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Perspective

Designing Non-Peptide Peptidomimetics in the 21st Century: Inhibitors Targeting Conformational Ensembles

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Historical Perspective of Ligand Binding

During the past 100 years ligand binding has been described via two basic rationales. Emil Fisher first proposed in 1894 the *lock and key* rationale[1] to describe ligand−receptor binding. In this model, the receptor (this term also includes enzymes) is symbolized by a rigid lock into which the symbolic key, or ligand, must precisely fit (Figure 1). This was the sole model used to describe ligand binding events for over 50 years until Koshland in 1958 proposed an *induced fit* model[2] to describe ligand−receptor binding events that seemed to proceed in a zipper-like fashion. He hypothesized that binding of the "substrate causes a change in the 3-dimensional relationship in the active site" leading to a fit that "occurs only after the changes induced by ligand binding." Over the years, conformational changes of the receptor ascribed to an *induced fit* binding have ranged from the very subtle movements of single amino acid side chains to large conformational changes involving movement of entire protein domains.

These two complementary models have been utilized to describe most of the structural data presently available in the literature. The *lock and key* rationale describes the binding event if, after inspection of the ligand−receptor complex, the observed receptor conformation resembles the unbound-receptor conformation. Conversely, if the conformation of the bound-receptor is different than the unbound-receptor (no matter how subtle the differences), then *induced fit* rationalizes the observed ligand binding process.

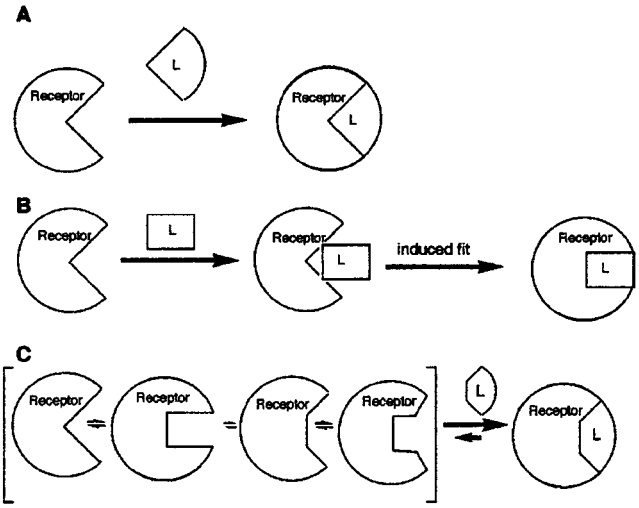

Figure 1. Ligand binding models: (A) lock and key; (B) induced fit; (C) stabilization of conformational ensembles (Ligand, L).

Recently, *stabilization of receptor conformational ensembles*[3−5] has emerged to rationalize a range of ligand binding events without necessitating either the *lock and key* or *induced fit* mechanisms. This model assumes that macromolecules exist as multiple, equilibrating solution conformations that can be described by mechanical laws with standard statistical distributions. The process of ligand binding effectively shifts this equilibrium to the bound-receptor conformation from the statistical distribution of native conformations. In this view, ligands bind to the ensemble of *pre-existing* receptor conformations. Productive binding shifts the

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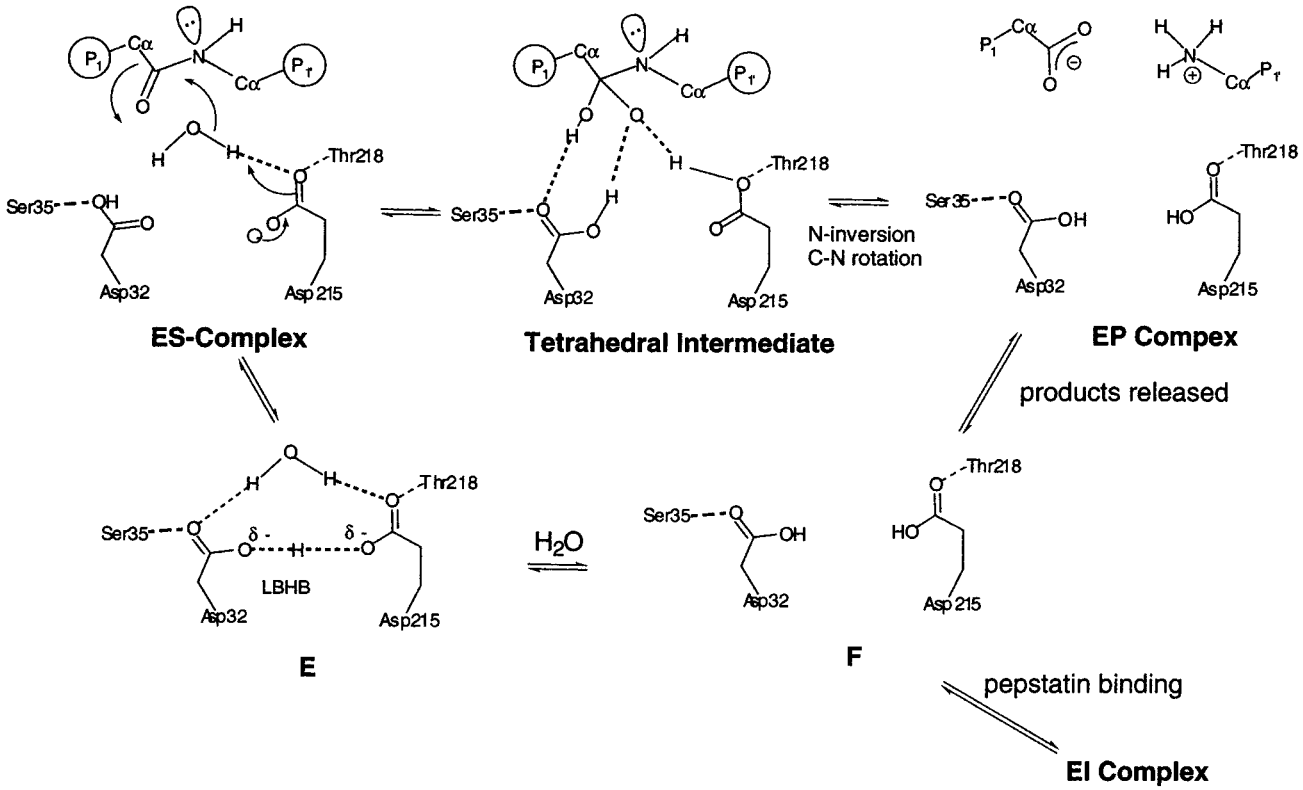


Figure 2. General acid−base catalytic mechanism for aspartic peptidases.

overall dynamic equilibrium to stabilize the bound-receptor conformation.

This concept of conformationally mobile receptors (and ligands) is not new, but arose shortly after the discovery of modern conformational analysis.[6,7] Almost 40 years ago, Straud stated[8] "the conformation of an enzyme in solution is regarded to be a statistical average of a number of conformations, the protein structure oscillating between these conformations." Since then, conformational mobility of biologically active proteins has been repeatedly demonstrated via biophysical methods. Nevertheless, due to computational limitations, current molecular modeling and drug design efforts treat proteins as static models even though they are clearly dynamic macromolecular structures constantly in motion. In general, the static models portray either the native protein conformation or the protein conformation tightly bound to a potent peptide-derived inhibitor. Some modeling studies accommodate small changes in protein and ligand side chain conformations or hydrogen bonding interactions that occur in a process called "soft lock and key",[9] and these small changes have subsequently been utilized to modify inhibitor design. But other, significantly altered protein conformations are rarely considered although biophysical methods have established their existence. Successful design of structurally novel inhibitors may necessitate targeting receptor conformations located outside the narrow window of conformational ensembles presently exploited via current inhibitors. Systematic exploration of *pre-existing* receptor conformations within the ensemble has not been the focus of structure-based drug design strategies.

The goal of this Perspective is to outline for medicinal chemists the potential impact of receptor conformational mobility on rational drug design. While others have postulated receptor-based conformational selection of ligands[10] and successfully designed inhibitors to emulate the β-strand binding motif of native ligands,[11,12] we show that *novel protein conformations* (not observed in either native or enzyme−inhibitor complexes) can be exploited to create non-peptide enzyme inhibitors. We begin with a review of the development of peptidomimetic aspartic peptidase inhibitors and conclude with a new proposal for discovering fundamentally novel non-peptide peptidomimetics by targeting conformational ensembles. Although we focus on the aspartic peptidases, the lessons derived therein are applicable to other peptidase and receptor−ligand systems.

Aspartic Peptidase Knowledge Base

The aspartic peptidases are an extensively characterized class of enzymes with a large number of native and enzyme−inhibitor crystal structures presently available.[13] This structural information has led to a more precise understanding[14−17] of this therapeutically important class of enzymes. The aspartic peptidases consist of two structural domains (N- and C-terminus) that define the active site, along with a hairpin turn structure, or "flap" region, that covers the enzyme active site (HIV protease contains two flaps). It has become evident from structural considerations that during catalysis the flap must be open to allow substrate entry into the active site and must close to promote catalysis. Finally the flap must re-open to allow diffusion of products from the active site. The aspartic peptidases catalyze the cleavage of a peptide amide bond via a general acid−base mechanism (Figure 2).[18] These peptidases have two Asp-Thr-Gly sequences lining the active site and contain a water molecule bound between the two catalytically active Asp residues. This substrate water molecule is activated by the aspartic acids for

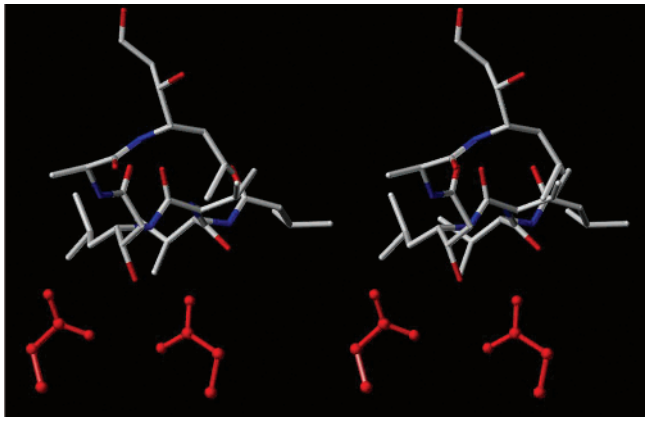


Figure 3. Hydroxyl unit of pepstatin binding to Rhizopus pepsin.[38]

nucleophilic attack on the substrate amide carbonyl to generate the tetrahedral intermediate, which is observed as a low-energy species in molecular dynamic-ab initio calculations.[19] The amide nitrogen is eventually protonated leading to the collapse of the tetrahedral intermediate and subsequent release of the amide bond hydrolysis products from the active site.

Two additional enzyme forms have been added to the mechanisms for catalysis and inhibitor binding. Carloni and co-workers calculated that a low-barrier hydrogen bond (LBHB)[20] is formed between the two aspartic acid carboxyl groups in the HIV protease ground state (Figure 2, **E**).[19,21,22] Northrop utilized this LBHB to explain aspartic peptidase pH titration curves and transpeptidation chemistry.[23,24,20] Additional kinetic isotope studies[23] identified a post product-release enzyme form (Figure 2, **F**), the key intermediate necessitating the "isoenzyme" mechanism.

Development of Aspartic Peptidase Inhibitors

Aspartic peptidase inhibitors have been designed to treat hypertension, malaria, AIDS, and Alzheimer's disease, and this list is expected to increase as genomic sequencing continues. Over the years a number of native and enzyme−inhibitor crystal structures have been solved for both the medicinally relevant aspartic peptidases (renin, plasmepsin, HIV protease, β-secretase, and cathepsin D) and model peptidases (penicillopepsin, endothiapepsin, chymosin, pepsin, and *Rhizopus chinensis* pepsin). Both peptide-derived and non-peptide inhibitors have been developed,[25] and the relationships between the different peptidomimetics can be analyzed in terms of enzyme−inhibitor crystal structure complexes.

A key structural element in most inhibitors of aspartic peptidases is a hydroxyl or hydroxyl-like moiety that binds to the two catalytically active aspartic acids (Figure 3) in place of the Asp-bound water molecule. For example, the unnatural amino acid statine (Figure 4, **1**) found in the peptide natural product pepstatin **2**[26,27] was postulated[28−30] to mimic the amide bond hydrolysis transition state (Figure 2). Since these results were obtained nearly 25 years ago, a number of mechanism pathway inhibitors (Figure 4, **3**−**9**) have been invented and subsequently developed into useful aspartic peptidase inhibitors.[25] Notable are the hydroxyethylene **4**[31−34] and the hydroxyethylamine **5**,[35,36] the latter

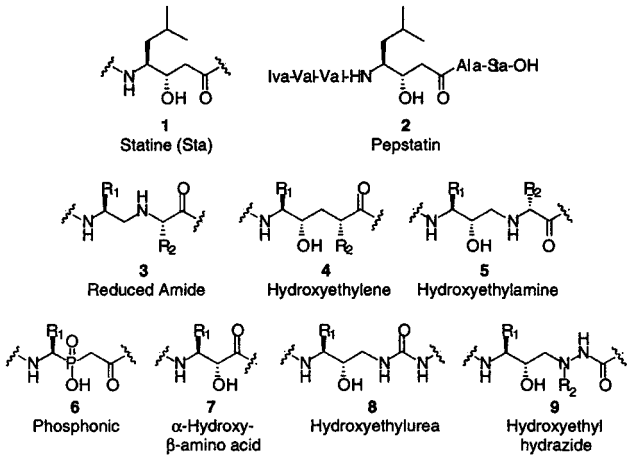


Figure 4. Some transition-state analogue (TSA) units effective for inhibiting aspartic peptidases.

