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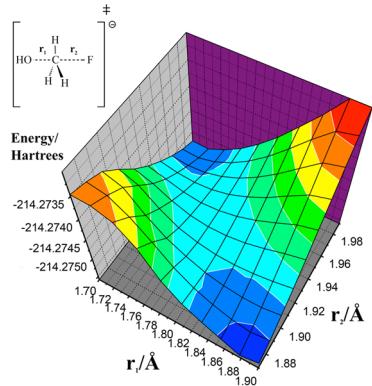
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Integrated Approach to Structure-Based Enzymatic Drug Design: Molecular Modeling, Spectroscopy, and Experimental Bioactivity

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1. INTRODUCTION

Analysis of the literature involving computational modeling of diseases and drug design with the aid of experimental spectroscopic techniques reveals that this specific combination of research areas calls for a thorough review paper. The impetus of this review is more directed toward experimentalists, those that perform drug synthesis and *in vitro* testing as well as those that are involved in biochemical studies of proteins. Therefore, more attention was given to the explanation of computational aspects of this field of study since most experimentalists would largely benefit from that. It is assumed that experimentalists have suitable background knowledge of spectroscopic techniques and therefore less attention was given to that.

Structure-based drug design is of great importance in the search for potential novel drugs. In this regard, computer-aided approaches are useful for applications ranging from interpretation and verification of experimental spectroscopic results to modeling the interaction of high-affinity inhibitors with a specific receptor site. Ongoing progress in the field of integrated computational, biophysical analysis, and biochemical/biological (*in vitro* and *in vivo*) approaches opens up new directions in structure-based drug design. This review deals with the contribution of computational modeling supplemented with applied spectroscopic techniques in an integrated approach to characterize drug–receptor interactions. We propose three integral components for optimal drug design:

- Molecular modeling (sections 2 and 3).
- Experimental binding activities, preferentially obtained from thermal titration calorimetry experiments (section 4).
- Biophysical analytical measurements that can be linked to experimental bioactivities and/or to a computational model (section 5).

A summary of the available tools for these fields is provided in Tables 1 (section 1), 2 (section 3), and 3 (section 5).

Although it is widely acknowledged that such an integrated approach is advantageous in drug design,^{1,2} this review demonstrates that actually few researchers are practicing this philosophy.

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Table 1. Computational Methods Used for Drug Design

methods	description	section
Bioinformatics	Diverse tools aimed at providing structural and statistical information about proteins including enzymes and inhibitor–enzyme complexes	2
Molecular graphics	Various software tools to create, present, and modify 3D molecular structures	3.1
QSAR	Mathematical framework which correlates physicochemical properties, the so-called descriptors, of lead compounds and their bioactivities; with QSAR studies it becomes possible to predict the activity of modified or related compounds; no information about the target is required	3.2.3
Homology modeling	Computational approach which generates reasonable protein 3D structures using a 3D model from a template protein sequence of one or more homologous proteins of which the structure is known	3.2.1
3D NMR solution structures	Structural information derived from short interproton NOE distances supplemented by torsion angles; dedicated software is used to derive a 3D protein structure (can take several months); a less accurate structure can be obtained using normal MD calculations with a number of carefully chosen interatomic constraints determined with NMR techniques (quicker method)	3.2.2
Molecular docking/virtual screening	Molecular docking is used for both exploring possible conformations of a ligand inside the binding site of a protein and estimation of their binding affinities; when the process is automated for a larger set of ligands, the approach is called virtual screening	3.3.3 and 3.4
Molecular dynamics	Powerful tool for exploring mechanisms, dynamics, and energetics of proteins and protein–ligand complexes	3 and 3.3.4
QM/MM	Hybrid computational approach for more reliable simulation of enzymatic reaction mechanisms; regions of the enzyme active site are treated at the QM level, while the rest of the enzyme and surrounding water molecules are treated with MM	3 and 3.3.4

Optimal success in drug design is essential for both academic researchers as well as pharmaceutical companies. Integration of the three components for drug design is proposed to optimize and speed up the process. The link between the first two approaches (molecular modeling and bioactivity results) is quite logical, and most researchers in the field ensure that molecular modeling results are calibrated against bioactivity data.

Integration of the third component is required to speed up the drug design process by linking bioassay results with a biophysical analytical method (such as spectroscopy, X-ray electrochemical methods, etc.) or if possible to provide information about the interactions between the inhibitor and the active site. For example, if a specific NMR signal is shifted when the inhibitor effectively reacts with the receptor site, then it will be much faster to screen new compounds for activities using this bioanalytical method. First prize would certainly be if the computational model is also linked to the analytical method. This is possible if the analytical method provides structural information about the drug/enzyme interaction or if the computational model also calculates spectroscopic data (or other biophysical characteristic). Another example is if the backbone dihedral angles of the inhibitor inside the active site can be deduced with biophysical methods (NMR, CD, etc.). NMR methods can also be used to determine the position of the inhibitor with respect to the enzyme active pocket. Various other examples are discussed in section 6.

The review starts off by providing background of rational drug design and the computational tools required for such research, followed by a number of case studies. It demonstrates that computer modeling coupled with experimental spectroscopic techniques offers a powerful strategy in studies of enzymatic inhibitors and drug–receptor interactions. This approach enables researchers to obtain quantitative binding models of drug–receptor interactions, which are validated with available spectroscopic observations and applicable to a wide range of diseases.

Unfortunately it appears that the variation in bioassay values (IC_{50}) and binding constant data (K_a) is severely hampering effective calibration of molecular modeling models (see discussion in section 4).

For the purpose of this review, the terms computational chemistry and molecular modeling are used interchangeably. The term *in silico* is commonly used in the literature and refers to experiments that are carried out on computers. It was first

used in public during the 1989 workshop “Cellular Automata: Theory and Applications” in Los Alamos, NM. Pedro Miramontes, a mathematician from the National Autonomous University of Mexico (UNAM), presented the report “DNA and RNA Physicochemical Constraints, Cellular Automata and Molecular Evolution”. In his talk, Miramontes used the term “*in silico*” to characterize biological experiments carried out entirely in a computer. The work was later presented by Miramontes as his Ph.D. dissertation.³

While there are an increasing number of reviews about computational studies of different categories of inhibitors, the same is not true for the combination of computational studies and spectroscopic experimental techniques and for the design of new enzyme inhibitors. A multitude of articles^{4–18} about structure-based drug design involving various computer-based methods to justify and confirm various types of spectroscopic data have been published but provide few examples where an integrated approach was followed.

It is clear that researchers are increasingly using the terms drug design, molecular modeling, and spectroscopy in their manuscripts (see Figure 1), suggesting that the concept of an integrated approach is taking off.

However, this review demonstrates that to date only about 150 publications have appeared that used an integrated approach where molecular modeling, spectroscopy, and drug design were combined. As is clear from a closer inspection of these 150 odd reports, a considerable number of them have employed all three proposed components but have not fully integrated all of them.

A number of reports on the combination of these topics have appeared^{6,7,19,20} but without truly integrating them. Hambley et al.¹⁹ discussed the combination of modeling Pt/DNA interactions in combination with NMR spectroscopy. Even though this paper is applicable to cancer drug design, it did not focus on that as such. Van Dongen et al. reviewed studies where NMR spectroscopy, homology modeling, and X-ray crystallography were used in the early drug discovery process.⁶ However, the review does not refer to any papers where an integrated drug design approach using both spectroscopic techniques and molecular modeling was applied. Rush et al.⁷ presented successful applications of X-ray, NMR, and molecular modeling in the design of inhibitors of matrix metalloproteinases (MMPs) and tumor necrosis factor-alpha (TNFalpha)-converting enzyme (TACE) from Wyeth, a pharmaceutical company. Although they combined these topics in their article,

Table 2. Biophysical Methods Used for the Analysis of Drug/Enzyme Interactions

methods	description	section
Circular dichroism spectroscopy	CD provides information about the secondary structure of peptides/proteins; it measures the optical activity of the molecule at variable wavelengths	1, 5, and 6
Fluorescence spectroscopy	Several different types of fluorescence techniques exist; in all cases a fluorescent tag should be attached to (typically) the guest molecule; the wavelength of the fluorescence emission changes as a result of host-guest interactions (sometimes the intensity of the signal also changes, or it is quenched)	1, 5, and 6
Infrared spectroscopy ^a	Signals from amide groups (CONH) in proteins are quite prominent and extensively used to determine structural information about proteins; phosphodiester groups are sensitive to hydrogen bonding; lipids can be studied through the ester group	1, 5, and 6
NMR spectroscopy	Measures ¹ H, ¹³ C, ¹⁵ N, and even some magnetic resonance signals of metal ions in biomolecules; it can provide structural information (through-space proton-proton interactions (NOE), backbone torsional angles, etc.) to study inhibitor/enzymes complexes; it provides solution-phase information and is a relatively fast technique	1, 3, 2, 2, 3, 4, 5, and 6
Surface plasmon resonance (SPR) spectroscopy	SPR makes use of a light beam that reflects from a surface covered with a biomaterial; a quantum resonance effect between the photons and the valence electrons of the biomaterial is observed; this will change when an inhibitor in solution is transported (pumped) over the biomaterial attached to the solid surface	1, 5, and 6
X-Ray crystallography	Method of determining the atomic and molecular structure of a crystal that includes biological systems (such as proteins, lipids, etc.), organic and organometallic compounds, minerals, and metals; X-ray crystallography has led to a better understanding of protein structures and mechanisms, extremely valuable if the structure of an inhibitor/enzyme complex is obtained; it is not facile to grow high-quality crystals of biomolecules	1, 2, 5, and 6
Several other biophysical methods were described in this paper		5 and 6

^aIt is well known⁵⁹⁴ that calculated IR data gives a 10–15% error toward higher frequencies. Some software automatically compensates for that.

the bigger part of the review covers their own reports on solution structures for these enzymes using NMR data with the NMRPipe software. They also described the faster method of NMR structure determination using NOE data with restricted MD calculations. Fanelli and De Benedetti^{20,21} reported a combination of computational modeling, X-ray, and NMR to study the structure-function analysis of G protein-coupled receptors (GPCRs). In their 2005 review²¹ they referred to one paper where spectroscopic information was used to validate a 3D-generated model²² and one group^{23–25} that used NMR data to model the seven helix bundle of several GPCRs. In the extended 2011 review²⁰ they referred to a new case²⁶ where the mechanism of the conversion of rhodopsin to bathorhodopsin was investigated by QM/MM methods and supplemented by Raman spectroscopy data.

The main thrust of our review is therefore broader than the existing review papers in the field.

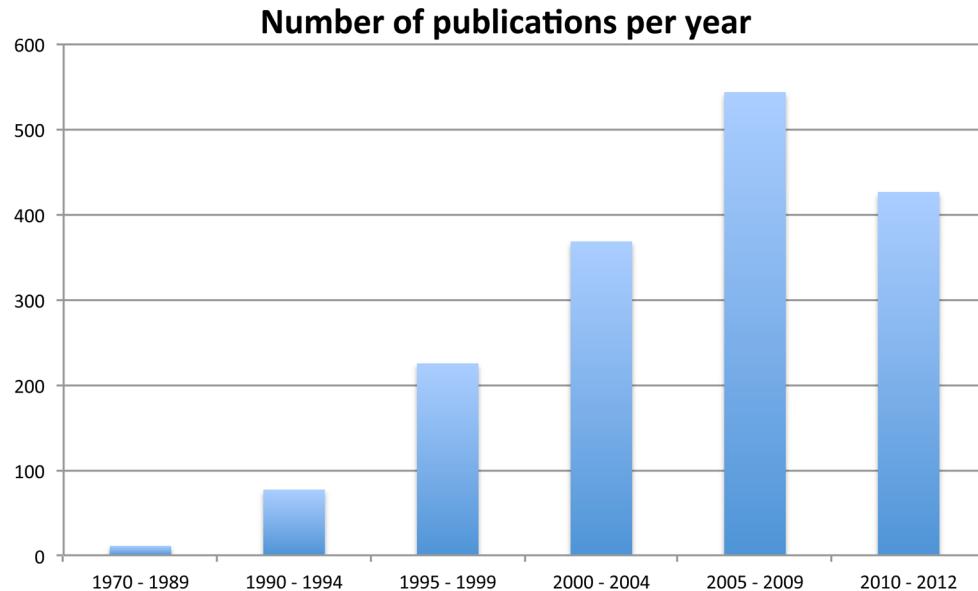
The ultimate goal of drug design is to identify and propose potential molecules that act against a specific target of a particular disease. Expansion of a library of receptor targets with known three-dimensional structures will motivate researchers to design novel potential inhibitors, which may function as drugs that bind to the receptor's active site(s).^{27–30} With this in mind, the ability of computational methods to calculate the binding affinity of inhibitors toward enzymes is of great importance to probe the structural determinants involved in recognition and stabilization of the enzyme-inhibitor complex.³¹

From the late 1980s to the mid 1990s unrealistic expectations were created for methods of computer-aided structure-based drug design. This was partly the result of the fast development and much improved computer hard- and software at the time. Fast expansion of computational resources created optimism to study larger and more complex biological systems which would then enable researchers and pharmaceutical companies to solve many problems experienced in the drug discovery process.^{32,33} It was widely believed that new computational techniques were powerful tools capable of predicting molecular properties that are inaccessible to experimental probes. Scientists have propagated that computational methods significantly minimize time and resource requirements of chemical synthesis and biological tests, thereby reducing the cost of preclinical and clinical studies.^{16,31,34} It was argued that the trend of a reduced number of new drugs entering the market during the middle-late 1990s could be linked to the diminishing focus on traditional methods at the beginning of the 1980s in favor of computational methods as the driving technology for drug discovery.^{35,36} It then became clear that these expectations about computational methods were overoptimistic. Our review demonstrates that the field of rational drug design with the aid of molecular modeling has matured in the past decade, and it is now realized that an integrated experimental and theoretical approach is essential for optimum impact. Successful collaboration between scientists of various disciplines (biochemistry/microbiology/pharmacy, synthesis/analysis, and molecular modeling) is required to accelerate development of new and effective products in the drug discovery process.³⁷

Computer-aided drug design forms part of the research strategy called structure-based design, which is an iterative combination of experimental and computer-based approaches. The results of both of these approaches can be combined to generate hypotheses and ideas (and test them) about the

Table 3. In Vitro Methods To Determine the Bioactivities of Inhibitors

methods	description	section
IC ₅₀	Bioactivities are commonly reported as the half-maximal inhibitory concentration; half-maximal inhibitory concentration (IC ₅₀) is a measure of the effectiveness of a compound in inhibiting a biological or biochemical	1 and 4
Isothermal titration calorimetry (ITC)	Most reliable quantitative technique that can directly measure the binding affinity (K_a), enthalpy changes (ΔH), and binding stoichiometry (n) of the interaction between two or more molecules in solution; from these initial measurements Gibbs energy changes (ΔG) and entropy changes (ΔS) can be obtained	1 and 4
K_a and K_d	Association and dissociation equilibrium constants of the inhibitor/enzyme complex	1 and 4
High-throughput screening (HTS)	Experimental technique mostly adopted in pharmaceutical industry in order to screen thousands of drug-like compounds for binding or biological activity against disease-infected cells (ligand-based drug design) or against specific target enzymes (receptor-based) in order to deduce an optimal binding pattern or a SAR	2, 3.4, and 4

**Figure 1.** Number of publications per year that include the following keywords: drug design, molecular modeling, and spectroscopy. (Search results from the Web of Knowledge database, Dec 2012.)

binding modes of a series of inhibitors to a given macromolecular target such as an enzyme.³⁷

Receptors are often classified as mechanoreceptors, thermoreceptors, nociceptors, chemoreceptors, and photoreceptors that respond when subjected to mechanical displacement, temperature change, pain response, chemicals, and light, respectively. A drug that binds to a pathogenic receptor is often also called an inhibitor or ligand. There are several terms to describe the inhibitor, such as a guest or key, while the receptor is often called the host or lock.³⁸ In 1894 Emil Fischer³⁹ compared the exact fit of a substrate to the catalytic center of an enzyme with the picture of a “lock-and-key”.^{40–42} This term perhaps best describes the complementary nature, which is required for effective receptor–inhibitor interactions.

Inhibitors generally bind to a variety of receptors such as hormones, neuromodulators, DNA, or neurotransmitters and acetylcholine receptors (AChR).^{43,44} This review will focus mainly on enzyme-linked receptors where the binding of a ligand causes inhibition of the enzymatic activity.

An inhibitor that binds to an enzyme and blocks the activity can be either a reversible or an irreversible inhibitor. Reversible inhibitors bind through noncovalent interactions such as hydrogen bonds, hydrophobic interactions, and ionic bonds. These inhibitors do not undergo chemical reactions during the binding process. Such inhibitors can be removed by dilution or dialysis.⁴⁵ In contrast, irreversible inhibitors usually react with the enzyme and change it chemically via covalent bond formation. These inhibitors modify key amino acid residues

needed for enzymatic activity. Reversible inhibitors can further be classified as follows:^{45,46}

- Competitive (which results from the inhibitor having a relatively strong affinity for the active site of an enzyme; substrate and inhibitor compete for access to the enzyme’s active site);
- Noncompetitive inhibitors where the inhibitor binds only to the substrate–enzyme (S–E) complex (since this binding is in equilibrium, the inhibitor prevents conversion toward product);
- Mixed inhibitors where the inhibitor binds to the enzyme at the same time as the enzyme’s substrate;
- Noncompetitive inhibitors which is a form of mixed inhibition where binding of the inhibitor to the enzyme reduces its activity but does not affect binding of the substrate. The inhibition potency depends only on the concentration of the inhibitor.^{46,47}

The field of drug design is divided in two major categories: ligand-based⁴⁸ and receptor-based⁴⁹ design. The first is related to knowledge of molecules that bind to the given biological target. This is normally used when information of the 3D structure of the target enzyme is not available. A key advantage is that novel lead compounds are obtained through slight chemical modifications to the active molecule, followed by changes in bioactivity. Knowledge of the structure–activity relationship (SAR) enables researchers to design more active compounds, based on the lead compound.

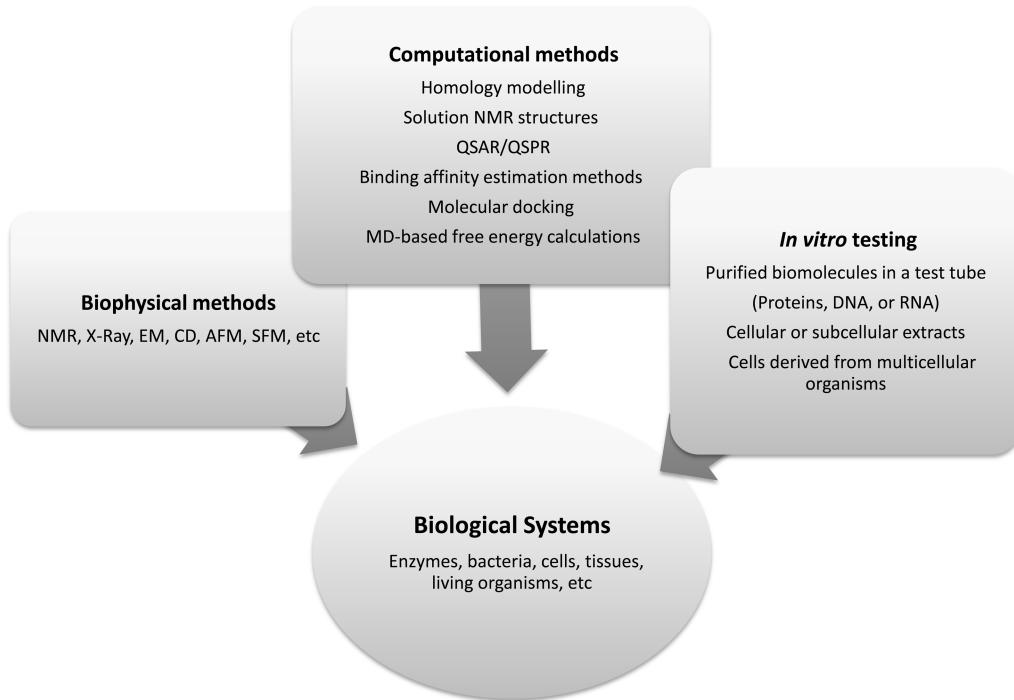


Figure 2. Flow chart of biochemical/microbial and computational approaches used to study biological systems during the drug design process.

The latter category relies on knowledge of the three-dimensional structure of the biological receptor yielded by X-ray crystallography, homology modeling, or NMR spectroscopy methods.⁴⁹ The key advantage is that novel structures can be proposed, based on the ability to fit the active site of the receptor. These two techniques contribute significantly to drug design and development and refinement of therapeutic agents.^{48,49}

In order to speed up identification of novel lead compounds, high-throughput screening (HTS) techniques were developed.^{50–52} HTS can be performed for both categories of drug design (ligand- or receptor-based methods). This is an experimental drug discovery process in which batches of drug-like compounds are tested for binding or biological activity against disease-infected cells (ligand-based drug design) or against specific target enzymes (receptor based) in order to deduce an optimal binding pattern or a SAR. HTS requires parallel screening of large numbers of chemicals with respect to genetic or pharmacological tests, and it has been very successful in providing numerous starting points for the design of new drugs.^{53,54} Combinatorial synthesis of inhibitors has also contributed significantly to the rapid expansion of the field of HTS.

To identify the bioavailability properties for compounds Lipinski's rules⁵⁵ are applied. These rules cannot predict whether a certain compound will be pharmacologically active; it merely gives an indication of whether the compound will be bioavailable. Poor absorption or permeation is more likely when there are more than 5 H-bond donors (nitrogen or oxygen atoms with one or more hydrogen atoms), 10 H-bond acceptors (nitrogen or oxygen atoms), the molecular weight (MWT) is greater than 500, and the calculated octanol–water partition coefficient⁵⁶ or Log P (C Log P) is greater than five (or M log P > 4.15).

Veber's drug-like filter appears to be an improvement and more general method for prediction of the bioavailability of a

new drug.⁵⁷ They suggest compounds which meet only the two criteria:

- Ten or fewer rotatable bonds;
- Polar surface area equal to or less than 140 Å² (or 12 or fewer H-bond donors and acceptors) will have a high probability of good oral bioavailability in animals/rats.

