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Harnessing Nature's Insight: Design of Aspartyl Protease Inhibitors from Treatment of Drug-Resistant HIV to Alzheimer's Disease†

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Introduction

Historically, nature has provided an incredible variety of structurally complex and biologically important molecules. Indeed, a great many of today's clinical medicines are obtained directly from natural products or from their derivatives.[1] Natural products continue to be a very important source for modern drug discovery and development.[2] Their seemingly limitless structural features coupled with their novel biological properties have provided enormous inspiration for the development of new reactions and methodologies en route to natural product synthesis and structural variations for drug discovery.[3]

Beginning in the fall of 1994, we initiated a research program aimed at synthesizing bioactive natural products with interesting structural features. This endeavor resulted in the total synthesis of numerous targets, covering over two dozen or so different structural families. Some notable examples of our accomplished bioactive targets include novel and exceedingly potent microtubule stabilizing agents laulimalide (**1**)[4] and peloruside A (**2**);[5] a potent microtubule destabilizing agent cryptophycin 52 (**3**);[6] anticancer agents amphidinolide T (**4**),[7] amphidinolide W (**5**),[8] and lasonolide A (**6**);[9] antibiotic agent madumycin II (**7**);[10] pancreatic lipase inhibitor tetrahydrolipstatin (**8**);[11] novel actin inhibitory agents doliculide (**9**)[12] and jasplakinolide (**10**);[13] novel antibacterial agent platensimycin (**11**);[14] and a histone deacetylase inhibitor largazole (**12**)[15] (see Figure 1). The unique structural features of these natural products required the development of new synthetic tools and methodologies for their synthesis. In the context of our synthesis of various bioactive targets, we have developed a variety of new and practical asymmetric reactions based on intermolecular and intramolecular metal chelation.[16] Notable carbon−carbon bond forming synthetic methodologies include highly diastereoselective *syn*- and *anti*-aldol reactions,[17] asymmetric inter- and intramolecular Diels−Alder and hetero Diels−Alder reactions,[18] and asymmetric multicomponent reactions.[19] The scope and utility of these methodologies have been demonstrated through the synthesis of a variety of bioactive molecules.

Another important objective of our synthetic endeavors is to carry out detailed biological studies and explore the medicinal potential of these target molecules. This crossover, to focus on the biological aspects of these natural products has led to a number of unexpected but very significant results. For example, following the synthesis of marine sponge-derived laulimalide, we defined the mode of action of this novel microtubule stabilizing agent in collaboration with Dr. Ernest Hamel of the National Cancer Institute. Subsequently, we have established that laulimalide binds to a hitherto unknown drug-binding site on tubulin and that laulimalide shows a synergistic effect with paclitaxel. Peloruside A has now shown similar properties as laulimalide.[20] We are currently involved in further exploration of the chemistry and biology of these novel microtubule stabilizing agents with the ultimate goal of developing novel anticancer therapeutic agents based on laulimalide and peloruside A. In another instance, using synthetic doliculide, we have shown that doliculide is an enhancer of actin assembly.[21] More recently, in collaboration with Dr. Yves Pommier of the National Cancer Institute, we have investigated the biological mechanism

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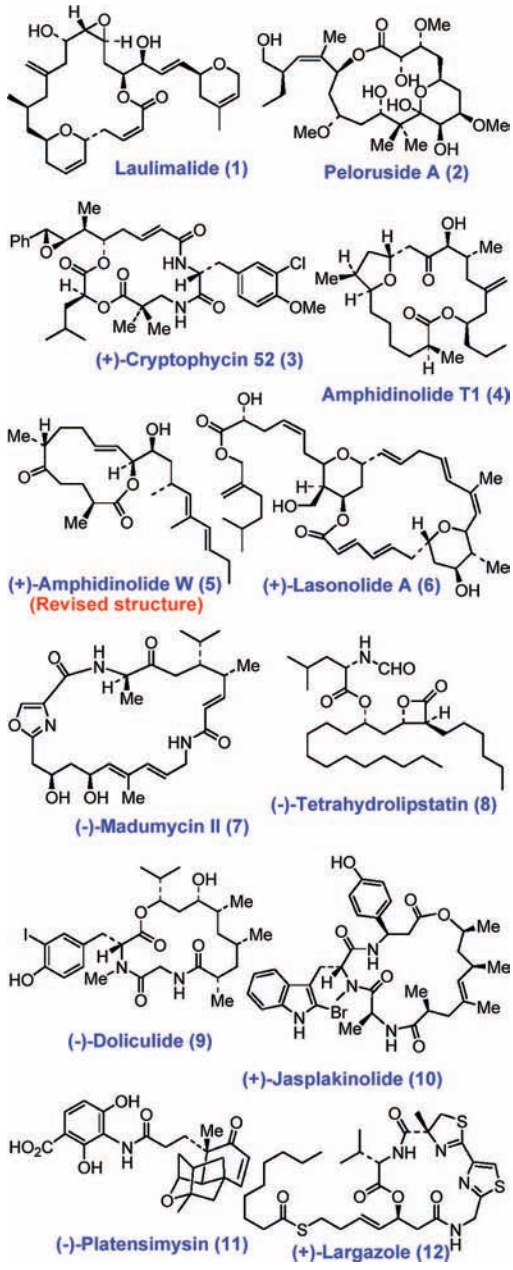