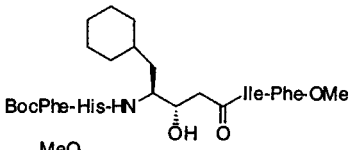
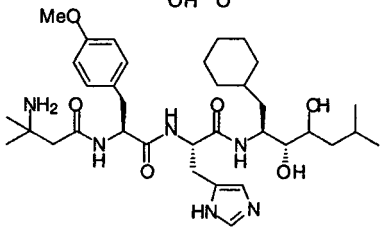
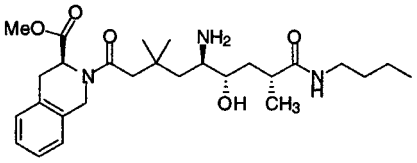
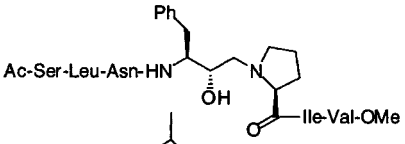
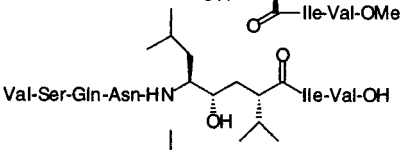
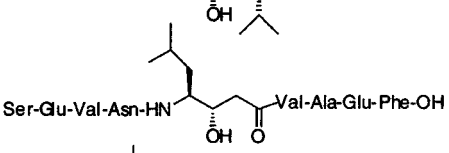
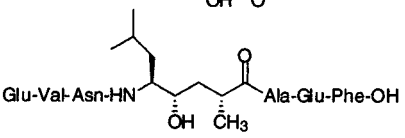
employed extensively in the development of HIV protease inhibitors.[37]

These inhibitors were originally designed to be transition-state analogues (TSA) for the enzyme-catalyzed amide bond hydrolysis. However, recent calculations by Carloni have shown the critical inhibitor hydroxyl group binds to the HIV protease bis-protonated form of the catalytic dyad, not to the monoprotonated form implicated in the catalytic mechanism.[22] Carloni has suggested these inhibitors bind to the *isomechanism* form (Figure 2, **F**) of the enzyme proposed by Northrop.[22] Consequently, pepstatin and, by analogy, other effective peptide-derived inhibitors of aspartic peptidases contain elements of collected-substrate inhibitors as proposed earlier.[30] These findings are consistent with the fact that the best inhibitors of aspartic proteases are one-atom extensions of an isosteric replacement of the substrate backbone, i.e., hydroxyethylamines (**5**) and related analogues.

Selective aspartic peptidase inhibitors have been designed by replacing the specific peptidase substrate dipeptidyl cleavage site with a TSA dipeptide mimic (Figure 4). The principles of this strategy were first utilized to develop selective inhibitors for the model aspartic peptidases and have been extended (Table 1) to renin, HIV protease, and β-secretase, the therapeutically promising aspartic peptidases.[39] Replacement of the dipeptidyl cleavage site of a native substrate with a TSA effectively generates an inhibitor specific for the peptidase that recognizes the TSA side chains plus amino acid side chains both up- and downstream from the cleavage site. The enzyme active site is buried in a deep cleft capable of accommodating up to nine amino acid residues of the substrate/inhibitor with the inhibitor's exquisite selectivity obtained by the complementary interaction between the enzyme binding sites (S_6−S_3') with the inhibitor (the P_6−P_3') residues. Some renin inhibitors have also been shown to bind to a new subpocket (S_3^{sp}), which provides a method to increase both inhibitor potency and selectivity.[40]

Unfortunately, very few of the first generation of peptide-based aspartic peptidase inhibitors proved clinically useful due to limited oral bioavailability. As a result, the first pharmaceutical attempts to convert an aspartic peptidase (renin) inhibitor into a clinically useful treatment for hypertension via TSA-based inhibi-

Table 1. Examples of Selective TSA Inhibitors of Aspartic Peptidases

Enzyme Target	Selective Inhibitor
Renin	
Renin	
Renin	
HIV Protease JG-365	
HIV Protease U-85548e	
β-secretase	
β-secretase	

tors proved to be a monumental failure.[41] It was eventually realized after extensive modifications to the ancillary peptide functionality that developing bioavailable peptide-derived inhibitors critically depended on the molecular weight of the inhibitor. In contrast, developing inhibitors for HIV protease was substantially easier than for renin because HIV protease recognizes a significantly smaller minimum substrate sequence. Some of the highly modified HIV protease inhibitors[37] now in clinical use (Figure 5) have excellent oral bioavailability and establish that application of this design process can be very successful in favorable cases.

Up to this point we have used the term *peptidomimetic* without providing a working definition. Recent literature describing the development of peptidomimetic inhibitors rarely defines peptidomimetic, and this word is often applied to a variety of different structural types.[42] For example, peptide analogues that contain one or more amide bond replacements (defined as a *pseudo peptide* by Spatola[43]) have sometimes been called peptidomimetics. Peptide analogues that contain a conformationally restricted amino acid unit or other conformational constraint[44] have been called peptidomimetics. These types of peptidomimetics are essentially amino acid mimetics pieced together in a linear fashion to

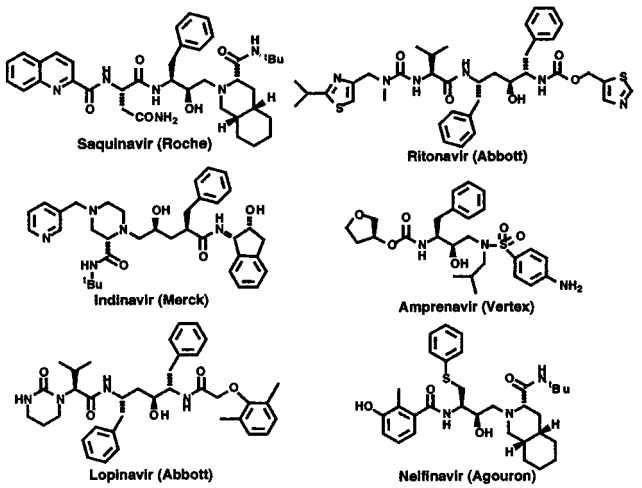


Figure 5. Peptide-derived TSA inhibitors of HIV protease used in AIDS therapies.

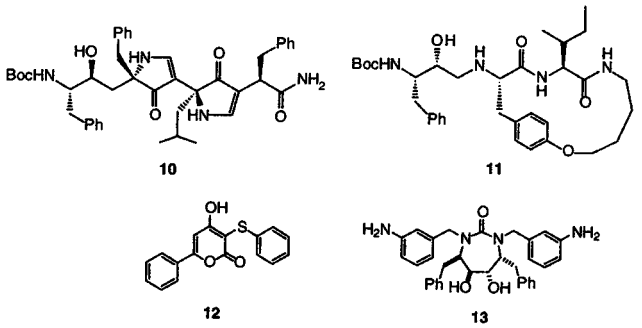


Figure 6. Examples of newly defined peptidomimetics (**10** and **11**: peptide-derived; **12** and **13**: non-peptide).

mimic the normal biologically active peptide substrate. In contrast, Farmer[45] initially proposed the term peptidomimetic to describe potentially novel scaffolds *designed to replace the entire peptide backbone* while retaining isosteric topography of the enzyme-bound peptide (or assumed receptor-bound) conformation. Heterocyclic natural products or screening leads that bind to peptide receptors also have been called peptidomimetics by virtue of their mimicking (or antagonizing) the function of the natural peptide.[46] Although confirmation of mimicry via structural data is rarely available for receptor-bound ligands,[42] ample evidence establishes that some heterocyclic inhibitors do mimic the extended β-strand of enzyme-bound substrate-derived inhibitors (vide infra). In these cases, the term peptidomimetic as defined by Farmer is appropriate even though the inhibitor lead structure was not designed.[47]

For peptidomimetic peptidase inhibitors we recently suggested[48] a definition based on the topography of the inhibited enzyme active site and the chemical composition of the inhibitor. Many peptidase inhibitors, e.g., **10** and **11** (Figure 6), are actually amino acid and transition-state mimics pieced together to emulate the ligand-bound extended β-strand substrate conformation of other peptide-derived inhibitors and consequently retain considerable peptide character.[11,49] For example, the cocrystal structure of **11** bound to HIV protease[50] demonstrated that the enzyme-bound inhibitor successfully mimicked the extended β-strand binding conformations found for a variety of other HIV protease inhibitors (Figure 7). We define these inhibitors as

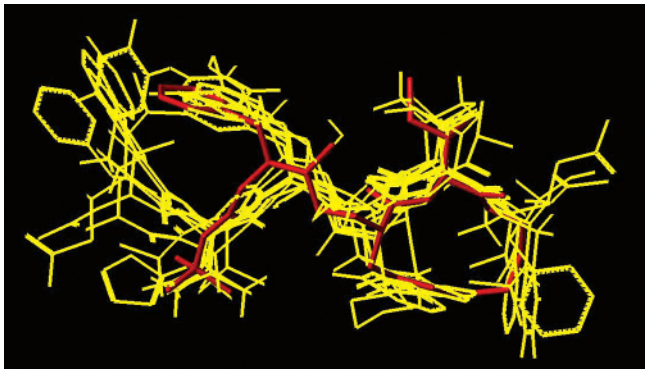


Figure 7. Comparison of HIV-protease bound conformation of acyclic inhibitors[54] with peptide-derived peptidomimetic **11**[55] designed to emulate the β-strand binding ligand conformation.

peptide-derived peptidomimetics to highlight their close structural relationship with the enzyme-bound peptide-substrate conformation. Notably the elegant pyrrolinone mimics of enzyme-bound extended β-structures (e.g., **10**) developed by Hirschmann and Smith[11,12] illustrate a sophisticated version of this type of peptidomimetic. Their mimetic stabilizes both intra- and intermolecular hydrogen bonds, and the ability to switch between these two conformers is thought to provide improved bioavailability.[51]

Structurally distinct heterocyclic aspartic peptidase inhibitors, e.g., **12** and **13**, have been discovered either via high-throughput screening (HTS) or rational design methods and subsequently developed into useful HIV protease inhibitors.[52,53] We define these inhibitors as *non-peptide peptidomimetics* to account for their remote structural relationship to native peptide substrates.

Most importantly, comparison of these two distinct peptidomimetic classes reveals that the active site topography accessed by the non-peptide peptidomimetics is nearly identical to that accessed by the peptide-derived peptidomimetics. In effect, these structurally distinct compounds selectively stabilize the *same enzyme conformation* within the complete ensemble of enzyme conformations. This is not completely surprising since the structural evolution of the non-peptide inhibitors was guided by consideration of the bound-conformations of known peptide-derived inhibitors.

Ideally, a major goal of the medicinal chemist is to discover novel structures with pharmacodynamic properties that enable both oral and CNS bioavailability and suitable duration of action. This necessitates developing new compounds that circumvent the multiple export and metabolism mechanisms that exist to control levels of active peptides in vivo. We propose that designing structures to target the ensemble of conformations not accessed by previously designed inhibitors may be a way to achieve this goal. Since some recent non-peptide peptidomimetics have been demonstrated to stabilize previously unobserved enzyme active site conformations,[48,56] we now review different types of conformational changes detected in and around the active site of aspartic peptidases.

Conformational Changes Revealed via Peptide-Derived Inhibitors

The first indication that aspartic peptidase active sites are conformationally flexible arose from studies of the model enzyme penicillopepsin in the late 1970s. James and co-workers solved the structure of a known irreversible inhibitor of the aspartic peptidases, 1,2-epoxy-3-(*p*-nitrophenoxy)propane (EPNP), covalently attached to penicillopepsin in 1977.[57] This structure demonstrated that a molecule of EPNP was covalently bound to both catalytically active aspartic acid residues (Asp32 and Asp215) and that the bound water molecule was displaced from the enzyme active site. More interesting from our perspective was the major conformational change observed for the Tyr75 side chain. The rotation of the Tyr75 phenolic ring led James to hypothesize participation of Tyr75 as a proton donor in the enzyme-catalyzed amide bond hydrolysis,[57] in analogy to the role of Tyr48 in carboxypeptidase A. When later enzyme−inhibitor crystal structures demonstrated the location of Tyr75 in its unrotated native position, James withdrew his proposal[58] and the rotation of Tyr75 in penicillopepsin has not been further examined. Yet this was the first detailed demonstration of conformational flexibility in the aspartic peptidases. The potential of Tyr75 to rotate into alternate locations has important implications for the development of novel inhibitors as we shall see later.

In 1982 an important conformational transition was discovered from the X-ray structure of a statine-based peptide inhibitor bound to penicillopepsin. Although a variety of pepstatin-based peptide inhibitors had been synthesized in the Rich group, only one peptide, Iva-Val-Val-StaOEt,[59] cocrystallized with penicillopepsin in a form suitable for X-ray structural determination. James and co-workers solved the structure of the complex[58] and found that the critical 3S-hydroxyl group was hydrogen bonded to the catalytic aspartic acid groups and displaced an enzyme-bound water molecule. This binding mode was similar to the pepstatin-pepsin cocrystal structure,[60] but an important structural change was observed for the first time. In the native penicillopepsin structure,[61] the "flap region" (comprising residues Trp71-Gly83) was found in an "open" conformation that did not obstruct the enzyme active site cleft. However, the crystal structure of the enzyme−inhibitor complex showed significant conformational changes in the flap that enabled this segment to close upon the inhibitor (Figure 8) bound within the active site. The binding resulted in a movement of the tip of the β-hairpin turn structure by approximately 4 Å toward the catalytic groups, thereby trapping the inhibitor. Related inhibitors synthesized in the Rich group incorporating a LySta analogue into pepstatin-derived inhibitors also showed a similar flap stabilization but demonstrated a high degree of selectivity based on the Lys electrostatic interactions with the enzyme flap.[62] A similar flap movement[63] has also since been detected in the binding of pepstatin to cathepsin D, a related mammalian aspartic peptidase. The discovery that the flap subunit can move 4 Å has important consequences for the development of novel inhibitors.

Crystal structures of chymosin, a bovine aspartic peptidase closely related to other mammalian aspartic peptidases, revealed additional unexpected conformational changes. In the native enzyme, the flap was rotated into an "open" conformation[64,65] similar to native penicillopepsin, but more surprising was the observed

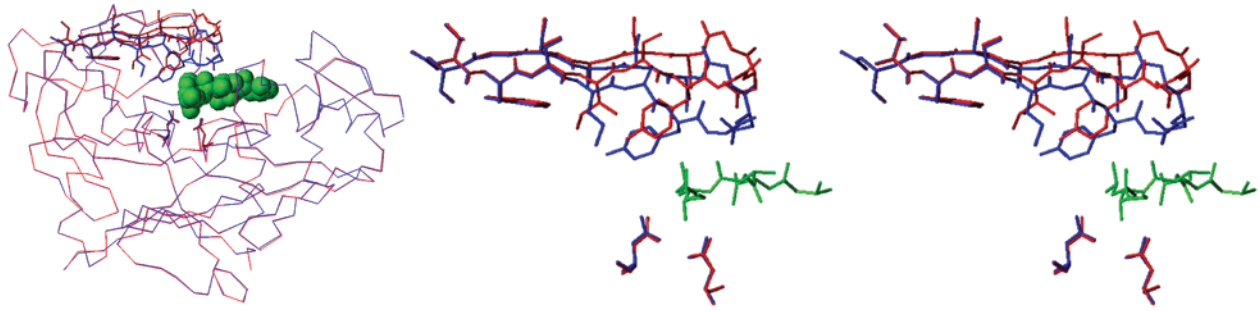


Figure 8. Comparison of penicillopepsin conformations.[67] Overlap of both native and inhibitor-bound enzyme conformations with a cross-stereo of native and Iva-Val-Val-StaOEt-bound enzyme conformations (native conformation, red; inhibitor-bound conformation, blue; inhibitor, green).

