ENZYME INHIBITORS

1. Introduction

The inhibition of enzyme activity is one of the major regulatory devices of living cells and one of the most important diagnostic procedures of an enzymologist. It provides not only valuable information on fundamental aspects of enzymatic catalysis and metabolic pathways, but also on the implications for pharmacology and toxicology. An indepth study of enzyme inhibition supplies information about the specificity of an enzyme, the physical and chemical architecture of the active site, and the total description of the enzyme–substrate (ES) and

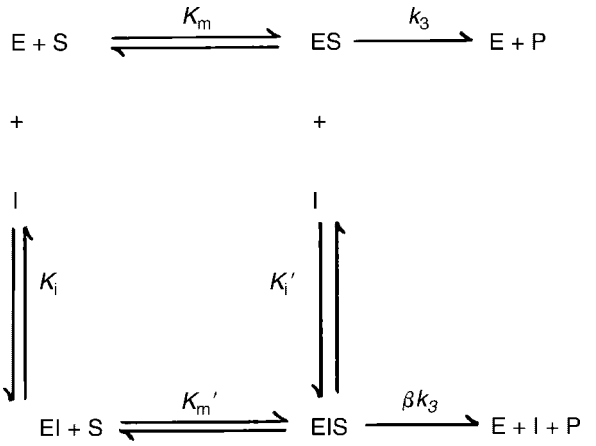

Fig. 1. Reversible enzyme inhibition.

enzyme–product (EP) complexes (1). Any substance that reduces the velocity of an enzyme-catalysed reaction can be considered to be an "inhibitor". In everyday life, enzyme inhibitors masquerade as drugs, antibiotics, preservatives, poisons, and toxins.

Reversible enzyme inhibition (noncovalent binding) is divided into two types: complete (linear), in which the plots of reciprocal velocity versus inhibitor concentrations give a straight line, and partial (hyperbolic) in which this plot is a hyperbola. With complete enzyme inhibition, the velocity tends to zero when the concentration of the inhibitor increases; with partial inhibition, the enzyme is converted into a modified, but still functional, enzyme–substrate–inhibitor (ESI) complex (Fig. 1) ($\beta =$ degree of partiality; K_i, $K_{i'}$, K_m, and $K_{m'}$ are defined as respective dissociation constants) (2). The analysis of the enzymatic kinetic data consists of several steps, in which the result of a precedent step determines a subsequent one: data reduction, analogue data plot inspection, kinetic model construction, derivation of kinetic equations, and the regression analysis for the determination of all relevant kinetic parameters.

There are three types of inhibition: competitive, noncompetitive, and uncompetitive. The first deals with a substance that combines with free enzyme in a manner that prevents substrate binding ($K_{i'} = \infty$) (Fig. 1); the second has no effect on substrate binding as the substrate and inhibitor bind reversibly, randomly and independently at different sites. In this case, since $K_i = K_{i'}$, the Haldane relationship requires that $K_m = K_{m'}$; the third is illustrated by a compound that binds to the enzyme–substrate complex to yield an inactive ESI complex ($K_i = \infty$).

2. Inhibitor Parameters

Graphical interpretations of the types of inhibition are needed to calculate the necessary parameters. Some graphic methods, including those of Lineweaver-Burk (3), Dixon (4), and Cornish-Bowden (5) are used for kinetic analysis of

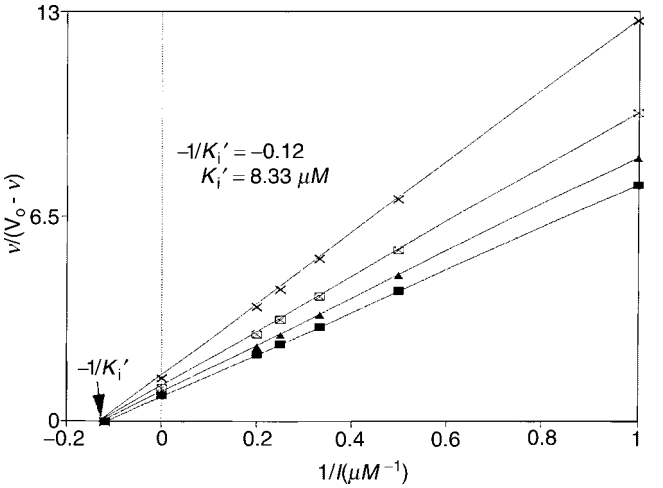

Fig. 2. Fractional velocity plots showing partial competitive inhibition of glutamine synthetase by alanine at different levels of glutamate [2.0 μM (+), 2.5 μM (▲), 3.33 μM (×) and 5 μM (x)].

complete or linear inhibitor action; these plots are not valid for partial inhibition. If precise estimates of the data are required, it is important to take into account relative variability in the data points, and to use objective and unbiased procedures (6,7). Only a few analytical methods for the investigation of the action of partial inhibitors have been published (1,8,9). Despite its limitations in assuming that the enzyme is unireactant, the plot of fractional velocity [$v/(V_o$-$v)$] versus reciprocal of inhibitor concentration [$1/I$], where V_o and v are the rates of enzyme reaction attained by the system in the presence of a fixed amount of substrate,

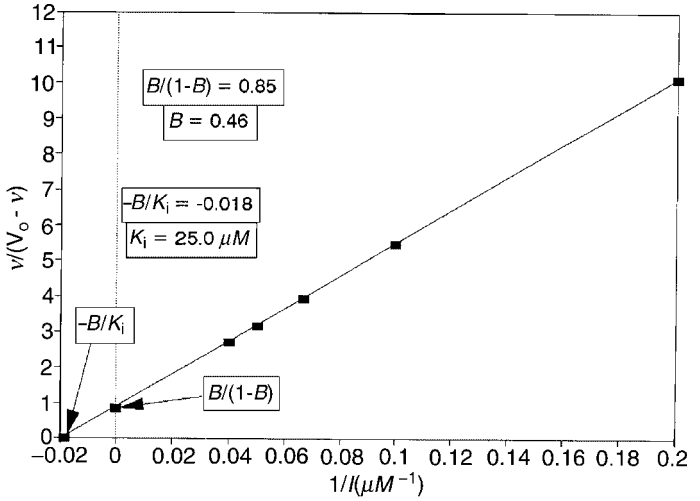

Fig. 3. Fractional velocity plot showing partial noncompetitive inhibition of hexokinase by adenosine diphosphate (ADP).

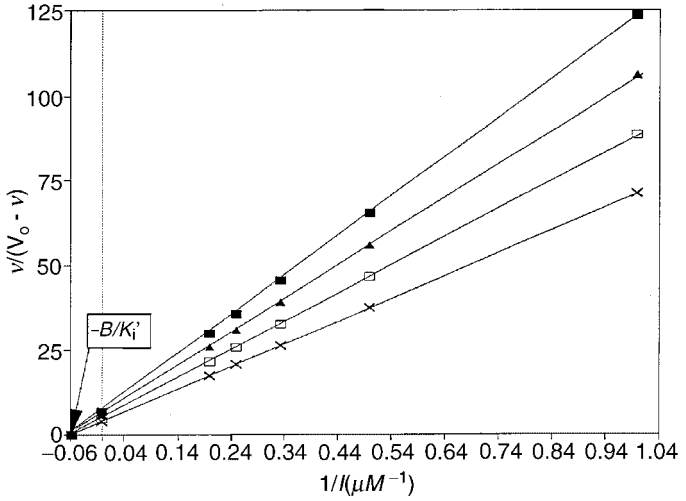

Fig. 4. Fractional velocity plots showing partial uncompetitive inhibition of mevalonate diphosphate decarboxylase by β-methylene adenosine 5′-triphosphate at different levels of 3-phospho-5-pyrophosphomevalonate (PPPM) [2.0 μM (+), 2.5 μM (▲), 3.33 μM (×) and 5 μM (x)].

and in the absence and presence of inhibitor, respectively, offers a unique solution for measuring kinetic parameters in both linear and hyperbolic inhibition (1). Partial inhibition affords straight lines that converge on the $1/I$ axis, at a point away from the origin (Figs. 2–4); complete inhibition gives straight lines that pass through the origin (Figs. 5–7); For competitive inhibition, the slopes of the lines increase with increasing substrate concentration (Figs. 2 and 5);

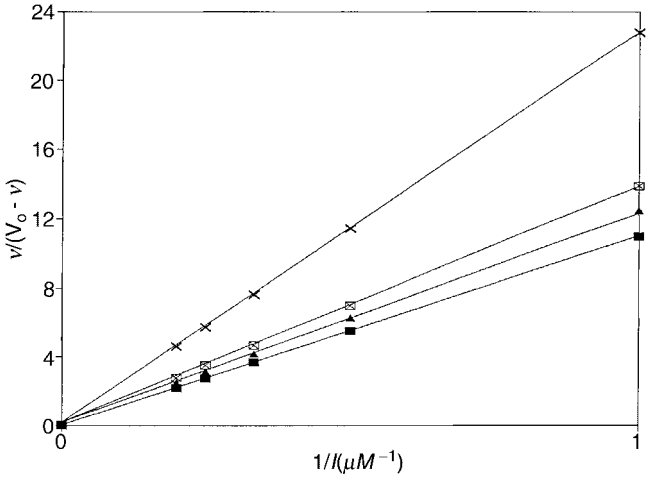

Fig. 5. Fractional velocity plots showing complete competitive inhibition of the sulfatase enzyme by galactose-6-sulfate at different levels of *p*-nitrocatechol sulfate [1.0 μM (+), 1.5 μM (▲), 2.0 μM (×) and 5 μM (x)].

