It proves that TACE is also responsible for the release of active TGF-α from its inactive precursor [183]. Apart from TGF-α, TACE is responsible for the shedding of a number of proteins, such as L-selectin, TNF-R2, Amyloid Precursor Protein (APP), Type II IL-1 receptor etc. But, it has also been shown that TACE is not a universal sheddase [178].

Hence the question arises, what makes a given protein a TACE substrate? The sequences cleaved by TACE in various substrates are highly variable. Moreover, the structure of the substrate-binding cleft of the enzyme does not suggest a strong interaction between the substrate to be cleaved and TACE. It has been suggested that interactions distant to the cleavage site are required and in some cases the substrate and the enzyme both have to anchor to cell membrane [178].

1.2.1.5 Regulation of TACE

The regulation of TACE activity is poorly understood. It has been observed that the shedding rate of TACE increases within minutes of addition of cell activators like phorbol esters. Inhibitors of Mitogen Activated Protein Kinase (MAPK) cascade block the increase in shedding rate in a number of cases where TACE is a primary sheddase [184]. However, the mechanism of action of MAPK cascade is not clear. Interestingly, a small protein TIMP-3 (Tissue Inhibitor of Metalloproteinase-3) that inhibits most matrix metalloproteinases, also inhibits TACE. But whether TIMP-3 acts as a physiological regulator of the enzyme remains unknown.

1.2.2 Neuraminidase as a target for the swine flu

The impact of influenza infection is felt globally every year when the disease develops in approximately 20% of the world's population [185] Advances in understanding of the molecular and cellular biology of influenza have led to identification of several molecular targets for the design of agents against this disease. The influenza virus has three genera: A, B and C. All can infect humans, but only A is responsible for illness on the pandemic scale. So, it is A that attracts attention. The virus comes in many different guises. The version currently prevalent worldwide i.e. swine flu is more properly called H1N1 2009, the letters refer to important glycoproteins dotted over the surface of the viral envelop. H stands for hemagglutinin, a molecule that anchores the virus to any cell it enters. No anchorage means no entry. Because of the key role of hemagglutinin, it is used as the antigen to prepare anti-flu vaccine. And then there is N i.e. neuraminidase. The

most advanced clinical agents described to date are inhibitors of neuraminidase, a glycoprotein residing on the surface of the influenza virus [186].

The 2009 swine flu virus (H1N1pandemic), which can be transmitted from one person to another, has become a threat to the global health and economy [187]. The current seasonal flu vaccine, which targets a different H1N1 strain, provides little or no protection against H1N1pdm. In terms of medication, the two FDA approved antiviral drugs, Tamiflu (oseltamivir) and Relenza (zanamivir), are effective against this new type of virus [188]. Unfortunately, such virus types are known for their quick mutations and gene assortments which enable them to escape the host immune systems and resist drugs. In specific, a case of swine flu resistant to Tamiflu was observed in Denmark on June 29, 2009 for the first time, and soon after that in Japan and Hong Kong [189].

Hemagglutinin and neuraminidase (NA), the two glycoproteins located on the surface of the influenza virus, have long been considered as potential targets for the development of antiviral agents. NA is responsible for viral release from infected cells and viral transport through the mucus in the respiratory tract. NA, also called sialidases, cleave terminal sialic acid residues from glycol-conjugates, promoting the release of newly formed virus particles from infected cells. The three pathogenic viruses, the 1918 Spanish flu (H1N1), the 2003 Avian flu (H5N1) and the recent 2009 Swine flu (A/H1N1), all share the same subtype 1 (N1) of neuraminidase. Thus, influenza neuraminidase has been considered as an attractive target for the development of new drugs for influenza [190].