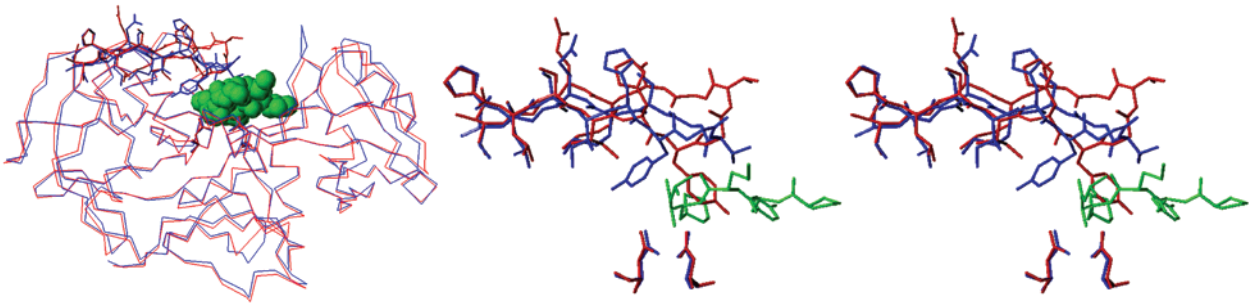


Figure 9. Comparison of chymosin conformations.[68] Overlap of both native and inhibitor-bound enzyme conformations with cross-stereo of native and CP-113971-bound enzyme conformations (native conformation, red; inhibitor-bound conformation, blue; inhibitor, green). Note: Native flap residue Tyr75 would obstruct inhibitor binding.

180° rotation of Tyr75 into the region normally occupied by an inhibitor/substrate P_1 substituent. For several years, attempts to obtain chymosin−inhibitor complexes had failed; only native enzyme crystals were obtained. This could be attributed to the rotation of Tyr75 into a position that blocked substrate-like molecules from binding.

Eventually, Groves and co-workers obtained crystals of the known renin inhibitor CP-113971 bound to chymosin and solved the crystal structure[66] and found a remarkable conformational mobility of Tyr75. Comparison of native and enzyme−inhibitor crystal structures (Figure 9) revealed the aspartic peptidase flap had "closed" over the inhibitor in the active site by moving approximately 4 Å toward the catalytically active groups compared with the native enzyme structure. However, the lack of electron density for Tyr75 established that this important residue was conformationally mobile, even in the enzyme−inhibitor complex. Therefore, the enzyme−inhibitor structure not only demonstrated a new location for the flap and Tyr75 but also established the important conformational mobility of these structural features.

Additional aspartic peptidase−inhibitor structures have revealed other conformational changes in or near the active site with important implications for inhibitor design. Endothiapepsin, a fungal aspartic peptidase, has been utilized as a model aspartic peptidase system with more than 20 X-ray structures[69] solved for both native and endothiapepsin−inhibitor complexes. The crystallographic evidence suggests endothiapepsin exists in a delicate equilibrium between two observable forms[70,71] that have considerable differences in active site topography. The two forms are believed to interconvert as a result of environmental conditions, with a rigid body movement largely affecting the S_3 binding pocket. The two different conformations are selectively stabilized by inhibitor binding in the S_3 subsite. This structural flexibility, which has now been identified in other aspartic peptidases,[72] has been proposed[71] to be potentially important for the function of aspartic peptidases.

Yet another type of conformational change near the active site of an aspartic peptidase has been observed with the binding of the iodophenylalanine-containing inhibitor A66702 to pepsin. The bulky iodine atom protrudes into the enzyme a3 helix to stabilize a conformation in which this helix was displaced about 1 Å from the position in the native structure. As a result of these movements, the active site volume increased significantly.[73]

Rate of Movement of "Flap" Regions

The hairpin turn structure, or "flap" region, in aspartic peptidases covers the enzyme active site. During catalysis the flap must first open to allow substrate entry to the active site, the flap must then close for catalysis to occur, and finally the flap must re-open to allow diffusion of products from the active site. Clearly, flap mobility is critical for aspartic peptidase function. This process must be as fast or faster than k_{cat}, the rate constant describing all events after formation of the ES complex.[74]

The opening and closing of the flap region necessitates a reconfiguration of active site functional groups as interactions between specific interdomain residues are disrupted. Consequently, H-bonds seen between residues, including Tyr75, in the X-ray complexes of enzymes with and without peptide-derived tight-binding

inhibitors must break during catalysis. It follows that breaking the observed H-bonds is not significantly costly, as the opening and closing of the active site is both a fast and low-energy process,[75] and the conformational mobility of these residues increases with the opening of the flap and breaking of conserved H-bonds.

Development of Peptide-Derived Therapeutic Aspartic Peptidase Inhibitors

Renin. The aspartic peptidase renin plays a pivotal role in the biosynthesis of the potent vasoconstrictor angiotensin II, and inhibitors of this enzyme have been sought for 40 years as potential antihypertensive drugs.[41] Historically, renin inhibitors were developed by replacing the dipeptidyl cleavage site of substrate sequences with an appropriate TSA before any structural data were available to guide structure-based design; only more recently have a few X-ray structures of renin−inhibitor complexes been solved. While many substrate-derived renin inhibitors showed promise in vitro, none were successfully developed into antihypertensive drugs due to the poor pharmacokinetic properties associated with these peptide-derived inhibitors. However, the principles established in the renin studies facilitated the discovery of HIV protease inhibitors. Most of the known TSAs (Table 1) were first developed for inhibition of renin.

HIV Protease. The most intensely studied aspartic peptidase during the last 20 years has been HIV protease, the aspartic peptidase needed for viral replication implicated in AIDS.[76,77] A variety of peptide-derived peptidomimetic inhibitors of HIV protease have been successfully developed. Numerous X-ray structures of inhibitors bound to HIV protease have been obtained, and the structural information has played a central role in the successful development of AIDS drugs. Many previous studies as reviewed in detail by Babine and Bender[78] have examined the interactions of peptide-based inhibitors with HIV protease. Herein we highlight the enzyme−inhibitor interactions associated with enzyme conformational mobility.

Historically, the first crystal structures of inhibitors bound to HIV protease were the reduced amide analogue MVT-101[79] and the hydroxyethylamine analogue, JG-365.[80] MVT-101 lacked the critical hydroxyl group of a TSA inhibitor but clearly illuminated the S_3-S_3' binding sites accessed by the peptidyl inhibitor in the expected extended β-strand binding conformation. The crystal structure of JG-365, a 200-fold more potent inhibitor, bound to HIV protease provided a high-resolution view of the critical TSA hydroxyl group interaction with the protease catalytic machinery.

The information gained from both cocrystal structures was immediately utilized in structure-based design processes leading to the currently available AIDS drugs. Both JG-365 and Ro 31-8959[81] (Roche drug marketed as Saquinavir) were developed as HEA analogues of the Phe-Pro substrate cleavage site (Figure 10A). Surprisingly, the hydroxyl group stereochemistry of these potent HIV protease inhibitors was reversed; JG-365 contained an (*S*) hydroxyl group while Ro 31-8959 contained an (*R*) hydroxyl group. Molecular modeling led to the hypothesis that the two diastereomeric peptides adopted different binding modes at the C-terminus of the binding site.[82] This model was confirmed by the X-ray crystal structures of the diastereomeric inhibitors bound to HIV protease.[83] Interestingly a related stereochemical paradox had been discovered earlier in the Rich group during the synthesis of pepstatin-based pepsin inhibitors.[84] The addition of a methyl group to C3 of statine-based inhibitors (**14**) generated a series of Me3Sta analogues in which the preferred hydroxyl group stereochemistry was reversed (**15**, **16**), with the more potent inhibitors containing the (*R*)-Me3Sta unit (Figure 10B).

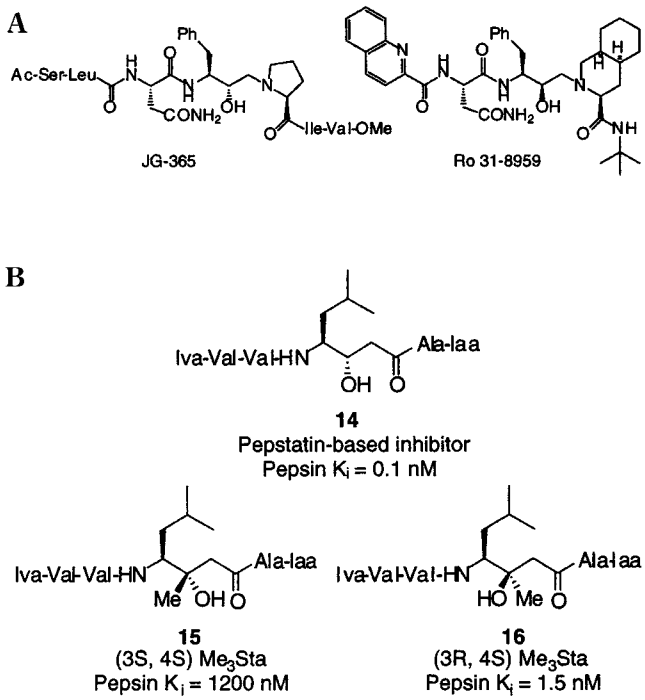


Figure 10. (A) JG-365 and Ro 31-8959. (B) Structural comparison of Me3Sta inhibitors.

Changes in flap position and geometry have also been demonstrated for HIV protease. The X-ray structure of Ro 31-8959 bound to HIV protease revealed a new enzyme conformation with an altered flap conformation. This stabilization produced a larger S_1 binding site relative to JG-365 that was complementary to the DIQ moiety.[78] Similar conformational stabilizations were observed with other of HIV protease inhibitors, including Nelfinavir, bound to HIV protease by researchers at Agouron.

Non-Peptide Peptidomimetic Aspartic Peptidase Inhibitors

Farmer's proposal[45] that non-peptide peptidomimetics might be identified to maintain the topography and function of biologically active peptides stimulated much research to identify and/or design such compounds. However, only a few non-peptide peptidomimetic aspartic peptidase inhibitors have been reported, and most were obtained by applying structure-based design methods to compounds identified via HTS methods.

The seven-membered cyclic urea HIV protease inhibitors (Figure 11) were designed[85] to stabilize the normal extended β-strand binding enzyme conformation but also to displace a second water molecule stabilizing the enzyme flaps. A potential pharmacophore was designed,[86] and then virtual screening of the HIV active

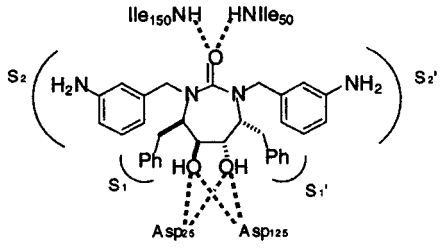


Figure 11. Rationally designed non-peptide peptidomimetic HIV protease inhibitor.

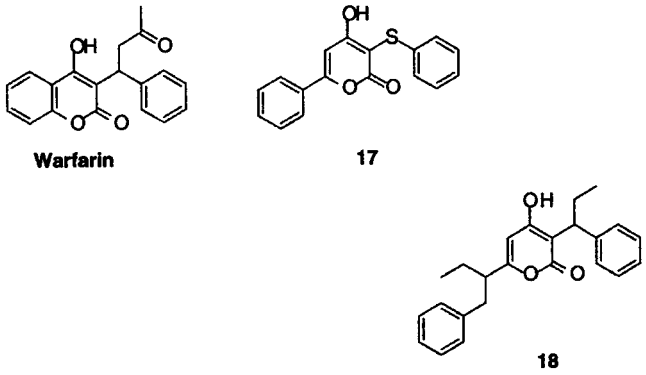


Figure 12. HTS non-peptide peptidomimetic HIV protease leads.

site in the extended β-strand binding conformation with water displaced led to a non-peptide lead structure which was further modified. Inspection of the resulting enzyme−inhibitor crystal structure clearly demonstrates the optimized inhibitor is a non-peptide peptidomimetic that stabilizes the normal extended β-strand binding enzyme conformation. Interestingly, NMR studies on the inhibitor−enzyme complex revealed a dynamic conformational mobility not evident in the crystallographic studies.[87]

Discovery efforts at Parke-Davis[88] and Pharmacia[89] simultaneously and independently developed the related pyrone-based HIV protease inhibitors (Figure 12, **17** and **18**). Both groups applied structure-based design methods to the anticoagulant Warfarin, identified as an HIV protease inhibitor via HTS. Conversion of the lead compounds into clinically useful drugs was achievable only after high-resolution enzyme−inhibitor crystal structures were obtained. Subsequent optimization eventually afforded the clinically useful HIV protease inhibitors **19** and **20**. Surprisingly, enzyme−inhibitor crystal structures (Figure 13) demonstrated that the optimized inhibitors bind in opposite directions, but both stabilize the standard peptide-derived β-strand binding enzyme topography.

Stabilization of Fundamentally Different Enzyme Active Site Conformations

Recently, researchers at Roche discovered a series of novel non-peptide inhibitors of renin that bind to a new enzyme active site conformation.[56,90−92] The 3,4-disubstituted piperidines, **21** and **22** (Figure 14), inhibit human renin at low micromolar and nanomolar concentrations, respectively. Most importantly, the inhibitors stabilize an enzyme active site *other* than the previously accessed β-strand binding enzyme conformation. It is instructive to compare the structures of **21** and **22** with structures of previous peptide-derived

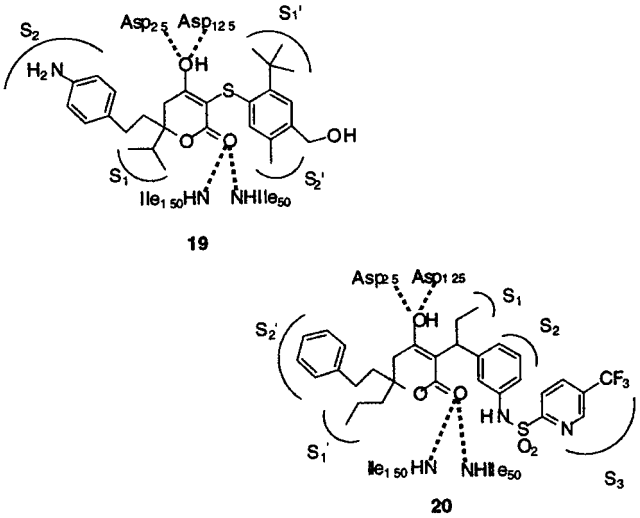


Figure 13. Binding of **19** and **20** in HIV active site.