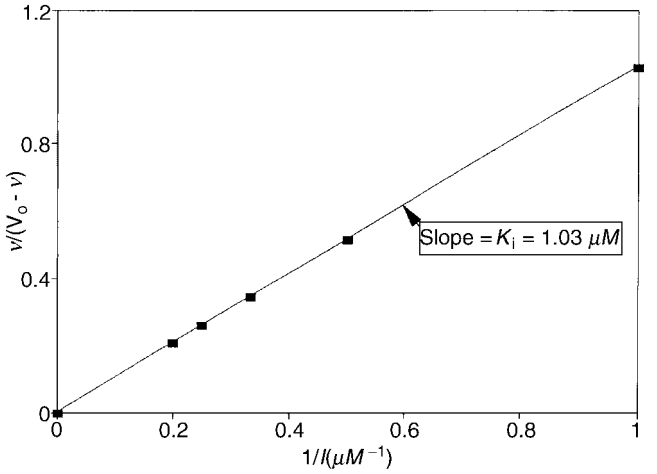

Fig. 6. Fractional velocity plot showing complete noncompetitive inhibition of liver alcohol dehydrogenase by adenosine monophosphate (AMP).

with noncompetitive inhibition, the slopes are independent of substrate concentration (Figs. 3 and 6); with uncompetitive inhibition, the slopes of the lines decrease with increasing substrate concentration (Figs. 4 and 7). The kinetic parameters, K_m, K_i, $K_{i'}$, and B (degree of partiality) can best be determined from respective secondary plots of slope and intercept versus substrate concentration, for competitive and noncompetitive inhibition (Figs. 8–10) or slope and intercept versus reciprocal substrate concentration for uncompetitive inhibition. (Figs. 11–13).

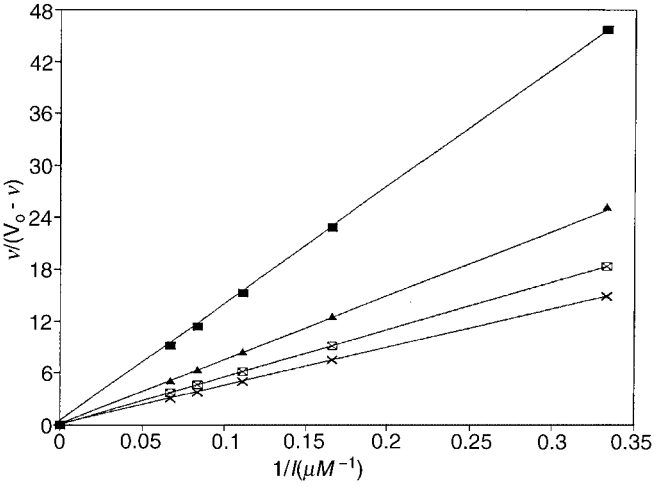

Fig. 7. Fractional velocity plots showing complete uncompetitive inhibition of the amino peptide enzyme by bestatatin at different levels of L-leucine *p*-nitro anilide [2.0 μM (+), 2.5 μM (▲), 3.33 μM (×) and 5 μM (x)].

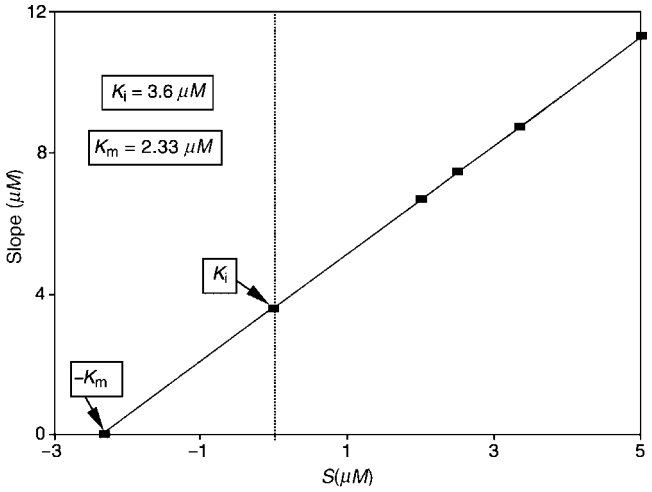

Fig. 8. A secondary plot of the slopes obtained from the lines from Figure 2 versus glutamate concentration.

Irreversible enzyme inhibition (covalent binding) creates a covalent bond between the inhibitor and the enzyme and may be divided into two phases: The inhibitors first bind to the enzyme in a noncovalent fashion and then undergo subsequent covalent bond formation. There are two fundamental classes: Suicide inhibition (10) (Fig. 14) and mechanism-based inhibitors. (Fig. 15) (11).

When selective irreversible inhibitors are used to label active site residues of an enzyme to aid in their identification, they are called affinity labels. A suicide inhibitor, on the other hand, is an affinity label that is unreactive until it is acted upon by the enzyme, at which point it binds irreversibly. The fundamental

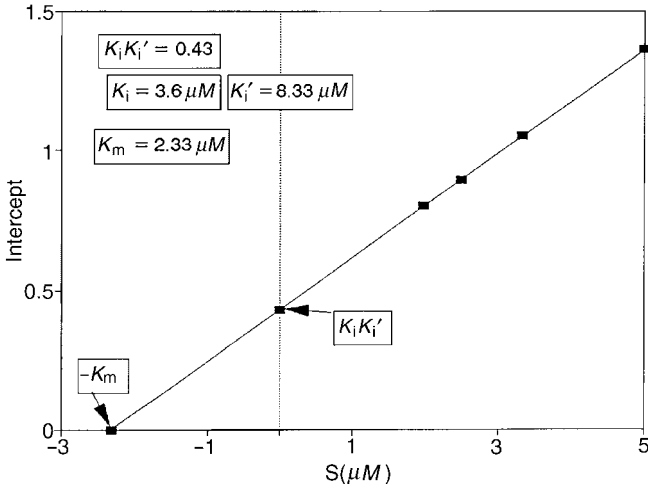

Fig. 9. A secondary plot of the intercepts on the y axis of Figure 2 versus glutamate concentration.

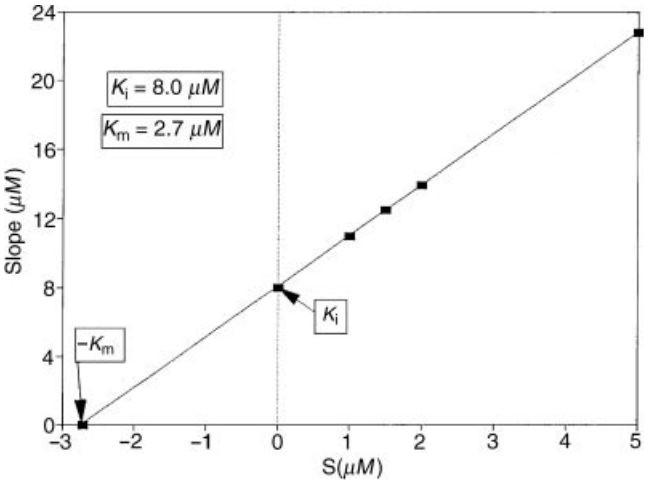

Fig. 10. A secondary plot of the slopes obtained from the lines from Figure 5 versus *p*-nitrocatechol sulfate concentration.

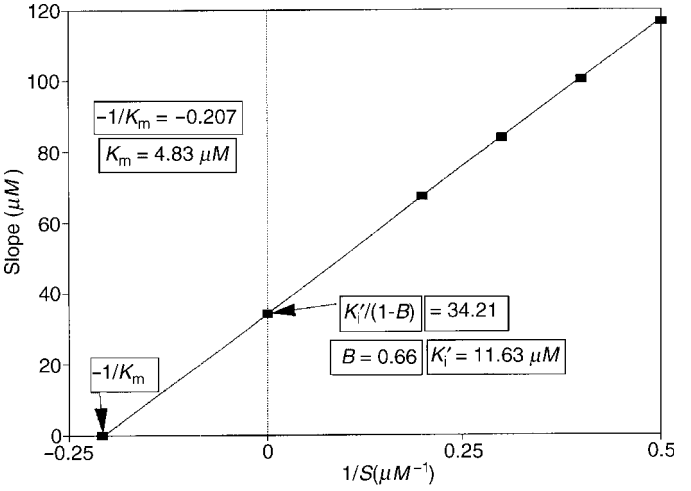

Fig. 11. A secondary plot of the slopes obtained from the lines from Figure 4 versus the reciprocal of 3-phospho-5-pyrophosphomevalonate (PPPM) concentration.

action of a mechanism-based inhibitor depends on the enzymes catalytic mechanism as the inhibitor, which is a substrate analogue, irreversibly modifies the enzyme at a particular step in the catalytic cycle (12). A knowledge of the catalytic mechanism of the enzyme with its normal substrate, and the introduction of an appropriate latent functional group into the substrate is important as a fairly unreactive species is converted into a highly reactive one only during the specific catalytic step. Furthermore the inhibitor must fulfil the binding specificity requirements for the ligand recognition site.