1.2.2.1 Mechanism of action of neuraminidase inhibitors

The neuraminidase inhibitors zanamivir and oseltamivir interfere with the release of progeny influenza virus from infected host cells, a process that prevents infection of new host cells and thereby halts the spread of infection in the respiratory tract (**Figure 1.5**). Since replication of influenza virus in the respiratory tract reaches its peak between 24 and 72 hours after the onset of the illness, drugs such as the neuraminidase inhibitors that act at the stage of viral replication must be administered as early as possible. As a class, neuraminidase inhibitors are effective against all neuraminidase subtypes and therefore, against all strains of influenza, a key point in epidemic and pandemic preparedness and an important advantage over the adamantanes, which are effective only against sensitive strains of influenza A. These new drugs, if used properly, have great potential for diminishing the effects of influenza infection.

1.2.2.2 Development of neuraminidase inhibitors

All influenza viruses bear two surface glycoproteins, a hemagglutinin and a neuraminidase, which are the antigens that define a particular strain of influenza. The variation in hemagglutinin and neuraminidase molecules over a period of time permits the virus to evade human immune responses and therefore necessitates the formulation of a new vaccine each year. The hemagglutinin is a sialic acid receptor–binding molecule and mediates entry of the virus into the target cell. Neuraminidase, the target molecule for neuraminidase inhibiting compounds cleaves the cellular-receptor sialic acid residues to which the newly formed particles are attached (**Figure 1.5**). This cleavage releases the viruses, which can now invade new cells. Without neuraminidase, infection would be limited to one round of replication, rarely enough to cause disease. Neuraminidase may also facilitate viral invasion of the upper airways, possibly by cleaving the sialic acid moieties on the mucin that bathes the airway epithelial cells [191].

The ability of transition-state analoges of sialic acid to inhibit the influenza neuraminidase was first recognized in the 1970s [192-194]. Potent inhibitors such as zanamivir

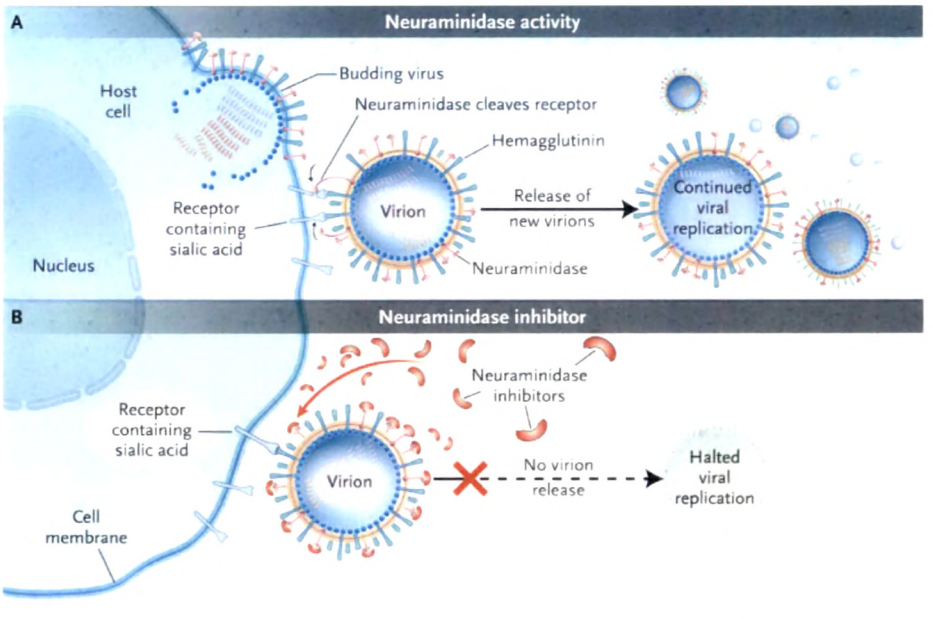

Figure 1.5 Mechanism of Action of Neuraminidase Inhibitors. Panel A shows the action of neuraminidase in the continued replication of virions in influenza infection. The replication is blocked by neuraminidase inhibitors (Panel B), which prevent virions from being released from the surface of infected cells.

closely mimic the natural substrate, fitting into the active site pocket and engaging the protein in the most energetically favorable interaction [195-197].