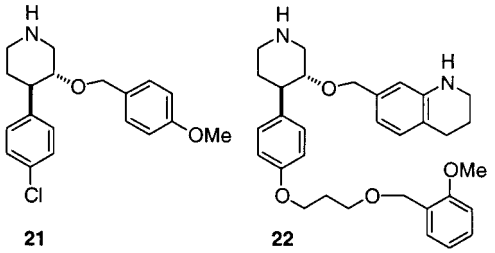


Figure 14. Roche piperidine-based aspartic peptidase inhibitors.

inhibitors, as this can help guide future design of non-peptide peptidomimetics from peptides.

Portions of both lead structure **21** and optimized analogue **22** bind in the active site of human renin in a mechanism-based fashion. The binding of the piperidine nitrogen to the enzyme catalytic carboxyl groups is similar to the binding of the statine hydroxyl[30] and aminostatine nitrogen[93,94] in peptide-derived inhibitors. In addition, the 3-alkoxy group resides in the contiguous S_1-S_3 enzyme subsite near the normal binding location of the side chains in peptide-derived inhibitors. *Piperidines **21** and **22** clearly are non-peptide peptidomimetic inhibitors that stabilize an enzyme conformation not previously observed for this enzyme.*

In these complexes, the aspartic peptidase flap structure is stabilized in an open conformation and the Tyr75 side chain is rotated by 120° from its position in the native enzyme, similar to the conformations noted herein with other aspartic peptidases. The stabilization of the open flap with concomitant Tyr75 rotation disrupts a conserved hydrogen bond between Tyr75 and Trp39 believed to be important for keeping the flap closed during catalysis. The breaking of this conserved hydrogen bond allowed the Trp39 side chain to rotate from its usually observed position within one hydrophobic environment into another local hydrophobic environment. This Trp39 side chain rotation opened access to another previously unobserved hydrophobic pocket into which the piperidine 4-phenyl 4′-substituent bound. These results clearly demonstrate how important conformational mobility is for binding of these piperidines to aspartic peptidases (Figure 15).

Comparison of the active site topography of the enzymes stabilized by peptide-derived and piperidine-

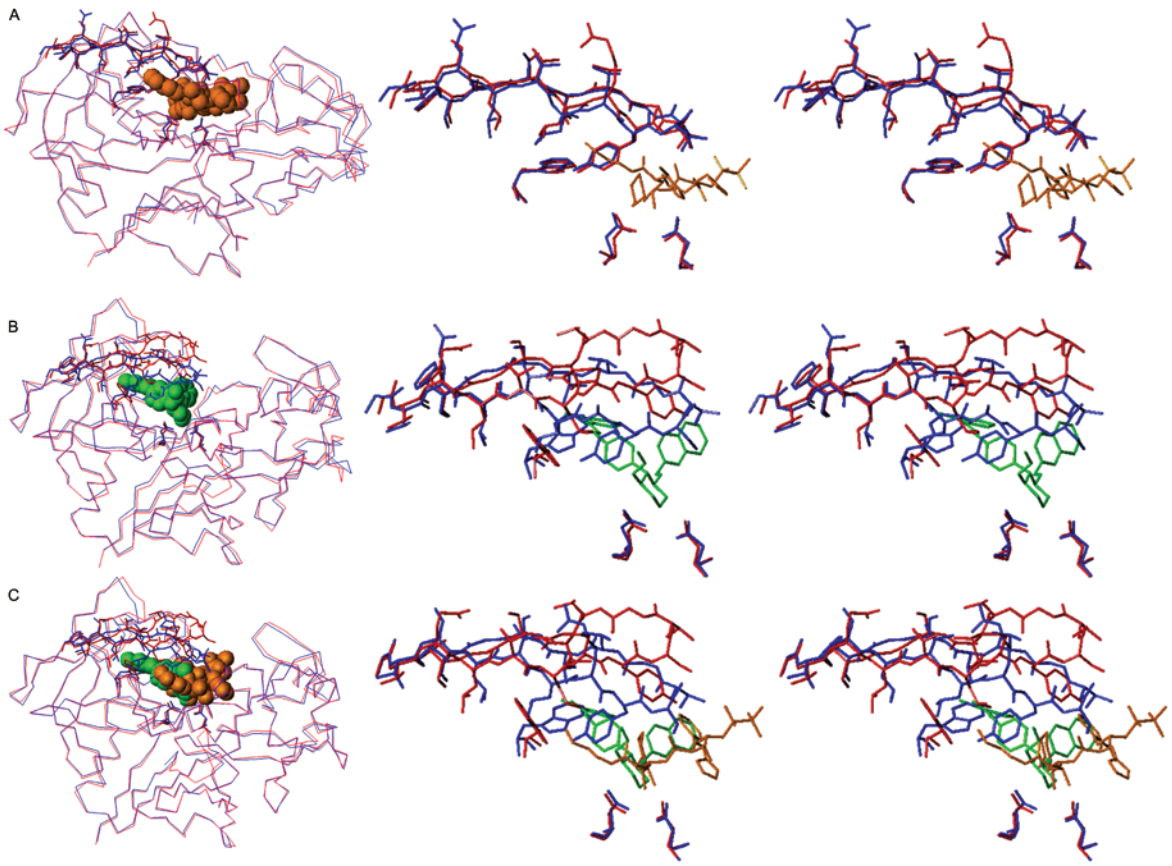


Figure 15. Comparison of observed renin conformations.[95] (A) Overlap of native and peptide-derived inhibitor-bound enzyme conformation (native conformation, blue; inhibitor-bound conformation, red; inhibitor, orange) with cross-stereoview. (B) Overlap of native and piperidine-bound enzyme conformation (native conformation, blue; piperidine-bound conformation, red; inhibitor, green) with cross-stereoview. (C) Overlap of peptide-derived inhibitor-bound enzyme conformation and piperidine-bound enzyme conformation (peptide-bound conformation, blue; piperidine-bound conformation, red; peptide inhibitor, orange; piperidine, green). Note: Hydrogen bond between Trp39 and Tyr75 in A; broken in B; distinction shown in C.

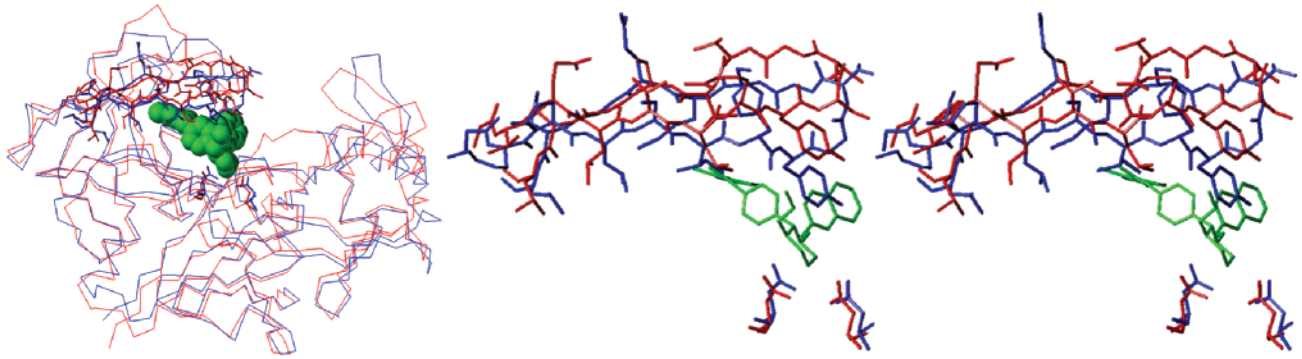


Figure 16. Comparison of observed renin and chymosin conformations.[96] Overlap of piperidine-bound renin conformation and native chymosin conformation (piperidine-bound conformation, red; native chymosin conformation, blue; inhibitor, green) with cross-stereoview. Note: Similar rotation of Tyr75 yet different locations due to slightly unique flap movements.

derived inhibitors shows a fundamental difference in topographies. The piperidines bind to an enzyme active site that is significantly different from the β-strand binding enzyme conformation (Figure 15C). Interestingly, this conformation is remarkably similar (Figure 16) to the observed native chymosin conformation reported as a self-inhibited conformation. Rotation of Tyr75 destroys much of the binding surface comprising the S_1 subsite. Consequently, the peptide-derived inhibitors would not adequately stabilize this conformation within the ensemble. In contrast, the piperidine-derived inhibitors are effective stabilizers of this enzyme form because the piperidine 4-phenyl substituent binds into the space previously occupied by the Tyr75 aromatic ring to regenerate an aromatic cluster. The binding of the 4-phenylpiperidines to the enzyme extended β-strand binding-site is precluded by the superposition of the 4-phenyl group with the Tyr75 aromatic group. Thus, *Tyr75 is acting as a "gate keeper" that determines which type of inhibitor can bind.* In the observed native enzyme conformation and enzyme conformation stabilized by peptide-derived inhibitors, the piperidines cannot bind;

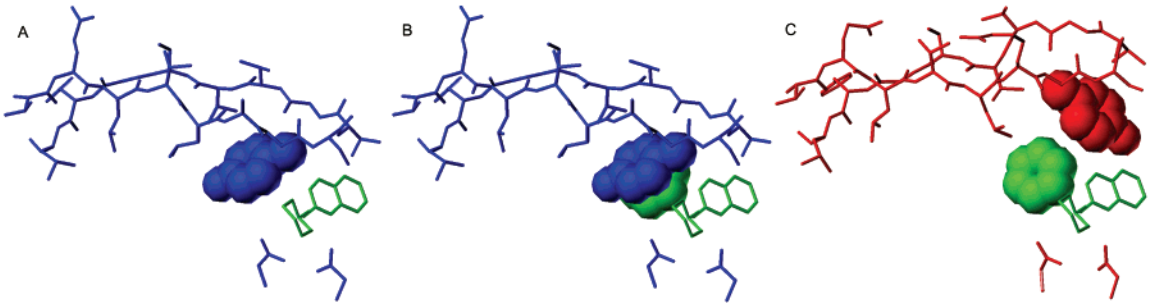


Figure 17. GRAB peptidomimetics in action. (A) Gatekeeper Tyr75 (spacefill model) prevents the piperidine 4-phenyl substituent from binding to native renin conformation. (B) GRAB overlay of piperidine 4-phenyl and gatekeeper Tyr75 of Renin native flap conformation. (C) Gatekeeper Tyr75 binding site replacement by 4-phenyl piperidine (renin native flap conformation, blue; piperidine-binding flap conformation, red; piperidine-based inhibitor, green).

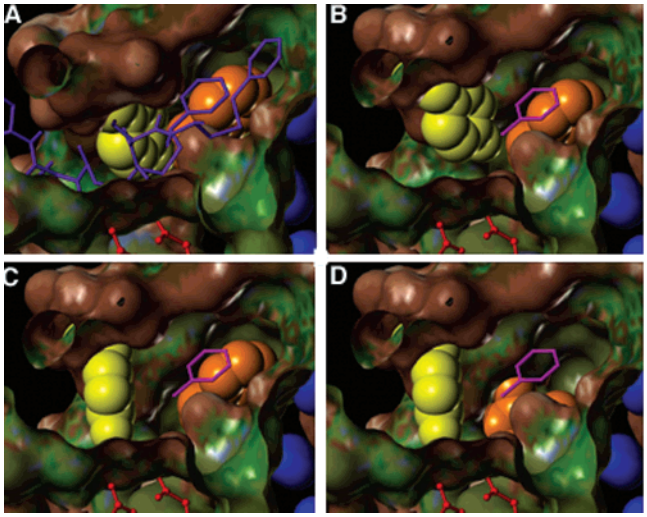


Figure 18. Evolution of mechanistically altered porcine pepsin active site for GrowMol-based inhibitor design (flap, light brown; peptide−inhibitor, purple; Asp32 and Asp215, red; Tyr75, yellow; Trp39, orange). (A) Inhibitor bound in active site. (B) Excise inhibitor and raise flap 1 Å. (C) Rotation of Tyr75 χ^1 by −120°. (D) Rotation of Trp39 from local hydrophobic to another hydrophobic environment.

in the alternate piperidine-binding mode, substrate-derived inhibitors cannot bind.

Group Replacement Assisted Binding Peptidomimetics (GRAB Peptidomimetics)

The piperidine inhibitors stabilize an enzyme conformation not seen in the ensembles stabilized by all inhibitors designed to emulate the β-strand binding mode. Consequently, the piperidine inhibitors constitute a new class of non-peptide peptidomimetics. The differences can be seen by careful comparison of the two active sites (Figure 15) in which different renin−inhibitor complexes are superimposed and their corresponding flap regions highlighted. The enzyme conformation stabilized by a peptide-derived inhibitor is compared to the observed native enzyme (A); the enzyme conformation stabilized by piperidine-derived inhibitor is compared to the observed native enzyme (B); and the enzyme flap conformations stabilized by the piperidines and the peptide-derived inhibitors are compared (C). What is striking is the close proximity of the piperidine C4 phenyl group to the space vacated by the rotation of Tyr75. In effect, the piperidine phenyl group has replaced the Tyr75 aromatic ring. *We define this stabilization process as group replacement.*

Side chain group replacement has not been used to design peptidase inhibitors but has been used to design peptidase substrates. In 1987, Wells and co-workers[97] invented substrate-assisted catalysis using serine peptidases to illustrate the concept. Site-directed mutagenesis to remove the active site histidine from subtilisn rendered the mutant enzyme catalytically inactive. But catalytic activity was regained when an imidazole group was placed in a synthetic substrate at a point designed to replace the missing enzyme imidazole group. The mutated enzyme could not cleave normal substrate sequences but did cleave the designed histidine-containing substrate. The substrate imidazole group replaced the enzyme imidazole group when the substrate was bound to the mutant subtilisn.

As we have highlighted, the piperidine inhibitor 4-phenyl (Figure 17) occupies the space vacated by rotation of Tyr75. More precisely, the piperidine phenyl group replaces the vacated residue to assist the piperidine stabilization of the enzyme complex. We define this new class of non-peptide peptidomimetic inhibitors as "group replacement assisted binding" peptidomimetics (*GRAB peptidomimetics*).

Computer Generation of GRAB Peptidomimetics

The Holy Grail in peptidomimetic research is to devise methods to rationally transform peptide-derived information into non-peptide inhibitors. Strategies for converting peptide-based inhibitors into non-peptides would revolutionize the drug discovery process because a major stumbling block in drug design is obtaining compounds with appropriate bioavailability. For several years, we have attempted to utilize structure-generating programs to design novel non-peptide peptidomimetics. We had utilized successfully the structure-generating program GrowMol[98,99] to design a variety of aspartic peptidase inhibitors with novel scaffolds. GrowMol was used to "rediscover" known tight-binding peptide-derived inhibitors related to pepstatin[100,101] as well as to generate other novel peptide-derived and non-peptide-derived inhibitors.[102−104] During these earlier studies, the inhibitors were designed to complement the β-strand binding active site topography of the aspartic peptidases.