Despite the considerable success of HTS and combinatorial synthesis methods, these are still time consuming and relatively expensive. One of the shortcomings in the current drug discovery processes is that in too many cases not enough information is known about the specific target of the disease. Due to a gradual move toward rational drug design and enormous progress in various bioanalytical methods, the 3D structural information of more drug targets is becoming readily available. This has contributed significantly to the evolution of the rational drug design process with the aid of computational techniques.^{6,17,42,58,59} This approach has gradually become more popular in drug development since the first applications were reported in the 1950s. It often employs molecular software to construct and examine three-dimensional models of drugs and their interaction with known biological targets.^{60–62} The success of biophysical experimentalists to determine structural information such as single-crystal X-ray structures of ligand–receptor complexes as well as performing applied research at the interface of computational structural biology, biophysics, and biomaterials⁶³ really contributed to the acceleration of the field of rational drug design over the last couple of decades.

It is important to note that the crystal structure of an enzyme (or the enzyme–inhibitor complex) represents an average over all molecules in the crystal.³³ Hence, in the case of conformational differences for a side chain or ligand in the crystallographic structure, one must decide which conformation to use for simulation studies.⁶⁴ Theoretical researchers should also be reminded that any crystal structure of an enzyme complex represents only one snapshot of perhaps hundreds or

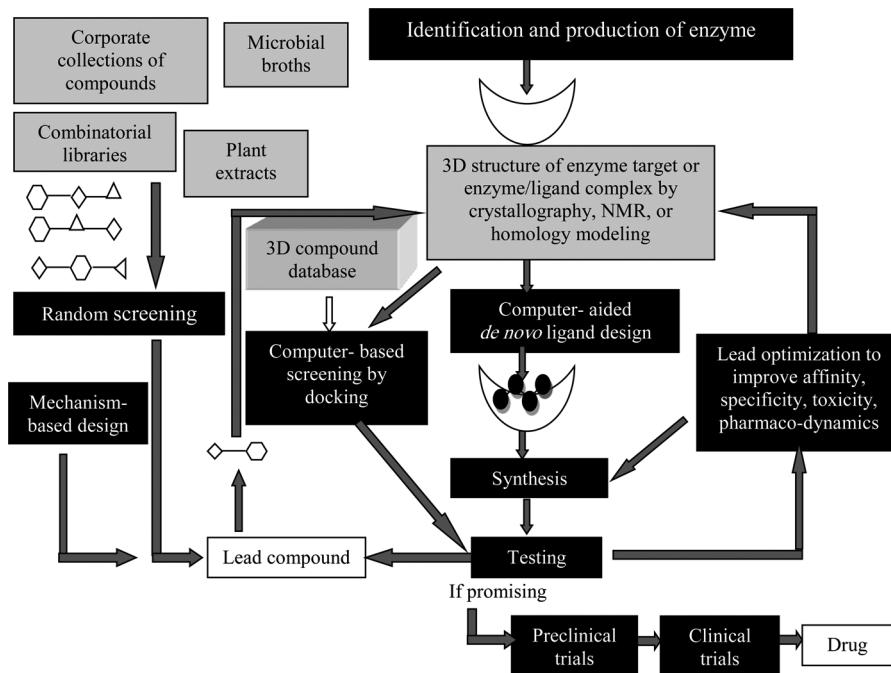


Figure 3. Structure-based drug design process. (Reprinted with permission from ref 37. Copyright 1998 American Physiological Society. Reprinted with permission from ref 100. Copyright 1994 Elsevier.)

more different pictures from the dynamic movement of atoms and molecules in such systems.^{10,33} In addition, the crystal data of the protein can have missing residues or point mutations. In such cases homology modeling is a logical option. Despite several potential pitfalls, in most cases computational-derived structures (gas phase or in solution) actually correspond extremely well with solid-state X-ray structures.⁶⁵

Scientists also employ a variety of other biophysical experimental methods such as NMR, electron microscopy (EM), atomic/scanning force microscopy (AFM/SFM),⁶⁶ circular dichroism (CD),⁶⁷ and surface plasmon resonance (SPR)⁶⁸ to obtain more structural information of ligands and ligand–receptor complexes.^{69–71} See section 5 for more details with respect to biophysical analytical methods.

A simple flow chart demonstrates the combination of biochemical/biological (*in vitro* and *in vivo*)^{72–74} and computational approaches for a better understanding of biophysicochemical drug–receptor interactions and practical applications in biomaterials and medicine (Figure 2).

As mentioned earlier, the ultimate goal of computer-aided design of various types of inhibitors is prediction of compounds that are expected to strongly bind to the receptor site, thereby interfering with or altering the normal activity of the bioactive host. Integration of computational and biochemical/biological (*in vitro* and *in vivo*) methods can eventually lead to effective prediction of structural information of the inhibitor–receptor complexes.^{75,76} For rational drug design,^{60,62,77} as many as possible experimental techniques such as bioassays, analytical (including structural determination) and physical chemistry (including biophysical analytical) techniques, and synthesis are combined with theoretical or computational methods.² The purpose of our review is to specifically look at the combination of molecular modeling and experimental techniques (bioassay data, structural methods, and spectroscopy) to facilitate the process of drug development.

Over the past decade, the majority of computational review articles have covered structural features of numerous inhibitors with particular attention to a better understanding of the interactions in receptor–inhibitor complexes which are summarized briefly. In this regard, various common computer-based techniques have been covered. These include QSAR approaches,^{78–83} free binding energy calculations,^{84,85} conformational search and energy minimization,^{86,87} ab initio^{88,89} (including DFT),^{90,91} molecular mechanics,⁸⁸ molecular dynamic simulations,^{30,54,59,85,92,93} homology modeling,^{94–96} and molecular docking^{54,82,86,97} among others.

Several recent papers^{37,60,98,99} have indicated that the combination of structural knowledge related to pharmacologically relevant targets with an experimental and computational approach can be effectively used for structure-based design of specific high-affinity inhibitors.³⁷ Figure 3 provides a schematic overview of this process. Comprehensive analysis of experimental and computational binding data provides new insight into the nature of the interactions between the inhibitor and the receptor or enzyme.

Numerous interesting papers^{37,101–105} have highlighted the synergies between experimental and computational methods, and one can expect more of these studies in future.

2. BIOINFORMATICS AND DATABASES: TOOLS FOR STRUCTURAL INFORMATION ABOUT ENZYMES AND ENZYME–INHIBITOR COMPLEXES

Data and databases are crucial to both bioinformatics and chemoinformatics. Undoubtedly, without easily accessible electronic data, the majority of searches would be fruitless and most kinds of predictive or analytical software may not have been developed or assessed.¹⁰⁶ Following the improvement of experimental X-ray crystallography, homology modeling, and NMR methods, the 3D structural data of thousands of bioactive receptors and protein crystal structures have appeared in the literature. Various experimental and

computational techniques also provide improved information about the structural dynamics and electronic properties of various types of ligands and enzymes. These factors have led to the rapid development and dramatic increase in structure-based drug design efforts.⁴⁹

It should be noted that even though several experimental techniques can provide information about drug–receptor interactions, high-throughput *in vitro* screening to identify probable interaction modes between a receptor and thousands of ligands is still relatively expensive.¹⁰⁷ In most cases the experimental methods also require utilization of supplementary modeling techniques. Unfortunately it appears that few current researchers choose to combine experimental techniques with computational chemistry methods to improve their understanding of the interaction between drugs and receptor sites.

Structural data of protein and ligand compounds obtained by crystallography,¹⁰⁸ homology modeling,^{95,109–115} or NMR^{116,117} is deposited in suitable 3D databases. Databases for these purposes include¹¹⁸ the UniProt Knowledgebase for protein sequences,¹¹⁹ ModBase,¹²⁰ and the Swiss-Model Repository¹²¹ for 3D protein structures generated with homology modeling. If the target protein is not available in any of these locations then homology modeling, which is discussed in the next section, can be used to predict/generate the 3D structure of the former protein. It is also important to obtain information, in the literature, about the active site of the specific target protein and if possible which amino acids segments are crucial in the mechanism of action of the enzyme–substrate reaction/interaction. This is required since it is normally expected that competitive inhibitors occupy a prominent position in close proximity of these amino acid segments inside the active site.

The second challenge is to find structural sources of potential ligand/inhibitor compounds. There are several databases¹²² of purchasable compounds including the following:

- Symyx Screening Compounds Directory (SCD);¹²³
- MDL Screening Compounds Directory;¹²⁴
- ChemNavigator iResearch library;¹²⁵
- National Cancer Institute database;¹²⁶
- The ligand info database,¹²⁷ which is a collection of numerous public databases of small molecules, i.e., ChemBank,¹²⁸ PDBe,¹²⁹ KEGG,¹³⁰ NCI,¹³¹ AKos GmbH,¹³² Asinex Ltd.,¹³³ and TimTec, which contains over 1.1 million entries;¹³⁴
- GDB database¹³⁵ (i.e., the Generated Database of Chemical Space of Small Molecules) which contains 26.4 million compounds, the vast majority of which have never been synthesized;
- ZINC database.¹³⁶

3. ENZYME–INHIBITOR INTERACTIONS: A COMPUTATIONAL POINT OF VIEW

Over the past three decades significant advances in computer-based techniques used for rational drug design have been made. This is the result of the almost logarithmic enhancement of computer performance due to improved hardware developments,^{137,138} supported to a lesser extent by enhanced computational code and software.^{139,140} There are two main levels of theory used in computational chemistry research, namely, force field and electronic structure methods.

The most practical of these two methods for modeling of enzymes involves molecular mechanics (MM) force field

software¹⁴¹ that is based on the classical laws of physics. These methods can model very large biological systems such as enzymes at a fraction of the cost (in terms of hardware and time) of electronic structure methods (ab initio and DFT methods). DFT methods are used for smaller biochemical systems,^{142–146} but hybrid methods^{143,146,147} are required for large biomolecular systems. The fundamental theory of these methods is well documented in various textbooks.^{138,140,148–152}

MM methods make use of severe approximations with respect to the electronic and steric nature of atomic interactions.¹⁵³ These approximations are based on parameters that are normally built on information from X-ray structures or extrapolated from high-level ab initio or DFT calculations on smaller model systems.

The approximations are embedded in the force field description for the MM calculation. This latter method is not suitable for calculations that involve bond formation/dissociation, as is the case for transition states¹⁵⁴ in chemical (see Figure 4) or biochemical reactions. It normally also overestimates through-space atom/atom interactions, which can only be accurately calculated with electronic structure methods.

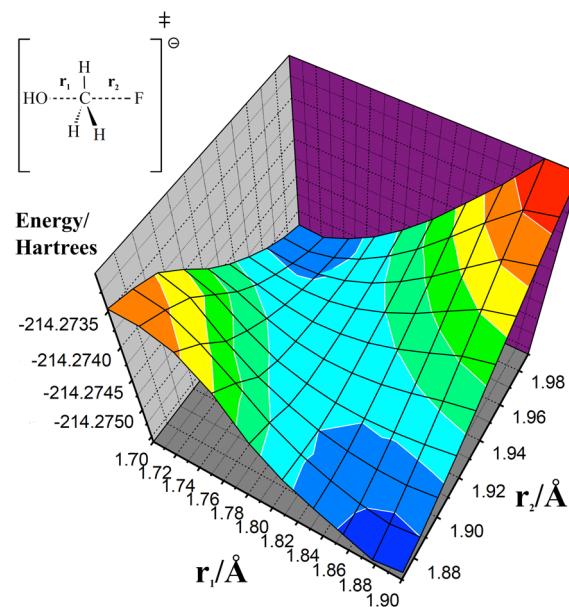


Figure 4. Typical saddle point energy profile of the reaction where F⁻ performs an S_N2 attack on CH₃OH. Data was generated with a relaxed scan calculation using the Gaussian software. A reasonable starting point for a transition-state optimization can be extracted from the scan output where $r_1 \approx 1.82$ and $r_2 \approx 1.92$ Å. Picture was created with Statistica.¹⁵⁵ (See section 3.3.4 for an example of a multidimensional free energy scan with QM/MM/MD.)

MM methods are parametrized methods designed normally to obtain static structures and energies relative to idealized systems. Force fields should therefore be chosen with caution as they are normally designed for a certain family of molecules. Force field development is an active field of research.^{88,156–158}

Numerous computational software¹⁵⁹ for MM modeling exist: Amber,^{160,161} Charmm¹⁶² (Chemistry at Harvard Molecular Mechanics), Dragon,¹⁶³ Gromacs,¹⁶⁴ MDScope (NAMD and VMD, Visual Molecular Dynamics),^{165,166} and Sybyl.¹⁶⁷ Most of these packages also include molecular

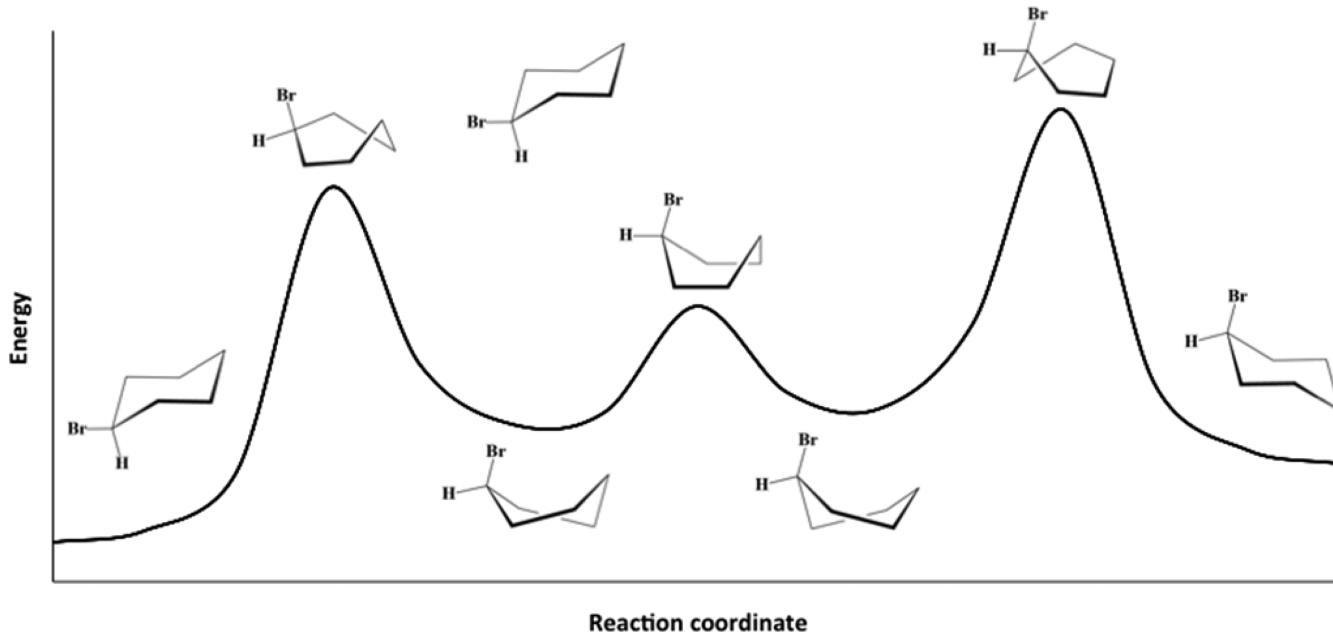


Figure 5. Theoretical energy profile for interconversion of bromo–cyclohexane conformations at room temperature.

dynamics (MD) options, and some of these packages also have quantum mechanical (QM) modules.

MD methods simulate the movement of the molecule(s) at a certain temperature (mostly at 300 K for biological systems). However, it is important to note that the MD temperature scale for sterically hindered compounds is not well calibrated. A well-known case is the conformational interconversion of cyclohexane which is experimentally known to occur at 300 K.¹⁶⁸ With MD methods at 400 K the twist boat form of cyclohexane oscillates between the different twist forms (see Figure 5), while at 600 K the molecule contains sufficient kinetic energy to convert between one of the rigid chair forms and the twisted form. Further increase of the temperature up to 1000 K yields both chair conformations as well as the twisted conformations. It is unclear whether it is a problem with the force field or with the MD algorithm. We tested this with various force fields (Amber, MM3, MFF, etc.), and all of them give the same result.

Proteins appear to reflect experimental observations with MD simulations at 300 K. However, AMBER force field studies from our group^{169–174} with severely hindered non-natural cage amino acids revealed that much higher temperatures (600–900 K) are required to explore the conformational space of short peptides involving extremely hindered cage amino acids.

When flexible shorter peptides or proteins are studied with MD methods, it should be realized that hundreds or even thousands of low-energy conformations are possible to be observed.^{169–174} Simulated annealing methods¹⁷⁵ can be used to complement MD studies. Statistical methods are required to ensure that the chances of finding new unique conformations are rather small (see Figure 6).¹⁷¹

Electronic structure or quantum mechanical (QM) methods based on quantum chemical theory are the most accurate but most expensive calculations in terms of computational resources and time. Such methods include *ab initio*^{176,177} including density functional theory (DFT)^{90,91,142–147,178–189} and semiempirical calculations.^{152,190,191} For more accurate QM calculations, electron correlation methods such as MP2, CCSDT, etc., are required. DFT methods approximate electron correlation.^{91,144,182–184} QM methods are currently only used

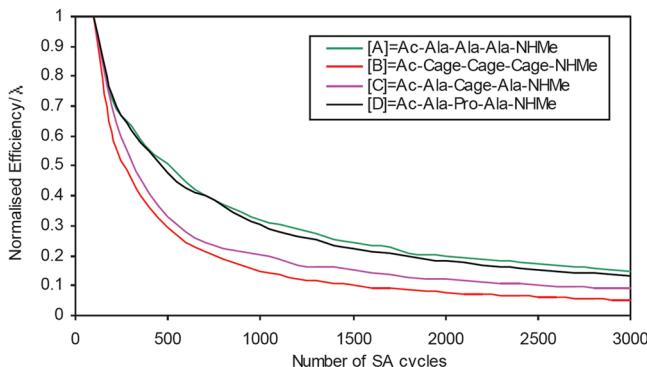


Figure 6. Plot of the normalized efficiency parameter (λ) against the number of simulated annealing cycles for a number of peptides. Graph gives an indication of the statistical chances to find new unique conformations after 3000 cycles (i.e., less than 10% for case B). (Reprinted with permission from ref 170. Copyright 2006 Elsevier.)

for smaller systems, due to the large demand for computer resources and the long time required for these calculations.

Semiempirical methods use QM calculations only for the valence electrons in the system and make drastic approximations for the other electrons as well as the behavior of the atom nuclei. As is the case for MM force fields, semiempirical methods (AM1, MNDO, PM3, PM6, etc.) are not universally applicable to different molecular systems. A literature search should be performed to confirm the applicability of the method for a specific system. Alternatively, it should be benchmarked against an affordable QM method.

In order to obtain the best of both computational methods, hybrid QM/MM methods were developed^{192–206} and applied.^{85,196,197,199,200,202,204,205,207–229} Most of these methods also include a module to perform QM/MM/MD simulations. The larger part of the protein is treated at the force field level, while the essential amino acid fragments in the active pocket as well as the inhibitors are treated with QM (in many cases using a semiempirical method). These methods are normally included in MM software. A related method in QM software

was developed, which was called Oniom (our own *n*-layered integrated molecular orbital and molecular mechanics).^{176,230–235} This method also uses different layers for the system but is operated from a QM package, usually without MD functionality. It is quite complicated to generate the correct input files for Oniom calculations. The Gausview software is very user friendly in this regard.

Several QM packages,¹⁵⁹ such as DMol3, Gamess, Gaussian, Jaguar, Molpro, Mopac, NWChem, Turbomol, etc.,²³⁶ are used by researchers. Pointing out the advantages and disadvantages of different software packages falls outside the scope of this review. In general, most of the comparable software, built on the same theory, can achieve similar results, if not identical; the same theoretical foundation and algorithms will ensure identical results, provided the same specific settings and input data are used. Differences in terms of user friendliness, specific applications, and perhaps implementation of faster codes are subtler and will require a more in-depth literature study and/or hands-on experience with the software. Beginners are advised to read the most recent literature and see which software appears to be more popular with researchers in the field. Note that proprietary software is in general more user friendly but more restricted in terms of freedom or flexibility to change settings, etc. It may be advantageous to start with proprietary software, especially if support for open source software is not readily accessible.

The question is often asked what exactly the calculated energy values mean or how do the numbers correlate with experimental binding energy data. In most cases it is better to work with relative energy differences since the total energies from a given program can only be compared relative to other energies with the same program and the same version using the exact same method and conditions.

Free energy ab initio calculations, which include both entropy and enthalpy contributions (and zero-point energy corrections), are the most reliable but in most cases not practical for very large systems. A rule of thumb is that a gain of about 5.7 kJ/mol (1.4 kcal/mol) according to the computational model corresponds to a factor of 10 in an equilibrium constant at 298 K (an increase in experimental binding affinity).³³

Most often calculated structures (MM and QM, gas phase or in solution) compare quite closely to X-ray data from the molecule in the solid state.^{65,237} In some cases, extreme packing forces can cause distortion of the expected 3D structure of the molecule. This can normally be explained if the packing in crystal structure data is carefully inspected.

In the next section, various computational tools that are most commonly used for drug design are described.

3.1. Interactive Molecular Graphics

Visualization software emerged in the late 1980s^{238–240} in order to create and explore graphical representations of computer science concepts. With these tools, data can be represented in two (see Figure 7), three, or even higher dimensions.²⁴¹ This software can read and display standard 3D structural data and allow for manual manipulation of the chemical structures, and it can be used to manually dock candidate ligands in the binding site of various enzymes.²⁴²

It should be noted that some of these packages can also perform molecular optimizations with MM or QM methods and are also commonly used to visualize large molecules due to their user-friendly features. Such software¹⁵⁹ include Argus-

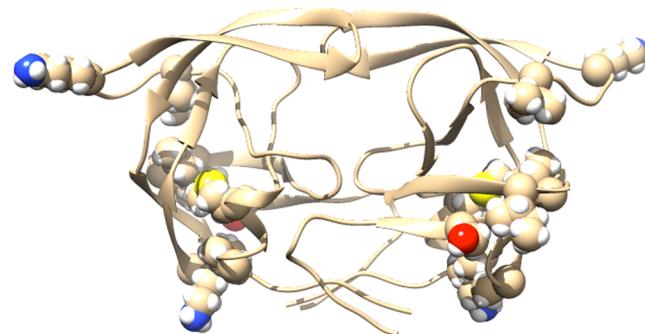


Figure 7. Different methods to present enzymes are possible. This is a representation of the C-SA wild-type HIV PR with the mutation positions (with respect to subtype B) highlighted using van der Waals radii. VMD software was used for this presentation.¹⁶⁶

Lab,²⁴³ Avogadro,²⁴⁴ ChemDraw,²⁴⁵ Chimera,²⁴⁶ DeepView,²⁴⁷ GaussView,²⁴⁸ Hyperchem,²⁴⁹ MacroModel,²⁵⁰ Molecular Operating Environment (MOE),²⁵¹ Ortep,²⁵² Pymol,²⁵³ Swiss PDB viewer,²⁵⁴ and VMD (Visual Molecular Dynamics).¹⁶⁶

As a result, interactive molecular graphics methods are used extensively and remain the principal tool for initial ligand preparation for most modeling purposes. These programs have been reviewed in great detail.^{34,58,60,255} Most of the software listed can also be interactively used to construct ligands or proteins by connecting atoms or small molecular fragments, based on standard interatomic distances, angles, and dihedral angles.