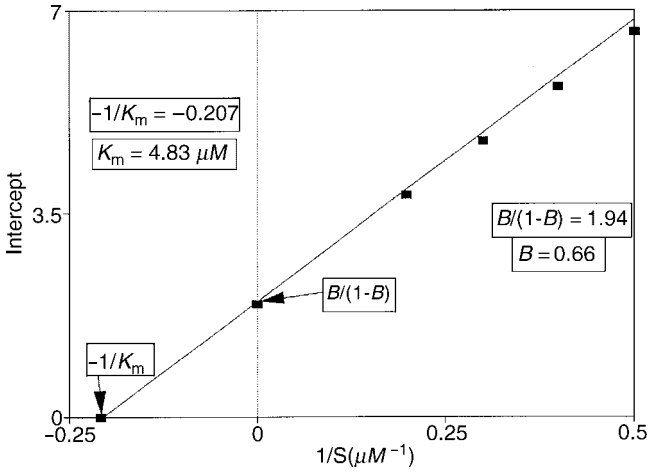

Fig. 12. A secondary plot of the intercept on the y axis of Figure 4 versus reciprocal of PPPM concentration.

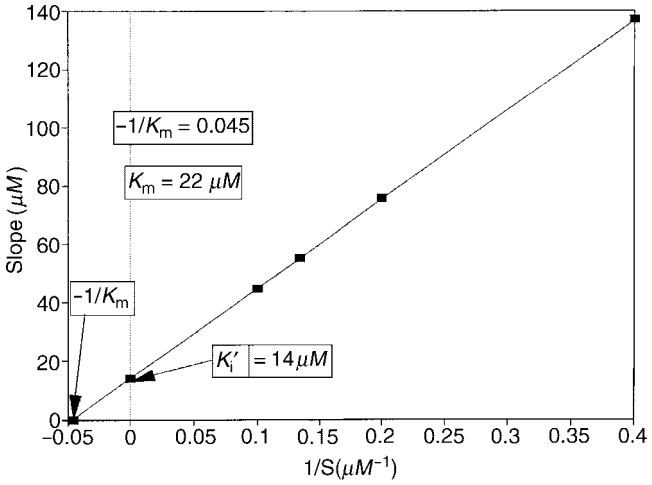

Fig. 13. A secondary plot of the slopes obtained from the lines from Figure 7 versus the reciprocal of L-leucine *p*-nitro anilide concentration.

3. Inhibitor Design

3.1. De Novo. The rational design of enzyme inhibitors is still being pursued as the most viable and fundamental for the design of drugs. First, the de novo design of inhibitors requires the three-dimensional (3D) structure of the target enzyme or of a model constructed from related enzymes; or the biological activities and structures of related inhibitors for the particular enzyme; or the pharmacore, which consists of the chemical groups of a ligand and their relative

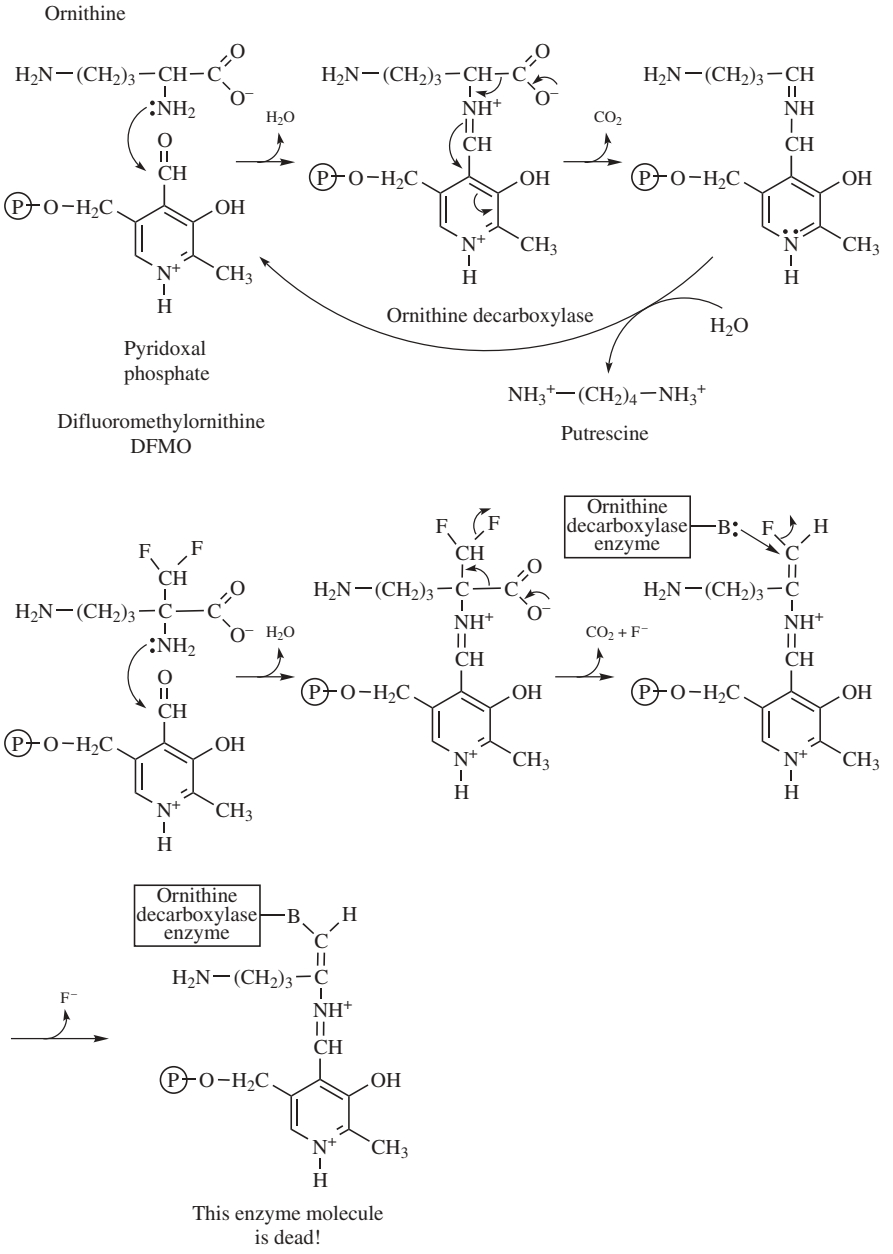

Fig. 14. Suicide inhibition of ornithine decarboxylase by difluoromethylornithine.

orientations that are important for binding. Second, a compound is then designed that will bind to a molecular site on the enzyme in such a way that it will alter its behavior. Third, this compound is chemically synthesised and tested. A structure–activity relationship is needed to determine the properties of a molecule based on its structure and this will be followed by building a structure that is based on the desired properties (13).

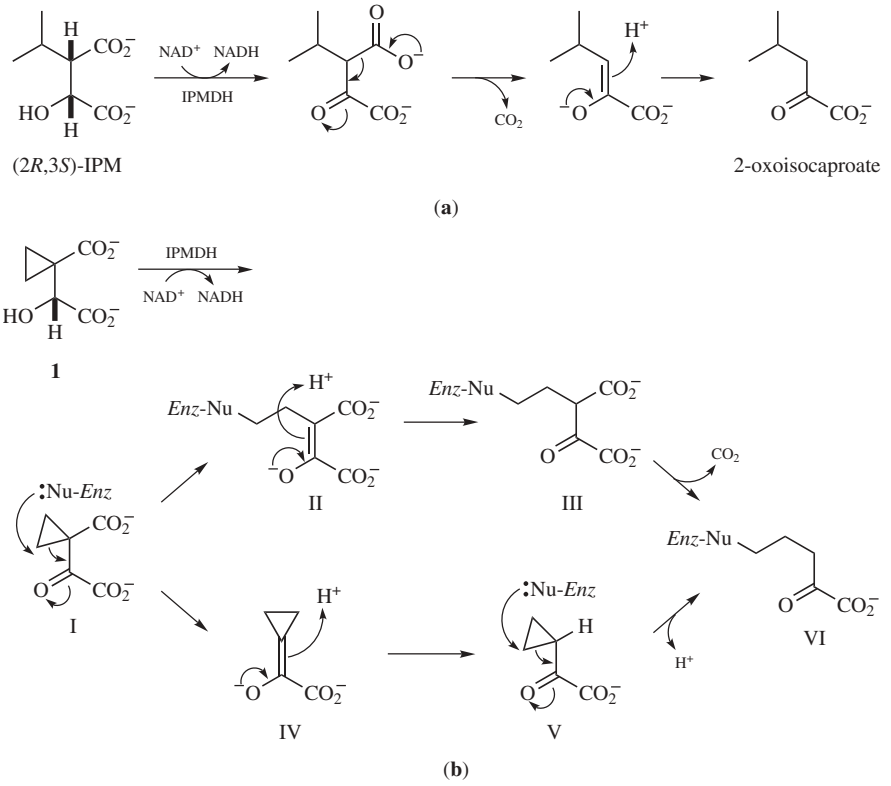

Fig. 15. Mechanism-based inhibition of 3-isopropylmalate dehydrogenase by 3-cyclopropylmalate. (**a**) actual mechanism, (**b**) basic concept of mechanism-based inhibition.

3.2. Computer Assisted. Computer-assisted inhibitor design represents more recent applications of computers as tools in the inhibitor design process. In most current applications, attempts are made to find an inhibitor (drug) that will interact favorably with an enzyme that represents the target site. Binding of the inhibitor to the enzyme may include hydrophobic, electrostatic, hydrogen-bonding interactions, and solvation energies that optimizes the fit of an inhibitor to an enzyme. Many computer aided inhibitor design systems choose to predict the properties of either the inhibitors (inhibitor based) that operate on the enzyme or the enzyme itself (enzyme based), but not usually both.