1.2.3 Aromatase as a target for the treatment of breast cancer

Breast cancer is a malignant (cancerous) growth originating from breast tissue, most commonly from the inner lining of milk ducts or the lobules that supply the ducts with milk [198]. Worldwide, breast cancer is the most common cancer in women after skin cancer, representing 16% of all female cancers. In 2009, breast cancer caused 464,000 deaths worldwide (7% of cancer deaths) [199].

The biology of breast carcinoma is complex with multiple factors contributing to its initiation and progression. The biomarkers that play key role in breast cancer development are steroid receptors [estrogen receptor (ER), progesterone receptor (PgR), retinoic acid receptor (RAR-β)) and selected suppressor/susceptibility genes (p^{53}, BRCA1, BRCA2)]. Prolonged exposure to circulating estrogens increase risk of breast cancer by mutation in selected tumor suppressor genes (p^{53}, BRCA1, BRCA2) accounting for 25% of all breast cancers [200,201]. Approximately 70-80% of breast tumors are ER positive (ER+). There are two types of estrogen receptors, ER-α and ER-β. Estrogens are the main ligands for these receptors. Prolonged exposure to circulating estrogens contributes to increased incidences of breast cancer. Removal of estrogens via oophorectomy decreases the risk of breast carcinoma [202]. In postmenopausal women, large part of estrogens in the breast tissue is derived from *in situ* biosynthesis using androgens as a substrate. Major sites of estrogen production are ovaries in premenopausal women, placenta in pregnant women and peripheral tissues such as adipose, muscles and breast tissues in postmenopausal women [203].

1.2.3.1 Estrogens

Estrogens are essential regulators of many physiological processes including maintenance of the female sexual organs, the reproductive cycle and numerous neuroendocrine functions. In breast cancer, through binding to their target receptors, they promote proliferation of breast cancer cells [204]. Estrogens enhance growth and proliferation of certain target cells, such as breast epithelial cells and estrogen-dependent mammary carcinoma cells [205]. The binding of estrogens to receptor is the initial step in estrogen-mediated stimulation of DNA synthesis and cell replication in target tissue [206].

The etiology of breast cancer has a strong hormonal component. Once an epithelial cell of ductal system is transformed into a malignant phenotype, it is no longer subject to normal growth controlling mechanisms. A malignant cell may also be non-

invasive, i.e. unable to penetrate the basement membrane (*in situ* cancer) [207]. Ductal carcinoma *in situ* is the most common histological variant of the non-invasive stage of breast cancer. Similarly, invasive or infiltrating duct carcinoma is the commonest form of breast cancer accounting for 85 to 90% of all cases.

1.2.3.2 Estrogen biosynthesis

The initial step in biosynthesis of estrogens is cleavage of cholesterol side chain to yield pregnenolone which is catalyzed by desmolase (**Figure 1.6**). Subsequent multi-step process gives androgens, androstenedione and testosterone. Final and rate limiting step in