A number of useful visualization tools¹⁵⁹ for X-ray crystal structures are also available, such as Crystallographica,²⁵⁶ CrystalMaker,²⁵⁷ CrystalDiffract,²⁵⁸ COOT,²⁵⁹ Mercury,²⁶⁰ Olex2,²⁶¹ Single-Crystal,²⁶² and VICS-II.²⁶³

3.2. Drug Design Approaches in the Absence of Knowledge about the 3D Structure of the Target Enzyme

There are many cases where the 3D structures of target enzymes are still unknown. Three computational techniques are then used for drug design, namely, homology modeling, computational methods in combination with NMR, and QSAR calculations.

3.2.1. Homology Modeling. A good understanding of the function and physiological role of a therapeutic target is necessary for a successful drug discovery process.²⁶⁴ Despite technical advances in X-ray crystallography, multidimensional NMR spectroscopy, and other structural identification methods, the lack of high-resolution 3D structural information for the vast majority of therapeutic enzymatic targets still remains an ongoing challenge.^{17,95,112,265,266} Development of accurate 3D molecular models with the ability to perform computational structure prediction can provide a cost-effective alternative in the absence of experimental structures. Hence, the contribution of homology modeling plays a vital role in structure-based drug discovery to predict and generate reasonably accurate 3D models for many of these targets. Homology modeling is able to generate reasonable protein structures by providing a 3D model from a template protein sequence based on the structures of one or more homologous proteins of which the structure(s) have been reported.

In order to create a model, the following steps are normally followed.¹⁰⁹

- The 3D structure(s) of a protein that have a reasonably similar primary structure (higher than 25% homology)²⁶⁷ than the protein with unknown structure is required.
- The sequence of the two proteins is then aligned.
- Structurally conserved and variable regions are identified.
- Coordinates for the core (structurally conserved) residues of the unknown structure are created from those of the known template structure(s).
- Conformations for the loops in the unknown structure are created.
- The side chain conformations for the modeled protein are created.
- The unknown structure is refined and evaluated.

Researchers should note that in most instances when there is significant sequence alignment, the 3D structures should normally be very similar. However, it may still be possible in a few rare cases that the 3D structures of these two proteins are in fact quite different. Application of more than one template can sometimes solve this issue.¹¹⁴

When the appropriate single-crystal X-ray structure of your target enzyme has been published, it is possible to verify if your homology model is in fact accurate. This operation can be performed by examining whether the generated structure displays a RMSD of 1–2 Å with the overlay of the backbone atoms of the published experimental structure.

Development and improvement of refinement tools in the context of drug discovery and homology modeling is an active area of research.¹¹¹ An interesting ongoing project (10th Community Wide Experiment on the Critical Assessment of Techniques for Protein Structure Prediction, CASP)²⁶⁸ to boost this field of research should be noted. At the beginning of each year, structures about to be solved by crystallography or NMR are identified, and their sequences are made available to researchers in the field of homology modeling. Tens of thousands of models submitted by approximately 200 prediction groups worldwide are then processed and evaluated toward the end of the year. Independent assessors in each of the prediction categories bring objectivity and balanced and independent insight to this process. Tools for visualization, comparison, and analysis of submitted models are available at this site. The results of the CASP experiments are annually released.²⁶⁸

In this regard, computational resources,¹⁵⁹ like Modbase,¹²⁰ Protein Model Portal,²⁶⁹ Swiss-Model,²⁵⁴ generate 3D protein models using various automated methods. Other commercial packages are mentioned in the literature.¹¹² These tools provide 3D models that serve as starting points for biologists/experimentalists to assist in structural genomics (SG) and biomedical research projects. Homology modeling has been successfully employed for generation of receptor sites and identification of inhibitor hits for high-throughput docking (HTD).^{53,54}

A number of challenges exist with template identification, namely, accurate sequence alignment and refinement methods which require a wider application of computational generated models in the drug discovery process.²⁷⁰

A report about the latest developments in terms of homology modeling appeared in 2010. State of the art homology modeling as well as common problems and opportunities in this field were described.¹¹⁵ The usefulness of both this method (using the Modeler program)^{271,272} and spectroscopic methods (NMR, Fourier transform infrared spectroscopy, X-ray fiber

diffraction, cryoelectron microscopy, site-directed spin labeling, and site-directed introduction of fluorescence probes) to determine the structure of the M13 protein for biomedical research was presented. Also, the accuracy of the homology models was analyzed with true blind experiments. Despite the fact that this method provides accurate 3D structures of many proteins, they concluded that “*the protein folding problem is far from solved. The best models are still built by those who got the alignment right in the first place, which unfortunately implies that structural diversity is often missed: one cannot yet ignore the difficult-to-align regions and simply predict them with ab initio folding instead. The sequence alignment problem will thus remain an active research field for years to come.*” This paper clearly revealed the significant mutual contribution of homology modeling and spectroscopy in drug discovery. In some cases the experimental spectroscopic results are not a prerequisite to analyze the model, while in other cases the model appeared helpful for the spectroscopic experiments, and in some cases both are required.

Thus, although experimental structures of many suitable template proteins are available for unknown structures, not in every case are all of the residues able to be refined with homology modeling. A good example where this is demonstrated is the peanut allergen Ara h 2 (3OB4).^{113,273–275} In highly variable loop regions of this protein one does often find a string of residues completely missing. Also, there are examples of N- and C-terminal regions that cannot be resolved and even artifacts in those regions of synthetic constructs added to aid in the growing of useful crystals for structure determination. As an example, the “one and only” experimental structure of the peanut allergen Ara h 2 (3OB4)²⁷⁶ has both a synthetic construct and a completely unrelated protein attached to the N-terminus. This does deform/compress the N-terminal region of this structure and limits the usefulness of this structure in analyses. 3OB4 also has an undefined loop region, and these residues are missing.

Power et al. (2013)²⁶⁷ described these subtle aspects in great detail by comparing 10 homology modeled structures of similar proteins, including Ara h 2. They have shown that not all 3D models have the same reliability (between 1 and 2 Å in structured regions is considered good). They demonstrated that current quality assessment tools can help users in avoiding overinterpreting of the structural information. The user should be especially wary of regions that do not align to the template or those based on templates with a large content of disordered or flexible loops. Proteins with large disordered regions require improved tools to determine accurate models. They demonstrated that one of the most challenging aspects of homology modeling is to assess how the model is doing in the absence of an experimental structure.

There is a great demand to develop better methods to reduce modeling errors in the cases where target and template macromolecule share lower sequence similarities. Several comprehensive reviews^{94,109,275,95,110,277–285} and textbooks^{95,110,286} exist on this topic and its applications^{286–300} in the drug discovery process.

3.2.2. Solution Structures from NMR Data. Use of NMR measurements to obtain structural data of proteins has developed enormously in the last decades^{301–306} due to the difficulty in getting suitable X-ray structures for the millions of disease-related enzyme targets.^{307–325} There are two basic NMR methods to determine the 3D solution structure of proteins or enzymes. The first is a direct determination of high-

resolution solution NMR structure of the enzyme with dedicated software, and the second³⁰⁹ is an indirect method based on a combination of NMR data and constrained MD calculations.^{305,306,326–328} Both methods rely on crucial structural information derived from short interproton distances supplemented by torsion angles. The distances are derived from NOE measurements and the torsional angles from an analysis of a three-bond coupling constant, which are related to dihedral angles.^{329–331}

The first method makes use of designated software¹⁵⁹ such as ASDP,³³² CABM NMR software,³³³ computer-aided structure elucidation,³¹⁸ Crystallography and NMR System (CNS),³³⁴ CYANA,³³⁵ DIANA,³³⁶ DISTGEOM,³³⁷ NMRPipe,³³⁸ and Rosetta,³³⁹ etc. The software employs mathematical algorithms that automatically translate all experimental 2D NMR data into a 3D structure that matches the data.

The second method uses normal MD software packages which make use of selective NMR-derived constraints (manually introduced to the calculation) to obtain modeled solution structures for the specific enzyme. The quality of the approximated enzyme structure depends on the strategic structural importance of the manually chosen distance constraints, the number of constraints, and the accuracy thereof.

Other NMR structural software packages were discussed by Wang et al.³²³ Some of these software packages can also simulate NMR spectra from 3D structures (including 2D NMR data).

Despite many successful 3D solution structures having been obtained, the first direct method is cumbersome and takes several months or even up to a year to resolve one complex enzyme structure.⁷ The resolution is less accurate than X-ray structures obtained from solid-state crystals.³⁴⁰ The second method is more rapid but provides less accurate results.

It is important to note that NMR methods for 3D structure determination of proteins is only useful for proteins up to 25–30 kDa since for larger proteins the NMR sensitivity drops and peak broadening makes proper resolution of signals impossible.^{33,327}

To date (Aug 2012) 8286 protein structures³⁴¹ have been elucidated with these NMR techniques, compared to about 1400³¹¹ up to the year 2000.

QM software can also be used to calculate NMR data of peptides and proteins.³⁴² It appears that QM methods (including DFT) can be used for calculation of NMR data for enzymes,³⁰⁶ although due to size limitations approximations of smaller model systems³⁴³ and hybrid methods³⁴⁴ are generally still used. Carbon 13 simulated NMR data is in general less sensitive to conformational changes/differences. Simulated proton data is much more sensitive to conformation differences, and such data should be treated with much more caution. In reality, experimental NMR data represents the average spectrum of various conformations due to rapid interconversion of most dihedral angles at room temperature. Low-temperature NMR experiments can trap molecules in one or more conformations.

A second type of NMR simulation software (such as the ACD laboratories software,³⁴⁵ Chemdraw,²⁴⁵ ChemWindows, etc.) makes use of existing NMR data in order to make predictions. Such software can be trained on a library of compounds when suitable NMR output is not by default produced.

3.2.3. Quantitative Structure–Activity Relationship (QSAR).

The necessity of QSAR techniques becomes apparent when families of bioactive compounds are found with little knowledge about the structure of the target. If a mathematical QSAR framework between some of the physicochemical properties of the bioactive compounds and the bioactivity of a family of inhibitors or drugs is found,³⁴⁶ it becomes possible to predict the activity of modified or related compounds. The underlying principle of these methods is that molecular structures have inherent features responsible for its physical, chemical, and biological properties. QSAR models were designed to find the correlation between a calculated value related to a physicochemical property and experimental biological/pharmacological data.^{78,81,83,347–354}

QSAR modeling is also useful for the screening of effective drugs and anticipating potential problems in development of new agents before performing receptor docking and empirical determination of toxicity both *in vitro* and *in vivo*.⁸¹

Almost all QSAR techniques rely on two-dimensional (2D) and three-dimensional (3D) molecular descriptors, which serve as a useful mathematical representation of physicochemical information of bioactive molecules. These descriptors facilitate determination of the correlation between molecular structure and one or more biomolecular properties of the specific family of compounds.⁸¹ Two-dimensional descriptors are related to functional groups and atomic properties of the compounds including octanol/water partition coefficient ($\log P$), polarizability, van der Waals volume, the approximated surface area, and molecular weight among others. Three-dimensional descriptors depend on the structure and conformation of the molecules.¹⁰⁵ If the mathematical relationship between the descriptors of a family of drugs and the experimental bioactivity of the compounds is not satisfactory, then refinement of the variable that is used to contribute to the descriptors is required.

The following software¹⁵⁹ are widely used in QSAR analysis: C-QSAR,³⁵² Dragon,¹⁶³ Hyperchem,²⁴⁹ MATLAB,³⁵⁵ RECKON,³⁴⁹ and SIMCA multivariate analysis package.³⁵⁶ Statistically based programs such as MCASE³⁵⁷ and TOPKAT³⁵⁷ use multiple QSARs on small and homogeneous sets of data. Review papers^{350,358} and several textbooks^{347,351,354} describe the principles and applications of QSAR methods.

Mizukoshi et al. (2012)³⁵⁹ described a new NMR experiment to achieve accurate pharmacophore mapping required for QSAR methods. They called it the “Difference of Inversion RECoverY rate with and without Target IrradiatiON” (DIRECTION) method. Most current experimentalists exploit nuclear Overhauser effects (NOEs) such as transferred NOE, saturation transfer difference (STD), pumped NOE, and water–ligand observed by gradient spectroscopy (water-LOGSY), etc.) to perform ligand pharmacophore (or epitope) mapping. Since the difference of the longitudinal relaxation of each ligand proton severely interferes with the derived pharmacophore mapping result, it is important to evaluate intermolecular cross-relaxation terms for accurate pharmacophore mapping. They proposed a simple and rapid approach for pharmacophore mapping experiments, which utilizes the difference between the longitudinal relaxation rates of ligand protons with and without irradiation of the protons of the target protein.

3.3. Drug Design Approaches When the 3D Structure of the Target Enzyme Is Known

The first prize will always be to have a 3D structure of the enzyme target. Advances with single-crystal X-ray studies of enzymes and enzyme/substrate or inhibitor complexes really enabled the advancement of rational drug design in the last two decades. Apart from NMR techniques and homology modeling, a new technique that is expected to bring exciting new information of living organisms at the molecular level is called cryo-electron tomography (CET). It is a branch of cryo-electron microscopy and rebuilds a 3D structure from 2D images of a frozen cellular sample. Rapid freezing preserves the aqueous environment of the biosample. A high-resolution CET 3D image at the molecular level of *Treponema pallidum* (the causative agent of syphilis) was recently reported.³⁶⁰ Unfortunately, it seems that several difficulties experienced with this technique are still hampering the wide use of it.

The next sections present a general overview of computational approaches that are used to obtain more information about the inhibitor/enzyme interactions.

3.3.1. Binding Affinity Estimation Methods. Theoretical determination of the drug–enzyme binding mode and energy was one of the challenging parts in the early stages of drug design.¹⁰⁵ Quantum mechanical methods can calculate binding affinities *de novo*, but force field methods required development of algorithms, such as empirical energy functions, to provide an estimation of the interaction between ligands and enzymatic targets. In terms of drug design, several methods were developed as useful applications. The next sections discuss various types of methods that calculate binding affinities commonly used in the field of drug design.

3.3.2. Empirical Energy Functions. Empirical energy functions explain a molecule's energy as a function of the atomic coordinates and include conformational, nonbonded interaction energy terms, van der Waals and electrostatic functions, crystallographic and noncrystallographic symmetry interactions, and explicit hydrogen-bond terms.^{361,362}

Empirical energy functions form the basis of all molecular mechanical methods (including docking). Improvement of the quality of these calculations is an active field of research.

Application of empirical energy functions was initially applied to small molecules; however, nowadays more advanced empirical force fields, derived from a variety of experimental data, have been developed, and it enables extended use of these functions toward much larger systems such as biological macromolecules.^{363–365} These energy functions are effective tools for computing systems containing thousands of atoms, such as proteins or nucleic acids even in aqueous solution.³⁶⁶ Prediction of hydrogen positions in proteins is possible by empirical energy placement and comparison of the theoretical results with experimental neutron diffraction studies.³⁶⁷

Empirical force fields use atomistic models in which atoms are considered the smallest particles in the biosystem rather than the electrons and nuclei used in quantum mechanics.³⁶³ A suitable energy function has two requirements. First, for specific ligands, it should have its global minimum close to the experimentally observed conformation of the complex, and second, such a function should also rank different ligands in terms of their binding affinity.¹⁰⁵

Once a suitable solution to the problem of determining an estimate for the interaction between ligands and enzymes was found, more advanced methods were developed to assist in the drug design process.

3.3.3. Molecular Docking. Understanding of the binding mode between small ligand molecules and a therapeutic receptor is of intense interest to molecular scientists and forms the basis for the field of computer-aided drug design.⁹⁷ Molecular docking is used for both exploring possible conformations of a ligand inside the binding site of a protein as well as estimation of the strength of ligand–protein interaction.³⁶⁸ A virtual screening method utilizing a combination of docking and homology modeling was recently reported by Fan et al.³⁶⁹ They described an integrated modeling and docking protocol, combining comparative modeling utilizing Modeler and virtual ligand screening with Dock (a rigid docking method). It should be noted that automation for virtual screening is easier when rigid docking is performed. This is only practical for small rigid molecules.

The nature of the interactions between ligand molecules and their receptors are dynamic and relies on the understanding of molecular recognition as a lock and key mechanism.³⁹ There have been some significant advances and improvements in the computational tools that are used in rational drug design by means of ligand–protein docking since the mid-1980s.⁹⁷

It is important to confirm the flexibility of the enzyme target^{370–376} and whether or not it has several possible conformations.^{371–373,376–387} Flexible targets can potentially undergo changes upon binding with an inhibitor,^{376,377,380,383,385,388–390} which has implications for the accuracy of using rigid enzyme structures and also whether the enzyme has one or more possible active sites.^{390–392}

During the docking process, the Cartesian coordinates of the individual receptor and the ligand are used to predict the best-fit conformation of the ligands for the resulting ligand–receptor complex (see Figure 8). The binding affinities of various ligands

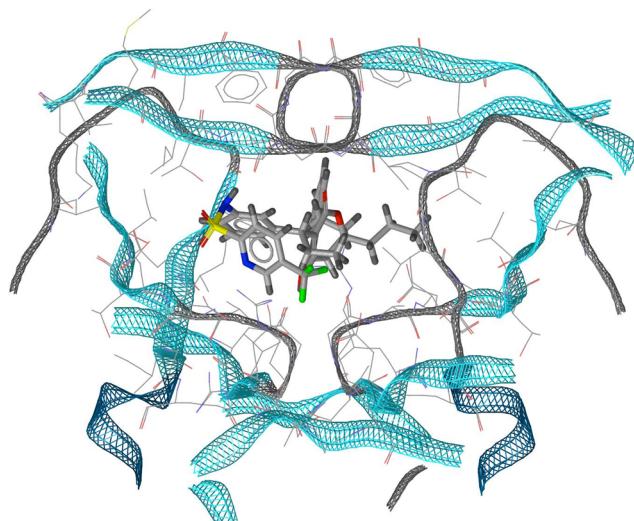


Figure 8. Docked tipranavir/C-SA complex presented using the VMD¹⁶⁶ software.

to the receptor are compared and normally provide a correlation between the bioactivity of the inhibitor against the specific enzyme. The correlation between docked binding energies and bioactivities is not always ideal due to two reasons. First, bioactivities may vary from laboratory to laboratory (see section 4), and second, the docking binding energies are quite crude for reasons described later in this section.

All docking programs have¹⁰⁷

- An algorithm that performs a conformational search for the specific inhibitor and search for the best fit of the inhibitor in the active enzymatic pocket and
- A scoring function that ranks them in terms of intermolecular binding energy, i.e., more negative values reveal a higher score for the interaction between the inhibitor and the receptor.

Note that most of the software simplify (bias) the docking process by providing an option to quite accurately indicate the position of the active site for exclusive use during the docking process.

As mentioned before, it is important to obtain as much information as possible in the literature about the active site of the specific target protein and if possible which amino acids segments are crucial in the mechanism of the enzyme–substrate reaction/interaction. This is required since it is normally expected that competitive inhibitors occupy a prominent position in close proximity of these amino acid segments, inside the active site. If information about the potential interaction between the inhibitors and the active site is known, then visual inspection of the docked results can be effectively used to eliminate nonsensible output. The MM algorithm sometimes induces unreal structural changes to the inhibitors, and visual inspection of the bond angles, dihedral angles (amide bonds normally have the NH proton in a trans position with respect to the carbonyl oxygen), chirality, etc., should be done for the best docked structures.

Initial rational drug design projects involved the screening of databases containing thousands of small molecules with automated docking methods in order to characterize those that involve the best fit for a specific target receptor site. This approach yielded a number of documented successes in lead drug discoveries.^{393–395} The scoring function that ranks the geometry of the ligand–receptor site complexes depends on either the calculated potential in the binding site or a similarity function that biases the design toward known ligands.^{396,397} Most docking software makes use of force field (molecular mechanical) calculations that approximate the binding energy based on both high-level calculated QM and experimental data. It should be noted that accurate binding energies can only be calculated from basic principles with ab initio quantum mechanical (including density functional theory) calculations.

Various common modeling programs implement a specific type of docking strategy for ligand design.¹⁵⁹ Some of them incorporate an energy minimization procedure to facilitate identification of complexes with good geometries and reasonable ligand–receptor interactions with significant improvement of the results.³⁹⁸ DOCK, one of the first docking programs, is based on rigid docking and the use of geometrical criteria to evaluate the complementarities between the receptor and the ligand.⁵⁸ The program also uses spheres complementary to the receptor molecular surface to create a space-filling negative image of the receptor site.³⁹⁹ Rigid docking is only practical when the molecule does not have multiple rotatable bonds and subsequent different conformations. An exception is when complexation of a smaller protein, with a known and relative rigid 3D structure, to a larger protein is studied.

Flexible docking varies rotatable bonds of both the inhibitor and the amino acid segments (such as side chains) in the active site in order to find the best possible fit of the ligand. The degree of flexibility is normally manually controlled since

introduction of more rotatable bonds requires more computational resources and time. This docking method obviously gives superior results to rigid docking methods.