The former approach is applicable when the structure of the enzyme site is unknown, but when a series of compounds have been identified that exert the activity of interest. To be used most effectively, one should have structurally similar compounds with high activity, with no activity, and with a range of intermediate activities. In recognition site mapping, an attempt is made to identify a pharmacophore, which is a template derived from the structures of these compounds and is represented as a collection of functional groups in 3D space that is complementary to the geometry of the enzyme.

In applying this approach, conformational analysis will be required, the extent of which will be dependent on the flexibility of the compounds under

investigation. One strategy is to find the lowest energy conformers of the most rigid compounds and superimpose them. Conformational searching on the more flexible compounds is then done while applying distance constraints derived from the structures of the more rigid compounds. Ultimately, all of the structures are superimposed to generate the pharmacophore that is then used as a template to develop new compounds with functional groups in the desired positions. In applying this strategy, one must recognize that one is assuming that it is the minimum energy conformers that will bind most favorably in the receptor site though there is no a priori reason to exclude higher energy conformers as the source of activity.

The second half of the challenge in developing a new inhibitor molecule is to take the important properties and determine the structure that will have them. Computational and rule-based equations suffer from drawbacks due to combinatorial complexity of the search space, design knowledge acquisition difficulties, nonlinear structure–property correlations, and problems incorporating higher level biological knowledge.

The enzyme-based approach applies when a reliable model of the enzyme is available, as from X-ray diffraction, nuclear magnetic resonance (nmr), or homology modeling. With the availability of the enzyme, the problem is to design inhibitors that will interact favorably at the site. Enzyme-based inhibitor design incorporates a number of molecular modeling techniques and though it is not the intention of this review to present an exhaustive account of all of the commercial packages available it is pertinent to tabulate and mention a few.

Structure–Activity Relationship. Table 1 shows structure-activity relationships.

Table 1. **Structure-Activity Relationships**

QSAR with CoMFA (15)	QSAR with CoMFA provides tools to build statistical and graphical models of biological activity from molecular structures, and uses these models to make predictions for the activity of untested compounds.
Advanced CoMFA (15)	Advanced CoMFA offers specialized CoMFA fields and clustering tools that assist in refinement of predictive models.
ClogP/CMR (15)	ClogP uses a fragment-based approach to calculate octanol–water partition coefficients of compounds for use as descriptors in QSAR studies. CMR uses an atom-based approach to calculate molar refractivity.
Distill (15)	Distill classifies compounds according to their common substructures and organizes the results in a display that enables visualization of structure–activity relationships.
HQSAR (15)	HQSAR uses molecular fragment information and statistical methods to automatically generate structure–activity relationships without requiring alignment of ligands.
Molconn-Z (15)	Molconn-Z computes a wide range of topological indices based on molecular structure.
TSAR (16)	TSAR finds trends in data using statistical and visual analysis tools.
CoMSIA	CoMSIA uses Gaussian function to assess steric, electrostatic, hydrophobic, and hydrogen-bond donor–acceptor fields.
EDDFA	EDDFA uses steric, electrostatic, electronic, kinetic energy densities, fukui function, laplacian, local average ionization potential and binary node potential fields to build molecular structures.

QSAR. A detailed introduction to the physical parameters, mechanisms, methodology, applications, and properties of QSAR is presented elsewhere (14).

Enzyme-inhibitor design is an iterative process that begins with a compound that displays an interesting biological profile and ends with optimizing its activity profile. The process is initiated when a hypothesis is established that relates the chemical features of the molecule (or if a series of process(es) is responsible for activity, the hypothesis generally is refined by examining structural molecules) to the biological activity without a detailed understanding of the biochemical similarities and differences for active and inactive molecules. Rules can be used for a series of compounds to evaluate new chemical entities (eq. 1).

$$\text{Biological activity} = \text{const} + \text{C1 P1} + (\text{C2 P2}) + (\text{C3 P3}) + \cdots \quad (1)$$

where the parameters P1 through Pn are computed for each molecule in the series and the coefficients C1 through Cn are calculated by fitting variations to the parameters and the biological activity. The factors that influence and predict are (a) binding data measured with sufficient precision to distinguish between compounds; (b) a set of parameters that can be easily obtained and that are likely to be related to enzyme affinity; (c) a method for detecting a relationship between the parameters and binding data (the QSAR), and (d) a method for validating the QSAR. There must be information about the size and shape of the interface and physical properties in the active site of the enzyme–inhibitor complex in order to understand how enzymes recognise small inhibitor molecules (17).

Enzyme inhibitors may exert their biological effects by participating in a series of events that include transport to the enzyme's active region, binding with the enzyme, and subsequent metabolism to an inactive species. Since the interaction mechanisms between the inhibitor and the putative enzyme are unknown in most cases inferences are made, to explain these interactions, from molecular properties and descriptors for known molecules. Once the relationship is defined, it can be used to aid in the prediction of new or unknown molecules. After biological data has been collected, it must be expressed in terms of free energy changes that occur during the biological response. The free-energy terms E (energy), G (free energy), H (enthalpy), and S (entropy), are represented by a series of parameters that could be derived for a given molecule. When examining the potency of an inhibitor (the dosage required to produce a biological effect), the change in standard free energy is proportional to the inverse logarithm of the concentration of the compound (C) (eq. 2).

$$\log 1/[\text{C}] = -2.3\, RT \log K = G_0 \quad (2)$$

Since the purpose of a QSAR is to highlight relationships between activity and structural features, it is necessary to find one or more structural features that relate these molecules to their associated activity. Additionally, it would be necessary to find a parameter that works consistently for all molecules.

There are several potential classes of parameters used in QSAR studies. Electronic effects such as electron-donating and electron-withdrawing tendencies, partial atomic charges and electrostatic field densities are defined by Hammett sigma values, resonance parameters (R values), inductive parameters

(F values), and Taft substituent values (*, Es). Steric effects such as molecular volume and surface area are represented by values calculated for molar refractivity and the Taft steric parameter. Enthalpic effects are calculated using partition coefficients (log P) or the hydrophobic parameter, which is derived from this partition coefficient. In addition, an assortment of structural indices are used to describe the presence of specific functional groups at positions within the molecule. The linear equation that describes the relationship between activity and this parameter set is the Hansch equation (eq. 3)

$$\log 1/[\mathrm{C}] = A(\log P) - B(\log P)^2 + C(\mathrm{Es}) + D(^*) + (E) + \cdots \tag{3}$$

Multiple linear regression analysis is used to derive the values of the coefficients. In general, Hansch-type studies are performed on compounds that contain a common template (usually a rigid one such as an aromatic ring) with structural variation limited to functional group changes at specific sites. While there are limits to the Hansch approach, it permits complex biological systems to be modeled successfully using simple parameters. The main problem with the approach was the large number of compounds that are required to adequately explore all structural combinations. Further, the analysis methods did not consider conformational effects (18). If the association between the parameter(s) selected and activity is strong, then activity predictions will be possible as it is assumed that parameters can be calculated more accurately than activity can be measured. The relationship between biological activity and specific functional groups at specific locations on parent molecules can be ascertained (19) (eq. 4).

$$\text{Activity} = \mathrm{A} + ij\ G_{\mathrm{ij}}X_{\mathrm{ij}} \tag{4}$$

where A is defined as the average biological activity for the series, G_{ij} is the contribution to activity of a functional group i in the jth position, and X_{ij} is the presence ($i = 1$, $j = 0$) or absence ($i = 0$, $j = 0$) of the functional group i in the jth position. The procedure uses equation 4 to build a matrix for a series of inhibitor molecules and represents this matrix as a series of equations. Substituent constants then were derived for every functional group at every position and statistical tests used to test the importance of these constants. If the models were shown to be valid, the model was used to predict activity values for compounds that had not been prepared. In general, while a large number of compounds are required to explore the effects of multiple substitution patterns, the Free-Wilson approach substantially reduces the number of analogues required. The method, however, demands that the effects of substituents are additive.

CoMFA. This analysis utilizes partial least squares (PLS) and cross-validation, to develop inhibitor models for activity predictions. The approach used in the CoMFA procedure requires that alignment rules for the series of inhibitor molecules are defined that overlap the putative pharmacophore for each molecule. Once aligned, each molecule is fixed into a 3D grid and the electrostatic (estat) and steric (ster) components of the molecular mechanics force field, arising from interaction with a probe atom, are calculated at intersecting lattice points within this grid.

PLS essentially relies closely on the fact that the correlations among parts of a molecule are similar so that the real dimensionality is smaller than the number of grid points. Since these coefficients are position dependant, substituent patterns for the series are elucidated that define regions of steric bulk and electrostatic charge associated with increased or decreased activity. The number of components needed for the best model and the validity as a predictive tool are assessed using cross-validation (20–23) (Fig. 16).

QSAR with CoMFA (15,24). This provides tools to build statistical and graphical models of activity from molecular structure, and uses these models to make accurate predictions for the activity of untested compounds. It organizes structures and their associated data into molecular spreadsheets, calculates molecular descriptors, structural, conformational, geometric, electronic, thermodynamic, hydrophobic, molar refractivity, highest occupied molecular orbital (HOMO) or lowest unoccupied molecular orbital (LUMO) values and specialised 2D fingerprints (HQSAR) and performs sophisticated statistical analyses that reveal patterns in structure–activity data (25). LeapFrog (26) uses a CoMFA model as the basis for de novo ligand design, and can optimize lead compounds or generate novel structures.