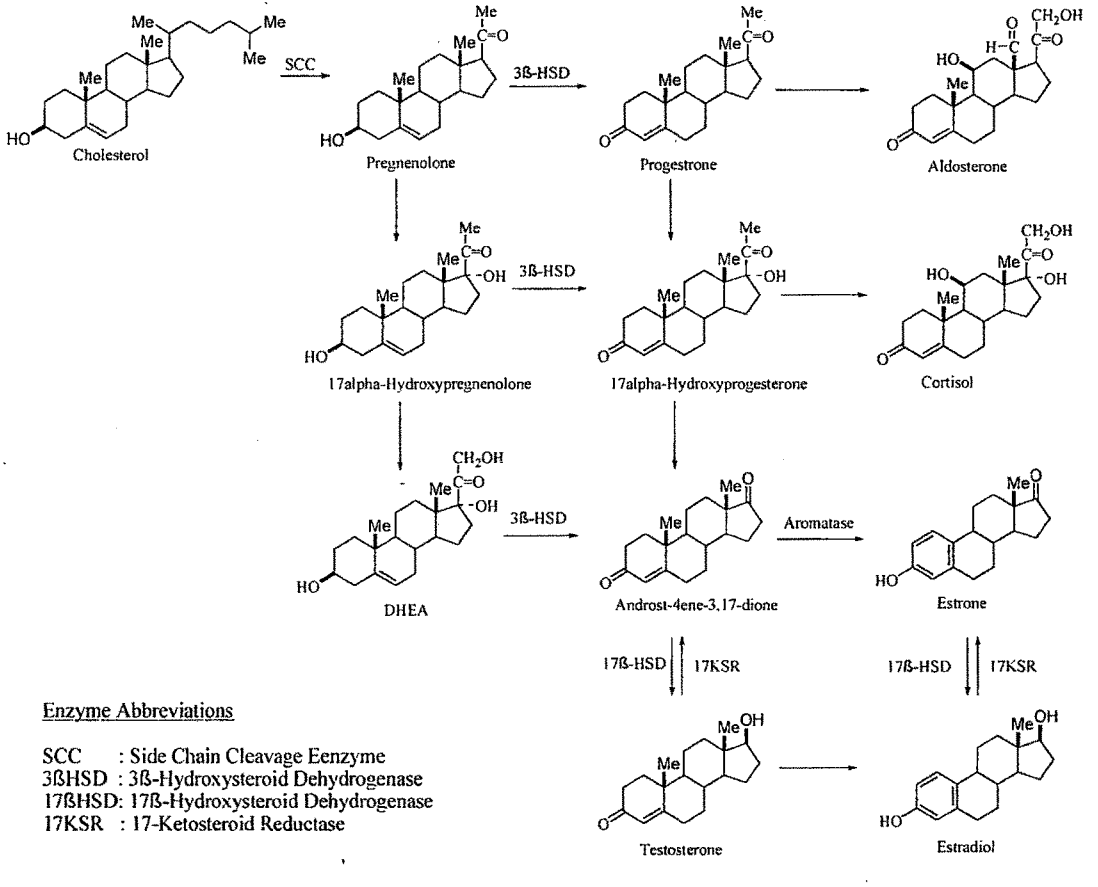

Figure 1.6 Steroidogenic Pathway

estrogen biosynthesis is aromatization of ring A and removal of C-19 of androgens. This is catalyzed by a cytochrome P450 enzyme complex called aromatase [208]. Estradiol is the most potent endogenous estrogen which can either be directly synthesized from testosterone or through interconversion between estrone and estradiol catalyzed by 17β-hydroxysteroid dehydrogenase. Very little amount of estrogens may be available from

estrogen sulfate by action of estrogen sulfatase. Steroid sulfatase catalyses conversion of inactive sulfate conjugated steroids to active nonconjugated forms. Estrone sulfate is considered as a reservoir for active estrone due to its prolonged half life and increased concentration in plasma [209].

1.2.3.3 Aromatase

Aromatase was first isolated by Ryan in 1959 from the microsomal function of fresh human placental tissue [210]. Aromatase is the cytochrome P450 enzyme that converts androgens including androstenedione and testosterone to the estrogen products estrone and estradiol, respectively [211]. This enzyme plays a key role in the regulation of these sex steroids. The aromatase gene, designated as CYP19, encodes the cytochrome $P450_{arom}$ and consists of 10 axons, with the exact size of the gene exceeding 70 kilobases. The gene is located on chromosome 15q21.1. The full length cDNA of 3.4 kilobases encodes for a protein of 503 amino acids with a molecular weight of approximately 55,000 Daltons [212]. The amino acid sequence of $P450_{arom}$ is distinct from other members of the P450 cytochrome family and drugs have been developed with selectivity towards the cytochrome P450 moiety in aromatase, permitting more specific inhibition.