Flexx⁴⁰⁰ is an efficient docking program for medium-sized flexible ligands. It docks molecules in a fragment by fragment basis and uses the Bohms empirical function⁴⁰¹ for scoring. The torsion angle database⁴⁰² is used to generate different poses of a fragment, and then the selected fragment is placed in the active site of the protein and the alignment processed to demonstrate probable interactions. It is effective for the screening of small databases of ligands and has been extended to predict the position of water molecules in the binding site.⁴⁰³

Gold⁴⁰⁴ implements a genetic algorithm that encodes the approximate conformation of the ligands and uses a simple least-squares fitting procedure to generate the final conformation. It also allows flexibility around bonds to hydrogen-bond donors and acceptors in the receptor.⁴⁰⁵ The differences between docking programs boils down to the function of the searching algorithm, the weight each method assigns to the different types of intermolecular interactions, and the steric overlaps that are considered for calculation of the docking score.¹⁰⁷ Both Gold⁴⁰⁶ and AutoDock⁴⁰⁷ use a genetic algorithm (GA) to estimate the poses of the ligand inside the protein's active site.

Autodock also uses the Lamarckian GA, where the changes in induced conformations are used as a make up for offspring poses. Geisteger charges are computed and the Autodock atom types are defined using the Autodock Tools graphical user interface supplied by MGL Tools.⁴⁰⁸ With this program an active site is selected, based on the position of the native ligand structure in the active site (if it is known). AutoDock Vina⁴⁰⁹ is about 2 orders of magnitude faster in producing docked results than the AutoDock software.⁴¹⁰ It also has an improved code for binding mode predictions. This program automatically employs a gradient optimization method in its local optimization procedure, and it calculates the grid maps and ranks the conformations based on the implemented scoring function. The accuracy of binding energy results is also better than for Autodock.

Docking studies play a vital role in rational drug design, and some excellent reviews^{411–418} are recommended. There has been a noticeable increase in research output in this field over the past few years.^{97,104,368,419}

Docking calculations can be achieved on benchtop computers since it uses low-level calculations. Even though docking methods have contributed tremendously to rational drug design, it should be acknowledged that the conformational and energetic information obtained are rather crude.³⁶⁸ In most cases solvent effects are neglected, and real dynamic movement of the inhibitor–enzyme complex is not possible. Also, docking techniques were designed to provide an estimation of the binding affinity of the inhibitor upon finding the best fit inside the active enzymatic pocket. Scoring failures in docking indicate the inability (or inaccuracy) of the energy function to fit the most compatible score to a correct sampled conformation out of the generated ensemble. Choosing a more accurate energy function implemented in the software may improve the overall results. The effectiveness of the docking algorithm decreases as a function of the number of rotatable bonds.⁴²⁰

Since most docking software (such as Autodock) “remove” the protons of the enzyme and inhibitors, more useful information can be obtained from MD studies where the protons (and water molecules as the solvent) are considered.

Instead of a single possible complex orientation, the average interactions distances (such as hydrogen bonds etc.) can be investigated from a MD simulation. MD-based average free energy calculations provide more accurate⁴²¹ binding affinity results,^{422–428} and in our view more researchers should at least confirm the validity of a representative number of docked results with MD average free energy calculations. Alternatively, it is advised to use reliable bioassay data as a benchmark for a number of compounds in the family of docked structures.

It is therefore not surprising that most computational studies have currently evolved to also include MD studies of the inhibitor–enzyme complex involving solvent. A good standardized platform⁴²⁹ for the most accurate method to calculate binding energies of HIV PR inhibitors has appeared recently from our laboratory.

3.3.4. MD-Based Free Energy Calculations. In most molecular dynamics (MD) software packages,¹⁵⁹ such as X-PLOR⁴³⁰ and CHARMM,¹⁶² the empirical energy function is developed to approximate the *potential energy* of the system. A variety of specific parametrizations for empirical energy functions are available in the X-PLOR program, which is applied to computational structural biology and also for exploration of the conformational space of macromolecules that are restrained (by an algorithm) to regions allowed by a combination of empirical energy functions results and experimental data.^{431–433}

Calculation of accurate free binding energies of ligands complexed to macromolecular targets is thus combined with MD methods. A stable MD simulation for at least 2 ns is required for accurate average free binding energies. A RMSD plot of the complex should be used to confirm that the system has reached equilibrium and whether the system is stable under the MD conditions. An example of a RMSD plot of the MD simulation of an enzyme is presented in Figure 9.

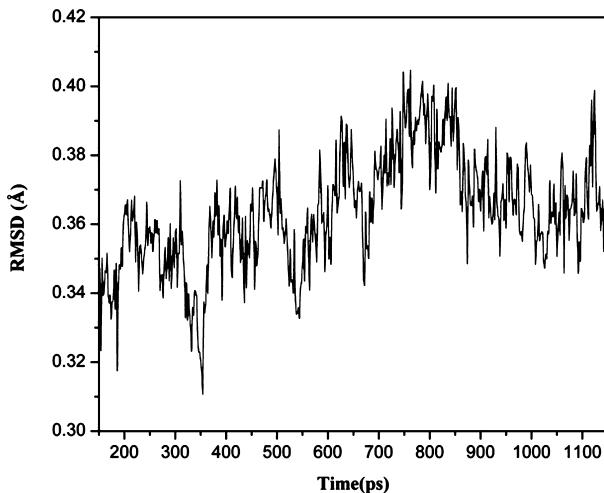


Figure 9. Typical RMSD plot from a MD simulation of an enzyme over time.

These methods also provide a molecular view of the interaction between the inhibitor and the enzyme, giving an enhanced understanding of the experimental observations. MD-based free energy calculations are useful for identification of some biological properties such as binding mode, the protonation state of certain residues, and the flexibility of the molecule.^{30,59} The free binding energy calculations consider

both enthalpic and entropic contributions of the system.^{105,434} It is also possible to analyze the inhibitor/enzyme complex for various types of interactions between them (see Figure 10).

Improved binding energies and mechanistic aspects of the interaction between the inhibitor and the enzyme can also be calculated with hybrid QM/MM/MD methods.⁴³⁶ Since the majority of biochemical processes are not occurring in the gas phase, MM/MD methods including explicit solvent molecules were effectively introduced.^{176,207} These methods evolved to a hybrid QM/MM/MD approach in which the system is divided into two regions: one uses a MM force field, and the other is described at an appropriate QM level, Figure 11.^{436,437}

A very elegant method to generate a multidimensional free energy surface for an enzymatic reaction mechanism involves calculating potentials of mean force (PMFs) using the method of umbrella sampling^{438–441} during a MD simulation. An example by Soliman et al.^{442,443} involved a study of glycoside hydrolysis. In short, the method uses two reaction coordinates (ξ_1 and ξ_2) in a specific mechanism, wherein each coordinate involves at least 3 atoms. The position of the center atom is calculated based on the constraints imposed on the two terminal atoms.

An umbrella potential $U_i \nu_i$ of harmonic form (eq 3) is defined for each coordinate $\xi_i \xi_i^0$, where k_i is a force constant and ξ_i^0 is a constant reference value.

$$\xi_1 = d(O_{lg} \cdots C_{ano}) - d(O_{nuc} \cdots C_{ano}) \quad (1)$$

$$\xi_2 = d(O_{don} \cdots H_{don}) - d(H_{don} \cdots O_{lg}) \quad (2)$$

$$U_i(\xi_i) = \frac{1}{2} k_i (\xi_i - \xi_i^0)^2 \quad (3)$$

The result is a multidimensional free energy surface plot of the reaction, Figure 12.

Several review articles^{30,216,396,444–465} and textbooks^{466–468} describe the theory and application of MD free energy calculations to biochemical phenomena using Amber,¹⁶¹ Sybyl,¹⁶⁷ Discovery from Insight II (later taken over by Accelrys),⁴⁶⁹ NAMD,⁴⁷⁰ ICM,⁴⁷¹ and several other computational software.¹⁵⁹

3.4. Virtual Screening Techniques

Virtual screening is the computational mirror image of the experimental high-throughput screening (HTS),^{51,472} and it proved an efficient implementation in the drug design and discovery process.^{122,369,473} Advances in computational and bioinformatics techniques and hardware solutions have allowed for fast mining of chemical and biological data to search for novel compounds with desired properties from commercial and private chemical databases^{474,475} (see section 2 for a discussion of available structural databases).^{472,473}

Virtual screening approaches rely on either the information of a known active ligand (pharmacophore/ligand based VS)^{381,474,476–478} or the knowledge of the 3D structure of the target protein (target, receptor-based VS)^{478–484} and estimate the binding affinities of small molecules toward this. Both approaches, pharmacophore-based or structure-based VS, are often complementary, and various hybrid techniques have been adopted to exploit their complementary nature.^{485–491}

Receptor/structure-based virtual screening normally makes use of docking techniques.⁴⁸⁴ Most of the normal docking software can be used for this purpose (see section 3.3.3). A

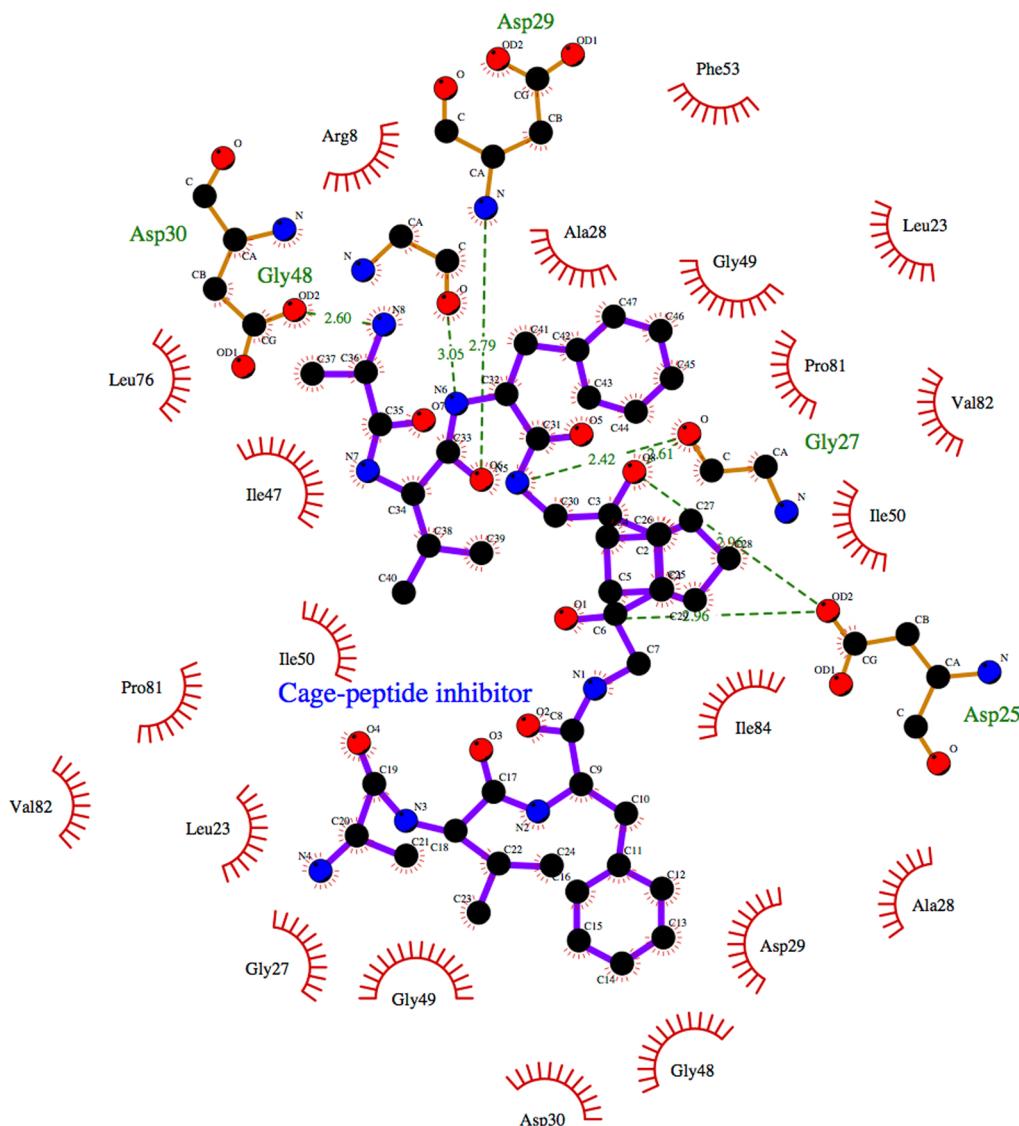


Figure 10. Average electrostatic and hydrogen-bonding interactions calculated from a MD simulation between a PCU cage peptide and the nearby residues of C-SA HIV PR active site (some atoms were removed to provide a clearer picture). This plot was created with the Ligplot⁴³⁵ software.

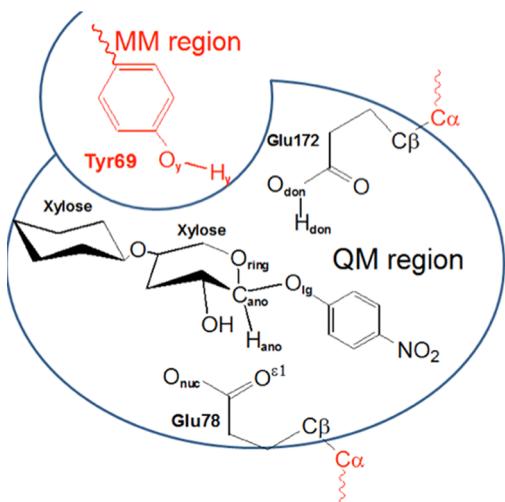


Figure 11. QM/MM regions for the mechanism of glycoside hydrolysis.⁴⁴²

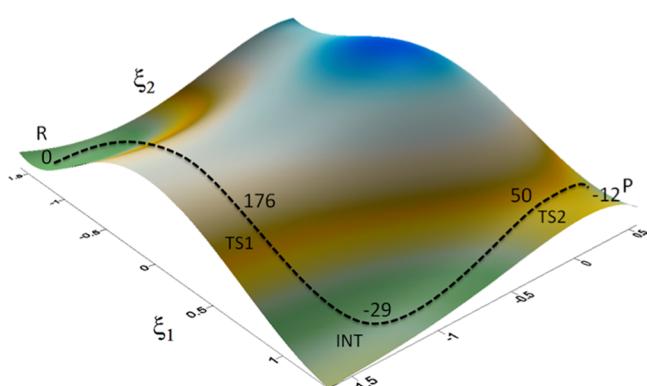


Figure 12. AM1/OPLS-calculated multidimensional free-energy profile for glycosylation as a function of the coordinates for nucleophilic substitution ξ_1 and proton transfer ξ_2 (relative energies in kJ mol^{-1}).⁴⁴²

recent review discusses many of the relevant issues involving virtual screening.⁴⁹²

Virtual screening can also be combined with biophysical methods such as X-ray crystallography, isothermal titration calorimetry (ITC),⁴⁹³ which represents the most accurate and consistent results for any comparison purposes (see section 3 of this manuscript for a discussion about this with references), fluorescence spectroscopy, NMR,⁵¹ and other methods. It was pointed out in 2007¹²² that “the number of virtual screening publications considering ‘biophysical methods’, that is, mainly X-ray crystallography, NMR spectroscopy, isothermal titration calorimetry (ITC) and surface plasmon resonance (SPR) spectroscopy, is rather low and sum to approximately 10%; however, a clear upward trend in publications on this subject area is occurring.”

Several recent VS papers have appeared using circular dichroism,⁴⁹⁴ fluorescence,^{494–509} ITC,^{510–513} NMR,^{473,510,513–526} and SPR^{496,526–528} spectroscopy.

In our opinion, reviewers and editors should require a more robust approach for computational studies, where calibration or validation of the observations is demonstrated from a comparison with experimental results (bioassay data as well as experimental structural data, including biophysical analytic methods).

The last part of this review will only focus on reports that have combined a spectroscopic/molecular modeling approach for design of new drugs.

4. EXPERIMENTAL BIOACTIVITIES OF ENZYME/INHIBITOR INTERACTIONS

General reviews^{529–533} on this topic are not common. Review papers involving in vitro assays are quoted for the two major diseases reported herein, namely, cancer^{533–540} and HIV.^{532,541–544} It is important to note that the toxicity of the inhibitor toward a human cell line is also reported. This gives an indication of the selective nature of the inhibitor. The toxic side effects of currently marketed HIV drugs are becoming a huge problem for patients undergoing prolonged treatment.^{545–556}

New researchers in the field are recommended to perform a literature search of available bioassay methods for the specific enzyme they want to study. It is common to report in vitro activity results of the purified enzyme in a buffer solution. This is also used for mechanistic studies of inhibitors since protein binding,⁵⁴¹ metabolic degradation, trans-membrane transport, and other complications occur during infected whole cell tests. In most cases observed activities are reported as the half-maximal inhibitory concentration (IC_{50}) results.

However, infected whole cell activity tests and in vivo bioassay results provide a more realistic picture of the bioavailability of the inhibitor. Many more complications are possible for oral or intravenous dosages of the drug. See section 1 for a discussion about bioavailability including the Lipinski rules⁵⁵ and Veber’s drug-like filter.⁵⁷

For the purpose of rational drug design based on structural information about the inhibitor/enzyme complex that is also linked to the experimental bioactivity of the inhibitor, it is obviously more advantageous to obtain such data from studying the interaction of the inhibitor in the presence of a buffered solution of the pure enzyme.

We recently reported⁴²⁹ that most experimental HIV PR activity data (IC_{50} , binding constants, etc.) varies quite significantly in different publications, even when the data has been acquired in the same laboratory but at different times. We concluded from a literature survey that experimental binding free energy data (obtained from thermal titration calorimetry

experiments) represents the most accurate and consistent HIV PR activity results for any useful comparison purposes. The lack of such data is severely hampering the advancement of new drug HIV PR design. This is certainly also true for other enzyme targets. Biochemists should be encouraged to drastically expand such experimental results for more targets and inhibitors.

These ITC data can be obtained from two database sources apart from the open literature. The first is the BindingDB by the group of Gilson.^{557–559} The second is the Structure/Calorimetry of Reported Protein Interactions Online database (SCORPIO) of published isothermal titration calorimetry studies, established by Ladbury.⁵⁶⁰

Nevertheless, researchers should take care with isothermal titration calorimetry experiments to ensure still more precision. Matulis and co-workers⁵⁶¹ have shown that some data have relatively high errors in the measured enthalpies of protein–ligand binding reactions. They propose universal validation standards for titration calorimeters.

A recent review by Carbett et al. concerning experimental thermodynamic studies demonstrates the importance of high-throughput calorimetric methods for effective drug design.⁵⁶²

5. BIOPHYSICAL ANALYTICAL TOOLS FOR DRUG DESIGN

A range of biophysical methods for the study of protein interactions has been recently reviewed.^{14,419,563–579} Several drug design-related review articles have also included biophysical methods as a section of the manuscript.^{569,574,580–593}

Spectroscopy⁵⁶⁵ plays an important role in the study of protein interactions and drug design. The following spectroscopic techniques have been reviewed: AFM and scanning tunneling,⁵⁹⁵ cellular electrical impedance,⁵⁹⁶ circular dichroism,⁵⁹⁷ electrical impedance,⁵⁹⁶ fluorescence,^{598–603} infrared,^{604–606} linear dichroism,⁵⁹⁷ magnetic resonance morphometry and MR spectroscopy (MRS),⁶⁰⁷ NMR,^{315,317,566,608–612} nuclear quadrupole resonance (NQR),⁶¹³ tomographic diffuse optical,⁶¹⁴ optical,⁶¹⁵ Raman,^{616–618} and surface plasmon resonance,^{580,619–626} and synchrotron radiation spectroscopy.⁶²⁷

The following specific nonspectroscopic biophysical methods have recently been reviewed: capillary electrophoresis,⁶²⁸ centrifugation,^{629,630} electrochemical methods,⁶³¹ cryoelectron microscopy,⁶³² labeling techniques (spin labeling, double labeling, fluorescent labeling, triplet labeling, Moessbauer spectroscopy),⁶³³ mass spectrometry,^{633–637} molecular imprinting,^{584,638} nanoparticles,^{624,639–644} optical tweezers,⁶⁴⁵ scanning electrochemical microscopy,⁶⁴⁶ size characterization,⁶⁴⁷ thermophoresis,⁶⁴⁸ and X-ray crystallography^{649–655} as well as crystal growing techniques.^{656,657}

Please note that the techniques mentioned in this section are not exhaustive; more methods can be found in the literature.

6. INTEGRATED MOLECULAR MODELING AND SPECTROSCOPIC TECHNIQUES IN DRUG DESIGN

As indicated in the Introduction, the impetus of this review is directed more toward experimentalists, and it is assumed that they have suitable background knowledge of spectroscopic and in vitro techniques. Less emphasis was therefore placed on the latter.

It appears that the first study involving a combination of the disciplines of spectroscopy and molecular modeling was presented in 1990.⁶⁵⁸ The authors studied the vibrational spectroscopy of earosil and compared it with calculated data. The first drug-inhibitor-related paper that included both computational and spectroscopic techniques seems to have appeared in 1992 by Fesik et al.⁶⁵⁹ They reported a combination of NMR techniques and molecular modeling methods to obtain the conformation of receptor-bound ligands. This is in principle a very powerful method since the behavior of the inhibitors in the active site can be monitored. If this information is suitably reported to the computational chemists, then verification of the computational model can be achieved. In turn, this can then be used to potentially predict the behavior of new inhibitors.

It is also clear from the literature that several drug design review papers have appeared in which the authors have combined separated spectroscopic and molecular modeling reports to demonstrate how this combination can assist with the drug design process. However, these articles did not refer to studies where this combined approach was executed as such, but the notion of the advantages of combining spectroscopy and molecular modeling is propagated. Such cases will be included at the end of each subsection.

In 2005, a review by Lundqvist³³ discussed common challenges in the field of lead identification toward application of virtual computational screening techniques combined with X-ray 3D structural information and NMR spectroscopy. The author motivates why a combination of biophysical experimental techniques, molecular modeling, and drug screening at the early stages (*in vitro*) is beneficial. Even though the author advocates the advantages of such an integrated approach, he did not refer to any computational studies where spectroscopic methods were utilized. Real advantages and improved results will only be achieved when theoretical drug design is built around well-defined experiments involving a biophysical analytical method. This will illuminate the interactions between the inhibitor and the target and where suitable bioassay data on the activities of the inhibitors are measured, especially if it happens to correlate with the other two components.