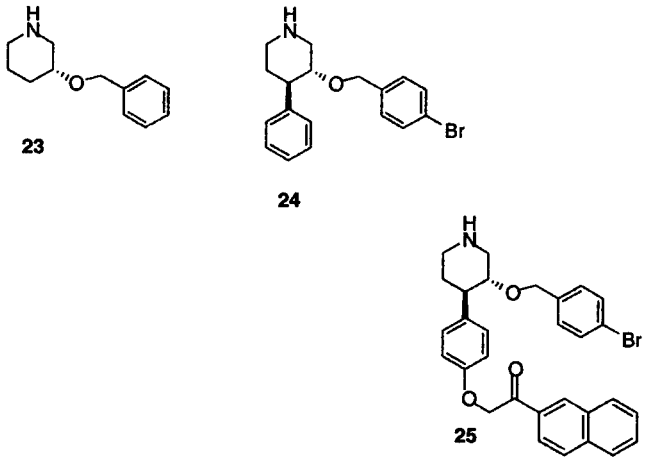


Figure 19. GrowMol generated piperidines.

The Roche inhibitors presented a unique challenge because the binding modes of the piperidines are fundamentally different than the peptide-derived peptidomimetics. Would it be possible to find the piperidine class of inhibitors by use of GrowMol when starting with a peptide-derived peptidomimetic? Successful demonstration of this strategy would be extremely important, as it would provide a means to develop fundamentally novel inhibitors based on the information obtained with the peptide-derived inhibitors. When we used GrowMol to generate potential inhibitors in the renin active site, we eventually succeeded in regenerating 3,4-disubstituted piperidine inhibitors, but *the process was extremely difficult*. After extensive experimentation, we found it necessary to move the flap and rotate the Tyr75 side chain in accordance with changes described for other aspartic peptidases reviewed in previous sections.[105]

We then turned to two aspartic peptidases for which no piperidine inhibitors had been reported in order to determine if this same stabilization process would work for other aspartic peptidases. The detailed strategy is described for pepsin but it is essentially the same one we used for all three enzymes.

Beginning with the X-ray structure of a known statine-based peptide inhibitor bound to pepsin (Figure 18A), we attempted to grow the piperidine unit from the P_1 S-benzyl side chain. However, growth from this point on CySta toward the catalytic carboxyls only generated straight chain amines. On the basis of the literature describing flap movements, we raised the flap about 1 Å (Figure 18B), and subsequently generated a series of piperidines (**23**) which lacked the C4 substituent. Molecular modeling revealed that a −120° rotation of χ^1 in Tyr75, similar to the rotation seen in chymosin and renin, would provide the space needed for the piperidine C4-phenyl (Figure 18C). After implementing these active site conformational changes, GrowMol created the 3,4-disubstituted piperidine **24**, a direct analogue of the Roche HTS lead **21**. Further rotation of Trp39 in a fashion similar to that observed with the renin−piperidine structures (Figure 18D), followed by structure generation, gave the acetonaphthone analogue **25** (Figure 19). This structure is closely related to the optimized Roche inhibitors. After synthesis and testing, piperidines **24** and **25** inhibited *R. chinensis* and porcine pepsin with IC_{50} values of 2 and 0.2 μM, respectively. The modeling and inhibition data suggest that GRAB peptidomimetic stabilization may be a general process for these aspartic peptidases.

The "rediscovery" of the 3,4-disubstituted piperidines as renin inhibitors and the extension of the strategy used to find them to other aspartic peptidases demonstrates that the structural information provided from the peptide-based inhibitors can be used to design non-peptide peptidomimetics. However, successful generation of the GRAB peptidomimetics required exploration of an enzyme active site topography not apparent in the crystal structures of either native enzyme or enzyme−peptide-derived inhibitor complexes. While this new active site conformer could be found by altering the active site in mechanistically rational ways, *we would not have found these inhibitors using the current methods available without a priori knowledge of their existence.*[105] The discovery of the Roche inhibitors and their correlation with peptide-derived inhibitors is a peptidomimetic "Rosetta stone." Understanding how two distinctly different inhibitor structures can fit into two enzyme active site topographies differing in substantial but mechanistically related ways made it possible to extend the process to two other aspartic peptidases, pepsin and *R. chinensis* pepsin. It must be emphasized that these GRAB peptidomimetics stabilize an enzyme conformation that is different from the extended β-strand binding conformation that binds all previous peptide-derived and non-peptide inhibitors. Therefore, we propose that methods for *rational* inhibitor design need to be revolutionized to include enzyme conformations outside the normally observed conformations; medicinal chemists should attempt to target the entire conformational ensemble.

As receptor conformational mobility is likely to occur in all biological targets, the design of GRAB peptidomimetics should be quite general. At any interface between enzyme and substrate or at a protein−protein interface, it should be possible to determine if host protein residue side chains can rotate into new positions to generate structural voids into which potential inhibitors could fill. Inhibitors can be designed and synthesized to complement each new binding site, and in favorable cases potent inhibitors will be formed. It should be noted however, that the optimized renin inhibitors bind to an enzyme active site conformation formed by synchronous movements of three side chains. The C4-phenyl group binds to the enzyme to replace Tyr75 which has rotated to another position. Interestingly, Leu73 rotates to fill some of the Tyr75 pocket, and this in turn allows Trp39 to occupy a new site formed in part by the vacated Leu73. This cascade of conformational transitions in the renin example allows the optimized inhibitor to stabilize an enzyme conformation with multiple alterations not observed with binding of the classic substrate-derived peptidomimetics.

Rational Drug Design for the 21st Century: Targeting Conformational Ensembles

Without a screening lead, the rational design of non-peptide peptidomimetics has had limited success to date. The novel, symmetric, urea-based inhibitors of HIV protease (Figure 11) designed from peptide-derived

inhibitors by researchers at Dupont-Merck[85] actually represents a different type of a GRAB peptidomimetic in which a key enzyme-bound water molecule was replaced by the urea carbonyl group. In the case of the 3,4-disubstituted piperidines, GRAB peptidomimetic inhibitors of pepsin and *R. chinensis* were designed to target an enzyme conformation not evident in the enzyme−peptide-derived inhibitor structures.

The GrowMol design strategy enabled the rational design of non-peptide peptidomimetic inhibitors from the crystal structure of an enzyme-bound peptide-derived peptidomimetic. The evolution of our design process now provides the foundation for rational design of novel non-peptide peptidomimetic inhibitors targeting enzyme conformational ensembles based upon the wealth of structural information generated via peptide-like enzyme−inhibitors. We think that design of future inhibitors must target not only the presently observable bound-receptor conformations, but also the complete ensemble of pre-existing receptor conformations. *The successful design of structurally and fundamentally novel inhibitors may necessitate targeting receptor conformations located outside the narrow window of the conformational ensembles presently exploited with peptide-derived inhibitors.*

Automated Identification of Novel Inhibitors Based on Conformational Ensembles

Conformational ensembles already are used to clarify the binding of compounds to conformationally mobile receptors.[106−111] Researchers at Agouron have studied the binding of known peptide-derived inhibitors to HIV protease conformational ensembles utilizing Monte Carlo simulations.[112] Their methods have been extended in a parallel fashion to allow simultaneous and independent Monte Carlo simulated dynamic studies[113] of a known peptide inhibitor binding to HIV protease conformational ensembles.

It seems possible to develop a computerized-automation process to generate fundamentally novel inhibitors targeting receptor conformational ensembles, a process beyond the current docking of known structures to known active site conformers. To do this will require a significant expansion of the number of enzyme conformers in the conformational ensemble beyond the numbers now generated to model the extended β-strand binding site. For example, allowing the mobile flap in an aspartic peptidase to open in 0.1 Å increments could add 40 sets of additional conformers to the ensemble, each containing some number of active site conformers complementary to a new (for example) heterocyclic scaffold. Further, we have found that inhibitor structures were best grown from active sites stabilized by nonoptimal peptide-derived inhibitors; we assume this occurs because the active sites are not "shrink wrapped" about the inhibitors. Whether these two processes will prove general for other enzyme systems remains to be experimentally determined. But with aspartic peptidases the number of potential conformational ensembles will expand beyond the roughly 40-fold initial estimate. Calculating potential scaffolds for each of the potential conformations will be a massive calculation. But if it can be done, it should greatly increase the number of non-peptide structures that could serve as scaffolds for inhibitor design and optimization.

This process is computationally intensive. What are the chances new structures exist that have not already been evaluated by existing approaches? Bohacek and McMartin have calculated[99] the number of possible small molecules with molecular weights below 500 is greater than 10^{62}, which is about 10^{52} times the known organic compounds in this molecular weight range. Thus, to date the potential conformational space for novel ligands has hardly been probed. It is of course impossible to synthesize all these compounds (not enough starting material exists in the universe)[101] but also it will not be necessary. Even today we have perhaps 1000 useful drugs culled from about 10 billion small organic molecules. Clearly we do not need to make all organic structures to find new, better drugs.

We do need to find methods to design improved structures that satisfy the necessary goals of inhibiting the target receptor in vivo by obtaining selective, orally active inhibitors with appropriate lengths of duration. Today, one of the major stumbling blocks in drug discovery remains identifying orally active inhibitors. Interfacing structure-generating programs with traditional medicinal chemistry inhibitor design will help focus the many combinatorial synthetic methods on more promising low molecular weight, non-peptide drug-like structures.

One attractive strategy to identify novel inhibitors targeting conformational ensembles is via computerized ligand-generating programs that generate and evaluate novel ligands within receptor binding sites.[114] Two categories of programs, *placement-connection or fragment-growth strategies*, are known. Both methods utilize high-resolution receptor structures and evaluation programs to rank generated structures for their ability to bind to the target. The placement-connection type of ligand-generation program places molecular fragments from a designated fragment library into the receptor binding site[115,116] complementary to the binding site and then utilizes other programs[117−122] to connect these fragments. The major disadvantages of this method include limitations on the types of novel fragments used as well as complicated issues with fragment linker connections.

A second type of ligand-growth program utilizes the fragment-growth method[123−126] to generate substructures from a ligand already bound to the receptor by connecting designated fragments. This allows a wide variety of interactions to be generated as the program grows novel inhibitors into the active site, but the fragments come from a defined fragment library, which again limits the potential for structural diversity. To overcome this limitation, *ligand-generating programs that grow structures via single-atom growth units have been developed*. These programs are capable of generating novel inhibitors with the greatest amount of molecular interactions and structural diversity—they are also the most computationally intensive and require accurate and fast methods to evaluate generated structures. The single-atom ligand-growth programs include LEGEND,[127] GenStar,[128] and GrowMol.[98] An automated design methodology to identify fundamentally novel potential inhibitors can be based upon single-atom growth programs but would have to be augmented to

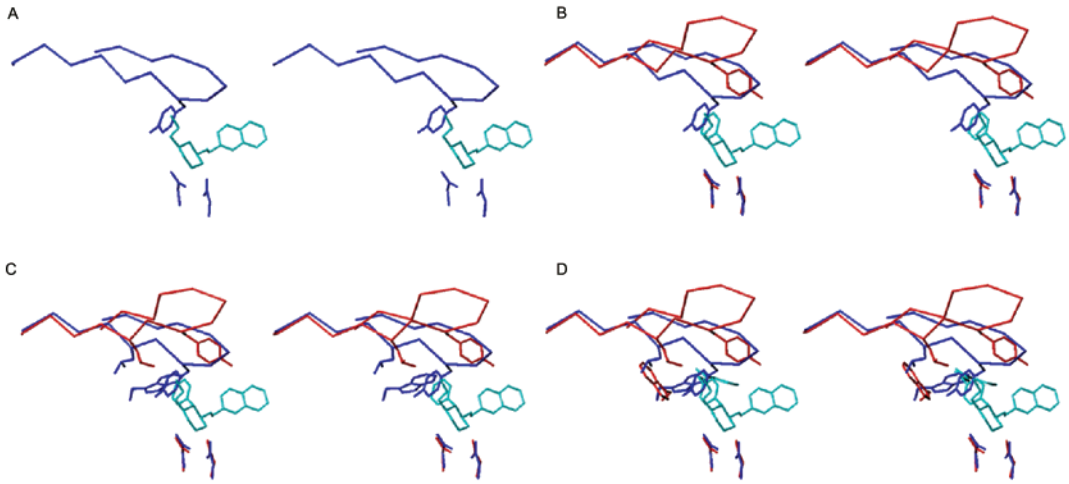


Figure 20. Domino conformational transition generation of piperidines by structure-generating program (native renin conformation, blue; piperidine-bound renin conformation, red; inhibitor, turquoise).

handle the multiple enzyme active site conformations in the ensemble.

A third approach to design peptidomimetics that target conformational ensembles might be to develop new programs for probing active site mobility in a progressive fashion. As described for the renin inhibitors discovered by Roche scientists, the optimized renin inhibitors fill an active site formed by major conformational transitions beyond the standard extended β-strand topography. The peptidase flap opens partially, and Tyr75, Leu73, and Trp39 all rotate away from positions observed in cocrystal structures of peptide-derived inhibitors. Ensemble theory predicts the selective stabilization of this preexisting conformer by inhibitor binding, but it is unclear whether these conformational transitions arise independently of each other or in concerted fashion. Either way, we believe computer programs capable of calculating such "domino conformation transitions" should be possible and would be especially effective for identifying novel active site conformers. These would be used in conjunction with ligand-generating programs to create novel inhibitor structures.

The potential design of inhibitors utilizing "domino conformational transitions" is shown below (Figure 20). Initially, the structure-generating programs would generate the piperidine scaffold docked in the active site (A). The subsequent conformational transition of flap raising and Try75 rotation could be found by such programs and into this receptor conformation the program could generate the 4-phenyl substituent (B). Then, the domino conformational transition occurs in which Leu73 is rotated into space previously occupied by Tyr75 (C), which allows Trp39 to rotate into that vacated space, which enables the 4′-substituent to fill the newly created binding site (D).