Figure 1. Structures of recent bioactive targets.

of action of lasonolide A.[22] It turns out that lasonolide A exerts its antitumor activity by promoting chromosome condensation. Our detailed biological studies began as an outgrowth of our natural products syntheses but have expanded to great significance.

Our exploration of the chemistry and biology of natural products brought a unique perspective to our research interests in the area of design and synthesis of molecular probes for biological systems. Particularly, our synthesis work serves as an important inspiration to our seemingly more academic approach to medicinal chemistry. As can be seen in Figure 1, the majority of molecules are devoid of any peptidic features, and yet these molecules bind to their biosynthetic enzymes as well as to the respective target macromolecules with high affinity. Therefore, it is quite conceivable that certain structural features, scaffolds, and templates of bioactive natural products can be incorporated into the design of molecular probes for bioactive peptides. We were particularly intrigued by the possibility of mimicking peptide binding with stereochemically defined cyclic ethers and heterocyclic templates present in many bioactive natural products. Indeed, nature has been optimizing such templates over a very long natural selection process and making them compatible with various biological microenvironments. On the basis of these premises, we aspired to design and incorporate such natural-product-derived templates in the structure-based design of inhibitors for aspartyl proteases. Initially, we focused our efforts in two areas: (1) HIV-1 protease inhibitors for the treatment of HIV/AIDS and (2) design of β-secretase inhibitors as a possible treatment of Alzheimer's disease. Of particular note, our research endeavor focused on protein−ligand X-ray structure guided design, based on the molecular insights in the enzyme's active site. Our broad synthetic experience and expertise along with our critical analysis of protein−ligand interactions have empowered our molecular design capability. Graduate students and research fellows in my laboratories often find motivation and inspiration through the stereochemical complexity of our target molecules, distinct synthetic challenges, and the implication of potential applications of these molecular designs to important problems in human medicine. After all, the teaching and training of students as tomorrow's scientists is a very important part of my academic endeavor.

The Need for Conceptually Novel HIV-1 Protease Inhibitors

The advent of HIV protease inhibitors in late 1995, was hailed as a major step forward in the battle against HIV/AIDS.[23] The drug combination in highly active antiretroviral therapy (HAART) involving HIV-1 protease inhibitors has revolutionized the treatment of AIDS.[24] HAART treatment regimens have significantly reduced viral load, increased CD_4^+ lymphocyte cell counts, and arrested the progression of HIV/AIDS. As a result, there has been a significant reduction in morbidity and mortality caused by HIV/AIDS in the U.S. and other industrialized nations.[25] Despite these important advances, effective long-term antiretroviral therapy has been a very complex issue. There are serious limitations to all FDA approved first generation protease inhibitors, including debilitating side effects, drug toxicities, higher therapeutic doses due to peptide-like character, and expensive treatment costs. Perhaps most concerning of all is the emergence of viral strains resistant to approved antiviral drugs. In the era of early HAART, nearly 40−50% of those patients who initially achieved favorable viral suppression to undetectable levels rapidly went on to experience treatment failure.[26] Additionally, 20−40% of antiviral therapy-naive individuals infected with HIV-1 had persistent viral replication despite early HAART, possibly because of the transmission of multidrug-resistant HIV-1 variants. In addition to the issue of drug resistance, tolerance and adherence to complex medical regimens continue to be critical problems. The drugs need to be taken in gram quantities daily because of low oral bioavailability. As a consequence, complex side effects such as peripheral lipodystrophy, hyperlipidemia, and insulin resistance have been very serious.[27] With respect to the current multitude of problems, there is a critical need for the development of a new generation of PIs that exhibit improved pharmacokinetic properties and drug-resistance profiles. In this context, our research focus has been on the design of non-peptidyl PIs and