CoMSIA (Comparative Molecular Shape Indexes Analysis) (27). This method is similar to CoMFA, but uses a Gaussian function rather than Coulombic and Lennard-Jones potentials to assess steric, electrostatic, hydrophobic, and hydrogen-bond donor–acceptor fields. If the correct conformation of a molecule is not known, multiple conformers can be stored in the molecular spreadsheet to allow alternative conformers to be considered in a CoMFA or CoMSIA analysis. Statistical tools in QSAR with CoMFA include principal component analysis (PCA) (28) for uncovering relationships between descriptors, PLS (29) regression for analyzing continuous response data (IC_{50}, etc), and soft independent modeling of class analogy (SIMCA) (30) for analyzing data that is categorical rather

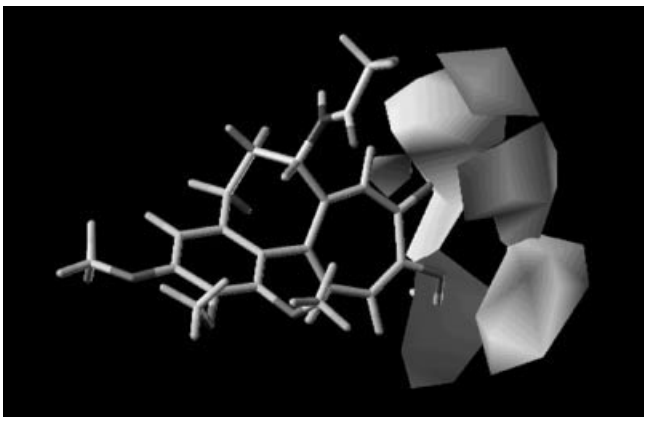

Fig. 16. Colchicine inhibitor in the CoMFA steric and electrostatic fields. Steric map indicating areas where bulk is predicted to increase (green) or decrease (yellow) activity. Eletrostatic map indicating where high electron density (negative charge) (red) and low electron density (positive charge) (blue) regions are expected to increase activity.

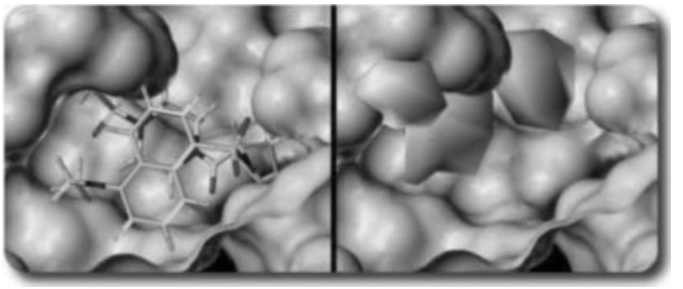

Fig. 17. A CoMSIA analysis of thrombin inhibitors. On the left, an inhibitor is positioned in the site based on X-ray coordinates. On the right, CoMSIA contours show regions predicted to prefer steric bulk (green) and other regions (yellow) where steric interactions are unfavorable. The sterically favorable contour lies within the pocket, while the sterically unfavorable contours intersect the surface, confirming the CoMSIA analysis.

than continuous (ie, active vs. inactive). The results of CoMFA or CoMSIA analyses are displayed as color-coded contours around molecules, allowing visual identification of regions responsible for favorable or unfavorable interactions with the receptor (Fig. 17).

HQSAR (31). This method does not require exact 3D information for the inhibitors but is reflected by a molecular fingerprint that encodes the frequency of occurrence of various molecular fragment types. The fragment size controls both the minimum and maximum length of the fragments to be included in the fingerprint. Molecular holograms are produced by generating all the linear and branched fragments such as atoms, bonds, number of hydrogen atoms, and chirality parameters. HQSAR identifies the patterns of substructural fragments related to activity in sets of bioactive molecules by identifying color coded molecular fragments that have positive or negative impact on activity.

EDDFA. This method is an improved CoMFA that utilizes eight molecular property fields instead of two. EDDFA uses steric, electrostatic, electronic, kinetic energy densities, fukui function, laplacian, local average ionization potential, and binary node potential fields. Each property field is rapidly generated using properties of the transferable atom equivalent (TAE) electron density distribution. Since inhibitor molecules would interact with enzymes via noncovalent interactions involving electron densities, descriptors using this principle would show high correlation to biological activity. A fine-grained version of the cross-validated guided region section (R2-GRS) routine of Cho and Tropsha (32) is used to identify important regions of space surrounding each molecule in the dataset. Field values in the selected regions are used as descriptors in a PLS regression analysis.

Pharmacophore Perception. Table 2 presents pharmacophore perception data.

Apex-3D (16). A detailed description of the fundamental architecture for this computer assisted molecular modeling package has appeared (33). It is an automated pharmacophore identification system that can identify possible pharmacophores from a set of biologically active molecules using statistical

Table 2. **Pharmacophore Perception**

DISCO (30)	DISCO performs pharmacophore elucidation from precomputed conformations of active compounds that bind to the same target.
GASP (24)	GASP elucidates pharmacophore models while allowing ligand flexibility and without requiring prior knowledge of pharmacophore elements or constraints.
RECEPTOR (24)	RECEPTOR uses systematic search to determine the common, 3D bound geometry of pharmacophore elements in a set of receptor ligands.
HIPHOP (16)	HIPHOP matches the chemical features of a molecule against inhibitor candidate molecules in a 3D database.
CATALYST (33)	CATALYST develops 3D pharmacophore models or hypotheses from a collection of molecules possessing a range of diversities in structure and activities.
Apex 3D (16)	APEX 3D can identify possible pharmacophores from a set of biologically active molecules using statistical techniques and 3D pattern matching algorithms.

techniques and 3D pattern matching algorithms. The program classifies molecular structures using three methods:

1. The agreement inductive method identifies common structural patterns in compounds having similar activity.
2. The difference inductive method identifies structural patterns to differentiate active and inactive compounds.
3. The concomitant variations inductive method highlights variations in structural features that explain changes in biological activity for sets of compounds.

Pharmacophores are defined by different chemical centers (atom-centered functional groups) and the distances between these centers and they include aromatic ring centers, electron donor ability hydrogen-bonding sites, lipophilic regions, and partial atomic charge. The information for each molecule is stored in a knowledge base in the form of rules that can be used to predict the activity of novel structures. Depending on the type of biological activity available, it is possible to identify pharmacophores for different binding orientations, enzymes, or agonist versus antagonist activity by building a knowledge base using the following steps:

1. Identify all possible binding interaction centers for each compound in the data set.
2. Generate topological (2D) or topographical (3D) distance matrices based on the set of descriptors.
3. Identify possible pharmacophores from all pairs of molecules using clique selection algorithms.
4. Classify these pharmacophores based upon their occurrence in compounds in each activity class using Bayesian statistics and their nonchance occurrence.

5. Set thresholds for probability and reliability statistics associated with a pharmacophore so that all training set molecules are properly classified by the pharmacophore rules.
6. Align compounds containing high probability pharmacophores on the original pharmacophore.

Pharmacophores and enzyme active site models play a very important role in inhibitor design yet before they can be built, however, the lead molecules must be aligned so that the active functional groups of the molecules are overlapping in space. One reason why this particular step could be considered as being difficult is that many compounds have rotatable bonds, so that the "active" conformer must be found first and then the molecule rotated to align with the other molecules in the study. The main difficulty in alignment is that usually the active functional groups in the pharmacophore are unknown, so each of the above steps involves some guessing. Often it is necessary to build several enzyme–inhibitor models based on alternate pharmacophores. The alignment procedure used is referred to as atom–atom mapping in which similar atoms in the molecule are determined and used as a template for the alignment. The strength of the interaction of a potential inhibitor molecule with the active site of an enzyme is more likely to be sensitive to the position and type of functional groups.

Once the knowledge base has been constructed, it can predict biological activity for inhibitors not included in the training set. The pharmacophores defined above can be used to build 3D QSAR models by correlating indexes calculated for biophore sites, secondary sites, or whole molecule properties. Three-dimensional QSAR models in Apex are generated and screened using a modified scheme of multiple linear regression analysis with variable selection.

HipHop (16). This method searches for types of chemical functional groups within potential inhibitor molecules and chooses conformations and alignments that overlap the groups in space and ignores any intervening backbone atoms in making these comparisons. The result is a series of hypotheses and alignments of inhibitor molecules for possible pharmacophores that ultimately can be used to generate an enzyme–inhibitor model. The advantage of HipHop is that the use of chemical properties in generating alignments is often more realistic in reproducing the details of the molecular recognition mechanism, as compared to atom–atom matching procedures. HipHop and atom–atom matching are complementary techniques and both would be used in suggesting pharmacophores for future study.