A conceptually simple method currently to locate conformationally mobile residues and to identify potential GRAB peptidomimetics utilizes merged group binding (MGB) Searches. The beauty of this method involves a reversal of the normal docking strategy, and simply docks the enzyme around the inhibitor generated in the active site.[129] After first constructing the desired scaffold within the active site, potential growth points are visible but appear "blocked" by enzyme groups. By use of the GRAB peptidomimetic concept, potential movable groups can be identified. Adding functionality on the potential inhibitor so that it overlays with the movable enzyme residue provides a method to search for novel inhibitors with the potential to bind altered enzyme conformations. Enzyme docking and minimization experiments applying Flo99 (other Monte Carlo search programs might provide similar results)[130] to the merged enzyme−inhibitor structure quickly identified new side chain arrangements complementary to the merged inhibitor structure. The implementation of these MGB searches offers a rational and simple method for medicinal chemists to design novel ligands targeting altered conformations within the ensemble.

None of these design approaches can be automated without fast and accurate programs to evaluate (score) the binding affinity of the enzyme for grown structures. Developing fast scoring algorithms to accurately predict the binding affinity across different classes of compounds is an important and extremely difficult research problem, and it is not at all clear when this problem will be solved. Nevertheless, we believe computational advances, both in hardware and software, eventually will facilitate accurate estimations of the binding affinities of different classes of compounds to different enzyme conformations within the ensemble. To provide a better evaluation process, the overall rankings must be searchable in a user-friendly manner via user-defined features. For example, a search for cyclic nitrogen-containing ligands should be achievable by simple typing in "piperidines" or "pyrrolinones." The inclusion of an easily searchable ranking set would provide the user quick access to determine types of novel scaffolds generated within the active site.

The strategies we have outlined for designing novel scaffolds via structure-generating programs capable of probing conformational ensembles are not currently available. We believe incorporating these strategies into a single ligand-generating program could provide the means to design inhibitors based upon conformational ensembles by an automated strategy. This process

should greatly accelerate the discovery of novel inhibitors with potentially enormous impacts on the future of medicinal chemistry efforts.

Future Prospects

We have shown that the Roche piperidine renin inhibitors are a "peptidomimetic Rosetta stone" that establish a logical connection between peptide-derived and non-peptide-derived inhibitors of aspartic peptidases. We deduced the structural pathway connecting these two classes of peptidomimetics by analyzing plausible conformational changes that must occur during proteolysis. Tyr75 was identified as the "gatekeeper" residue, and the mobility of Leu73 and Trp39 also was utilized to generate new binding sites not visible in the crystal structures of either the native or inhibitor-bound enzymes. "Gatekeeper" residues in other enzyme systems probably will be identified by a similar analysis, by utilizing MGB searches, or by considering side chains abutting solvated areas, such as bulk solvent or aqueous pockets within the folded protein. Potentially mobile residues can also be detected by global searching of enzyme conformational ensembles or more efficiently by focusing on residues with high thermal factors in the crystal structures.

Non-peptide peptidomimetics breaking from the extended β-strand conformational mold provide the potential to design smaller, more conformationally constrained molecules. Lam and co-workers in their landmark paper describing the design of non-peptide inhibitors of HIV protease have enumerated the advantages of these types of inhibitors. The penalties of conformational entropy is "prepaid" during synthesis of novel cyclic scaffolds designed to stabilize the receptor active site as opposed to during actual binding.[131] The group replacement concept (in their case, replacement of a water molecule) is also favorable from a thermodynamic perspective. Finally, hydrophobic interactions can be optimized between ligand and receptor with preferred conformation and stereochemistry built into the scaffold.

Recent reports in the literature have described the binding of ligands to dynamic receptor conformational ensembles. This process, which has become the new paradigm for receptor binding, causes an equilibrium shift of the pre-existing receptor conformations. This concept also provides the basis for a new paradigm for the design of medicinally useful ligands. Using the aspartic peptidases, we have described the conformational mobility observable in enzyme−inhibitor X-ray structures and shown how these examples provide the basis for designing ligands with other protein−ligand systems. For the design of fundamentally novel inhibitors based upon conformations presently unobservable, we have outlined the use of a computer-automation strategy that utilizes single-atom structure-generating and ligand evaluation programs and other computer methods capable of probing "domino conformational transitions." While current technologies are not capable of performing some of these strategies, we believe automated design of fundamentally novel ligands based upon conformational ensembles is on the horizon.

The implementation of the design strategy presented here may become a new paradigm for the design of medicinally useful organic structures in the 21st century. While we have focused on aspartic peptidases, literature precedent suggests this strategy will be useful for designing inhibitors of other biological systems. For example, Babine and Bender[78] describe the development of selective MMP inhibitors based on selective binding to different conformations in MMP active sites. Here, portions of an enzyme active site also appear to function in a "gatekeeper" role and have been exploited to develop selective inhibitors by altering the size of the ligand's P_1' substituent.

The extension of these ideas to other receptor systems seems likely. Recently, Volkman and co-workers have demonstrated the two-state behavior of nitrogen regulatory protein C (NtrC), a single-domain signaling protein, believed to be activated via a phosphorylation-triggered conformational change.[132] This study clearly demonstrates NtrC exists in equilibrium between functionally important conformations with phosphorylation or active mutations shifting this equilibrium to activate the protein. These researchers summarize their results by proposing that "stabilization of pre-existing conformations may be a fundamental paradigm for ligand binding."

Our new design strategy may also help to identify small molecules that regulate protein−protein interactions. Wells and co-workers[133] have shown that human growth hormone−receptor interactions are dominated by a relatively small number of critical residues—designated as "hot spots" in the receptor. Small conformational changes in "hot spot" residues in some receptor systems and subsequent application of the "group replacement" strategy might lead to useful GRAB peptidomimetics functioning at that receptor. Indeed designing inhibitors of protein−protein interactions has been a long and often unfruitful process, but our new strategy might provide new avenues of future research in this area.

It is even possible these strategies may find use for developing inhibitors of G-protein coupled receptors. Inooka and co-workers determined[134] the conformational states of the peptide hormone pituitary adenylate cyclase activating polypeptide (PACAP) binding to the G-coupled protein PACAP-specific hormone receptor via high-resolution NMR experiments in conjunction with molecular modeling-based conformational calculations. They detected a two-step ligand binding process in which PACAP first binds nonspecifically to the membrane to promote a shift of the ligand conformational ensemble in its N-terminal region that allows for specific binding to the PACAP receptor. The binding sites of GPCR contain many more amino acid side chains than peptidase active sites and potentially offer many more productive conformations for ligand binding. Structural data for GPCR are not available today, but in time it may be possible to calculate ensembles of potential binding sites for systems this complex. Of course these speculations can only be fully explored after structural data for GPCR interactions become available.

Until now a rational mechanism for transforming peptide-derived peptidomimetics into non-peptide peptidomimetics has not been evident. We propose that targeting conformational transitions associated with enzyme catalysis will guide future rational design of

non-peptide peptidomimetics. Structure-generating programs can facilitate this process by probing the steric constraints within an active site in order to identify enzyme groups with potentially important mobility and by generating diverse arrays of molecules from which medicinal chemists can extract suitable potential lead structures for further evaluation and synthesis. GrowMol-like programs represent a combinatorial design process that can best be exploited when coupled with powerful synthetic methods for optimization. Focused combinatorial synthesis of molecular scaffolds is likely to be a particularly effective way to optimize lead structures into tight-binding biologically active inhibitors. We believe the combinatorial design of ligands targeting conformational ensembles, coupled with combinatorial synthesis, will lead to important new classes of therapeutically useful molecules in the coming century.

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Biographies

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References

(1) Fischer, E. Einfluss der Configuration auf die Wirkung der Enzyme. *Ber. Dtsch. Chem. Ges.* **1894**, *27*, 2985.
(2) Koshland, D. E. Application of a theory of enzyme specificity to protein synthesis. *Proc. Natl. Acad. Sci. U.S.A.* **1958**, *44*, 98.
(3) Kumar, S.; Ma, B.; Tsai, C. J.; Wolfson, H.; Nussinov, R. Folding funnels and conformational transitions via hinge-bending motions. *Cell Biochem. Biophys.* **1999**, *31*, 141—164.
(4) Ma, B.; Kumar, S.; Tsai, C. J.; Nussinov, R. Folding funnels and binding mechanisms. *Protein Eng.* **1999**, *12*, 713—720.
(5) Tsai, C. J.; Kumar, S.; Ma, B.; Nussinov, R. Folding funnels, binding funnels, and protein function. *Protein Sci.* **1999**, *8*, 1181—1190.
(6) Barton, D. H. R. Conformational analysis. *Experientia* **1950**, *6*, 316—320.
(7) Hart, P. A.; Rich, D. H. Stereochemical aspects of drug action I: Conformational restriction, steric hindrance and hydrophobic collapse. In *Pract. Med. Chem.*; Wermuth, C., Ed.; Acad. Press: London, U.K., 1996; pp 393—412.
(8) Straub, F. B. Formation of the secondary and tertiary structure of enzymes. *Adv. Enzymol.* **1964**, *26*, 89—114.
(9) Sowdhamini, R.; Srinivasan, N.; Guruprasad, K.; Rufino, S.; Dhanaraj, V.; Wood, S. P.; Emsley, J.; White, H. E.; Blundell, T. Protein three-dimensional structure and molecular recognition: a story of soft locks and keys. *Pharm. Acta Helv.* **1995**, *69*, 185—192.
(10) Fairlie, D. P.; Tyndall, J. D. A.; Reid, R. C.; Wong, A. K.; Abbenante, G.; Scanlon, M. J.; March, D. R.; Bergman, D. A.; Chai, C. L. L.; Burkett, B. A. Conformational selection of inhibitors and substrates by proteolytic enzymes: implications for drug design and polypeptide processing. *J. Med. Chem.* **2000**, *43*, 1271—1281.
(11) Smith, A. B.; Keenan, T. P.; Holcomb, R. C.; Sprengeler, P. A.; Guzman, M. C.; Wood, J. L.; Carroll, P. J.; Hirschmann, R. Design, synthesis, and crystal structure of a pyrrolinone-based peptidomimetic possessing the conformation of a β-strand: potential application to the design of novel inhibitors of proteolytic enzymes. *J. Am. Chem. Soc.* **1992**, *114*, 10672—10674.
(12) Smith, A. B.; Hirschmann, R.; Pasternak, A.; Cuzman, M. C.; Yokoyama, A.; Sprengeler, R. A.; Darke, P. L.; Emini, E. A.; Schleif, W. A. Pyrrolinone-based HIV protease inhibitors. Design, synthesis, and antiviral activity: evidence for improved transport. *J. Am. Chem. Soc.* **1995**, *117*, 11113—11123.
(13) A search of the pdb for aspartic proteinase/protease revealed 121 structures had been deposited as of August 2001.
(14) Fruton, J. S. The mechanism of the catalytic action of pepsin and related acid proteinases. *Adv. Enzymol.* **1976**, *44*, 1—36.
(15) Pearl, L.; Blundell, T. The active site of aspartic proteinases. *FEBS Lett.* **1984**, *174*, 96—101.
(16) Suguna, K.; Padlan, E. A.; Smith, C. W.; Carlson, W. D.; Davies, D. R. Binding of a reduced peptide inhibitor to the aspartic proteinase from Rhizopus chinensis: implications for a mechanism of action. *Proc. Natl. Acad. Sci. U.S.A.* **1987**, *84*, 7009—7013.
(17) James, M. N. G.; Sielecki, A. R.; Hayakawa, K.; Gelb, M. H. Crystallographic analysis of transition state mimics binding to penicillopepsin: difluorostatine- and difluorostatone-containing peptides. *Biochemistry* **1992**, *31*, 3872—3886.
(18) Hyland, L. J.; Tomaszek, T. A., Jr.; Meek, T. D. Human immunodeficiency virus-1 protease. 2. Use of pH rate studies and solvent kinetic isotope effects to elucidate details of chemical mechanism. *Biochemistry* **1991**, *30*, 8454—8463.
(19) Piana, S.; Carloni, P.; Parrinello, M. Role of Conformational Fluctuations in the Enzymatic Reaction of HIV-1 Protease. Manuscript submitted.
(20) Northrop, D. B. Follow the protons: A low-barrier hydrogen bond unifies the mechanisms of the aspartic proteases. *Acc. Chem. Res.* **2001**, *34*, 790—797.
(21) Piana, S.; Carloni, P. Conformational flexibility of the catalytic Asp dyad in HIV-1 protease: an ab initio study on the free enzyme. *Proteins: Struct., Funct., Genet.* **2000**, *39*, 26—36.
(22) Piana, S.; Sebastiani, D.; Carloni, P.; Parrinello, M. Ab Initio Molecular Dynamics-Based Assignment of the Protonation State of Pepstatin A/HIV-1 Protease Cleavage Site. *J. Am. Chem. Soc.* **2001**, *123*, 8730—8737.
(23) Cho, Y.-K.; Rebholz, K. L.; Northrop, D. B. Solvent Isotope Effects on the Onset of Inhibition of Porcine Pepsin by Pepstatin. *Biochemistry* **1994**, *33*, 9637—9642.
(24) Cho, Y.-K.; Northrop, D. B. Transpeptidation by porcine pepsin catalyzed by a noncovalent intermediate unique to its isomechanism. *J. Biol. Chem.* **1998**, *273*, 24305—24308.
(25) Rich, D. H. Peptidase Inhibitors. In *Comprehensive Medicinal Chemistry. The Rational Design, Mechanistic Strudy and Therapeutic application of Chemical Compounds*; Hansh, C., Sammes, P. G., Taylor, J. B., Eds.; Pergamon Press: 1990; pp 391—441.
(26) Umezawa, H.; Aoyagi, H.; Morishima, H.; Matsuzaki, M.; Hamada, M.; Takeuchi, T. Pepstatin, a new pepsin inhibitor produced by acetinomycetes. *J. Antibiot.* **1970**, *23*, 259—265.
(27) Kunimoto, S.; Aoyagi, T.; Morishima, H.; Takeuchi, T.; Umezawa, H. Mechanism of inhibition of pepsin by pepstatin. *J. Antibiot.* **1972**, *25*, 251—255.
(28) Marshall, G. R. Structure—activity relations of antagonists of the renin-angiotensin system. *Fed. Proc., Fed. Am. Soc. Exp. Biol.* **1976**, *25*, 2494—2501.
(29) Marciniszyn, J., Jr.; Hartsuck, J. A.; Tang, J. Mode of inhibition of acid proteases by pepstatin. *J. Biol. Chem.* **1976**, *251*, 7088—7094.
(30) Rich, D. H. Pepstatin-derived inhibitors of aspartic proteinases. A close look at an apparent transition-state analog inhibitor. *J. Med. Chem.* **1985**, *28*, 263—273.
(31) Szelke, M.; Jones, D. M.; Atrash, B.; Hallett, A.; Leckie, B. J. Novel transition-state analog inhibitors of renin. *Pept.: Struct. Funct., Proc. Am. Pept. Symp., 8th* **1983**, 579—582.
(32) Szelke, M.; Jones, D. M.; Hallett, A. Enzyme inhibitors. European Patent Application EP 45665, 1982.
(33) Holladay, M. W.; Rich, D. H. Synthesis of hydroxyethylene and ketomethylene dipeptide isosteres. *Tetrahedron Lett.* **1983**, *24*, 4401—4404.