In a comprehensive review published by Cherry et al.⁶⁶⁰ the NMR and X-ray crystallography methodologies as well as computational screening methods (high-throughput docking, receptor and ligand-based pharmacophore, QSAR models, structure-based searching) were explored on a number of kinase targets. The review did not refer to any papers where a combination of spectroscopy and molecular modeling were used for drug design. Various tools of the trade for NMR and virtual screening are discussed and demonstrated with a number of case studies. It was concluded that integration of high-throughput screening tools combined with ongoing technological advances in computational chemistry, X-ray crystallography, and NMR spectroscopy has led to an increase in structure-guided approaches at the hit-finding stage of drug discovery. However, an integrated combination of all three components of drug design (molecular modeling, biophysical analytical methods, and bioactivity studies) was not demonstrated in this report.

6.1. Case Studies

This section predominantly focuses on recent advances in structure-based computational screening approaches and highlights the successful integration of computational methods with

common spectroscopic techniques and bioactivity studies. Applications in cancer research, HIV, Alzheimer's, and miscellaneous diseases are presented in order of the number of reports for each case. An evaluation of these reports was made with respect to the integration of the three proposed components, namely, molecular modeling, bioactivity results, and biophysical analytical techniques (spectroscopy). Cases where this was successfully achieved will be indicated.

We realize that we may be moving onto perilous ground with such an evaluation, since few of these reports have claimed to achieve that. We trust that the applicable authors will read our potentially negative "criticism" in the light of the scope of this review paper.

6.1.1. Cancer. The first publications in this field that combined computational techniques with spectroscopy seem to have appeared in 1994.^{661,662} Zhang et al. compared the X-ray and solution structure of cancer inhibitors with the force-field- and semiempirical-calculated structures. The crystal structure of one of the inhibitors was used as the starting point for conformational analysis using several commercially available software packages. The lowest energy conformation generated with a force field method disagreed with the X-ray structure. On the other hand, semiempirical calculations showed that the X-ray structure had a lower energy than the lowest force-field-calculated conformation. Subsequent data from NMR studies agreed well with the X-ray structure. Although this study reported bioactivity results, computational studies, and spectroscopy studies, they did not attempt to correlate any of the biophysical measurements to the bioactivities of the inhibitors or gain structural information about the inhibitor/enzyme complex. As such, the approach was not optimal in terms of an integrated drug design approach as proposed throughout the review.

Varnum et al.⁶⁶² determined the 3D structure of the active peptide using a combination of NMR and computational techniques and compared the expected active part of the peptide with the active site of the somatostatin receptor. They studied the solution conformations of RC-160, the cyclic d-Phe-Cys-Tyr-d-Trp-Lys-Val-Cys-Trp-NH₂ peptide, an analog of the tumor antiproliferatory neuropeptide somatostatin. The solution structure of the latter, labeled with rhenium (Re-RC-160), was determined with 2D NMR spectroscopy (600 MHz) and restrained MD simulations. Re-RC-160 displays the same average solution conformation as the apo form. These results indicate that the orientation of the side chains essential for somatostatin receptor binding is maintained in Re-RC-160. The value of this study is that a simplified substrate can be used which is linked to a 3D calculated structure and a spectroscopic analytical technique. This situated the researchers in a powerful position in terms of subsequent activity studies with new inhibitors.

In 1995, a joint application of homology modeling and circular dichroism spectroscopy⁶⁶³ of synthetic peptides was applied to characterize the secondary structure of adenylate kinase (ADK) for cancer therapy.⁶⁶⁴ Various mixtures of a phosphate buffer (pH = 7), trifluoroethanol, and methanol were used for conformational analysis using CD spectroscopy. Ten peptides exhibited α -helical conformation in all solvents except in pure buffer solution. Interestingly, the derived 3D structures from homology modeling (using the Modeler program^{271,272}) correlated well with these observations. More importantly, the theoretical 3D structure was in good agreement with the X-ray structure of adenylate kinase. It was

concluded that synthetic peptides are suitable tools to investigate the secondary structure of protein segments and that conformations observed for them were frequently preserved in the intact protein. It was also realized that identification of secondary structures of small peptide segments via CD spectroscopy was helpful for experimental verification of protein models generated with homology studies. The value of this report is limited for future drug design approaches since the activities of a series of inhibitors were not linked to a measurable biophysical analytical method. However, they demonstrate the value of CD measurements to confirm the 3D structures generated with homology modeling software.

Wender et al.⁶⁶⁵ studied computer modeling, two-dimensional proton NMR spectra, and biological evaluation of synthetic analogs of bryostatins as a family of emerging cancer chemotherapeutic candidates. These compounds were isolated from marine bryozoa and showed high affinities for protein kinase C (PKC) isozymes. Comparison of the computed inhibitor structures of newly designed compounds with known solution and solid-state structures of bryostatins was shown to be a useful measure for the screening of potential analog candidates. The two rings of bryostatin serve partly as spacers to remotely control the orientation and mobility of the groups (C_1 , C_{19} , and C_{26}) presumably required for optimum recognition. NMR proton assignments were made by phase-sensitive NOESY and total correlation spectroscopy (TOCSY), and double quantum-filtered correlated spectroscopy (DQF-COSY) spectra of compounds were analyzed to identify the specific distances between protons throughout the structures. The NOESY cross-peak intensities obtained for these inhibitors were converted to internuclear distance constraints and applied to the lowest energy structure produced from molecular modeling of the selected compounds. The structure was then subjected to a constrained molecular dynamics simulation at 900 K⁶⁶⁶ using Macromodel.^{250,667} The low-energy structures obtained from constrained gas-phase MD simulations followed by minimization of the conformers satisfied all NOE constraints. Inspection of some considered conformations were in reasonable agreement with the NMR data. The advantage of the study is that a thorough integrated method is described to determine and compare the cyclic backbone structures of related compounds showing bioactivity. Since the action of the inhibitor and the target enzyme was not known, that was the best that could be done under these circumstances.

Comparative homology modeling and multidimensional NMR studies using structure-based iterative NOE assignments were performed in 2000 by Yuan et al.⁶⁶⁸ on the tumor suppressor protein INK4 (inhibitor of cyclin-dependent kinase INK4) family. This was an effort to refine the p16^{INK4A} structure and identify the structure of p15^{INK4B}. The first tertiary structure for the latter was constructed by means of comparative homology modeling using the Modeler program (with p16^{INK4A} as the template) followed by restrained energy minimization with NMR determined constraints (NOE and H bonds). The p15 calculations were carried out using a simulated annealing⁶⁶⁹ method with the X-Plor program.⁴³⁰ First, the simulated annealing structures were analyzed based on the experimental distances and dihedral angle constraints. The resulting structures were then subjected to a second stage of simulated annealing calculations where, in addition to the distance and dihedral angle constraints, the structures were refined against secondary $^{13}C_\alpha/^{13}C_\beta$ chemical shift restraints. The structures were analyzed with X-Plor and Procheck and

visualized with the MolMol⁶⁷⁰ and Insight II⁴⁶⁹ packages. The limitation of this study with respect to the integrated drug design theme is that the third component of linking bioactivity to modeling and/or a biophysical measurement is lacking.

Beger et al.⁶⁷¹ obtained ^{13}C NMR quantitative spectroscopic data–activity relationship (QSDAR) models of steroid inhibitors binding to aromatase based on the simulated NMR data⁶⁷² in 2001. The ^{13}C NMR spectrum of an inhibitor included frequencies that were related to the quantum mechanical properties of the ^{13}C nuclear magnetic moment. The quantum mechanical description of magnetic moment, in turn, depended mainly on its electrostatic features and geometry. Therefore, it was postulated that ^{13}C NMR data can be used in much the same way that QSAR uses comparative molecular field analysis (CoMFA) of constitutional, topological, geometrical, electrostatic, and quantum descriptors. Simulated ^{13}C NMR spectra⁶⁷³ were determined using the ACD C NMR Predictor software.³⁴⁵ Simulated ^{13}C NMR spectra were used to provide a QSDAR model that was completely computer driven since no experimental NMR spectra were employed. Calculated NMR data was used as a composite set of descriptors for CoSA. Statistica¹⁵⁵ was utilized for comparative spectral analysis,⁶⁷⁴ and a subsequent QSDAR model for the inhibitor compounds binding to the enzyme was generated. Three of the models were based on comparative spectral analysis (CoSA), and the two other models were based on comparative structurally assigned spectral analysis (CoSASA). The CoSA QSDAR model gave better results in comparison to the CoSASA model. This study successfully practiced an integrated approach with all three required components in place.

Copper(II) bis(thiosemicarbazone) complexes are known to react selectively with hypoxic cells. In 2001, Blower et al.⁶⁷⁵ used a combination of spectroscopy, cyclic voltammetry, and computational studies (density functional theory) to determine the characteristics of hypoxic-selective ligands. This enabled them to synthesize new Cu(II) complexes directed toward hypoxic-selective delivery of nitrogen mustard-type molecules. Their ultimate plan was to change the copper metal for the β -emitting Re-186.

In 2003, a cancer chemoprevention method using polyphenols found in tea leaves was reported by Leone et al.⁶⁷⁶ Polyphenols inhibit the antiapoptotic Bcl-2-family of proteins (Bcl-x_L and Bcl-2). The authors used a combination of NMR binding experiments, fluorescence polarization assays, and docking studies. Two-dimensional [$^{15}N, ^1H$]-transverse relaxation optimized spectroscopy (TROSY) spectra were obtained for ^{15}N -labeled Bcl-x_L. The 3D structure of Bcl-x_L complexed to the Bak peptide was used for chemical shift mapping and docking studies. Molecular modeling studies were conducted with Sybyl.¹⁶⁷ The docked structures were initially obtained with FlexX⁶⁷⁷ as implemented in Sybyl. The resulting best scoring structures were then energy minimized using the routine DOCK of Sybyl keeping the receptor site rigid. A limitation of the paper is that unfortunately a clear comparison between the experimental inhibition constants and the docked binding energies was not provided.

Sebag and co-workers⁶⁷⁸ explored the conformational studies of novel estrogen receptor ligands as effective therapeutic agents for breast cancer utilizing 1D and 2D solution NMR spectroscopy and computational methods. 1D and 2D NOESY studies and comparison of ^{13}C NMR chemical shifts with theoretical GIAO-calculated absolute shielding constants at the DFT level with the Gaussian⁶⁷⁹ program were performed for

these inhibitors. ^1H and ^{13}C assignments of ligands were determined by means of DEPT, COSY, and HMQC experiments. The conformations of the considered ligands were also calculated and correlated to their respective receptor binding affinities. Contributing conformers were identified from the predicted ^{13}C shifts of possible structures and found to be consistent with conformational conclusions derived from the NOE data. The authors formally used all three proposed drug design components, but the real benefit of the paper is rather limited in terms of predicting if different compounds with the same backbone conformation will really fit into the active site.

In 2004, Foulon et al.⁶⁸⁰ reported a study in which typical QSAR descriptors for aromatase inhibitors were calculated and compared with spectroscopic data. They determined the acidity constants ($\text{p}K_a$) of eight *N*-substituted imidazole derivatives that were tested as inhibitors for treatment of breast cancer. A capillary electrophoresis (CE) method, which is based on the migration time or the mobility of the ionic species over a range of pH values, was used to determine the acidity constants of the considered inhibitors. All $\text{p}K_a$ variations induced by chemical modifications were validated by comparison of the results with UV–VIS spectroscopy as well as computational data with the ACD/ $\text{p}K_a$ software.⁶⁸¹ Reasonable correlation between the CE and the spectroscopic methods was reported. Also, acceptable correlation between the CE results and the data calculated using the ACD/ $\text{p}K_a$ software was reported.

Comparison of the experimental FT-Raman spectra of platinum(II) anticancer drugs with the corresponding theoretical parameters derived from DFT calculations was made by Michalska et al. in 2005.⁶⁸² It is interesting to note that the DFT results derived from the Gaussian program⁶⁷⁹ referred to the isolated molecule in the gas phase at 0 K, whereas a comparison was made with the Raman spectrum of the solid molecule at room temperature. However, the simulated Raman spectra closely resembled the observed solid-state spectrum of carboplatin. Some of the differences between the calculated versus the experimental frequencies or intensities were ascribed to the hydrogen bonding in the solid and also from anharmonicity of certain vibrations. The theoretical $\nu(\text{Pt}-\text{N})$ frequencies obtained with the LanL2DZ basis set⁶⁸³ were in good agreement with experimental results. This enabled unequivocal assignment of all observed Raman bands.

Zhang et al.⁶⁸⁴ (2006) investigated the structural determinants of mammalian arylamine *N*-acetyltransferases (NATs) that catalyze the detoxification of several carcinogens. NMR spectroscopy and homology modeling with Modeler from Insight II⁴⁶⁹ with energy minimization were employed to reveal the structural determinants of arylamine acetylation by NATs. Residues that play a critical role in the substrate binding and catalysis were characterized using NMR techniques by means of the chemical shift perturbation analysis for ^{15}N and ^1H nuclei. This enabled them to assign the secondary structural elements in hamster NAT2. A homology-based model of the enzyme was generated with Modeler,^{271,272} and structural differences between hamster NAT2 and prokaryotic NATs were explored. Docking structures of hamster NAT2 or human NAT1 complexed with PABA were generated using the Haddock⁶⁸⁵ program. NMR data was consistent with knowledge about the NAT catalytic mechanism. The Ramachandran plot^{686,687} of the obtained hamster NAT2 structure with the Modeler software⁶⁸⁸ revealed that no residue occupied φ , ψ angles in the disallowed regions and that about 87% of the residues was grouped into the most favored φ,ψ conformation for their

residue type. Moreover, the side-chain atoms of the residues that are known to play important catalytic roles were found to be oriented toward the enzymatic cavity. Their findings suggested an important step toward predicting whether arylamines present in new products can be detoxified by mammalian NATs. An example of a Ramachandran plot is provided in Figure 13.

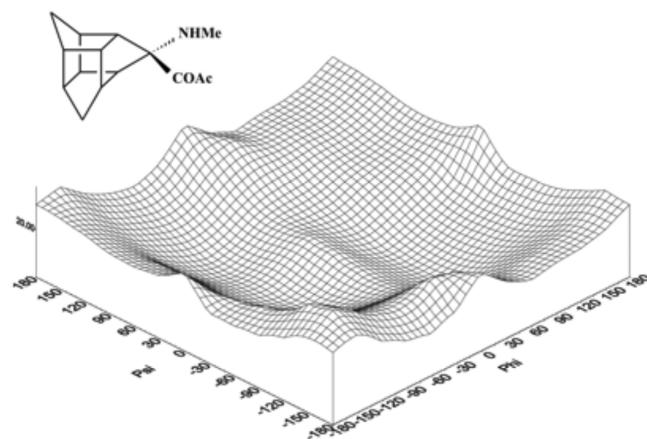


Figure 13. Example of a 3D Ramachandran plot from a trishomocubane amino acid. Data was generated with Amber. High-energy regions on the plot represent disallowed conformations. (Reprinted with permission from ref 689. Copyright 1998 Wiley.)

In 2008,⁶⁹⁰ the synthesis, biological assay, and molecular modeling combined with IR and ^1H and ^{13}C NMR studies of a series of biphenyl methylene imidazoles as CYP17 prostate cancer inhibitors against human 17 α -hydroxylase-17,20-lyase were investigated. The structures of the inhibitors were built with Sybyl,¹⁶⁷ and their structures were minimized using the MMFF94s force field.⁶⁹¹ The obtained geometries were then subjected to an ab initio calculation⁶⁹² at the DFT level (B3LYP) with the 6-31G basis set using the Gaussian^{679,693} program.⁵⁹⁴ Various inhibitors were docked into their CYP17 homology protein model generated with the Gold^{694,695} software. They observed a logical structure–activity relationship for biphenyl-type CYP17 inhibitors and observed that alkyl group substitution at the methylene bridge strongly improves the inhibitory potency. Moreover, it was reported that analogues with polar ring substituents appear to be potent inhibitors through effective hydrogen-bond formation with the active site.⁶⁹⁰

Several novel phosphatase producing liver-3 (PRL-3) inhibitors for cancer (IC_{50} values lower than 50 μM) were identified in a combined theoretical and spectroscopy study in 2008.⁶⁹⁶ Along with NMR spectroscopy, homology modeling of the target protein and structure-based virtual screening with AutoDock^{407,410,697} were applied. The effect of ligand solvation on the free binding energy of the formed complex was considered. The conformational motion of PRL-3 was also investigated with NMR techniques. Experimental data revealed high flexibility of the loop structure that includes the active site. Since the two NMR-derived structures of PRL-3 were not appropriate for docking, the X-ray crystal structure of PRL-1 holding a high sequence identity of more than 75% between the two PRL phosphatases was used as template for homology modeling. Detailed binding mode analysis from the docking simulations confirmed the stabilization of the inhibitors by both

formation of multiple hydrogen bonds and van der Waals contacts between the inhibitor and the active site as well as with the peripheral binding site. For the molecular solvation calculations, free energy atomic parameters developed by Kang et al.⁶⁹⁸ (unavailable in the AutoDock program) were used. It was concluded that docking simulations with an improved free binding energy function is a useful tool to elucidate the activities of the given inhibitors and can increase the accuracy of virtual screening results from libraries of biologically active molecules. Advances in computational techniques, in conjunction with progress in X-ray crystallography and NMR spectroscopy, have resulted in development of efficient procedures to design a diversity of potential anticancer inhibitors.⁶⁹⁶

One year later in 2009, structural modeling, MD calculations, free energy simulations, and biochemical characterization combined with CD spectroscopy⁶⁷ of RET and MET kinases in human cancer were investigated.⁶⁹⁹ Structural modeling of M918T RET and M1250T MET mutations was performed using Modeler,^{271,272} and subsequent refinement⁷⁰⁰ of the side chains of the homology modeled structure was performed using the SCRWL3⁷⁰¹ program. MD simulations and docking studies were performed using the homology-modeled protein structure and made use of the evolutionary search algorithm and knowledge-based energy model reported previously.⁷⁰² Also, evaluation of the free binding energy of the inhibitor with the oncogenic and drug-resistant RET mutants with NAMD⁴⁷⁰ molecular mechanics software were performed. The results from CD spectroscopy experiments revealed no significant variations in the secondary or tertiary structure of the M918T RET mutant. Interestingly, the calculated protein stability differences between the wild-type and cancer kinase mutants were in good agreement with the results from CD spectroscopy and consequently provided a molecular rationale of the observed inhibition. Their findings supported the molecular mechanism of activation, which induces an imbalance in the dynamic equilibrium shifted toward the active form of the enzyme. Hence, computer simulations of the inhibitor complexed to the oncogenic and drug-resistant RET enzyme were shown to provide a valuable molecular rationale for the observed inhibition profiles.⁶⁹⁹ The report appeared to have effectively integrated all three proposed components for rational drug design.

The binding site for 17- β -hydroxysteroid dehydrogenase type 1 as a drug target for breast cancer was explored by Michiels et al. in 2009.⁷⁰³ Due to difficulties with both crystallization of the enzyme–inhibitor complexes and observation of protein signals from the NMR experiments, an improved method was required to obtain structural information on this enzyme. Ligand-based NMR studies and molecular docking with Autodock were employed to obtain structural information on these complexes.^{407,410,697} Since 17-HSD1 exhibit limited solubility in water and a tendency to aggregate in aqueous solution, SALMON⁷⁰⁴ NMR spectra in water were acquired for screening and to characterize the orientation of the ligand binding to the enzyme. Salmon is a NMR method that utilizes waterLOGSY⁷⁰⁵ to determine the orientation of a ligand bound to a protein by mapping its solvent accessibility. The results of these NMR experiments strongly suggested the existence of an additional 17-HSD1 binding site for phytoestrogens which is neither the substrate nor the cofactor binding site. Apigenin was docked into the active site (dimer) generated from the crystal structure, and interestingly, these interactions also

matched the NMR-derived data from a structural viewpoint. Also, a satisfactory rationale for the observed effects in ligand binding was proposed which revealed the possibility of a binding site at the dimer interface. Their research bore fruitful ideas for the design of 17-HSD1 inhibitors as breast cancer therapeutics that also target a second potential binding site of the protein.⁷⁰³

In 2010, Baul et al.⁷⁰⁶ explored the interaction of a series of novel tributyltin(IV) 2/4-[*(E*)-2-(aryl)-1-diazetyl] benzoates with selected enzymes associated with propagation of cancer, namely, ribonucleotidereductase, thymidylate synthase, thymidylate phosphorylase, and topoisomerase II. They employed QSAR methods with the C-QSAR program⁷⁰⁷ and docking using ArgusLab²⁴³ in combination with NMR and Mössbauer spectroscopy experiments. ¹H, ¹³C, and ¹¹⁹Sn NMR spectra were recorded. Isomer shifts were considered relative to Ca(¹¹⁹Sn)O₃ at room temperature. Mössbauer spectroscopy provided useful insight into the solid-state structures of specific complexes, for which no suitable crystals were available for X-ray crystallography analysis. In vitro studies suggested that the cytotoxic activity correlates to the tetrahedral geometry of the complexes in solution. This observation was confirmed by the docking results.⁷⁰⁶ This report thus used all three proposed components for optimal drug design. They also linked a general measurable biophysical analytical technique (Mössbauer spectroscopy) with bioactivities of the inhibitors. However, discrimination between the relative activities of inhibitors was not possible.

Stauch et al.⁷⁰⁸ in 2010 employed a combination of NMR spectroscopy and molecular docking using the Gold program^{404,695} to characterize the interaction mode of argyrin as an anticancer agent with the human S20 proteasome. For assignment of the argyrin A, HSQC, COSY, TOCSY, and NOESY spectra were recorded. Information about the solution structure was obtained using the observed NOE interactions in a molecular modeling approach. Information of the interaction of the inhibitor with the proteasome was also obtained from NMR data. First, the conformation of argyrin A in an aqueous environment was deduced from NMR spectroscopy in which a second set of resonances in a noncrowded part of the spectrum was observed. These additional resonances were assigned to both the second conformation of argyrin A and potential degradation products. The absence of exchange peaks between the two sets of resonances in a ROESY experiment supported the second hypothesis. Also, they evaluated the 100 lowest potential energy conformations of argyrin A in an aqueous environment by employing quantitative treatment of NOESY cross-peak intensities. The enzyme has three active sites, and a 3D model was created with homology modeling. Inhibitors were docked with the enzyme (each active site separately), and it was observed that argyrin interacts strongly with the canonical substrate-binding site of the protease which will lead to inhibition of substrate degradation. Furthermore, the considered atomic interaction model was in agreement with the structure–activity relationship data. Besides various conserved backbone interactions between argyrin and all three substrate-binding pockets and a large number of hydrophobic interactions, specific contacts were found between the two aromatic tryptophan moieties of argyrin and variable regions of the proteasome binding pocket. These results can potentially facilitate the rational changes of ligand substitution to enable proteasome–subunit specificity.⁷⁰⁸ The report appears to have

effectively integrated all three proposed components for rational drug design.