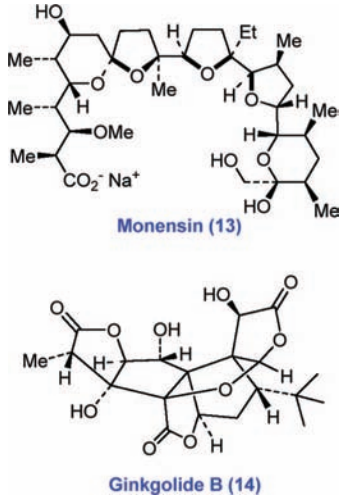


Figure 2. Structures of monensin and ginkgolide B.

optimization of structural elements to maintain potency against mutant strains resistant to currently approved PIs.

Peptide Bond Mimic: Design Inspiration from Polyether Natural Products

The X-ray structures of various peptidomimetic inhibitors bound to HIV-1 protease have provided a wealth of information regarding ligand-binding site interactions. Initially, we were particularly interested in saquinavir because it was a potent approved inhibitor with detailed structure−activity studies available and the protein−ligand X-ray structure had been determined.[28] On the basis of this available information, we initially set out to reduce molecular weight and peptidic features by designing conformationally constrained cyclic or heterocyclic structures that could mimic the binding of the peptide bonds. Saquinavir contains four amide bonds and has a molecular size of 679 Da.[28] Saquinavir is an exceedingly potent inhibitor (IC_{50} = 0.23 nM); however, its oral bioavailability is rather poor, possibly because of the presence of multiple amide/peptide-like bonds. On the basis of the X-ray structure, we speculated that a number of amide carbonyl bonds could be replaced with a cyclic sulfone or cyclic ether template where the ether oxygen may be positioned to interact with the viral enzyme similar to the carbonyl oxygens of saquinavir. Our motivation toward this design emerged from our synthesis and structure−activity studies of numerous bioactive natural products in my laboratory. We have been fascinated by the cyclic ether structural features of a collection of bioactive natural products that do not suffer from poor oral bioavailability problems typical of peptide and peptidomimetic drugs. Of special interest, both ionophore antibiotic monensin[29] (**13**, Figure 2) and platelet activating factor antagonist, ginkgolide B (**14**),[30] feature multiple cyclic ether subunits in their respective structures. Monensin has been used in cattle feed as an antibiotic and has shown good oral bioavailability in poultry and laboratory animals.[31] Ginkgolides have been part of herbal medicine for over 500 years used in the treatment of peripheral and cerebral circulation disorders. Ginkgolide B has been shown to exhibit good oral bioavailability in laboratory animals.[29] Inspired by these interesting pharmacological properties of ginkgolides and monensin, we intended

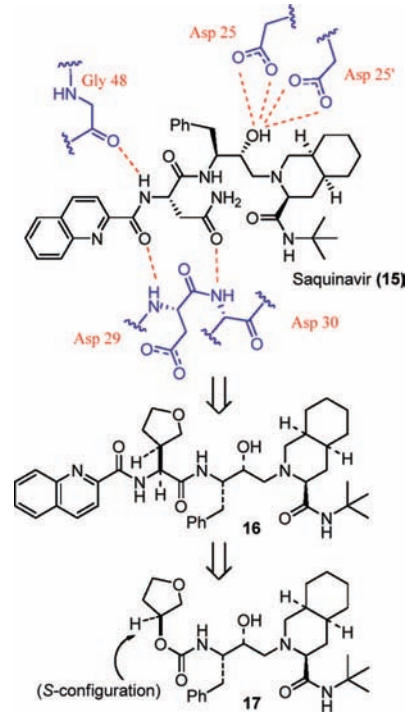


Figure 3. Design of cyclic ethers as novel P2-ligands.

to incorporate various cyclic ether structural features into our design of the next generation of PIs to improve pharmacological properties.