The enzyme complex is bound in the endoplasmic reticulum of the cell and is comprised of two major proteins [213, 214]. One protein is *cytochrome* $P450_{arom}$, a hemoprotein that converts C19 steroids (androgens) into C18 steroids (estrogens) containing a phenolic A ring. The second protein is *NADPH-cytochrome P450 reductase*, which transfers reducing equivalents to cytochrome $P450_{arom}$. Three moles of NADPH and three moles of oxygen are used in the conversion of one mole of substrate into one mole of estrogen product [215, 216].

1.2.3.4 Mechanism of aromatase

Aromatization of androstenedione proceeds via three successive oxidation steps, with the first two being hydroxylations of the angular C-19 methyl group (**Figure 1.7**). First step is hydroxylation at angular C-19 methyl group to afford 19-hydroxy intermediate; the reaction occurs with classical mechanism of P450 enzymes [217,218]. The second hydroxylation reaction stereoselectively removes the 19-pro-*R* hydrogen without an apparent kinetic isotope effect to yield the 19-gem-diol, which may dehydrate to the 19-aldehyde [219]. The third step oxidatively cleaves the C_{10}-C_{19} bond, resulting in aromatization of the steroid A-ring and release of formic acid. A number of mechanisms

have been proposed for the aromatization step. One of the mechanisms which received significant favor involves nucleophilic attack on the 19-aldehyde by the reduced ferrous dioxygen intermediate. The resulting peroxo hemiacetal is suggested to decay via a process by which the proximal oxygen atom removes the 1β-hydrogen, resulting in aromatization of the steroid A ring and release of formic acid [220].

Recently, a novel alternative mechanism was examined by Hackett *et al* in which the widely accepted cytochrome P450 oxidant iron oxene catalyst abstracts the 1β-hydrogen atom from compound I initiating the aromatization and deformylation cascade (**Figure 1.8**). The steroid models that contain the 2,3-enol moiety have a strikingly low barrier for 1β-hydrogen atom abstraction (< 7 kcal/mol) due to the ability of the enolized A ring to delocalize the impending radical. The transition states containing the 2,3-enol