In 2011, an integrated theoretical and high-resolution NMR as well as IR spectroscopic study was performed on the novel and selective inhibition of glycosyl carbonic anhydrase IX, which is overexpressed in many tumors.⁷⁰⁹ The conformational behavior of 4,6-di-O-acetyl-2,3-dideoxy-D-threo-hex-2-enopyranosyl sulfamide (SAE) and its IR vibrational and NMR magnetic properties (¹H and ¹³C chemical shifts) were evaluated from a computational point of view. The conformational space of SAE was investigated using MD simulations²⁴⁹ as implemented in the HyperChem package using a semi-empirical AM1 calculation. Four different starting conformations of SAE were considered, based on the orientation of the sulfamide group. Each of these conformations were built in both α - and β -anomeric forms. The AM1-optimized geometries were further optimized using density functional theory⁷¹⁰ with the Gaussian⁶⁷⁹ program. The eigenvalues of the Hessian matrix were converted to vibrational frequencies and used to help in the assignment of the experimental IR spectrum of the SAE models. ¹H and ¹³C isotropic shielding tensors were calculated⁷¹¹ relative to tetramethylsilane (TMS). To evaluate the different anomeric effects over the relative stabilities of the conformers, a natural bond orbital (NBO) analysis was also carried out. Detailed analysis of some of the geometric parameters shed light on the conformational behavior of the sulfamide in view of both exo- and endo-anomeric effects and antiperiplanar relationships. Optimized geometries and NBO results agree well with the reported crystallographic data. Theoretical calculations of ¹H and ¹³C chemical shifts seemed useful for a better understanding of the experimental spectra. IR spectra of the compounds were recorded, and assignment of some of the most important bands was accomplished using the calculated harmonic vibrational frequencies. It is well known that calculated IR data gives a 10–15% error (higher frequency).⁵⁹⁴ Some software automatically adjusts the calculated results.

Comparison of the NMR chemical shift calculations of choline (Cho) with experimental *in vitro* high-field ¹H and ³¹P NMR spectroscopy (for measurement of concentrations of individual choline derivatives) were made by Alcorn et al.⁷¹² for a wide variety of cancer types. Quantification of total choline from its proton spectrum has the potential to be a diagnostic tool for the presence of cancer and an accurate early indicator of the response of cancer to treatment. Experimental ¹H NMR signals of choline were obtained from the Human Metabolome Data Base (HMDB). The calculated (PBE0⁷¹³ and B3LYP functionals in Gaussian) NMR chemical shifts⁷¹¹ and coupling constants of gas-phase-optimized choline were compared to experimental results. It was concluded that for both the PBE0 and the B3LYP correlation functions the NMR chemical shifts calculated by the GIAO method exhibited a lower mean absolute deviation than the CSGT method.⁷¹⁴ Solvent effects were evaluated with the IEF-PCM solvation model, which generated a reasonable geometry compared to the crystal structure of choline. The small differences between the computational data and the crystal structure (mostly the dihedral angles) were ascribed⁷¹⁴ to obvious differences between the crystalline and the aqueous environments. Moreover, the B3LYP hybrid functional yielded the most accurate chemical shifts, though the mean absolute deviation agreed well with that of the PBE0⁷¹⁵ functional. The largest difference in the mean absolute deviation reported in this study

was 0.06 ppm, which was on par with the absolute errors between the calculated and the experimental chemical shifts. Slight differences in chemical shifts were also explained as the result of explicit solvation effects (hydrogen bonding).

Zohoorian-Abotorabi et al. (2012)⁷¹⁶ examined the interaction of two antbreast cancer drugs, i.e., fluoxymesterone (FLU) and cyclophosphamide (CYC), with human serum albumin (HSA) using spectroscopy, zeta potential, and molecular docking techniques. Resonance light scattering (RLS) was utilized to investigate the effect of the two anticancer drugs on changes of the protein conformation. Enhancement in RLS intensity was attributed to formation of a new complex between the two compounds and the protein. Both drugs demonstrated a powerful ability to quench the fluorescence of HSA, and the fluorescence quenching action was more intense with a mixture of the two drugs. Docking studies were also performed and demonstrated that the inhibitors are binding close enough to the fluorescent groups to cause quenching. The effect of both drugs on the conformation of HSA was analyzed using synchronous fluorescence spectroscopy. The fluorescence quenching of HSA appears to originate from the Trp and Tyr residues which demonstrated a conformational change of HSA with addition of both drugs. The binding distances between HSA and the drugs were estimated by Forster theory, and it was revealed that nonradiative energy transfer from HSA to both drugs occurred with a high probability. According to CD measurements, the influence of both drugs on the secondary structure of HSA in aqueous solutions was also investigated and illustrated that the α -helix content of HSA decreased with increasing drug concentration in both systems. Moreover, the zeta-potential experiments revealed that both drugs induced conformational changes on HSA. The report has effectively integrated all three proposed components for rational drug design. A 2009 review paper⁷¹⁷ has combined separated spectroscopic and molecular modeling reports on HSA and AGP drug binding.

Two review papers were found that have combined separate reports on spectroscopic and molecular modeling to demonstrate how this combination can assist with the drug design process.

In a 2004 review by Rush et al.⁷ application of X-ray, NMR, and molecular modeling of potential inhibitors for matrix metalloproteinases (MMPs) and the TNF α -converting enzyme (TACE) from Wyeth (a pharmaceutical company) were presented. This review is concerned with application of experimental structural information and molecular modeling to design inhibitors for MMPs as an active target for cancer and arthritis. The larger part of the review covers direct creation of 3D solution structures for target enzymes from NMR data using software such as NMRPipe.⁷¹⁸ However, since this method is very time consuming they also used crucial structural data from NOE measurements to create models using MD calculations where these interatomic distances are restricted; the second method produces faster but less accurate structural information. The authors then referred to several in-house studies using these 3D models for subsequent drug design efforts using normal computational techniques. None of the articles covered in the review actually combined all three of the desired components for optimal drug design.

In 2010, polymorphic binding of bioactive isoquinoline alkaloids to nucleic acid as effective anticancer/antitumor agents was reviewed.⁷¹⁹ To elucidate the mode and mechanism

of action of topoisomerase inhibitory activity in terms of structure–activity relationships, the interaction of isoquinoline alkaloids with polymorphic nucleic acid structures was studied with a number of experimental techniques as well as computational chemistry methods. The experimental techniques included spectrophotometry, spectrofluorimetry, thermal melting, CD, NMR spectroscopy, electrospray ionization mass spectrometry (ESI-MS), viscosity, isothermal titration calorimetry, and differential scanning calorimetry. Molecular modeling studies focused on berberine binding to various short oligonucleotide duplexes in an effort to rationalize their mechanism of action. It made use of NOE data used as constraints in MD simulations of the inhibitor complexed to the enzyme. The authors referred to structure–activity relationships using previously reported methodology, also involving computational methods. They only referred to one paper⁷²⁰ where a combined spectroscopic (NMR and UV) and molecular modeling approach was used to study DNA intercalation of these compounds. The nucleic acid binding properties of complex formation of two groups of the considered alkaloids with different polymorphic nucleic acids were also analyzed in terms of their anticancer activity. It was concluded that the DNA binding of the planar sanguinarine and coralyne was stronger and thermodynamically more favored compared to the buckled structure of berberine and palmatine. This observation correlated well with the intercalative mechanism of sanguinarine and coralyne and the partial intercalation by berberine and palmatine. It was concluded that the differential binding of these alkaloids to various polymorphic nucleic acid conformations with specific biological functions is useful knowledge in nucleic acid-targeted drug design.⁷¹⁹ Only one reference in the review report has used all three proposed components for optimal drug design.

6.1.2. HIV. The AIDS epidemic is truly a global threat. Of the estimated 33 million people infected with HIV worldwide in 2010, 22 million are in Africa.⁷²¹ At the end of 2011 this figure was 24 million.⁷²² There are several HIV targets, and the inhibitors are classified accordingly: nucleoside reverse transcriptase inhibitors (NRTIs), nucleotide reverse transcriptase inhibitors (NtRTIs), non-nucleoside reverse transcriptase inhibitors (NNRTIs), protease inhibitors (PIs), fusion inhibitors, coreceptor inhibitors, and integrase inhibitors (INs). It is important to note that HIV integrase is very flexible, and at least 11 different conformations were initially reported.⁷²³ This complicates the situation considerably for static docking studies. Readers are again referred to the discussion in section 4 about exercising care with respect to the use of experimental bioactivity data for calibration of computational models.

HIV drug design that involves the study of molecular recognition and binding patterns with the aid of spectroscopic techniques and molecular modeling⁷²⁴ is reviewed in this section. Toward the end of this segment, review papers that have combined separate molecular modeling and spectroscopic reports to demonstrate how this combination can assist with the HIV drug design process are presented.

AZT was the first Food and Drug Administration (FDA) approved HIV drug (1987), and it acts as an inhibitor of reverse transcriptase (TR). The first study (1991) involving NMR spectroscopic studies and MM2 calculations⁷²⁵ of AZT in solution discovered discrepancies between the solution structure for AZT and that of the X-ray structure.⁷²⁶ The link with the bioactivity component of drug design was not made.

The HIV-1 protease enzyme has been a therapeutic target for FDA-approved commercial AIDS drugs as far back as 1995.^{727,728}

Several studies involving MD simulations of the HIV-1 protease, with and without inhibitors, have been published since 1990.^{729–732} Earlier efforts directed toward development of peptidic-based inhibitors of HIV protease did not involve combination of molecular modeling with spectroscopic techniques.^{733–736}

Several comprehensive articles have been published on structure-based HIV drug design with the aid of a combination of computational methods and experimental techniques (spectroscopic and *in vitro* experiments) within the last two decades.

For example, Podlogar et al.⁷³⁷ used NMR and molecular mechanics modeling to obtain structural information about a macrocyclic HIV PR inhibitor in 1994. They also modeled the interaction of the lead inhibitor (HnT PR inhibitor 1) with the PR and concluded that the conformation observed for the free inhibitor, from NMR and computational studies, is similar to the conformation of the enzyme-bound inhibitor. This report appears to have used all three proposed components for optimal drug design in an integrated fashion.

In 1997, ¹H and ¹³C NMR chemical shift assignments and molecular docking of bifunctional enols of *N*-protected phenylalanine HIV-1 protease inhibitors was investigated by Boulanger et al.⁷³⁸ In that study, NMR coupling constant measurements indicated that the NH–CH α dihedral angles are different for the *E* and *Z* conformational forms of the amino acid side chain enols. Molecular modeling calculations were carried out (Insight II, Discover, DGII, Docking, Sorption, later taken over by Accelrys)⁴⁶⁹ in an attempt to identify the fitting orientation of each inhibitor to the binding site of the HIV-1 protease. These calculations involved MM minimization of the inhibitors (CVFF) followed by docking to calculate the binding energies. The calculated intermolecular energies were found to correlate with the experimental inhibition coefficients. This report appears also to have used all three proposed components for optimal drug design in an integrated fashion.

In 1999, the X-ray structure and conformational dynamics of HIV-1 protease complexed with the statine-derived inhibitor SDZ283-910 were investigated. In this regard, general agreement of time-resolved fluorescence spectroscopy and MD simulations was observed.⁷³⁹ The 3D structure of HIV-1 protease and different inhibitor complexes was useful for understanding its mode of action. Further optimization of these inhibitors using structural detail of inhibitor-induced conformational changes of the HIV-1 protease from the MD simulations was performed, and the results were experimentally verified. MD computer calculations¹⁵⁹ using O-PLOR⁷⁴⁰ and X-PLOR⁷⁴¹ revealed the expected motions within subunits of the protease that are essential for the catalytic activity of this enzyme. Also, fitting experimental fluorescence spectroscopy and MD simulations data revealed very good agreement for both uncomplexed protease and the SDZ283-910 complex. Binding of the inhibitor induced faster decay of both the experimental and the computed protease fluorescence anisotropy signal decay. It was realized that in order to get more reliable MD results a longer simulation period⁷⁴² was required. This observation revealed the importance of a comparison of MD results with experimental data.⁷³⁹ This report appears to have used all three proposed components for optimal drug design in an integrated fashion.

In 2001 the interaction of derivatives of AZT with lipid membranes was studied utilizing a combination of NMR techniques and computational chemistry methods.⁷⁴³ This report used only two of the proposed components for optimal drug design, namely, molecular modeling and a biophysical analytical method (spectroscopy).

Later, in 2002 Dang et al.⁷⁴⁴ employed NMR spectroscopy and data from X-ray studies with constrained molecular minimization methods for the binding study of DG35-VIII as an HIV-protease inhibitor exhibiting nanomolar IC₅₀ values in different solvent media. For this purpose, they used the molecular mechanics algorithms in DYANA⁷⁴⁵ and MSI (Insight II, Discover, Docking, later taken over by Accelrys)⁷⁴⁶ to investigate the effectiveness of these software⁷⁴⁷ (see Monti et al.⁷⁴⁸ for another example where this was also done) and the conformational changes that the drug experiences upon interaction with the active site. The obtained theoretical low-energy structures of inhibitors were consistent with the experimental NMR data. To determine the 3D structure of these compounds, NOESY experiments were employed to identify proton internuclear distances. NOESY data for DG35-VIII in solution enabled them to identify two structures with similar energies of which only the “extended” structure, which was found in methanol, bound to the active site of HIV protease. In other words, the extended structure of DG35-VIII in comparison to closely related HIV-protease inhibitors (VX-478 and ABT-538) exhibited similar backbone structures with the functional isostere groups superimposed on each other. Having no X-ray structures of this drug with HIV protease, a model was constructed with the NMR software and constrained molecular mechanics in order to examine the binding of DG35-VIII to the active site of the HIV protease. The calculated binding energy of DG35-VIII with HIV-protease was determined by energy minimization of the complex and found to be comparable with VX-478 and ABT-538.⁷⁴⁴

Tajmir-Riahi and co-workers (2004) studied FTIR and UV-VIS difference spectroscopic methods in combination with molecular modeling to determine binding sites and binding constants of AZT to the nucleoside-binding site of the viral reverse transcriptase.^{749,750} It was shown experimentally that this interaction inhibits DNA replication of the host cell by chain termination. It appears they used all three proposed components for optimal drug design in an integrated fashion.

Later, in 2006, a combination of MD simulations with NMR spectroscopy data was used to study molecular models of the protein Vpu from HIV-1.⁷⁵¹ In other words, molecular models of the protein Vpu from HIV-1 were built using NMR-based structural models in solution which were then refined by MD simulations (6 ns) in a fully hydrated lipid bilayer using the Gromacs¹⁶⁴ package. Simulation results supported the findings that the orientation of the cytoplasmic domain in the phosphorylated models of HV1S1-rot appeared close to the membrane surface.

O'Meara et al.⁷⁵² in 2007 reported molecular modeling of inhibitors complexed to RT. They also employed NMR NOE techniques to determine structural information about the inhibitors in solution. The combined knowledge was used to design and test new inhibitors.

In 2007, Lee-Huang et al.⁷⁵³ studied the interaction of oleuropein (Ole) and hydroxytyrosol (HT) derived from olive leaf with viral targets (HIV-1 fusion and integrase inhibitors). They performed molecular docking with Autodock,⁶⁹⁷ MD simulations, and free energy calculations⁷⁵⁴ with Insight II from

the Discovery Studio program.⁴⁶⁹ To validate the modeling predictions, the effect of oleuropein and hydroxytyrosol on HIV-1 fusion complex formation was examined using CD spectroscopy. They concluded that oleuropein and hydroxytyrosol bind to the highly conserved hydrophobic pocket on the surface of the HIV-gp41 fusion protein. This hydrophobic surface exhibits hydrogen bonding with Q577 and hydrophobic interactions with I573, G572, and L568 on the gp41 N-terminal heptad repeat (NHR) peptide N36 and inhibits formation of the gp41 fusion-active core.⁷⁵³

Also, in 2007, Tintori et al.⁴⁹⁷ investigated application of an electron-ion interaction potential (EIIP) technique in combination with molecular modeling and experimental biological data for identification of new HIV-1 integrase (IN) inhibitors. Virtual screening was performed with Autodock^{407,410,697} to characterize any potential IN inhibition. An effective inhibitor forms a stable complex with the IN and hinders binding of the latter to viral-encoded DNA.⁷⁵⁵ The X-ray structure was energy minimized by means of Macromodel to avoid any steric clashes⁷⁵⁶ based on a method in the literature.⁶⁶⁷ The best compound displayed an IC₅₀ value of 69 μM for inhibition of the IN (experimental biological data). The activity was assessed with a fluorescence fluctuation assay (K_i value of 19 μM). These results were in agreement with modeling studies.⁴⁹⁷ An in vitro experiment was performed where the inhibitors were added to integrase in the presence of the viral-encoded DNA. Binding (or inhibition thereof) of the integrase to the viral-encoded DNA was monitored through attachment of a fluorescent molecule to the DNA. Virtual screening data was obtained using Autodock software. It was proposed that these compounds were able to prevent binding of IN to the DNA through interaction with the DNA binding domain of IN. This report appears to have used all three proposed components for optimal drug design in an integrated fashion. It appears that this report did not consider the flexible nature of the IN enzyme⁷²³ (see comments at the start of this section).

Andrianov and co-workers reported computer modeling of novel potential targets for anti-AIDS drugs with particular attention to their application to the HIV-1 gp120 V3 loop in 2007 and 2011.^{757,758} Modeling the 3D structures for V3 was performed on structural information obtained from NMR spectroscopy. The NMR-based 3D structural models for the amino-acid sequences of the V3 loop from the HIV-1 subtypes A, B, C, and D was generated using molecular modeling techniques. The models obtained revealed common structural motifs in the same portion of the gp120 envelope protein. Hence, the lowest energy 3D structures of V3 were computed by homology modeling with Modeler^{271,272} followed by simulated annealing methods⁷⁵⁹ using Gromacs¹⁶⁴ software. These structures were compared with each other as well as with those obtained by X-ray diffraction and NMR spectroscopy. Interestingly, joint analysis of the calculated and experimental data proved that despite the high amino-acid sequence variability, the HIV-1 V3 loop contains three structurally conserved segments. Hence, the gp120 V3 protein seems a useful target for the rational, structure-based design of new HIV-1 drugs.

In 2009 the synthesis and biological assay of various ACV ProTides holding three different aryl moieties, an amino acid, and the ester parts were explored by Derudas et al.⁷⁶⁰ ¹H, ¹³C, and ³¹P NMR spectra were recorded. They monitored the enzyme activity with ³¹P NMR. To obtain an improved understanding of the antiviral behavior of these compounds,

molecular docking studies with Autodock^{407,410,697} software using a crystal structure of the enzyme with different potential inhibitors were performed. These were then evaluated against the observed ³¹P NMR measured antiviral activities. Docking results revealed that the varied binding energies of the inhibitors correlated to the corresponding experimentally observed rates of metabolism.⁷⁶⁰ This report appears to have used all three proposed components for optimal drug design in an integrated fashion.

Identification of flap conformations for two drug-resistant HIV-1 PR were explored by Galiano and co-workers.⁷⁶¹ Pulsed electron paramagnetic resonance (EPR) spectroscopy combined with MD simulations and distance measurements were employed for this purpose. Site-directed spin labeling (SDSL) and pulsed double electron–electron resonance (DEER) EPR spectroscopy was used to discriminate between conformations of the flaps in the inhibitor-bound “closed” state and the apo-state of HIV-1 PR. MD simulations precisely reproduced the experimentally observed DEER-based distance distribution profiles and provided a useful link that correlates the EPR distances to structural and dynamic features of the flaps. The average conformations of the enzyme were determined during the MD simulations, and interestingly, the degree of closure was in good agreement with the semipen crystal structure of apo HIV-PR. Combined analyses revealed that the average conformation of the flaps, the range of flap opening and closing, and their flexibility differ markedly in HIV-1PR as multiple mutations arise in response to antiviral therapy.

An extensive NMR study combined with a theoretical modeling investigation has recently been performed in our laboratory. Different families of pentacycloundecane (PCU) lactam peptides/peptoids and PCU diol peptides compounds were synthesized as potential inhibitors of the wild-type C-South African (C-SA) HIV-1 protease.

In our first study,^{762,763} a combination of NMR, molecular docking experiments using Autodock,⁴¹⁰ and MD studies (QM/MM/MD simulations⁴³⁶ using the DYNAMO package⁷⁶⁴) were applied to this family of promising PCU^{765–767} lactam^{768–772} peptidic C-SA PR inhibitors. Crucial information about the 3D structure of the corresponding inhibitors in solution was obtained with EASY-ROESY⁷⁷³ NMR experiments. The 3D structure of the peptide inhibitors was also calculated with MD techniques. Acceptable correlation between the inhibitory concentration (IC_{50}) data and the solution structure of the peptides was observed. This study for the first time revealed that the chirality of the PCU moiety and its ability to induce conformations of the coupled peptide side chain appears to influence the activity of these inhibitors. Docking results (Autodock) also correlated quite well with the experimental IC_{50} results.⁷⁷⁴ MD studies of the most potent inhibitor showed hydrogen-bond interaction between a hydroxyl group of the PCU-lactam with Asp25 of the dimeric catalytic triad residues Asp25-Thr26-Gly27 (A/B chains). Molecular modeling results suggest that the solution structure of the inhibitor is lost upon complexation with the PR, indicating an induced fit mechanism. This report has used all three proposed components for optimal drug design in an integrated fashion.

The second study⁷⁷⁵ involved NMR, biochemical, and computational methods for the eight PCU-lactam peptides and peptoids exhibiting reasonable inhibition of the wild-type C-SA HIV-protease with much less toxicity to human cells than the commercial control drugs. The PCU cage plays an

important role with respect to the peptide conformation. The interaction between these inhibitors and the HIV-PR was calculated with Autodock⁴¹⁰ and Autodock Vina,⁴⁰⁹ and docking results were too crude to be correlated with the relatively narrow range of experimental IC_{50} values (0.5–10 μ M). MD simulation results (AMBER)^{160,161} suggested that the peptoids exhibit much more conformational flexibility than the corresponding cage peptides as deduced from NMR NOE experiments.