Structure-Based Design of Cyclic Ethers and Sulfones as High-Affinity Ligands

The X-ray structure of saquinavir-bound HIV-1 protease revealed that the P2-asparagine carbonyl forms a hydrogen bond with the Asp 30 NH and the P3-quinaldic amide carbonyl oxygen forms a hydrogen bond with the Asp 29 NH.[32] On the basis of this molecular insight, 3(*R*)-tetrahydrofuranylglycine was developed as a novel P2-ligand for the asparagine side chain of saquinavir.[33] The corresponding inhibitor **16** (Figure 3) was exceedingly potent in both enzyme inhibitory and antiviral assay (IC_{50} = 0.05 nM; CIC_{95} = 8 nM). The stereochemical importance of the tetrahydrofuran has been shown to be critical to its potency. We speculated that the ring oxygen of tetrahydrofuran forms hydrogen bonds with the Asp 30 NH. In an attempt to reduce molecular size, the P3-quinaldic ligand was removed and a stereochemically defined urethane derivative was designed.[34] Inhibitor **17** has shown reduced potency (IC_{50} = 160 nM; CIC_{95} = 800 nM). However, inhibitor **17** is a small molecule inhibitor (515 Da) with a single amide bond.

Interestingly, incorporation of 3(*S*)-tetrahydrofuranylurethane in the hydroxyethylene isostere-derived inhibitor **18** (Figure 4) has shown an incredible potency enhancing effect in both enzyme and antiviral assay.[34] In contrast, cyclopentylurethane-derived inhibitor **19** has displayed a significant reduction of potency. Subsequently, researchers at Vertex Laboratories incorporated this 3(*S*)-tetrahydrofuranylurethane in their structure-based designed (*R*)-hydroxyethylsulfonamide isostere[35] and developed amprenavir[36] as a low molecular weight potent PI with improved pharmacological properties. As speculated previously, a protein−ligand X-ray structure of **20** indeed showed that the ring oxygen of the THF ligand makes weak interactions with Asp 29 and Asp 30 NHs.[37]

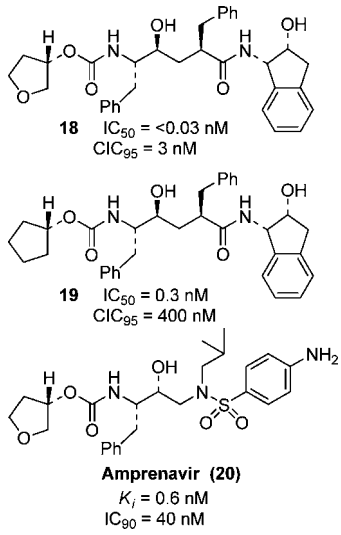


Figure 4. Tetrahydrofuranylurethane-derived potent PIs.

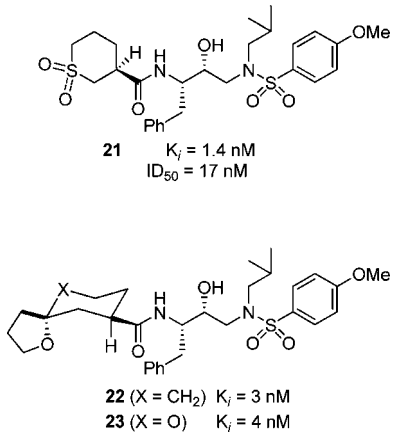


Figure 5. Cyclic sulfone, spiro-ether, and ketal-derived PIs.

The detailed structure−activity studies and the X-ray structure of saquinavir revealed the importance of the P2-asparagine.[32] Incorporation of a sulfone functionality in place of the carboxamide of the P2-asparagine provided an inhibitor with similar activity as saquinavir. Removal of the P3-quinaldic amide and replacement of the P2-asparagine of saquinavir by a 3(*S*)-1,1-dioxotetrahydro-2*H*-thiopyrancarboxamide provided an inhibitor with a loss of activity when compared to saquinavir.[39] However, incorporation of this ligand into the sulfonamide isostere resulted in the very potent inhibitor **21** (Figure 5).[38] A saquinavir-bound X-ray structure-based modeling study indicated that the trans-sulfone oxygen with respect to the carboxamide functionality aligned nicely with the Asp 30 NH. On the basis of this possible ligand-binding site information, we designed a stereochemically defined spirocyclic ether as the P2-ligand in inhibitor **22** (K_i = 2.9 nM) and then a spiroketal-based inhibitor **23** (K_i = 3.9 nM; ID_{50} = 1.2 μM).[41] Interestingly, this type of spiroketal functionality is inherent in many bioactive natural products including monensin.[29]