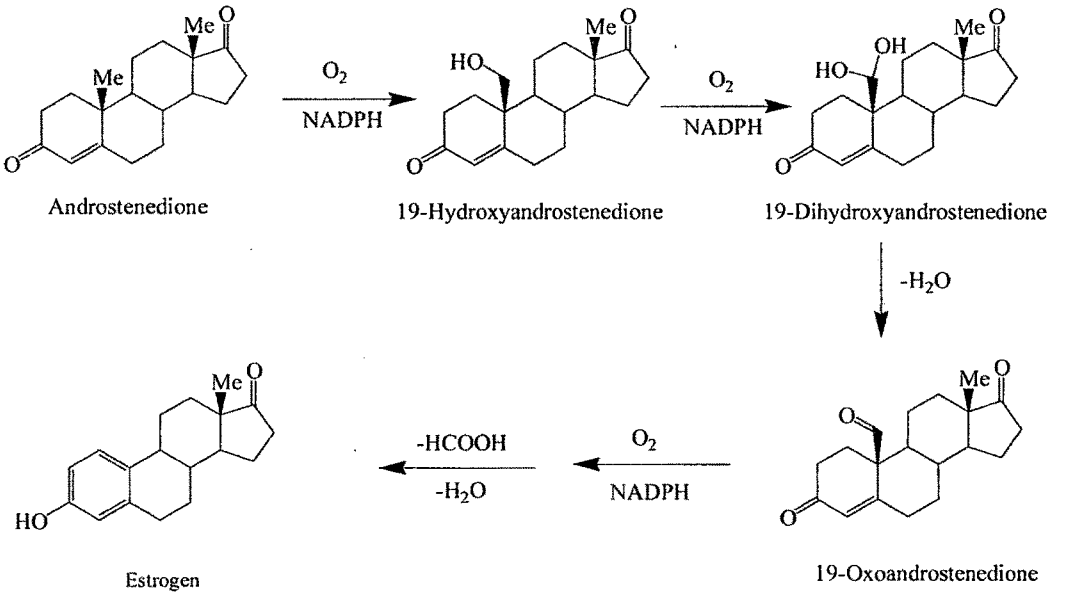

Figure 1.7 Reaction mechanism for estrogen biosynthesis by aromatase

moiety and the 19-gemdiol decay directly to the aromatized product, formic acid and the aqua bound model cytochrome P450 enzyme. Analysis of the reaction vectors indicate that the second hydrogen transfer occurs with a concerted nonsynchronous mechanism without an energy barrier. Thus, these calculations support a final catalytic step of aromatase involving the cytochrome P450 oxene intermediate, 1β-hydrogen atom abstraction and release of formic acid (**Figure 1.8**) [221].

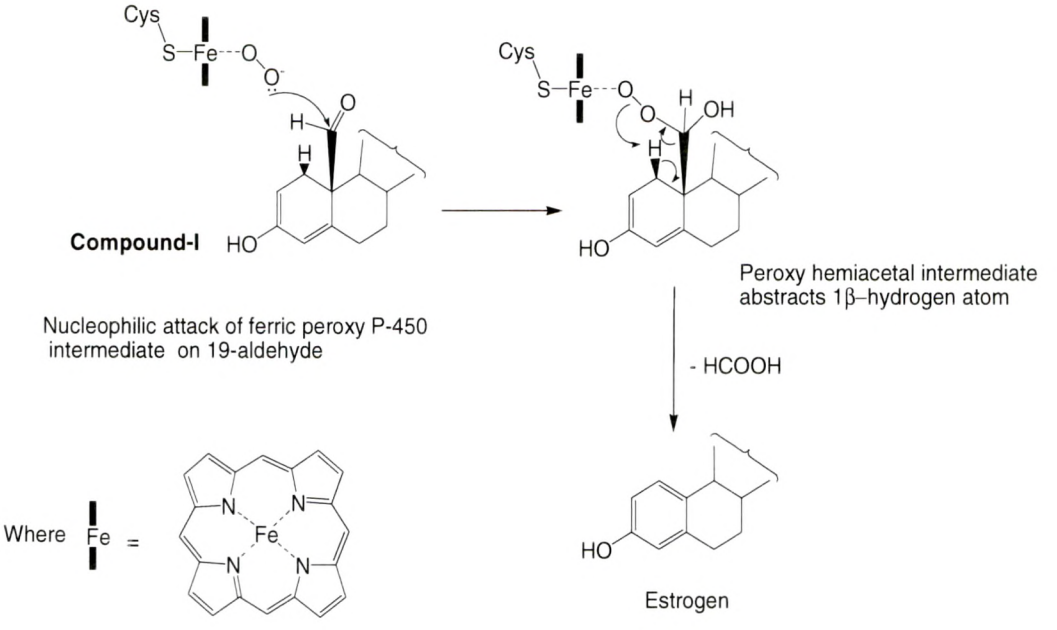

Figure 1.8 The third step of the oxidation mechanism of aromatase, proposed by Hackett *et al.* [221]

1.2.3.5 Structure of aromatase

Ghosh *et al.* identified the crystal structure of human placental aromatase enzyme having androstenedione as a co-crystallized ligand and deposited in protein data bank with ID 3EQM (**Figure 1.9a**) [222] The residues comprising the catalytic cleft of aromatase enzyme are Ile305, Ala306, Asp309 and Thr310 from the I-helix, Phe221 and Trp224