Our third study⁷⁷⁶ involved PCU diol^{766,767,771,777} peptide inhibitors against the wild-type C-South African HIV-1 protease. Docking results were in reasonable agreement with the measured IC_{50} values. MD simulations clearly showed that the inhibitor easily fits into the active enzyme pocket. It was evident from analysis of the hydrogen-bond interactions along the MD trajectories that the inhibitor forms hydrogen bonds between the cage hydroxyl groups and at least one of the two Asp25 carboxyl groups. As was the case before, the solution structure of these inhibitors as observed with ROESY techniques was not conserved upon complexation with the HIV-PR active pocket. MD studies revealed that the relative rigid interaction between the cage and the peptide side chain that was observed for the active cage lactam peptides was not observed for this family of cage diol peptides. This report has used all three proposed components for optimal drug design in an integrated fashion.

In our fourth study⁷⁷⁸ a new class of an nonscissile PCU ether-bridged-type isoster as potential HIV-1 wild-type C-SA protease inhibitors was reported. These inhibitors gave IC_{50} values ranging from 0.6 to 5.0 μ M. Docking and MD studies were performed. Docking and MD simulations suggest that the cage or part thereof occupies the S1/S1' subsite. EASY-ROESY experiments did not indicate rigid or stable inhibitor conformations.

Our most recent study⁷⁷⁹ reported a family of PCU diol peptoids as HIV protease inhibitors with IC_{50} values ranging from 6.5 to 0.075 μ M. Docking and MD studies were performed for inhibitors with the C-SA PR. NMR spectroscopy studies reveal that the peptoid side chains are more flexible than those of the corresponding peptides. This was confirmed by the MD results of the inhibitors in solution. The overall conclusion from our cage inhibitor modeling studies suggests that the solution structures of these inhibitors are not maintained upon binding to the active pocket of the enzyme and that an induced fit mechanism of binding most probably determines the mode of binding. This report has used all three proposed components for optimal drug design in an integrated fashion.

Two review papers combined separately spectroscopic and molecular modeling reports to demonstrate how this combination can assist with the drug design process.

In 1998, Wlodawer et al.⁷⁸⁰ reviewed the structure-based drug design of reversible HIV-1 protease inhibitors. The paper covers an in-depth discussion of structural information from crystallographic data and advances in determination of the solution structure of enzymes using advanced NMR techniques. Detailed analysis of a large ensemble of crystal structures of HIV-1 PR complexes from several databases provided a perspective of drug–target interactions that could be used for design of more efficient HIV drugs. Computational approaches such as MM calculations, molecular docking techniques, and free energy perturbation methods using MD simulations were also discussed. The whole review refers only to one paper by Podlogar et al.,⁷³⁷ who used a combined computational and

spectroscopic approach for HIV PR drug design. The review also provides general principles for inhibitor design, besides the correlation of experimental information with the computational results.⁷⁸⁰

In 2002, methods for developing HIV drugs as well as principal targets enzymes, namely, reverse transcriptase and protease, encoded by HIV and existing problems such as the rapid emergence of drug resistance were reviewed.⁹⁸ Specific features of development of RT-targeted drugs were also discussed in this review. The paper combines literature involving NMR of retroviral proteases and literature about computational methods including energy minimization using molecular mechanics,^{781,782} free energy perturbation calculations,^{783,784} and molecular docking.⁷⁸⁵ The authors demonstrated that a combination of computational simulations along with NMR data provides a strong basis for further structural modification and design. They again referred to only one 1994 publication⁷³⁷ which employed a combination of NMR and molecular modeling approach to study the structure of the inhibitors. This review emphasized that effective design of HIV-1 PR inhibitors requires an understanding of drug–target interactions on the molecular level, which can only be effectively achieved if theoretical and experimental techniques are combined.⁹⁸

6.1.3. Alzheimer's Disease (AD). In this section, we review several publications concerning an integrated spectroscopic and molecular modeling approach in structure-based drug design for Alzheimer's. Aggregation of amyloid peptides ($A\beta$) leads to fibril and plaque formation, and fibrillogenesis indirectly causes AD. This implies that one realistic approach to a therapy for Alzheimer's illness would involve inhibition of the aggregation of $A\beta$.^{786–788} Other potential targets will also be covered.

In 1994 the neurochemical evaluation of M₁ muscarinic receptor agonists for treatment of patients with Alzheimer's disease was investigated by Dunbar et al.⁷⁸⁹ utilizing a combined computational and spectroscopic (NMR and IR) approach. Molecular mechanics (AMBER) with charge scaling was carried out to examine the effects of the electrostatic environment on the interaction of the inhibitor with the receptor. MacroModel^{250,667} and Mopac⁷⁹⁰ were used for minimization of structures and calculation of the MNDO electrostatic potential (ESP) charges, respectively.⁷⁹¹ The results emphasized the utility of amidine systems in the development of ligands with activity at muscarinic receptors coupled to phosphoinositide metabolism in the central nervous system. Interestingly, the location of the ester substituent relative to the amidine system (determined with NMR) was consistent in each series examined, as confirmed by molecular modeling studies. It was also observed that structural modifications to the ester moiety might yield compounds with higher stability, affinity, and activity against various muscarinic receptor subtypes.

Cavillona et al.⁷⁹² in 1997 employed docking, NMR, and infrared spectroscopy to characterize the importance of the secondary structure of amyloid associated with Alzheimer's disease. As a potential model for the mechanism of fixation, macromolecular modeling techniques were used to achieve optimal tertiary structures of amyloid peptides 1-28 and 1-43. The distances obtained between certain groups were determined with NMR (little information was provided on the method used) and compared with the corresponding distances from minimized structures obtained from a standard

α -helical model that was modified based on homology of similar sequences with known 3D structure. Relatively small differences were observed in the orientations of certain hydrophobic parts of the molecule. Insignificant differences were detected for the starting N-terminal segment, and the C-terminal segment always adopts an extended β -strand conformation. Finally, docking calculations using the docking module in the Biosym package⁷⁴⁶ revealed that arginine-5 and lysine-6 are key residues involved in the interaction between congo red (as a potential inhibitor to prevent amyloid fibril formation) and amyloid proteins.

In 2001 an integrated application of molecular simulation and NMR spectroscopy in solution was reported⁷⁹³ in order to obtain a better understanding of the $A\beta(1-42)$ peptide. The coordinates of the peptide were experimentally generated using structural information obtained from solution-NMR spectroscopy data (limited information was provided about this approach in the paper), and the conformations were energy minimized using the MM+⁷⁹⁴ force field. This revealed that the central, first lipophilic core has an α -helical structure which is stabilized by intramolecular hydrogen-bonding forces. The second lipophilic core that exhibited a β -sheet structure experiences noncovalent forces with other β -sheets of $A\beta$ peptides where the β -strands run in an unequal amide-to-carbonyl direction. This study proposed a useful model system for understanding the conformations of the $A\beta(1-42)$ peptide and its simulated aggregation to dimeric and tetrameric peptides.

Also in 2001, Massi et al. performed a simulation study of the structure and dynamics of the Alzheimer's amyloid peptide in an aqueous environment combined with NMR measurements.⁷⁹⁵ NMR spectroscopy-derived amide bond order parameters and temperature-dependent α -proton chemical shifts were determined. ¹H-detected heteronuclear multiple quantum coherence NOESY spectra were recorded to determine the isotope-filtered ¹⁵N-edited spectra. The main purpose of this study was to validate the computational model through a direct comparison of simulated statistical averages with experimental observations of the peptide's structural dynamics. A second objective of the study was identification of key intrapeptide interactions that stabilize the central structural motif of the peptide [the central LVFFA hydrophobic cluster (17–21) region and the VGSN turn (24–27) region]. A reasonable correlation was observed for preservation of the structure of these elements and the interactions between the cluster and the turn regions when imposing the peptide monomer structure during the MD simulation with NMR NOE-derived constraints. The computational study provided a number of tests for the model related to the results of experiments probing the peptide structure, such as the rate of self-diffusion, conformational fluctuations, and key stabilizing interactions. Computed NMR⁶⁷³ order parameters (S_2) were in good agreement with experimentally measured values for three of the four simulations. They concluded that the LVFFA cluster and VGSN turn were cooperatively stabilized through intramotif hydrogen bonds and that the solvated structure is partly disordered in solution. They postulated that a basic understanding of the monomer solution structure was key to unraveling the mechanism of amyloid fibril formation.

In 2002, two β -sheet breaker (BSB) pentapeptide amides were designed for potential inhibition of Alzheimer's disease using the C-terminal sequence of the amyloid peptide as a template and biological assays were performed to evaluate their

efficiency.⁷⁸⁶ The mode of action of the peptides was rationalized by means of a combination of FT-IR spectroscopy and molecular modeling methods. Molecular docking of the β -sheet pentapeptide amides against amyloid protease using AutoDock and MD simulations (Gromacs)¹⁶⁴ was performed. Interestingly, analysis of the IR spectra indicated that $A\beta$ has a tendency to form β -sheet structures which matched the modeling findings that the pentapeptide amides are arranged in a β -sheet attached to the amyloid peptides. The docking results show hydrophobic interactions between the BSB and the $A\beta$ amino acids Ile-4 and Val-39. MD simulations⁷⁹⁶ confirmed the docking results.⁷⁸⁶

Fattorusso et al.⁷⁹⁷ in 2005 identified and optimized novel protein ligands and reversible nonpeptide caspase inhibitors with a combination of NMR-based screening and computational techniques. They used FlexX⁴⁰⁰ as implemented in Sybyl¹⁶⁷ for docking of the inhibitors from a chemical library. The 10 best docking results were confirmed with the Gold program.^{404,406} The docked binding energies (using an empirical binding energy scoring function) of the inhibitors complexed to the enzyme were in good correlation with the experimental inhibition constants previously reported.⁷⁹⁸ They referred to an integrated ¹⁹F NMR-based enzymatic assay and molecular modeling approach in the literature⁷⁹⁹ to discover and test a novel set of nonpeptidic reversible inhibitors. With NMR-based drug design strategies the best inhibitor is found from a library of scaffolds and then optimized either by derivation or linking two binders together as reported by Pellecchia et al.^{800,801} The difference in chemical shift of the trifluoromethyl substituent was exploited using ¹⁹F NMR as reported before.⁷⁹⁹ In other words, a novel class of reversible caspase inhibitors was developed through a structure-based iterative optimization approach. Ligand binding to select the initial hits from a scaffold library were monitored using NMR assays. Afterward, utilizing virtual docking a subset of the possible analogues was selected and tested either by NMR or with a traditional fluorimetric assay. This approach enabled them to identify novel hit compounds among hundreds of thousands of commercially available agents without relying on costly high-throughput screening techniques. Subsequently, they proposed new inhibitors with IC₅₀ values in the low micromolar range.⁷⁹⁷ This report appears to have used all three proposed components for optimal drug design in an integrated fashion.

In 2005, NMR measurements and molecular dynamics (MD) simulations of both wild-type and mutated amino acid sequences of Alzheimer's β -amyloid fibrils were investigated by Buchete et al.⁸⁰² All-atom molecular dynamics (MD) simulations with explicit solvent and multiple force fields were employed using the NAMD2⁴⁷⁰ program.¹⁵⁹ The structural stability and conformational dynamics of several solid-state NMR-based models of β -amyloid fibril structures were evaluated. The MD-derived mean values of the residue-specific backbone torsion angles (Ψ and ϕ) and of the side-chain angle χ_1 for finite $A\beta$ 9–40 fibril segments were estimated using the Talos⁸⁰³ program. These angles were extracted from the 10 ns MD trajectories of the four configurations and found to be very close to the corresponding experimental estimates. Since generally a broad distribution of torsional angles in the Ramachandran^{686,687} backbone exists for "floppy" proteins, in most cases the observed agreement between the average Ψ and ϕ values from experiments and simulations was accepted as

satisfactory, and the simulation structures were consistent to reported NMR and X-ray diffraction data.

Lee et al.⁸⁰⁴ employed NMR spectroscopy (DQF-COSY, TOCSY, and NOESY data), CD analysis,⁶⁷ and MD simulations using the Discover module of Insight II⁴⁶⁹ to investigate the interaction patterns of substrate peptides and β -Secretase (BACE). Three-dimensional structures of Sub W (a substrate peptide that consists of 10 amino acids, which are adjacent to the β -cleavage site of wild-type APP) and Sub M (Swedish mutant with double mutations on the one side of the β -cleavage site of APP) were studied by CD and NMR spectroscopy in aqueous solution. On the basis of key 3D data from the experimental methods, interaction patterns of β -secretase and substrate peptides were characterized using MD simulations.

In 2010, Katalinic et al.⁸⁰⁵ explored structural aspects of eight flavonoids (galangin, kaempferol, quercetin, myricetin, fisetin, apigenin, luteolin, rutin) as inhibitors against human butyrylcholinesterase (BChE), a known target for Alzheimer's disease. They applied UV–VIS absorbance spectroscopy and molecular docking analysis (Accelrys, Discovery Studio).⁴⁶⁹ Flavonoid conformers were generated with a Monte Carlo method⁸⁰⁶ and subjected to rigid docking. A correlation between the structural differences of the flavonoids and the inhibition potency of human plasma BChE was established. Docking experiments revealed that flavonoids bind to the BChE active site through numerous hydrogen bonds and π – π interactions. Multiple modes of interaction between flavonoids and the BChE catalytic residues were observed. The calculated binding energy of each complex was compared with the Gibbs binding energy obtained from the experimental enzyme–flavonoid inhibition constant (K_i) values using the equation $\Delta G = -RT \ln(K_i)$. The BChE–flavonoid complex whose calculated binding energy matched closest to the experimentally determined Gibbs binding energy was used to identify the active conformation of the flavonoid inhibitor.⁸⁰⁵ This report appears to have used all three proposed components for optimal drug design in an integrated fashion.

A similar paper⁸⁰⁷ also appeared in 2010 which studied the alkaloid structural pattern of lycopodane-type alkaloids from *L. annotinum* ssp. *alpestre* growing in Iceland for clinical efficacy in treatment of Alzheimer's disease, specifically in relation to inhibition of AChE. Solvent-dependent equilibrium constants between the ketone and the hemiketal forms of acrifoline that complicated the NMR spectra of acrifoline with exchange broadening of resonances were determined. Conformational equilibria for these alkaloids with a C₁₁–C₁₂ double bond were determined by NOESY spectroscopy. The most stable conformations of acrifoline were refined with molecular modeling. Interactions of AChE with lycopodane-type alkaloids were determined with molecular modeling and used to justify the weak AChE inhibitory activity observed for lycopodane-type alkaloids.⁸⁰⁷ Due to the lack of a high-resolution crystal structure of *E. electricus* AChE (EeAChE), the structure of *T. californica* AChE (TcAChE) complexed with huperzine B was used for the modeling studies. Phe-330 of TcAChE was replaced with Tyr-330 for docking (Glide)⁸⁰⁸ and MD studies⁷⁹⁶ (ChemBio3D Ultra).²⁴⁵ Flexible docking was applied to the ligands and the applicable amino acids of the protein. It was argued that the absence of strong hydrogen-bonding interactions between the inhibitor and Tyr-130 and Trp-84 in the catalytic domain appears to be the reason for the insignificant inhibitory activity observed with the in vitro

tests. Molecular modeling revealed that these alkaloids fit well in the binding site of huperzine. The observed binding pattern of anhydrolycodoline from the docking study opened the possibility of synthesis of analogues with increased potency.

Also in 2010, Khattab et al.⁸⁰⁹ investigated the synthesis, IR, ¹H, and ¹³C NMR spectral, and molecular modeling studies of quinoxaline (three ring aromatic backbone structures) based monoamine oxidase (MAO) inhibitors for Alzheimer's disease and evaluated their MAO inhibitory properties. Molecular docking studies as well as conformational alignment using the Molecular Operating Environment Dock (MOEDock)²⁵¹ package (MOEDock is based on the algorithms in AutoDock) were performed with simulated annealing used as the search protocol. The lowest energy conformations of MAO-inhibitors complexes were subjected to an energy minimization using the MMFF94X⁸¹⁰ force field in order to obtain more accurate binding energies. QSAR calculations with the hypothetical binding pattern of these compounds were performed using the crystal structure of human MAO-A. Binding modes of the docked compounds exhibited hydrogen-bond interactions with several residues (Ser-209, Tyr-69, and Gln-74) of the active site of the MAO-A enzyme. Hydrogen-bonding calculations and docking scores of inhibitors to the active site of the MAO-A enzyme correlated well with the observed experimental enzyme inhibitory activities. The inhibitory profiles of the newly proposed compounds were quite competitive when compared to experimental MAO-A selectivities, and the model was considered successful for design of new potential inhibitors.

6.1.4. Miscellaneous Diseases and Examples. Besides the reports related to HIV, Alzheimer's, and cancer diseases, several publications have appeared in the literature over the past few years concerning integration of computational, *in vitro*, and spectroscopic structure-based drug design for several other diseases of which a short overview is given in this section.

In 1999, structural characterization of the interactions of peptide inhibitors with the *N*-terminal proteinase domain of the Hepatitis C virus (HCV) NS3 protein, with known bioactivities, was explored by Cicero et al.⁸¹¹ using experimental NMR and molecular modeling studies. Two-dimensional ¹H-¹⁵N HSQC and transfer NOE experiments were recorded. In order to perform a comparison between the shifts induced by each peptide, data were normalized to 100% of complex formation and chemical shift corrections for the enzyme saturation were introduced. Force field energy minimization and molecular dynamics were performed using the BatchMin⁶⁶⁷ program and the molecular modeling package InsightII/Discover, respectively.⁷⁴⁶ Various structurally related peptides with different residues were considered, and the influence of each amino acid residue binding to the protease was elucidated. The change induced in the NS3 NMR spectra upon binding to different ligands was monitored and used to characterize the ligand-binding sites. Fast exchange relative to the NMR time scale between the bound and the free forms enabled identification of the correct position of the respective peaks in the complex. NMRPipe³³⁸ was used to process the experimental data and generate a 3D structure model. The loop connecting strands E₂ and F₂ was also identified in the S-binding region. The ligand formed an extended and antiparallel β -sheet conformation with strand E₂ of the protein, and it interacts with the P₁ carboxylate group in the oxyanion pocket of the enzyme. Similar observations were made from the MD results.⁸¹¹ This report appears to have used all three proposed components for optimal drug design in an integrated fashion.

A research project in 1999⁸¹² studied experimental electronic absorption spectroscopy for inhibitors of reversible MOA which is associated with depression. This data and the bioactivities were compared to theoretical ab initio electronic calculations for these compounds. Complexation of riboflavin with various analogues of befloxatone was observed in solution and caused a 1:1 charge transfer complex identified by a new UV absorption band at 492 nm. Substitution of the alkoxy group on the phenyl ring of phenyloxazolidinones induced a red shift of the absorption band (λ_{max}) characteristic of the intermolecular electron transfer in the complex; this was confirmed by the lower calculated ionization potential of these molecules (energy values of the HOMO). To evaluate the interaction between several reversible MOA inhibitors in the oxazolidinone series, they used the Moplot (Molecular Orbital Plot) subprogram implemented in the Motec package^{190,813} and the Gaussian⁶⁷⁹ set of programs⁶⁹³ to investigate the interaction between the substrates and the active site of the enzyme. Ab initio electronic properties (molecular orbital topologies and energies)⁸¹⁴ of befloxatone and the cofactor belonging to the phenyl oxazolidinone chemical family exhibited a primary interaction between befloxatone and the cofactor of the enzyme. Further, electronic absorption spectroscopy observations confirmed the possibility of a putative interaction of this inhibitor with the flavin cofactor of monoamine oxidase. The obtained model for reversible inhibition of MOA by befloxatone was partially confirmed by experimental observations.⁸¹² This report appears to have used all three proposed components for optimal drug design in an integrated fashion.

Structure-based inhibitor design for the human collagenase-3 (MMP-13) enzyme⁸¹⁵ was undertaken in 2000 by Chen et al.⁸¹⁶ A high-resolution NMR solution structure of the catalytic fragment of human collagenase-3 (MMP-13) was used as a starting point for structure-based design of selective inhibitors for MMP-13. On the basis of the extended nature of the MMP-13 S1' pocket, a potent and selective inhibitor for MMP-13 was attempted from an initial high-throughput screening (HTS) lead. CL-82198 was identified as a weak inhibitor against MMP-13 while demonstrating no activity against MMP-1, MMP-9, or the related enzyme TACE. The drug-like properties of CL-82198 made it an ideal candidate for optimization of enzyme potency and selectivity. On the basis of NMR binding studies it was shown that CL-82198 bound within the entire S1' pocket of MMP-13. This is the source of its selectivity against MMP-1, MMP-9, and TACE. A strategy utilizing this information was devised for designing new inhibitors that showed enhanced selectivity toward MMP-13. The design strategy combined the critical selectivity features of CL-82198 with the well-known potency of a nonspecific MMP inhibitor (WAY-152177) to generate a potent and selective MMP-13 inhibitor (WAY-170523 with an IC₅₀ of 17 nM for MMP-13) and showed >5800-, 56-, and >500-fold selectivity against MMP-1, MMP-9, and TACE, respectively. This report appears to have used all three proposed components for optimal drug design in an integrated fashion.

The 2004 review by Rush et al.,⁷ which was discussed in the cancer section, is also concerned with design of inhibitors for MMPs as an active target for arthritis.