Design and Development of Bis-THF and Cp-THF Ligands

As mentioned earlier, a protein-structure-based model and an X-ray structure of **17**-bound HIV-1 protease revealed that the tetrahydrofuran oxygen is oriented toward the backbone Asp 29 NH. However, the distance between the THF ring oxygen and the Asp 30 seemed to be somewhat marginal for hydrogen bonding to occur.[34] An amprenavir-bound HIV-1 protease revealed similar ligand-binding site interactions, and the interactions appeared to be marginal for hydrogen bonding (Asp29 NH, 3.4Å; Asp 30 NH, 3.5Å).[37] It appeared that the corresponding sulfolane oxygen may interact better with the active site aspartates. As shown in Figure 6, 3-(*S*)-sulfolane **24** (IC_{50} = 76 nM; CIC_{95} = 350 nM) is more potent than the corresponding 3(*S*)-tetrahydrofuranylurethane with the saquinavir-derived isostere.[40] Incorporation of 3-(*S*)-sulfolane in the (*R*)-(hydroxyethyl)sulfonamide isostere with a *p*-methoxysulfonamide as the P2′-ligand provided very potent inhibitor **25** (K_i = 1.2 nM; ID_{50} = 19 nM; saquinavir K_i = 1.4 nM and ID_{50} = 18 nM, same assay).[41] We have demonstrated preference for the 3-(*S*)-configuration. An energy-minimized model structure of **25** in the VX-478 inhibited (inhibitor **20**) active site indicates

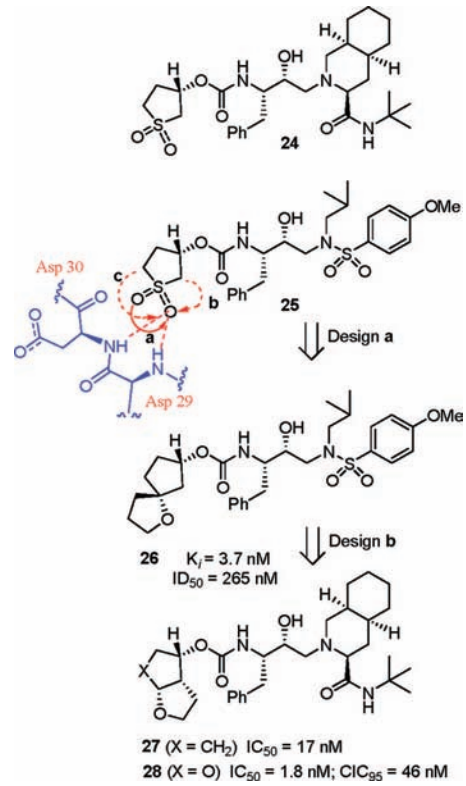


Figure 6. Design of bis-THF ligand as P2-ligand.

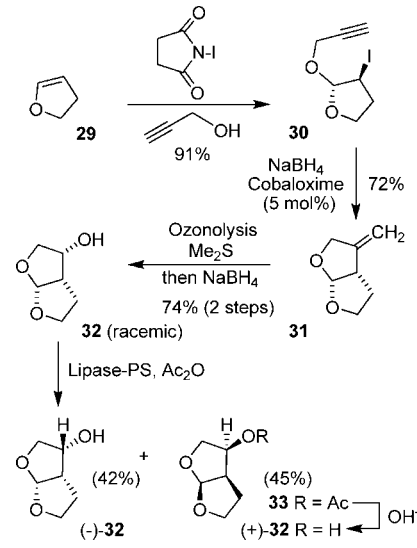


Figure 7. Convenient synthesis of (−)-bis-THF ligand.

that the cis-sulfone oxygen may be appropriately positioned to interact with the main chain atoms of the aspartate residues. Also, the sulfolane ring appears to fill the hydrophobic pocket in the S2-site effectively. On the basis of this molecular insight, we have designed a number of bicyclic ligands with ring oxygen(s) positioned to effectively interact with both the Asp 29 and Asp 30 NHs as well as fill in the pocket in the S2-site. As can be seen in Figure 6, the spiro-ether-derived P2-ligand (design **a**) is not very potent (inhibitor **26**).[42] Bicyclic 4-hexahydro-2*H*-cyclopentafuranylurethane in saquinavir isostere (**27**, $IC_{50} = 17$ nM; design **b**) is significantly more potent than either sulfolane derivative **24** or 3(*S*)-THF-derived inhibitor **17**.[43] Further design with the incorporation of a ring oxygen led to the fused bicyclic bis-tetrahydrofuran (bis-THF)-based P2 ligand shown in inhibitor **28** ($IC_{50} = 1.8$ nM; $CIC_{95} = 46$ nM).[43] It has exhibited marked potency enhancement compared to the 3(*S*) or 3(*R*)-tetrahydrofuranylurethane derivative.[33] The corresponding enantiomeric bis-THF ligand is less potent, which established that the ring stereochemistry of the bis-THF ligand is critical to the inhibitor activity. An X-ray structural analysis of **28**-bound HIV-1 protease revealed that both ring oxygens of the bis-THF ligand are close enough to hydrogen-bond with both main chain NHs of aspartates Asp 29 and Asp 30 in the S2-subsite. Enzyme inhibitory activity of the corresponding inhibitor with a 3-hexahydro-2*H*-cyclopentafuranylurethane is less potent ($K_i = 190$ nM), which further supports the involvement of both oxygens in ligand-binding site interactions.[43]