Catalyst (34). This method generates structure–activity hypotheses from a set of molecules of various activities. Once molecular connectivity and activity values are specified for all molecules, catalyst creates a set of generalized chemical functions (regions of hydrophobic surface, hydrogen bond vectors, charge centers, or other user-defined features) at specified relative positions (Fig. 18). Up to 10 hypotheses are produced and ranked by estimated statistical significance then examined graphically, "fitted" to a new molecules, or fed directly to a flexible 3D database search. In the first step of the process, a set of representative conformers is found that covers the low energy conformational space of each molecule. The second step locates a list of candidate hypotheses that are common among active and rare among inactive compounds. The theory

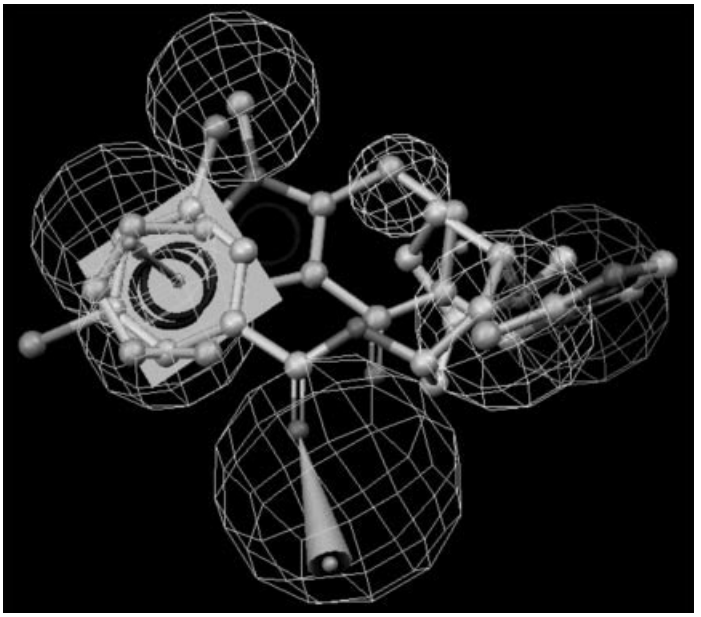

Fig. 18. Two 5HT3 inhibitors (green and yellow) mapped on to a six-feature hypothesis using the Catalyst algorithm. Cyan represents a hydrogen donor; magenta a hydrogen acceptor; brown a hydrophobic interaction; green an ionizable/charged group.

of minimum complexity estimation indicates that a predictive hypothesis will be the least number of descriptors required as well as a minimum of errors in the activities.

Molecular Modeling. See Table 3 for molecular modeling data.

MOLCAD (35). This method creates graphical images of an enzyme's active region to illustrate the properties of inhibitors that would be necessary

Table 3. **Molecular Modeling**

SYBYL/Base (24)	SYBYL/Base includes comprehensive tools for molecular modeling: structure building, optimization, and comparison; visualization of structures and associated data and force fields.
Advanced Computation (24)	Advanced computation provides a range of tools for conformational analysis of torsional states of a molecule or identify just its low energy conformations.
MOLCAD (35)	MOLCAD creates and displays molecule surfaces onto which it maps key properties, including lipophilicity, electrostatic potential, hydrogen-bonding sites, and local curvature.
MM3(2000) (24)	MM3(2000) is a molecular mechanics program that produces high quality 3D structures and computes molecular energy, vibrational spectra, and a variety of thermodynamic and spectroscopic quantities.
AMPAC (24)	AMPAC calculates structures and electronic properties of molecules using semiempirical quantum mechanical methods.
Confort (24)	Confort performs conformational analyses of inhibitor-sized molecules to identify the global minimum energy conformer, all local minima within a user-specified energy range, or a maximally diverse subset of conformers.

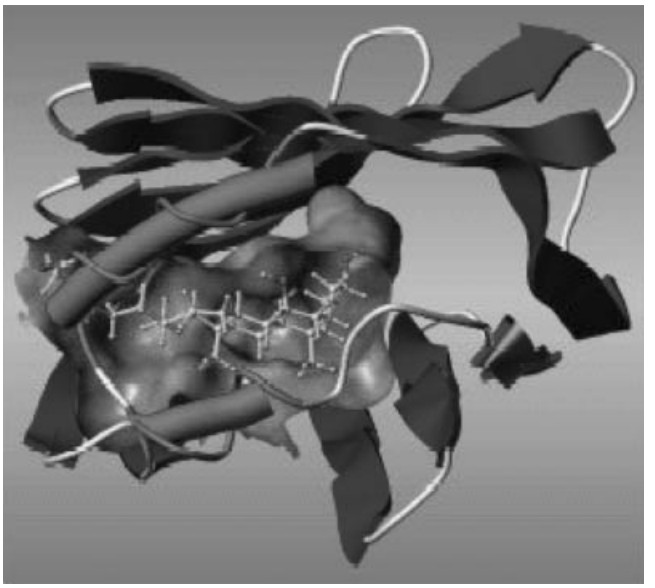

Fig. 19. The structure of a lipid binding protein complexed with glycocholate. The protein loop regions are rendered as a tube; blue arrows illustrate beta sheets, and red cylinders denote helices. The binding site [green] was located using MOLCAD's channel finding capability and is color coded by the lipophilic potential—cyan as negative and magenta as positive.

for molecular recognition (36). van der Waals and solvent-accessible enzyme surfaces can be shown and a broad range of properties such as lipophilic potential (37), electrostatic potential, hydrogen-bonding ability, local curvature, and distance. MOLCAD reveals the underlying secondary and tertiary structure, and maps onto these fundamental physical properties such as residue lipophilicity, flexibility based on atomic temperature factors, and the packing density. It can characterize the size, shape, and physical properties of intramolecular cavities and channels and examines the specificity of enzyme–inhibitor interactions (36,37) (Fig. 19).

Structure-Based Design. Table 4 gives information on structure-based designs.

SiteID (38) The recent explosion in the ability to predict and/or solve macromolecule structures has led to an increased interest in methods for modeling the interaction of inhibitors with enzymes. The first hurdle that must be surmounted, however, is to locate the active site or pocket in which the inhibitor binds. SiteID provides analysis and visualization tools leading to the identification of potential binding sites within or at the surface of enzyme targets. First, the enzyme active site is rapidly identified, while in the second, SiteID is used for a detailed analysis of the enzyme structure based on properties suitably identified for binding. In this mode, SiteID automatically creates a color-mapped database of solvent exposure, hydrogen-bonding character, hydrophobicity, and local surface curvature (Fig. 20).

Modeller. Knowledge of the 3D structure of an enzyme is a prerequisite for the rational design of site-directed mutations in the enzyme and can be of

Table 4. **Structure-Based Design**

Biopolymer (24)	Biopolymer delivers an extensive set of tools for building, visualizing, manipulating, and predicting the 3D structure of biological molecules.
Composer (24)	Composer builds 3D models of proteins from sequence using knowledge-based homology modeling methods.
ProTable (24)	ProTable uses SYBYL's Molecular Spreadsheet to deliver tools that analyze and assess the quality of enzyme structures.
SiteID (38)	SiteID provides analysis and visualization tools leading to the identification of potential binding sites within or at the surface of macromolecules.
LeapFrog (26)	LeapFrog uses a CoMFA model as the basis for de novo ligand design, and can optimize lead compounds or generate novel structures.
MODELLER (51)	MODELLER is used for comparitive modelling of protein 3D structures.
HOMOLOGY (16)	HOMOLOGY builds a 3D model of an enzyme from its amino acid sequence and the known structure of related proteins.
CONVERTER (16)	CONVERTER builds 3D models from 2D structural databases
SWISS-MODEL (53)	SWISS-MODEL is a fully automated comparative enzyme (protein) structure homology modeling server.

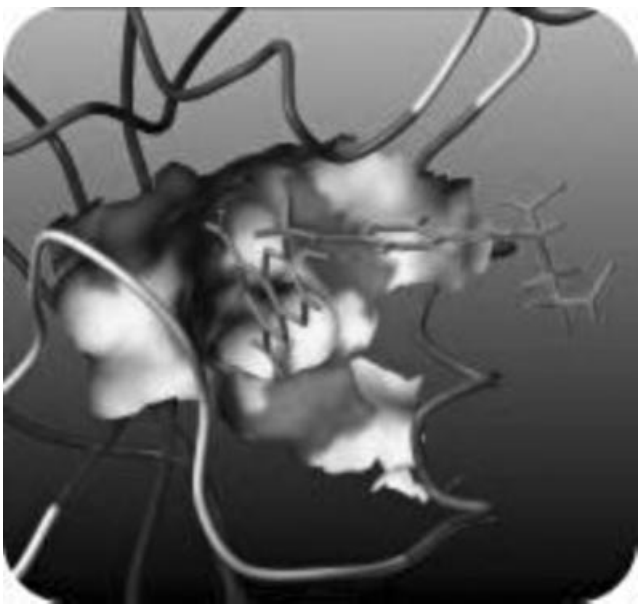

Fig. 20. The binding pocket of dihydrofolate reductase located by SiteID and shown as a MOLCAD surface. The red areas of the surface indicate contact atoms in the pocket, while the yellow areas show the residues in which those atoms are contained. The inhibitor (methotrexate) is shown in green.

great importance for the design of inhibitors. Structural information often greatly enhances understanding of how enzymes function and model-building on the basis of the known three dimensional structure of a homologous protein is at present the only reliable method to obtain structural information (39–45). Comparisons of the tertiary structures of homologous enzymes have shown that 3D structures have been better conserved during evolution than enzyme primary structures (46–48) and massive analysis of databases holding results of these 3D comparison methods indicate the feasibility of enzyme model-building by homology (49,50).

Modeller is a computer package used for homology or comparative modeling of protein 3D structures (51,52). A sequence alignment is modeled with a known related protein structure, by considering spatial restraints, and the programme automatically calculates and creates a model containing all non-hydrogen atoms.