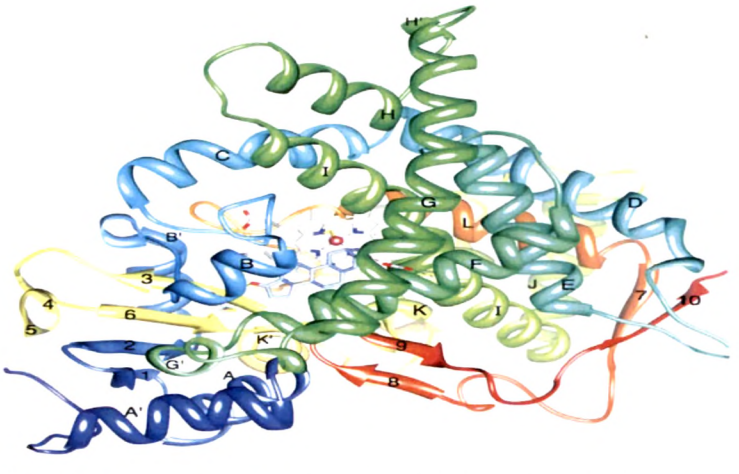

Figure 1.9a A ribbon diagram showing the overall structure. The N-terminus, starting at residue 45 is colored dark blue and the C-terminus ending at residue 496 is colored red. The α-helices are labelled from A to L and β-strands are numbered from 1 to 10. The heme group, the bound androstenedione molecule at the active site and its polar interactions are shown.

from the F-helix, Ile133 and Phe134 from the B-C loop, Val370, Leu372 and Val373 from the K-helix–b3 loop, Met374 from b3 and Leu477 and Ser478 from the b8–b9 loop. The 17-keto oxygen of the substrate makes a hydrogen bond (2.8 Å) with the back-

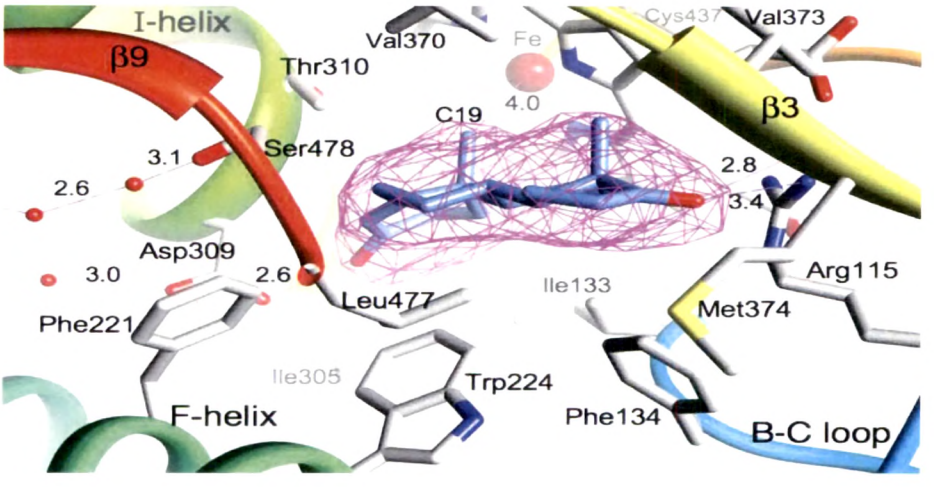

Figure 1.9b A close-up view of the active site showing the bound androstenedione molecule.

bone amide of Met 374 and a weak contact (3.4 Å) with NH1 of Arg115 (**Figure 1.9b**). The 3-keto oxygen is 2.6Å from the carboxylate $O_{\delta 2}$ of the Asp309 side chain indicating that the carboxylate moiety may be protonated.