(34) Greenlee, W. J. Renin inhibitors. *Med. Res. Rev.* **1990**, *10*, 173−236.
(35) Gordon, E. M.; Godfrey, J. D.; Pluscec, J.; Von Langen, D.; Natarajan, S. Design of peptide derived amino alcohols as transition-state analog inhibitors of angiotensin converting enzyme. *Biochem. Biophys. Res. Commun.* **1985**, *126*, 419−426.
(36) Rich, D. H.; Green, J.; Toth, M. V.; Marshall, G. R.; Kent, S. B. H. Hydroxyethylamine analogs of the p17/p24 substrate cleavage site are tight-binding inhibitors of HIV protease. *J. Med. Chem.* **1990**, *33*, 1285−1288.
(37) Lebon, F.; Ledecq, M. Approaches to the design of effective HIV-1 protease inhibitors. *Curr. Med. Chem.* **2000**, *7*, 455−477.
(38) PDB entry 6APR.
(39) Leung, D.; Abbenante, G.; Fairlie, D. P. Protease inhibitors: current status and future prospects. *J. Med. Chem.* **2000**, *43*, 305−341.
(40) Rahuel, J.; Rasetti, V.; Maibaum, J.; Rueger, H.; Goschke, R.; Cohen, N. C.; Stutz, S.; Cumin, F.; Fuhrer, W.; Wood, J. M.; Grutter, M. G. Structure-based drug design: the discovery of novel nonpeptide orally active inhibitors of human renin. *Chem. Biol.* **2000**, *7*, 493−504.
(41) Lien, E. J.; Gao, H.; Lien, L. L. In search of ideal antihypertensive drugs: progress in five decades. *Prog. Drug Res.* **1994**, *43*, 43−86.
(42) Ripka, A. S.; Rich, D. H. Peptidomimetic design. *Curr. Opin. Chem. Biol.* **1998**, *2*, 441−452.
(43) Spatola, A. F. Peptide backbone modifications: a structure−activity analysis of peptides containing amide bond surrogates, conformational constraints, and related backbone replacements. In *Chem. Biochem. Amino Acids, Pept., Proteins*; Weinstein, B., Ed.; Marcel Dekker: New York, 1983; pp 267−257.
(44) For a review of this strategy, see: Hart, P. A.; Rich, D. H. Stereochemical aspects of drug action I: Conformational restriction, steric hindrance and hydrophobic collapse. In *Pract. Med. Chem.*; Wermuth, C., Ed.; Acad. Press: London, U.K., 1996; pp 393−412.
(45) Farmer, P. S. Bridging the gap between bioactive peptides and nonpeptides: some perspectives in design. In *Drug Design*; Ariens, E. J., Ed.; Academic Press: New York, 1980; Vol. 10, pp 119−143.
(46) For an interesting compilation of the terminology, see: Fletcher, M. D.; Campbell, M. M. Partially modified retro-inverso peptides: development, synthesis, and conformational behaviour. *Chem. Rev.* **1998**, *98*, 763−795.
(47) For a discussion of the preference for designed peptidomimetics see: Olson, G. L.; Bolin, D. R.; Bonner, M. P.; Bös, M.; Cook, C. M.; et al. Concepts and progress in the development of peptide mimetics. *J. Med. Chem.* **1993**, *36*, 3039−3049.
(48) Bursavich, M. G.; West, C. W.; Rich, D. H. From peptides to nonpeptide peptidomimetics: design and synthesis of new piperidine inhibitors of aspartic peptidases. *Org. Lett.* **2001**, *3*, 2317−2320.
(49) Abbenante, G.; March, D. R.; Bergman, D. A.; Hunt, P. A.; Garnham, B.; Dancer, R. J.; Martin, J. L.; Fairlie, D. P. Regioselective structural and functional mimicry of peptides. Design of hydrolytically stable cyclic peptidomimetic inhibitors of HIV-1 protease. *J. Am. Chem. Soc.* **1995**, *117*, 10220−10226.
(50) Martin, J. L.; Begun, J.; Schindeler, A.; Wickramasinghe, W. A.; Alewood, D.; Alewood, P. F.; Bergman, D. A.; Brinkworth, R. I.; Abbenante, G.; March, D. R.; Reid, R. C.; Fairlie, D. P. Molecular recognition of macrocyclic peptidomimetic inhibitors by HIV-1 protease. *Biochemistry* **1999**, *38*, 7978−7988.
(51) Navia, M. A.; Chaturvedi, P. R. Design principles for orally active drugs. *Drug Discovery Today* **1996**, *1*, 179−189.
(52) Thaisrivongs, S.; Tomich, P. K.; Watenpaugh, K. D.; Chong, K.-T.; Howe, W. J.; Yang, C.-P.; Strohbach, J. W.; Turner, S. R.; McGrath, J. P.; Bohanon, M. J.; Lynn, J. C.; Mulichak, A. M.; Spinelli, P. A.; Hinshaw, R. R.; Pagano, P. J.; Moon, J. B.; Ruwart, M. J.; Wilkinson, K. F.; Rush, B. D.; Zipp, G. L.; Dalga, R. J.; Schwende, F. J.; Howard, G. M.; Padbury, G. E.; Toth, L. N.; Zhao, Z.; Koeplinger, K. A.; Kakuk, T. J.; Cole, S. L.; Zaya, R. M.; Piper, R. C.; Jeffrey, P. Structure-based design on HIV protease inhibitors: 4-hydroxycoumarins and 4-hydroxy-2-pyrones as non-peptidic inhibitors. *J. Med. Chem.* **1994**, *37*, 3200−3204.
(53) Lam, P. Y. S.; Jadhav, P. K.; Eyermann, C. J.; Hodge, C. N.; Ru, Y.; Bacheler, L. T.; Meek, O. M. J.; Rayner, M. M. Rational design of potent, bioavailable, nonpeptide cyclic ureas as HIV protease inhibitors. *Science* **1994**, *263*, 380−384.
(54) These inhibitors include PDB entries 1HIV, 1HPS, 1HSG, 1SBG, 1HVI, 1HXB, 4HVP, and 5HVP.
(55) PDB entry 1B6M.
(56) Oefner, C.; Binggeli, A.; Breu, V.; Bur, D.; Clozel, J. P.; D'Arcy, A.; Dorn, A.; Fischli, W.; Gruninger, F.; Guller, R.; Hirth, G.; Marki, H. P.; Mathews, S.; Muller, M.; Ridley, R. G.; Stadler, H.; Vieira, E.; Wilhelm, M.; Winkler, F. K.; Wostl, W. Renin inhibition by substituted piperidines: a novel paradigm for the inhibition of monomeric aspartic proteinases? *Chem. Biol.* **1999**, *6*, 127−131.
(57) James, M. N. G.; Hsu, I.-N.; Delabaere, L. T. J. Mechanism of acid protease catalysis based on the crystal strucutre of penicillopepsin. *Nature* **1977**, *267*, 808−813.
(58) James, M. N. G.; Sielecki, A.; Salituro, F.; Rich, D. H.; Hofmann, T. Conformational flexibility in the active sites of aspartyl proteinases revealed by a pepstatin fragment binding to penicillopepsin. *Proc. Natl. Acad. Sci. U.S.A.* **1982**, *79*, 6137−6141.
(59) Rich, D. H.; Salituro, F. G. Synthesis of analogues of pepstatin. Effect of structure in subsites P_1', P_2' and P_2 on inhibition of porcine pepsin. *J. Med. Chem.* **1983**, *26*, 904−910.
(60) Bott, R.; Subramanian, E.; Davies, D. R. Three-dimensional structure of the complex of the *Rhizopus chinensis* carboxyl proteinase and pepstatin at 2.5 Å resolution. *Biochemistry* **1982**, *21*, 6956−6962.
(61) James, M. N. G.; Sielecki, A. R. Structure and refinement of penicillopepsin at 1.8 Å resolution. *J. Mol. Biol.* **1983**, *163*, 299−361.
(62) Kuzmic, P.; Sun, C.-Q.; Zhao, Z.-C.; Rich, D. H. Long-range electrostatic effects in pepsin catalysis. *Tetrahedron* **1991**, *47*, 2519−2534.
(63) Baldwin, E. T.; Bhat, T. N.; Gulnik, S.; Hosur, M. V.; Sowder, R. C.; Cachau, R. E.; Collins, J.; Silva, A. M.; Erickson, J. W. Crystal structures of native and inhibited forms of human cathepsin D: implications for lysosomal targeting and drug design. *Proc. Natl. Acad. Sci. U.S.A.* **1993**, *90*, 6796−6800.
(64) Newman, M.; Safro, M.; Frazao, C.; Khan, G.; Zhdanov, A. S.; Tickle, I. J.; Blundell, T. L.; Andreeva, N. S. X-ray analyses of aspartic proteinases IV. Structure and refinement at 2.2 Å resolution of bovine chymosin. *J. Mol. Biol.* **1991**, *221*, 1295−1309.
(65) Gilliland, G. L.; Winborne, E. L.; Nachman, J.; Wlodawer, A. The three-dimensional structure of recombinant bovine chymosin at 2.3 Å resolution. *Proteins*: *Struct., Funct., Genet.* **1990**, *8*, 82−101.
(66) Groves, M. R.; Dhanaraj, V.; Badasso, M.; Nugent, P.; Pitts, J. E.; Hoover, D. J.; Blundell, T. L. A 2.3 Å resolution structure of chymosin complexed with a reduced bond inhibitor shows that the active site beta-hairpin flap is rearranged when compared with the native crystal structure. *Protein Eng.* **1998**, *11*, 833−840.
(67) PDB entry code 3APP was used for native enzyme and 1APU for inhibitor-bound conformation.
(68) PDB entry code 4CMS was used for native enzyme and 1CZI for inhibitor-bound conformation.
(69) Guruprasad, K.; Dhanaraj, V.; Groves, M.; Blundell, T. L. Aspartic proteinases: the structures and functions of a versatile superfamily of enzymes. *Perspect. Drug Discovery Des.* **1995**, *2*, 329−341.
(70) Sali, A.; Veerapandian, B.; Cooper, J. B.; Foundling, S. I.; Hoover, D. J.; Blundell, T. L. High-resolution X-ray diffraction study of the complex between endothiapepsin and an oligopeptide inhibitor: the analysis of the inhibitor binding and description of the rigid body shift in the enzyme. *EMBO J.* **1989**, *8*, 2179−2188.
(71) Sali, A.; Veerapandian, B.; Cooper, J. B.; Moss, D. S.; Hofmann, T.; Blundell, T. L. Domain flexibility in aspartic proteinases. *Proteins*: *Struct., Funct., Genet.* **1992**, *12*, 158−170.
(72) Abad-Zapatero, C.; Rydel, T. J.; Erickson, J. Revised 2.3 Å structure of porcine pepsin: evidence for a flexible domain. *Proteins*: *Struct., Funct., Genet.* **1990**, *8*, 62.
(73) Chen, L.; Erickson, J. W.; Rydel, T. J.; Park, C. H.; Neidhart, D.; Luly, J.; Abad-Zapatero, C. Structure of a pepsin/penin inhibitor complex reveals a novel crystal packing induced by minor chemical alterations in the inhibitor. *Acta Crystallogr.* **1992**, *B48*, 476−488.
(74) Rich, D. H.; Northrop, D. B. The dependence of enzyme inhibition of the distribution of enzyme forms. Implications for substrate and inhibitor structure−activity correlations. In *Computer Aided Drug Design*; Perun, T. L., Probst, C. L., Eds.; Marcel Dekker: New York, 1989; pp 185−250.
(75) Flap opening and closing is a low-energy process that may occur at rates up to 100 times per second with substrate K_{cat} for aspartic peptidases typically varring from 1 to 100 s^{-1}.
(76) Vella, S. HIV therapy advances. Update on a proteinase inhibitor. *AIDS* **1994**, *8* (Suppl 3), S25−29.
(77) Kitchen, V. S.; Skinner, C.; Ariyoshi, K.; Lane, E. A.; Duncan, I. B.; Burckhardt, J.; Burger, H. U.; Bragman, K.; Pinching, A. J.; Weber, J. N. Safety and activity of saquinavir in HIV infection. *Lancet* **1995**, *345*, 952−955.
(78) Babine, R. E.; Bender, S. L. Molecular recognition of protein−ligand complexes: applications to drug design. *Chem. Rev.* **1997**, *97*, 1359−1472.
(79) Miller, M.; Schneider, J.; Sathyanarayana, B. K.; Toth, M. V.; Marshall, G. R.; Clawson, L.; Selk, L.; Kent, S. B. H.; Wlodawer, A. Structure of complex of synthetic HIV-1 protease with a substrate-based inhibitor at 2.3 Å resolution. *Science* **1989**, *246*, 1149−1152.