Inhibitor Docking. See Table 5 for inhibitor docking information.

FlexX (54). This method is a fast algorithm for flexibly docking inhibitors, using incremental construction to actually build the inhibitors within the binding site of the enzyme. Taking advantage of the best ideas and techniques available for structure-based inhibitor design, FlexX incorporates enzyme–inhibitor interaction scores, fragmentation of the inhibitor along natural dividing points, inhibitor core placement in the active site, and reconstruction of the complete inhibitor from the fragments. FlexX includes conformational flexibility of the inhibitor—of critical importance because the low energy conformation is rarely the bioactive one (55–61) (Fig. 21).

Dock. More recent versions of DOCK (62) allow score parameters to be based on force fields, which include both van der Waals and electrostatic interactions (63,64). These results with DOCK illustrate the potential for programs to search objectively for inhibitors that are complementary to enzyme active sites,

Table 5. **Inhibitor Docking**

FlexX (54)	FlexX flexibly docks inhibitors into binding sites, allowing virtual screening of compound databases.
CScore (24)	CScore integrates multiple types of scoring functions for ranking the affinity of inhibitors bound to an enzyme.
FlexS (24)	FlexS is a program for automatic structural alignment of inhibitor molecules that can be used for shape-based screening in the absence of an enzyme structure, and to align molecules for 3D QSAR studies.
CombiFlexX (24)	CombiFlex applies a combinatorial approach to inhibitor docking.
FlexXPharm (24)	FlexX-Pharm enables pharmacophore-type constraints to be used in FlexX to guide inhibitor docking.
Insight II (16)	Insight II creates, modifies, manipulates, displays, and analyses molecular systems.
Binding Site Analysis (16)	Identifies and characterises an inhibitor binding site then uses them to find similar features in other known structures
Ludi (65)	Ludi is used to fit inhibitor molecules into the active site of an enzyme by identifying and matching complementary polar and hydrophobic groups.
Dock (62)	Dock uses parameters based on force fields and includes van der Waals and electrostatic interactions.
Affinity (66)	Affinity docks inhibitors to enzymes identifying low energy orientations.

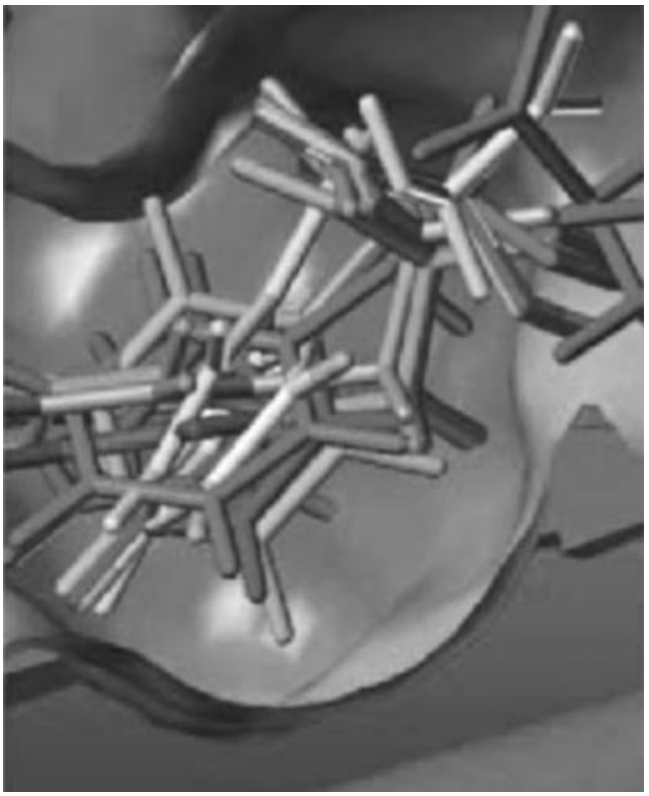

Fig. 21. A set of inhibitors docked on to the active site of carboxypeptidase A by FlexX.

thereby assisting researchers in identifying potential drugs that may be considerably different from existing ones. Once potential inhibitors have been identified by such methods, other molecular modeling techniques like geometry optimization may be used to "relax" the structures and to identify low energy orientations of inhibitors bound to enzyme active site. Molecular dynamics may also assist in exploring the energy landscape, and free energy simulations can be used to compute the relative binding free energies of a series of putative drugs. Many enzymes are membrane bound, making it extremely difficult to determine their 3D structure by nmr or X-ray crystallography. Furthermore complications may arise since enzymes may change shape as they bind, a process called "induced fit". Existing methods for constructing predictive models are unable to model steric interactions accurately, particularly when these interactions involve large regions of the molecular surface. Likewise, QSAR techniques are accurate only on a small scale, determining properties of specific regions but failing to produce an accurate global description of the molecule. Pharmacophore models attempt to combine some of the advantages of QSAR techniques with the idea of identifying substituents and advances in 3D QSAR have led to superior characterization of molecules and better calculation of their properties.

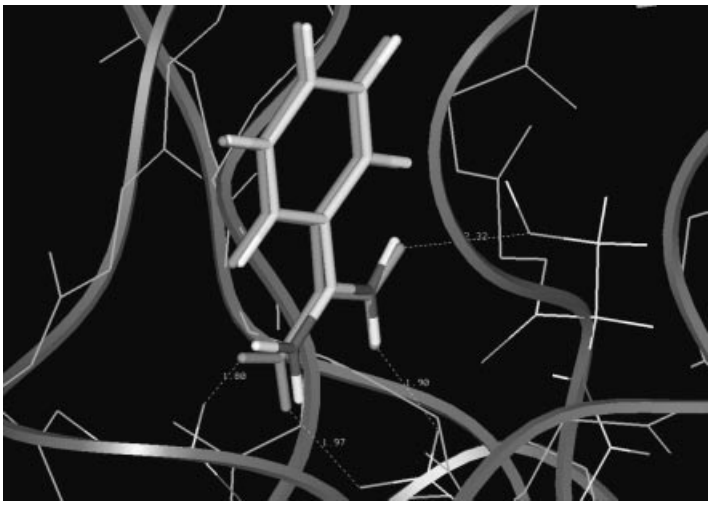

Fig. 22. Prediction by LUDI. Trypsin backbone (blue ribbon and purple atoms), the benzamidine X-ray coordinates (multicolored molecule) and a benzamidine fit by LUDI (red molecule).

LUDI. This method actually fits inhibitor molecules into the active site of an enzyme by matching complementary polar and hydrophobic groups and uses an empirical scoring function (65). Ludi can also suggest modifications that may increase the binding affinity between an existing inhibitor–ligand and the enzyme (Fig. 22).

Affinity. This method automatically docks inhibitors to enzymes identifying low energy orientations of the inhibitor within the active site and using force-field-based methods to automatically find the best binding mode (Fig. 23). This energy-driven method is especially useful in structure-based inhibitor design where the experimentally determined structure of an enzyme–inhibitor complex is often unavailable (66). The inhibitor binding may be accompanied by conformational change like side-chain movements or displacements of whole loop regions. Affinity allows exploration of these potential movements by defining flexible areas in the enzyme binding pocket as well as in the inhibitor. Affinity uses a two-step process to dock the inhibitors. First, initial placements of the inhibitor within the enzyme active region are made using a Monte Carlo type procedure to search both conformational and Cartesian space. Second, a simulated annealing phase optimizes the location of each inhibitor placement. During this phase, Affinity holds the "bulk" of the enzyme molecules (defined as atoms not in the user-specified binding site) rigid, while the binding-site atoms and inhibitor atoms are movable. Interactions between the bulk (nonflexible) and movable atoms are approximated by the very accurate and efficient molecular mechanical/grid (MM/Grid) method developed by Luty and co-workers (67), while interactions among movable atoms are treated using a full force-field representation. As an extension to Affinity within Insight II (68) product family is Interactive Docking.

Texture Mapping. The complexity of enzyme–inhibitor interactions leads to vast amounts of data generated by experiments or simulations (69). For a

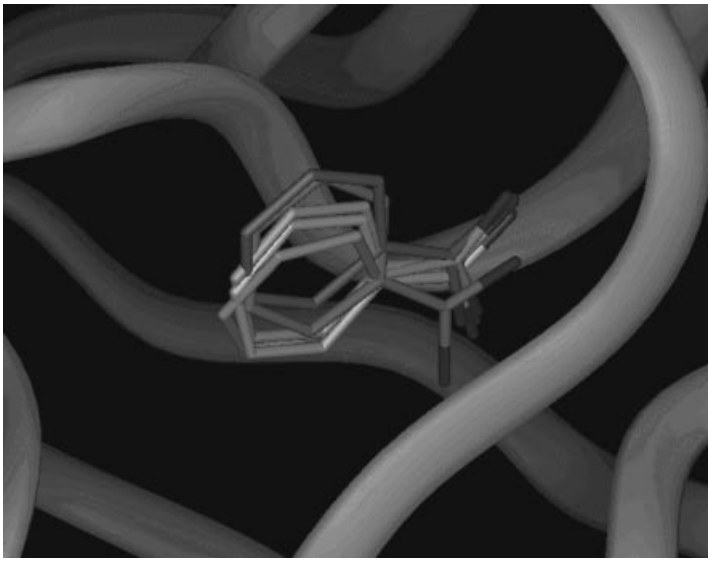

Fig. 23. Docking of benzamidine onto trypsin using Affinity. The crystallographically determined position for benzamidine is shown with green carbons, the other colored structures represent four of the lowest energy docked conformations calculated by Affinity. The green ribbons represent the protein backbone of trypsin.

better understanding of the structure and function of such systems, more advanced computer graphics capabilities are desirable, allowing one to go from a qualitative general overview toward more and more detailed aspects. With the advent of the technique of texture mapping (70,71) a new door for the visualization of enzyme–inhibitor interactions has been developed. Texture mapping is a graphic design by which a 2D surface, or texture map, is wrapped around a 3D object. It is a technique that applies a computer-derived image of an enzymes active region (texture space) by individual elements called texels (72,73). Two approaches, both capable of monitoring attractive and repulsive forces within enzyme–inhibitor complexes, are used to generate this visual information:

1. Discrete isocontour surfaces (74) that connect all points of a 3D property grid that are equal to a selected value.
2. Color coding for surface curvature (75).