1.2.3.6 Aromatase localization

In the case of humans and a number of higher primates, there is a more extensive tissue distribution of estrogen biosynthesis. Aromatase also occurs in the placenta and liver of the developing fetus, as well as in the adipose tissue of the adult. In premenopausal women, the ovaries are the principal source of estrogens which function as circulating hormones to act on different target tissues. In postmenopausal women and in men, estradiol does not appear to function as a circulating hormone; instead, it is synthesized in a number of extra-gonadal sites such as breast, brain and bone where its actions are mainly at the local level as a paracrine or intracrine factor. Thus, in postmenopausal women and in men, circulating estrogens are not the drivers of estrogen action; instead, they reflect the metabolism of estrogens formed in these extra-gonadal sites and they are reactive rather than proactive [223] Therefore, the aromatase enzyme can be found in many tissues including gonads, brain, adipose tissue, placenta, blood vessels, skin, bone, endometrium as well as in tissue of endometriosis, uterine fibroids, breast

cancer and endometrial cancer. The enzyme is located in the endoplasmic reticulum of the cell and its activity is regulated by tissue specific promotors that are in turn controlled by hormones, cytokines and other factors. Factors known to increase aromatase activity include age, obesity, insulin, gonadotropins and alcohol. Aromatase activity is decreased by prolactin, Anti-Mullerian Hormone (AMH) and smoking [224].

1.2.3.7 Aromatase in breast cancer

Approximately two-thirds of the newly diagnosed breast cancers in the U.S. are hormone dependent breast cancers, requiring estrogens for tumor growth. Aromatase activity has been demonstrated in breast tissue *in vitro* [225-228]. Furthermore, expression of aromatase is highest in or near breast tumor sites [226-229]. The regulation of aromatase expression varies due to the presence of different promoters in each tissue [230,231]. The increased expression of aromatase cytochrome $P450_{arom}$ observed in breast cancer tissues has been associated with a switch in the major promoter region utilized in gene expression [226]. The concentration of estradiol present in breast tumors of postmenopausal women is at least 20-fold greater than that present in the plasma[232]. With aromatase inhibitor therapy, there is a precipitous drop in the intratumoral concentrations of estradiol and estrone together with a corresponding loss of intratumoral aromatase activity [236]. This observation suggests that aromatase expression in the breast is the main source of estrogen driving breast cancer development.

1.2.3.8 Aromatase inhibitors in the treatment of breast cancer

Estrogens can influence the risk of breast cancer and also the growth of established tumors. Hormone-dependent breast cancer tumors depend on estrogens for growth. Two approaches treating these cases of breast cancer are either blocking the mechanism of action of estrogens or inhibiting their synthesis. These therapies are particularly helpful in postmenopausal women in whom hormone response is common and estrogen synthesis is primarily peripheral (adipose tissue, muscle and breast tissue) rather than in the ovaries. A large number of aromatase inhibitors have been developed and utilized in clinical studies over the last 20 years. This development was prompted by the recognition of the fact that the cytochrome P450 inhibitor aminoglutethimide is an aromatase inhibitor and exerts its therapeutic effectiveness in postmenopausal women with advanced breast cancer via the inhibition of aromatase [233, 234].

Aromatase inhibitors are generally used clinically for metastatic hormonal-dependent breast cancers, often in postmenopausal women when antiestrogens become inefficient or are not well tolerated [235].

1.2.3.9 Aromatase inhibitors

Aromatase inhibitors that have been used clinically can be categorized by the generation and by the mechanism of action. They are described as first, second and third generation inhibitors according to the order of their clinical development. They can also be classified as type I and type II according to their mechanism of action. Type I and type II inhibitors are also known as steroidal and nonsteroidal inhibitors, [235,236] respectively as shown in **Table 1.1.**

The type I agents are either competitive inhibitors which are structurally related to the substrate or suicide inhibitors derived from androstenedione like 4-OHA or exemestane. The type II agents behave as competitive inhibitors, coordinating one of their heteroatoms (N, S, and O) to the iron in the heme of the cytochrome.

Table 1.1 Types of aromatase inhibitors

Generation	Type-I Inhibitors (Steroidal)	Type-II Inhibitors (Nonsteroidal)
First	Testolectone	Aminoglutethimide
Second	Formestane	Fadrozole Rogletimide
Third	Exemestane	Vorozole Anastrozole Letrozole