In 2006, the influence of H₂O₂-mediated oxidation of epidermal antioxidant enzymes containing special target amino acids such as methionine (Met), tryptophan (Trp), cysteine (Cys), and selenocysteine (Sec) which are sensitive to

oxidation by reactive oxygen species (ROS), was studied with molecular dynamic computer simulations.⁹³ The effect of hydrogen peroxide-mediated oxidation of susceptible amino-acid residues affecting the enzyme active sites, cofactor binding, and dimerization/tetramerization domains was explored. The effect of H₂O₂-mediated oxidation on the enzyme active site, cofactor binding domains, and secondary structures within the three-dimensional (tertiary) structure of the enzymes enabled the researchers to estimate and confirm the deactivation or inhibition of each enzyme that was studied. Computer modeling predicted which parts of the enzyme active sites were altered by H₂O₂-mediated oxidation in thioredoxinreductase (TR) and acetylcholinesterase (AchE), whereas cofactor nicotinamide adenine dinucleotide phosphate (reduced form) binding is affected in both catalase and TR but not in glutathione peroxidase. This was achieved by calculating the thermodynamic stabilities of various possible oxidation products. These structural changes lead to impaired functionality. Fourier transform–Raman and fluorescence spectroscopy together with enzyme kinetics supported the theoretically determined structural changes caused by H₂O₂-mediated oxidation in TR and AchE as well as structural changes in cofactor nicotinamide adenine dinucleotide phosphate binding in both catalase and TR. It was concluded that computer modeling is more efficient when it is combined with experimental data from techniques such as enzyme kinetics, *in vivo* and *in vitro* FT–Raman and fluorescence spectroscopy, as well as *in vitro* immunofluorescence analysis. It was shown that computer-assisted modeling coupled with classical biochemical techniques provide a robust tool to explore oxidative stress-mediated metabolic changes of the skin.⁹³ This report appears to have used all three proposed components for optimal drug design in an integrated fashion.

In 2006, Duckworth et al.⁸¹⁷ characterized the structure of *Helicobacter pylori* enzyme XGPRTase for treatment of infection and compared the key structural features of the enzyme to other phosphoribosyltransferases. ³¹P nuclear magnetic resonance spectroscopy was applied to measure the XGPRTase activity in the cytosolic fraction of *H. pylori*. Bioinformatics was employed to analyze the phylogeny of XGPRTase, and a structural model of the enzyme was built using the LOOPP homology modeling⁸¹⁸ software. LOOPP is a fold recognition program based on the collection of numerous signals, merging them into a single score and generating atomic coordinates based on an alignment of the new 3D structure into a homologue template structure. The activity of purified recombinant XGPRTase was measured in the presence of guanine and xanthine. The results revealed the presence of XGPRTase activity in the bacterium and that the purified enzyme is a PRTase that catalyzes 6-oxopurines. Molecular modeling and bioinformatic tools including phosphoribosyltransferase amino acid sequence alignment with the ClustalW⁸¹⁹ homology modeling program were used to obtain a modeled 3D structure of the *H. pylori* XGPRTase. They identified important characteristics of the enzyme and compared them to other purine PRTases. ³¹P NMR was employed to measure xanthine–guanine phosphoribosyltransferase (XGPRTase) activity. The theoretical interactions of *H. pylori* XGPRTase with these transition-state analogues that were studied were based on the experimentally observed interactions of purine phosphoribosyltransferases with immunillin-GP. This study thus identified the enzyme as a promising therapeutic target for treatment of *Helicobacter pylori* and that

this complex (xanthine–guanine) is a potential lead compound for future drug design efforts.⁸¹⁷ This report appears to have used all three proposed components for optimal drug design in an integrated fashion.

Two papers reported the application of spectroscopic and computational techniques of nonpeptidic and allosteric inhibitors against the West Nile virus (WNV) and dengue infections.^{820,821}

In 2009,⁸²⁰ nonstructural 3 protease (NS3pro) inhibitors of the WNV were identified using automatic high-throughput fragment-based docking of about 12 000 compounds to the active site of the WNV protease. ¹⁵N-HSQC spectra of the corresponding inhibitor–enzyme complexes were recorded and the K_d value derived from the fitting curves. ¹D proton NMR spectra were useful in assessing any line broadening caused by the low molecular weight compounds, and ¹⁵N HSQC spectra were recorded to detect changes in the NMR shifts of the protein. Investigation of the free binding energy with electrostatic solvation was performed with the Daim (decomposition and identification of molecules),⁸²² Seed,⁸²³ and Ffld⁸²⁴ programs.⁸²⁵ DAIM was developed to automatically break up compounds in small-molecule libraries for fragment-based docking. The Seed software is used for docking libraries of fragments including a solvation energy evaluation. Ffld performs fragment-based flexible ligand docking and the results were validated using ¹⁵N HSQC NMR data. A total of 22 molecules were studied. The most probable binding mode of each inhibitor in the active site of NS3pro was confirmed by intermolecular NOEs observed from the NMR experiments. This information was used to propose three chemical modifications to improve the binding affinity of the best inhibitor.⁸²⁰ This report appears to have used all three proposed components for optimal drug design in an integrated fashion.

Likewise, in 2011, virtual screening of numerous ligands against the WNV-NS3 proteinase was performed.⁸²¹ They chose to study allosteric inhibitors capable of targeting the NS2B–NS3pro interface rather than the NS3pro active site with the Q-MOL⁸²⁶ molecular modeling package.⁸²⁷ Modeling studies were supplemented with fluorescence spectroscopy *in vitro* cell-based tests to determine the activities of the allosteric inhibitors against the enzyme. The computational results enabled them to identify a small subset of inhibitor/enzyme complexes. The calculated selectivity of the inhibitors was confirmed using *in vitro* cleavage assays with furin, a human serine proteinase (the substrate preferences of furin are similar to those of WNV NS2BNS3pro). The kinetic parameters (K_M and V_{max} values) were obtained using purified WNV NS2B-NS2pro samples and the pyroglutamic acid-Arg-Thr-Lys-Arg-7-amino-4-methylcoumarin fluorescence-labeled peptide substrate in the presence of various concentrations of the inhibitors. In agreement with previous studies,⁸²⁸ two compounds exhibited inhibition of flaviviral replication in cell-based experiments. This gave additional evidence in support of the predicted specificity. They proved that altering the interactions of the NS2B cofactor with the NS3 protease is a suitable approach for rational structure-based inhibitor development, and this method can readily be applied for identification of inhibitors of other flaviviral proteinases including DV NS2B-NS3pro.⁸²¹ This report appears to have used all three proposed components for optimal drug design in an integrated fashion.

Furthermore, synthesis, computational studies, and in vitro assay of carbonyl-based malaria protease inhibitors (endoperoxide carbonyl falcipain 2/3 inhibitors) and their endoperoxide hybrid parasites were performed by Gibbons et al.⁸²⁹ They employed EPR spectroscopy and molecular docking studies using the Autodock⁴¹⁰ program. Spectral and modeling studies on the ferrous-mediated decomposition of these antimalarial inhibitors revealed that these systems degrade through carbonyl inhibitor formation in tandem through carbon radical formation.⁸²⁹ This report appears to have used all three proposed components for optimal drug design in an integrated fashion.

In 2010, Chimenti et al.⁸³⁰ applied ¹H NMR spectroscopy and molecular modeling⁸³¹ of selective human monoamine oxidase inhibitors for regulation of emotion and other brain functions. ¹H NMR spectra were recorded at 400 MHz using DMSO-*d*₆ or CDCl₃ as solvents. *E*- and *Z*-isomers of the considered inhibitors were constructed with the Maestro GUI,⁸³² and a Monte Carlo conformational search was applied to all rotatable bonds of the inhibitors. The energies of the Monte Carlo structures were examined using the OPLS-AA force field⁸³³ implemented in Macromodel.^{250,667} The global minimum energy structures of the two isomers were subjected to docking with the hMAO-A and -B pockets using the Glide⁸³⁴ program. In agreement with the experimental data, the selected inhibitors exhibited a stronger interaction with hMAO-B than hMAO-A. The results in terms of the reversibility of the inhibition did not correlate well with the conformational analysis results from the docking studies. However, docking studies provided a suitable theoretical prediction of the initial possible inhibitor–enzyme interactions, and they were able to achieve a molecular rationale for the hMAO-A and hMAO-B selectivity of this novel set of 2-thiazolylhydrazone inhibitors.⁸³⁰ This report appears to have used all three proposed components for optimal drug design in an integrated fashion.

In 2011, Jun Yin et al.⁸³⁵ studied the reversible inhibition of tyrosinase by phthalic acid (PA). Tyrosinase is responsible for the skin disease oculocutaneous albinism. They investigated the prediction of the 3D structure of tyrosinase through homology modeling of the protein followed by protein–ligand docking with the Dock⁸³⁶ and Autodock⁴¹⁰ programs. They compared their theoretical results with experimental UV–vis and fluorescence spectroscopy data. A similar fluorescence spectrophotometric tyrosinase assay was performed, and the activity was recorded as the change in absorbance using UV spectrophotometry. Tryptophan fluorescence was measured following excitation. Changes in 8-anilino-1-naphthalenesulfonic acid (ANS) binding were studied by measuring the fluorescence observed for the tyrosinase. The relative steady-state fluorescence intensities in the absence and presence of quencher (PA) and the quencher concentration (Q), the values for the binding constant (*K*) and the number of binding sites (*n*) were derived. From the orientation of the docked inhibitors with respect to the active site, it was estimated that PA binds directly with several residues of tyrosinase (especially Leu-73). This fact suggests that PA could be a potent tyrosinase inhibitor due to its two hydroxyl groups that interact with the active pocket. The orientation of these groups was considered an important point for screening of potential tyrosinase inhibitors. Experimental observations were in reasonable agreement with the simulated data. It was concluded that the combination of inhibition kinetics with computational techniques enables effective identification of potential tyrosinase inhibitors and

the screening of their inhibitory mechanisms.⁸³⁵ This report appears to have used all three proposed components for optimal drug design in an integrated fashion.

In 2012, Tamer et al. reported a study “A combined crystallographic, spectroscopic, antimicrobial, and computational study of novel dipicolinate copper(II) complex with 2-(2-hydroxyethyl)pyridine”.⁸³⁷ Even though they used this combined approach, they did not correlate any specific calculated quantity or characteristic spectroscopic measurement with the observed bioactivities.

Later, D’Onofrio et al.⁸³⁸ utilized gadolinium complexes as MRI probes linked to an apolar fragment which were internalized into various cell types, including hepatocytes. Two lipid-functionalized gadolinium chelates were investigated for targeting human liver fatty acid binding protein (hL-FABP) as a means of increasing the sensitivity and specificity of intracellular-directed MRI probes. hL-FABP is the most abundant cytosolic lipid binding protein in hepatocytes, and it displays the ability to interact with multiple ligands involved in lipid signaling. It is believed to escort lipid-based drugs across the cell membrane. The interaction modes of a fatty acid and a bile acid-based gadolinium complex with hL-FABP have been characterized by relaxometric and NMR experiments in solution with close-to-physiological protein concentrations. Analysis of paramagnetic-induced protein NMR signal intensity changes were employed as a quantitative tool for determination of the binding stoichiometry. A few additional NMR-derived restraints were then sufficient to locate the ligand molecules in the protein binding sites using a rapid data-driven docking method. Relaxometric and ¹³C NMR competition experiments with oleate and the gadolinium complexes revealed formation of heterotypic adducts.

Ligand-efficient fragments binding to PDK1 were identified by an NMR fragment-based screening approach by Lee et al. (2012).⁸³⁹ PDK1 is a mitochondrial enzyme that codes for an isozyme of pyruvate dehydrogenase kinase (PDK). Computational modeling of the fragments bound to the active site led to the design and synthesis of a series of novel 6,7-disubstituted thienopyrimidin-4-one compounds, with low micromolar inhibitory activity against PDK1 in a biochemical enzyme assay.

Lee et al.⁸³⁹ in 2012 determined the structure of the translocator protein (TSPO) with NMR and computational techniques. TSPO is an important target for drug discovery and clinical molecular imaging of brain and peripheral inflammatory processes. PK 11195 [1-(2-chlorophenyl)-*N*-methyl-(1-methylpropyl)-3-isoquinoline carboxamide] is the major prototypical high-affinity ligand for TSPO. Elucidation of the solution structure of PK 11195 is of interest for understanding small-molecule ligand interactions with the lipophilic binding site of TSPO. Dynamic ¹H/¹³C NMR spectroscopy of this ligand revealed four quite stable but interconverting rotamers due to amide bond and 2-chlorophenyl group rotations. They used quantum calculations [B3LYP/6-311+G(2d,p)] to calculate proton and carbon ¹³ chemical shifts for the rotamers. Experimental NMR data were used to characterize the structures of rotamers for these ligands in organic solution. Energy barriers for both the amide bond and the 2'-chlorophenyl group rotation of PK 11195 were determined from dynamic proton NMR (ca. 17–18 kcal/mol), and they compared well with the calculated values. These results demonstrated that the *E* rotamer of PK 11195 is more stable in solution by about 0.4 kcal/mol. The authors claimed that these structural findings will aid future TSPO ligand design and

support the notion that TSPO prefers to bind ligands as amide *E*-rotamers.

The interaction of nonsteroidal anti-inflammatory drugs (NSAIDs) with cell membranes was studied⁸⁴⁰ by means of a number of biochemical, structural, computational, and biological systems including IR, NMR, and SPR spectroscopies and cell culture. This was achieved using a specific fluorescent membrane probe to demonstrate that NSAIDs have a strong affinity to form ionic and hydrophobic associations with zwitterionic phospholipids and specifically phosphatidylcholine (PC). These interactions are reversible and noncovalent in nature. A membrane model was studied using Gromacs (ffgmx), and the drug affinity for PC was demonstrated with MD studies of the drug immersed in the membrane model.

Most bacterial IspGs (a 4Fe4S protein involved in isoprenoid biosynthesis) contain two domains, a TIM barrel (A) and a 4Fe4S domain (B), but in plants and malaria parasites, there is a large insert domain (A*) whose structure and function are unknown. Liu et al. (2012)⁵⁰⁶ used an integrated approach to demonstrate that bacterial IspGs function in solution as (AB) (2) dimers and that mutations in either both A or both B domains block activity. Chimeras harboring an A mutation in one chain and a B mutation in the other have 50% of the activity seen in the wild-type protein as there is still one catalytically active AB domain. They employed molecular docking and electron microscopy to show that the A* insert domain has a TIM barrel structure that interacts with the A domain. This structural arrangement enables the A and B domains to interact in a “cup and ball” manner during catalysis, just as in the bacterial systems. EPR/HYSCORE spectra of the reaction intermediate, product, and inhibitor ligands bound to both two- and three-domain proteins are identical, suggesting the same local electronic structure. Docking results indicate these ligands bridge both the A and the B domains.

Pinheiro et al. (2012)⁸⁴¹ studied the antituberculosis drug rifabutin in combination with phospholipid Langmuir monolayers as a model for the lung surfactant monolayer. Zwitterionic 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) and anionic 1,2-dipalmitoyl-*sn*-glycero-3-phospho-(1'-rac-glycerol) (DPPG) were used as model phospholipids. A combination of *in situ* experimental techniques of Brewster angle microscopy, polarization-modulated infrared reflection-absorption spectroscopy, and UV-vis reflection spectroscopy with computer simulations was employed. Computer MD simulations were performed using a simple model of one DPPC molecule and one RBT molecule in vacuo. The RBT molecule is placed at different positions around the polar headgroup, therefore generating different starting configurations in order to determine the most likely position it will occupy upon MD simulation. The interactions between rifabutin and the DPPC and DPPG Langmuir monolayers were described as formation of an inclusion complex. The phospholipid rifabutin inclusion complex prevents penetration of the drug into the alkyl chain region of the phospholipids, leading to a disruption of the monolayer structure and a possible toxicological effect.

Saraboji et al. (2012)⁸⁴² reported high-resolution crystal structures of the carbohydrate recognition domain of galectin-3 (Gal3C) both in ligand-free and in complex states with lactose and glycerol. The positions of water and carbohydrate oxygen atoms in all three states were similar, indicating that the binding site of Gal3C is preorganized to coordinate oxygen atoms in an arrangement that is nearly optimal for recognition of β -galactosides. Deuterium NMR relaxation dispersion experi-

ments and molecular dynamics simulations demonstrate that all water molecules in the lactose-binding site exchange with bulk water in a relatively short time during MD. MD simulations identified transient water binding at sites that agreed with those observed by crystallography, indicating that the energy landscape of the binding site is maintained in solution. All heavy atoms of lactose are positioned like the corresponding atoms of glycerol in the Gal3C complexes. However, binding of glycerol to Gal3C is insignificant in solution at room temperature, as monitored by NMR spectroscopy or isothermal titration calorimetry under the conditions used (where lactose binding is readily detected). These observations make a case for protein cryocrystallography as a valuable screening method in fragment-based drug discovery and suggest that identification of water sites is important for future drug design efforts. The report has effectively integrated all three proposed components for rational drug design.

Song et al. (2012)⁸⁴³ studied the interaction of human serum albumin (HSA) with phillygenin employing fluorescence, circular dichroism (CD), UV-vis spectroscopic, and molecular docking methods. Fluorescence techniques were used to determine the binding constant. The results of UV-vis spectra show that the secondary structure of the protein changed in the presence of phillygenin. CD spectra demonstrated that the HSA conformation was altered by phillygenin with a major reduction of the α -helix and an increase in β -sheet and random coil structures, indicating a partial protein unfolding. The distance between donor (HSA) and acceptor (phillygenin) was calculated to be 3.52 nm, and the results of synchronous fluorescence spectra showed that the binding event can induce conformational changes in HSA. Molecular docking experiments demonstrated that phillygenin binds with HSA at the IIIA domain of the hydrophobic pocket. They were able to conclude that in the presence of copper(II), iron(III), and alcohol, the apparent association constant and number of binding sites of phillygenin on HSA were both decreased in the range of 89–92% and 16–19%, respectively.

In 2012, Yongye et al.³²⁸ demonstrated that NMR spectroscopy and molecular modeling methods (docking and MD studies) can be strategically combined to elucidate the molecular recognition features of the binding of threonine O-linked Thomsen-Friedenreich (TF) antigen to chimera-type avian galectin-3 (CG-3). Saturation transfer difference (STD) NMR experiments were used to determine where the interactions occur. The H4 protons of both the β -D-Galp and the α -D-GalpNAc moieties are unique. Two-dimensional-transferred NOE experiments combined with MD simulations suggested some differences in conformer populations between the free and the bound states. A dynamic binding mode for the TF antigen-CG-3 complex consisting of two different interactions was deduced. In the first case, interactions were formed between the terminal threonine residue and the receptor. In the second case, intermolecular interactions between the TF antigen and an internal region of GalpNAc are evident. The difference in the trend of some shifts in the heteronuclear single-quantum coherence titration spectra indicates some disparities in the binding interactions of CG-3 with lactose and TF antigen. The results provide a possible route for design of galectin-3 inhibitors with improved affinity and selectivity.

A combination of CD spectroscopy⁶⁷ and computational methods for noncovalent-based therapies utilizing photoactive drugs were demonstrated in a review by Monti et al. in 2011.⁷⁴⁸

Their review focused on the complex of a nalidixate anion and licochalcone A with the IIIA HSA-subdomain. They discussed an approach to use CD spectroscopy and computational techniques to investigate ligand–protein interactions. The sign and intensity of the circular dichroism bands were related to the equilibrium geometries of the associated structures that were examined.⁸⁴⁴ Conformations of low-energy complexes (in terms of the energetics and relaxation behavior of the system) were obtained using molecular mechanics and molecular dynamics with suitable solvent systems. They suggest that software¹⁵⁹ such as the AMBER,^{160,161} Charmm,^{158,162} Gromos,⁸⁴⁵ and Amoeba⁸⁴⁶ programs can be used for such calculations. The sign and intensity of the experimental CD bands related to the equilibrium geometries of the associated structures were then compared to the quantum mechanically computational rotational strengths of low-energy supramolecular complexes. This approach was useful for both chiral and achiral ligands in order to achieve a structure-based rationale of ground- and excited-state properties for the noncovalent biomolecular complexes in solution. This review combined the three proposed components for optimal drug design but did not refer to individual papers practicing this method.

A review by Trzaskowski et al. (2012)⁸⁴⁷ presented computational, spectroscopic (also other biophysical methods), and bioactivity methods employed to study the G protein-coupled receptors (GPCRs), also called 7TM receptors. These are membrane proteins that, upon activation by extracellular agonists, pass a signal to the cell interior. Ligands can bind either to extracellular N-terminus and loops (e.g., glutamate receptors) or to the binding site within trans-membrane helices (Rhodopsin-like family). These receptors are all activated by agonists, although spontaneous autoactivation of a receptor in the absence of any inhibitor has also been observed. They present various computational, spectroscopic, and biological methods on how best to study these molecular switches. Although all three proposed components of drug design are individually discussed in the review, they do not refer to any studies where an integrated approach was utilized.

7. CONCLUDING REMARKS AND FUTURE PROSPECTS

Drug design is a comprehensive interdisciplinary field, and the integration of disciplines such as molecular modeling, spectroscopy, and *in vitro* biochemical methods was demonstrated to be a very effective research tool. Computational methods play an important role in the drug design process since they do not only supplement experimental approaches but also contribute directly to the design and discovery of new drugs. Information provided by the three-dimensional structure of the receptor–inhibitor complex can be used to explain the results of the affinity data and guide the computer-aided search for improved inhibitors.

With the constant development of new computer hard- and software, it is expected that the next few years will see a dramatic increase of interdisciplinary rational drug studies. This review serves as a survey for effective integration of suitable molecular modeling drug design techniques with biophysical analytical methods and bioassay data. It has been demonstrated that the lack of reproducible HIV PR bioactivity data severely hampers theoretical advancement of new HIV PR drugs. This is likely the case for other diseases as well. We recommend that experimental binding free energies obtained from thermal titration calorimetry experiments be used as the standard biophysical data to which theoretical models are gauged. An

additional advantage is that binding free energies (experimental and calculated) provide information of both enthalpy and entropy contributions. Researchers in this field should therefore be actively encouraged to expand ITC studies for all possible target enzymes.

It seems most likely that future developments to study drug–receptor interactions will continue to build on not only the combination of biochemical and computational approaches but an integrated drug design approach involving molecular modeling and as many as possible experimental analytical techniques (biophysical methods) in combination with experimental binding free energy data.

It is clear that computer-aided rational drug design provides essential molecular insight into drug–host interactions that are not easily and fully accessible from conventional experimental techniques on their own. The possibility to improve the computational model in order to make better predictions when used in combination with experimental disciplines such as spectroscopy and *in vitro* results serves to aid and guide experiment and is poised to make far more significant contributions to drug development than has been possible in the past. This field of research will develop faster if reviewers and editors of applicable journals are better educated in this regard so that they will increasingly drive toward an integrated drug design approach involving molecular modeling, biophysical methods, and as far as possible experimental free binding energies for drug/enzyme interactions. In the case of docking studies, it is advisable that more conclusive evidence is provided, in particular, that the calculated binding energies correlate well with a representative experimental sample of *in vitro* bioactivities or alternatively the average binding free energies calculated from MD simulations.

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