(80) Swain, A. L.; Miller, M. M.; Green, J.; Rich, D. H.; Schneider, J.; Kent, S. B. H.; Wlodawer, A. X-ray crystallographic structure of a complex between a synthetic protease of human immunodeficiency virus 1 and a substrate-based hydroxyethylamine inhibitor. *Proc. Natl. Acad. Sci. U.S.A.* **1990**, *87*, 8805−8809.
(81) Redshaw, S.; Roberts, N. A.; Thomas, G. J. The road to Fortovase. A history of saquinavir, the first human immunodeficiency virus protease inhibitor. *Handb. Exp. Pharmacol.* **2000**, *140*, 3−21.
(82) Krohn, A.; Redshaw, S.; Ritchie, J. C.; Graves, B. J.; Hatada, M. H. Novel binding mode of highly potent HIV-proteinase inhibitors incorporating the (R)-hydroxyethylamine isostere. *J. Med. Chem.* **1991**, *34*, 3340−3342.
(83) Rich, D. H.; Sun, C. Q.; Prasad, J. V. N. V.; Pathiasseril, A.; Toth, M. V.; Marshall, G. R.; Clare, M.; Mueller, R. A.; Houseman, K. Effect of hydroxyl group configuration in hydroxyethylamine dipeptide isosteres on HIV protease inhibition. Evidence for multiple binding modes. *J. Med. Chem.* **1991**, *34*, 1222−1225.
(84) Rich, D. H.; Bernatowicz, M. S.; Agarwal, N. S.; Kawai, M.; Salituro, F. G.; Schmidt, P. G. Inhibition of aspartic proteases by pepstatin and 3-methylstatine derivatives of pepstatin. Evidence for collected-substrate enzyme inhibition. *Biochemistry* **1985**, *24*, 3165−3173.
(85) Lam, P. Y. S.; Ru, Y.; Jadhav, P. K.; Aldrich, P. E.; DeLucca, G. V.; Eyermann, C. J.; Chang, C.-H.; Emmett, G.; Holler, E. R.; Daneker, W. F.; Li, L.; Confalone, P. N.; McHugh, R. J.; Han, Q.; Li, R.; Markwalder, J. A.; Seitz, S. P.; Sharpe, T. R.; Bacheler, L. T.; Rayner, M. M.; Klabe, R. M.; Shum, L.; Winslow, D. L.; Kornhauser, D. M.; Jackson, D. A.; Ericson-Vitanen, S.; Hodge, C. N. Cyclic HIV protease inhibitors: synthesis, conformational analysis, P_2/P_2' structure−activity relationship, and molecular recognition of cyclic ureas. *J. Med. Chem.* **1996**, *39*, 3514−3525.
(86) DeLucca, G. V.; Erickson-Vitanen, S.; Lam, P. Y. S. Cyclic HIV protease inhibitors capable of displacing the active site structural water molecule. *Drug Discovery Today* **1997**, *2*, 6−18.
(87) Yamazaki, T.; Nicholson, L. K.; Torchia, D. A.; Wingfield, P.; Stahl, S. J.; Kaufman, J. D.; Eyermann, C. J.; Hodge, C. N.; Lam, P. Y. S.; Jadhav, P. K.; Chan, C. H.; Weber, P. C. NMR and X-ray evidence that the HIV protease catalytic aspartyl groups are protonated in the complex formed by the protease and a non-peptide cyclic urea-based inhibitor. *J. Am. Chem. Soc.* **1994**, *116*, 10791−10792.
(88) Tait, B. D.; Hagen, S.; Domagala, J. M.; Ellsworth, E. L.; Gajda, C.; Hamilton, H. W.; Prasad, J. V. N. V.; Ferguson, D.; Graham, N.; Hupe, D.; Nouhan, C.; Tummino, P. J.; Humblet, C.; Lunney, E. A.; Pavlovsky, A.; Rubin, J.; Gracheck, S. J.; Baldwin, E. T.; Bhat, T. N.; Erickson, J. W.; Gulnik, S. V.; Liu, B. S. 4-hydroxy-5,6-dihydropyrones. 2. Potent non-peptide inhibitors of HIV protease. *J. Med. Chem.* **1997**, *40*, 3781−3792.
(89) Turner, S. R.; Strohbach, J. W.; Tommasi, R. A.; Aristoff, P. A.; Johnson, P. D.; Skulnick, H. I.; Dolak, L. A.; Seest, E. P.; Tomich, P. K.; Bohanon, M. J.; Horng, M.-M.; Lynn, J. C.; Chong, K.-T.; Hinshaw, R. R.; Watenpaugh, K. D.; Janakiraman, M. N.; Thaisrivongs, S. Tipranavir (PNU-140690): a potent, orally bioavailable nonpeptidic HIV protease inhibitor of the 5,6-dihydro-4-hydroxy-2-pyrone sulfonamide class. *J. Med. Chem.* **1998**, *41*, 3467−3476.
(90) Guller, R.; Binggeli, A.; Breu, V.; Bur, D.; Fischli, W.; Hirth, G.; Jenny, C.; Kansy, M.; Montavon, F.; Muller, M.; Oefner, C.; Stadler, H.; Vieira, E.; Wilhelm, M.; Wostl, W.; Marki, H. P. Piperidine-renin inhibitors compounds with improved physicochemical properties. *Bioorg. Med. Chem. Lett.* **1999**, *9*, 1403−1408.
(91) Vieira, E.; Binggeli, A.; Breu, V.; Bur, D.; Fischli, W.; Guller, R.; Hirth, G.; Marki, H. P.; Muller, M.; Oefner, C.; Scalone, M.; Stadler, H.; Wilhelm, M.; Wostl, W. Substituted piperidines − highly potent renin inhibitors due to induced fit adaptation of the active site. *Bioorg. Med. Chem. Lett.* **1999**, *9*, 1397−1402.
(92) Marki, H. P.; Binggeli, A.; Bittner, B.; Bohner-Lang, V.; Breu, V.; Bur, D.; Coassolo, P.; Clozel, J. P.; D'Arcy, A.; Doebeli, H.; Fischli, W.; Funk, C.; Foricher, J.; Giller, T.; Gruninger, F.; Guenzi, A.; Guller, R.; Hartung, T.; Hirth, G.; Jenny, C.; Kansy, M.; Klinkhammer, U.; Lave, T.; Lohri, B.; Luft, F. C.; Mervaala, E. M.; Muller, D. N.; Muller, M.; Montavon, F.; Oefner, C.; Qiu, C.; Reichel, A.; Sanwald-Ducray, P.; Scalone, M.; Schleimer, M.; Schmid, R.; Stadler, H.; Treiber, A.; Valdenaire, O.; Vieira, E.; Waldmeier, P.; Wiegand-Chou, R.; Wilhelm, M.; Wostl, W.; Zell, M.; Zell, R. Piperidine renin inhibitors: from leads to drug candidates. *Il Farmaco* **2001**, *56*, 21−27.
(93) Sueiras-Diaz, J.; Jones, D. M.; Szelke, M.; Leckie, B. J.; Beattie, S. R.; Beattie, C.; Morton, J. J. Potent in vivo inhibitors of rat renin: analogs of human and rat angiotensinogen sequences containing different classes of pseudodipeptides at the scissile site. *J. Pept. Res.* **1997**, *50*, 239−247.
(94) Jones, D. M.; Sueiras-Diaz, J.; Szelke, M.; Leckie, B. J.; Beattie, S. R.; Morton, J.; Neidle, S.; Kuroda, R. New renin inhibitors containing novel analogs of statine. *J. Pept. Res.* **1997**, *50*, 109−121.
(95) PDB entry code 1BBS was used for native enzyme and 1RNE for peptide inhibitor-bound conformation.
(96) PDB entry code 1RNE was used for chymosin peptide inhibitor-bound conformation.
(97) Carter, P.; Wells, J. A. Engineering enzyme specificity by "substrate-assisted catalysis". *Science* **1987**, *237*, 394−399.
(98) Bohacek, R. S.; McMartin, C. Multiple highly diverse structures complementary to enzyme binding sites: results of extensive application of a de novo design method incorporating combinatorial growth. *J. Am. Chem. Soc.* **1994**, *116*, 5560−5571.
(99) Bohacek, R. S.; McMartin, C.; Guida, W. C. The art and practice of structure-based drug design: a molecular modeling perspective. *Med. Res. Rev.* **1996**, *16*, 3−50.
(100) Rich, D. H.; Bohacek, R. S.; Dales, N. A.; Glunz, P.; Ripka, A. S. Combinatorial design and combinatorial synthesis of enzyme inhibitors. *Actual. Chim. Ther.* **1996**, *22*, 103−111.
(101) Rich, D. H.; Dales, N. A.; Fan, F.; Flentke, G.; Glunz, P.; Keding, S. J.; Ripka, A. S.; Satyshur, K. Merging rational drug design with combinatorial chemistry: reasonable and unreasonable expectations. In *Medicinal Chemistry into the Millennium*; Campbell, M. M., Blagbrough, I. S., Eds.; Royal Society of Chemistry, Publ.: Cambridge, U.K., 2001; pp 16−24.
(102) Ripka, A. S.; Bohacek, R. S.; Rich, D. H. Synthesis of novel cyclic protease inhibitors using Grubbs olefin metathesis. *Bioorg. Med. Chem. Lett.* **1998**, *8*, 357−360.
(103) Dales, N. A.; Bohacek, R. S.; Satyshur, K. A.; Rich, D. H. Design and synthesis of unsymmetrical peptidyl urea inhibitors of aspartic peptidases. *Org. Lett.* **2001**, *3*, 2313−2316.
(104) Ripka, A. S.; Satyshur, K. A.; Bohacek, R. S.; Rich, D. H. Aspartic protease inhibitors designed from computer-generated templates bind as predicted. *Org. Lett.* **2001**, *3*, 2309−2312.
(105) Piperidines were found in about 1% of grown structures. Their predicted binding energies of −30 to −90 kJ/mol were consistent with low micromolar to sub-micromolar binding constants.
(106) Anderson, A. C.; O'Neil, R. H.; Surti, T. S.; Stroud, R. M. Approaches to solving the rigid receptor problem by identifying a minimal set of flexible residues during ligand docking. *Chem. Biol.* **2001**, *8*, 445−457.
(107) Schnecke, V.; Kuhn, L. A. Virtual screening with solvation and ligand-induced complementarity. *Perspect. Drug Discovery Des.* **2000**, *20*, 171−190.
(108) Carlson, H. A.; Masukawa, K. M.; McCammon, J. A. Method for including the dynamic fluctuations of a protein in computer-aided drug design. *J. Phys. Chem. A* **1999**, *103*, 10213−10219.
(109) Carlson, H. A.; McCammon, J. A. Accommodating protein flexibility in computational drug design. *Mol. Pharmacol.* **2000**, *57*, 213−218.
(110) Knegtel, R. M. A.; Kuntz, I. D.; Oshiro, C. M. Molecular docking to ensembles of protein structures. *J. Mol. Biol.* **1997**, *266*, 424−440.
(111) Ewing, T. J. A.; Makino, S.; Skillman, A. G.; Kuntz, I. D. DOCK 4.0: Search strategies for automated molecular docking of flexible molecule databases. *J. Comput.-Aided Mol. Des.* **2001**, *15*, 411−428.
(112) Verkhivker, G. M.; Rejto, P. A.; Bouzida, D.; Arthurs, S.; Colson, A. B.; Freer, S. T.; Gehlhaar, D. K.; Larson, V.; Luty, B. A.; Marrone, T.; Rose, P. W. Parallel simulated tempering dynamics of ligand-protein binding with ensembles of protein conformations. *Chem. Phys. Lett.* **2001**, *337*, 181−189.
(113) Bouzida, D.; Rejto, P. A.; Arthurs, S.; Colson, A. B.; Freer, S. T.; Gehlhaar, D. K.; Larson, V.; Luty, B. A.; Rose, P. W.; Verkhivker, G. M. Computer simulations of ligand-protein binding with ensembles of protein conformations: a monte carlo study of HIV-1 protease binding energy landscapes. *Int. J. Quantum Chem.* **1999**, *72*, 73−84.
(114) Bohacek, R. S.; McMartin, C. Modern computational chemistry and drug discovery: structure generating programs. *Curr. Opin. Chem. Biol.* **1997**, *1*, 157−161.
(115) Goodfod, P. J. *J. Med. Chem.* **1985**, *28*, 849.
(116) Tomioka, N.; Itai, A. J. GREEN − a package program for docking studies in rational drug design. *J. Comput.-Aided Mol. Des.* **1994**, *8*, 347−366.
(117) Lauri, G.; Bartlett, P. A. CAVEAT: a program to facilitate the design of organic molecules. *J. Comput.-Aided Mol. Des.* **1994**, *8*, 51−66.
(118) Eisen, M. B.; Wiley: D. C.; Karplus, M.; Hubbard, R. E. HOOK: A program for finding novel molecular architectures that satisfy the chemical and steric requirements of a macromolecule binding site. *Proteins*: *Struct., Funct., Genet.* **1994**, *19*, 199−221.
(119) Bohm, H. J. LUDI: rule-based automatic design of new substituents for enzyme inhibitor leads. *J. Comput.-Aided Mol. Des.* **1992**, *6*, 593−606.
(120) Bohm, H. J. The computer program LUDI: a new method for the de novo design of enzyme inhibitors. *J. Comput.-Aided Mol. Des.* **1992**, *6*, 61−78.
(121) Tschinke, V.; Cohen, N. C. The NEWLEAD program: a new method for the design of candidate structures from pharmacophoric hypotheses. *J. Med. Chem.* **1993**, *36*, 3863−3870.

(122) Ho, C. M. W.; Marshall, G. R. SPLICE: a program to assemble partial query solutions from three-dimensional database searches into novel ligands. *J. Comput.-Aided Mol. Des.* **1993**, *7*, 623−647.
(123) Gillet, V. J.; Myatt, G.; Zsoldos, Z.; Johnson, A. P. SPROUT, HIPPO and CAESA: tools for de novo structure generation and estimation of synthetic accessibility. *Perspect. Drug Discovery Des.* **1995**, *3*, 34−50.
(124) Moon, J. B.; Howe, W. J. Computer design of bioactive molecules: a method for receptor-based de novo ligand design. *Proteins* **1991**, *11*, 314−328.
(125) Rotstein, S. H.; Murcko, M. A. GroupBuild: a fragment-based method for de novo drug design. *J. Med. Chem.* **1993**, *36*, 1700−1710.
(126) Singh, J.; Saldanha, J.; Thornton, J. M. A novel method for the modeling of peptide ligands to their receptors. *Protein Eng.* **1991**, *4*, 251−261.
(127) Nishibata, Y.; Itai, A. Automatic creation of drug candidate structures based on receptor structure. Starting point for artificial lead generation. *Tetrahedron* **1991**, *47*, 8985−8990.
(128) Rotstein, S. H.; Murcko, M. A. GenStar: A method for de novo drug design. *J. Comput.-Aided Mol. Des.* **1993**, *7*, 23−43.
(129) We have utilized the Flo-99 software for these experiments; however, it seems other modeling software currently available would be capable of performing this simplistic search.
(130) Docking energies calculated for the piperidines varied between −30 and −90 kJ/mol, values consistent with low micromolar to sub-micromolar dissociation constants.
(131) For a discussion of conformational ensembles on the intrinsic K_i, see: Wiley: R. A.; Rich, D. H. Peptidomimetics derived from natural products. *Med. Res. Rev.* **1993**, *13*, 327−384.
(132) Volkman, B. F.; Lipson, D.; Wemmer, D. E.; Kern, D. Two-state allosteric behavior in a single-domain signaling protein. *Science* **2001**, *291*, 2429.
(133) Clackson, T.; Wells, J. A. A hot spot of binding energy in a hormone-receptor interface. *Science* **1995**, *267*, 383−386.
(134) Inooka, H.; Ohtaki, T.; Kitahara, O.; Ikegami, T.; Endo, S.; Kitada, C.; Ogi, K.; Onda, H.; Fujino, M.; Shirakawa, M. Conformation of a peptide ligand bound to its G-protein coupled receptor. *Nat. Struct. Biol.* **2001**, *8*, 161−165.

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