It is demonstrated that additional quantities describing local hydrophobicity, surface roughness, surface topography, and surface flexibility are introduced on the basis of empirical findings which are helpful quantities for understanding molecular recognition.

A rather useful application of multiple property texture mapping and enzyme–inhibitor construction is transparency illustrated by analyzing the local flexibility of a structure. As “floppiness” or flexibility of the enzyme active site domain increases so its transparency increases. Conversely, the more rigid

the structure the more opaque will be the active region. Such a transparency map may well be combined with any other color-coded property, as it is of interest to study the dynamic properties of a potential inhibitor molecule in many different contexts. By filtering property information on an enzyme active site molecular surface, one is able to distinguish between important and irrelevant information and to convert a qualitative property into a quantitative context in order to identify the binding site of an inhibitor.

Correct perception of the geometrical curvature shape of the enzyme–inhibitor structure implicated by electron density maps can be achieved with computationally much more demanding shading techniques such as the Gouraud (76) and Phong algorithms (77). The former often fails, however, as it destroys information in the highly curved regions of the surface because of linear interpolation of vertex colors that cannot account for local shadow variations. In contrast, the Phong shading approach interpolates the normal vectors for each geometric orientation, computing the lighting equation in the subsequent step. Attempts have been made to overcome some of the computationally intensive steps of the procedure (78), but their performance was insufficient to be a reasonable alternative to Gouraud shading in real-time applications. A much simpler solution is to use a special texture-mapping technique called normal mapping that is a valuable aid for visualisation of highly complex enzyme active surfaces. In contrast to Phong shading, the interpolation is not performed directly as "normals" are used to automatically generate texture coordinates based on the orientation of the surface vertices. The subsequent interpolation then takes place in 2D texture space, using the image of a perfectly rendered sphere as the texture. The visual result compares exactly to a surface computed with the original Phong approach.

3.3. Evolutionary Computing. Currently, most major pharmaceutical companies use rational inhibitor design and evolutionary techniques such as genetic algorithms or genetic function algorithm GFA as part of the inhibitor discovery process. SYBYL (79) offer genetic algorithm-based conformational search tools for exploring 3D shapes that inhibitors attain.

Neural network-based approach to modeling enzyme activity and designing new inhibitor compounds has also been formulated. A new hybrid method (GNN) (80) combining an artificial neural network and a genetic algorithm has been developed for quantitative structure–activity relationship (QSAR) studies. The genetic algorithm selects suitable sets of molecular descriptors to serve as inputs to neural network, in which model-free mapping of multivariate data is performed. Graphical description of the functional form of the descriptors (steric, electrostatic, hydrophobic) is presented and it is these that play an important role in determining activity of the inhibitor molecules. The effectiveness of GNN is tested by comparing its results with a benchmark obtained by exhaustive enumeration and different fitness strategies are examined from the evolution of genetic models, and QSARs with higher predictiveness are found.

Evolutionary Molecular Design (EMD). This method identifies the activity that is required for the enzyme and focuses on the inhibitor structure rather than the enzyme (81). Once the required activity has been identified for the enzyme, a molecular assembler is used to identify structures that meet the requirements identified for the enzyme. Genetic algorithms generate both the virtual enzyme and the new inhibitor.

GFA begins with a population of randomly constructed QSAR inhibitor molecule models that are rated according to relative predictiveness using an error measure (82,83). Experimental results against published data sets demonstrate that GFA discovers models that are comparable to, and in some cases superior to, models discovered using standard techniques such as stepwise regression, linear regression, or partial least-squares regression. As the number of computational and instrumentational sources of experimental data increase, the ability of GFA to perform variable reduction, to discover nonlinear relationships, and to present multiple models representing multiple interpretations of the data set, may become increasingly useful in data analysis and enzyme–inhibitor construction.

Genetic software techniques automatically design inhibitor molecules under the control of a fitness function that must be capable of determining which of two arbitrary molecules is better for a specific task. A population of random molecules is first generated and these are then evolved toward greater fitness by randomly combining parts of the better existing molecules to create new molecules that eventually replace some of the less-fit molecules in the population. A unique genetic crossover operator such as sets of atoms and connector bonds are represented by genetic graphs and can evolve any possible molecule given an appropriate fitness function. Inhibitors are generally small molecules and it is known that they fit precisely into enzyme active sites to block normal molecular processes that may occur in a living system. Furthermore, the inhibitor molecules must survive within such living system. One approach to inhibitor design is to find molecules that are similar to good drugs that have fewer negative side effects and consequently a candidate replacement drug is sufficiently similar to have the same beneficial effect but is different enough to avoid the side effects (84). Genetic software techniques used for enzyme–inhibitor design, describes the obvious parts of mapping standard genetic algorithm techniques to inhibitor design and the nonobvious portions: the crossover algorithm and fitness function (85). These use two parameters: the digestion rate that breaks bonds, and the dominance rate that controls how many parts of each parent appear in the descendants. Inevitably this algorithm produces fragments rather than completely connected molecules.

Enzyme–inhibitor design may be viewed as searching the space of all possible molecules for inhibitors with particular properties. The key point in deciding whether or not to use genetic algorithms for a particular problem centers around the question: What is the space to be searched? If that space contains structure that can be exploited by special-purpose search techniques, the use of genetic algorithms is generally computationally less efficient. If the space to be searched is relatively unstructured, and if an effective GA representation of that space can be developed, then GAs provide a surprisingly powerful search technique (86). It is reasonable to presume that searching the space of molecules using genetic graphs will be profitable in a number of domains.

4. Future Prospects

4.1. Molecular Nanotechnology. With particular reference to enzyme-inhibition, molecular nanotechnology can be defined as the 3D positional control

of molecular structure to create the enzyme inhibitors to molecular precision (87). Since the human body is comprised of molecules, the availability of molecular nanotechnology permits dramatic progress in human medical services and inhibitor design. More than just an extension of "molecular medicine," nanomedicine (88) is the preservation and improvement of human health using molecular tools and molecular knowledge and has extraordinary and far-reaching implications for the medical profession, for the definition of disease, for the diagnosis and treatment of medical conditions and ultimately for the improvement and extension of natural human biological structure and function. It must be necessary to establish a set of basic capabilities, including physical, chemical, thermodynamic, mechanical, physiological, immunological, cytological, and biochemical limits of molecular systems in order to recognize, sort and transport important inhibitor molecules; alter the shape or surface texture of the enzyme-active site, and perform computations, disable living cells, pathogenic bacteria, and viruses. The effectiveness of such systems critically depend on their biocompatibility with diseased human organs, tissues, and biochemical systems.

Progress must continue in the synthesis and use of nanostructures for the development of enzyme inhibitor implants delivered specifically to the site of action. Future considerations should be borne on technical requirements in the design and operation of biomedical nanobots that may not only be used as a molecular tool but could be inserted into analytical instruments for fundamental research and development on new drugs. A vast plethora of issues will be addressed. These include nanoinhibitor failure, side effects of nanomedical treatment, complex nanobotic systems for cell repair, tissue and organ manufacturing, rapid cardiovascular repair, treatments for pathogenic disease and cancer, response to physical trauma, burns and radiation exposure, spinal restoration and brain repair, improved nutrition and digestion, sex and reproduction, cosmetics, veterinary and space medicine, control of the ageing process, the future of pharmaceutical companies, and the medical profession.

Cures for the major life-threatening diseases could be in-sight within the next few years if such inhibitors against the enzymatic processes involved in the molecular basis of the diseases could be manufactured using such revolutionary nanotechnology. Computer-assisted nanoinhibitor design could be at the cutting edge of the technology and, in conjunction with nanobots, delivered at relatively high, though safe concentrations, to any biological active region. In this way they can "close-down" any external pathogen or virus, act as biosensors in the detection of a threat by biological warfare or prevent tumor growth.

4.2. Molecular Engineering. With the advent of molecular engineering, the principle of developing a structure for a nanoinhibitor and its "modus operandi" must satisfy prerequisites of being, at least, 100× smaller, 10,000× cheaper and 1000× faster (87). Though there are differences of opinion when referring to molecular engineering and the creation of microassemblers (89,90) the development of the nanoinhibitor as a microchip and the role of the computer as a delivery vehicle cannot be to far away into the future. Once molecular engineered machines (nanobots) are the order of the day then one can exploit this to make absolute copies of themselves thereby creating a second level of mass production limited only by the materials and information therein. Self-replicating microchip nanoinhibitors would be an absolute necessity to engineer molecules on a 10^9 scale.

There is no question that molecular engineering, nanoinhibitors and nanobots will be reality sooner than expected.

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