Our initial synthesis of optically active bis-THF ligands was carried out with (*S*)- or (*R*)-malic acid.[43] This initial synthetic route provided the stereochemical identity of the bis-THF ligands. However, this synthesis was not amenable for the preparation of large quantities of the bis-THF ligand required for detailed studies. We subsequently developed a very practical three-step synthesis of racemic bis-THF ligand.[44] Enzymatic resolution of the racemic alcohol using lipase PS-30 immobilized on Celite provided ample quantities of optically active bis-THF ligand **32** (Figure 7) in high enantiomeric excess (>96% ee) even though the bis-THF ligand contains three chiral centers.[44] This synthesis was utilized for the preparation of gram quantities of the ligand for in-depth studies. A number of other syntheses of optically active bis-THF ligand were reported from our laboratory.[45]

Development of Darunavir for Combating Drug-Resistant HIV

In an academic endeavor inspired by the polyether subunits of bioactive natural products, we have succeeded in designing a number of unprecedented cyclic-ether-derived non-peptide P2-ligands for the HIV-1 protease substrate binding site. As can be seen, incorporation of these ligands provided very potent and structurally novel PIs with substantially reduced molecular size. A number of inhibitors have also shown good pharmacokinetic properties in laboratory animals.[38,43] Our subsequent research objective focused on addressing the critical issue of drug resistance. Toward this aim, we planned to optimize PIs not only against wild type HIV-1 protease but also against a range of mutant proteases. Of particular interest, the X-ray structure of protein−ligand complexes of wild-type HIV-1 protease and a number of mutant proteases revealed only a small distortion in the backbone conformation of the enzyme.[46] This observation was of critical importance. On the basis of this insight, our design hypothesis was to maximize interactions in the active site of the protease with the backbone atoms. Particularly, we desired to design an inhibitor that makes

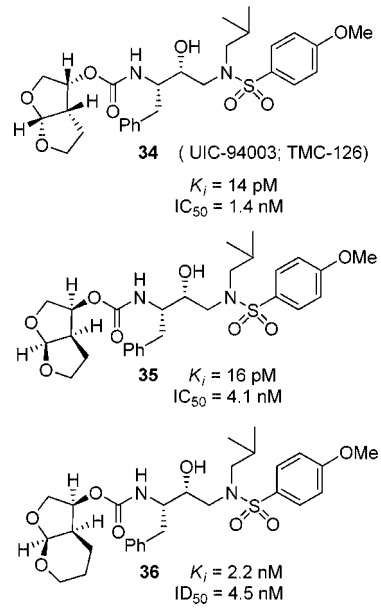


Figure 8. Bis-THF and THF-THP-derived potent inhibitors.

extensive hydrogen bonds throughout the active site protease backbone.[47,48] Conceivably, such PIs would retain these hydrogen-bonding interactions with mutant proteases and thereby maintaining potency against mutant strains. Our initial ligand design and optimization of ligand-binding were carried out with a saquinavir-derived hydroxyethylamine isostere. However, it appeared that the stereochemically defined 3(*S*)-THF and 3(*S*)-sulfolane ligands were even more effective in the (*R*)-(hydroxyethyl)sulfonamide isostere developed by Vasquez et al.[35] and Tung et. al.[36] We subsequently investigated the effectiveness of the bis-THF ligand in a number of other isosteres including the (*R*)-(hydroxyethyl)sulfonamide isostere with a *p*-methoxysulfonamide as the P2′-ligand, assuming that the methoxy group oxygen may interact with the aspartate residues in the other half of the dimeric enzyme. As shown in Figure 8, incorporation of 3(*R*),3a(*S*)-,6a(*R*)-bis-THF ligand provided inhibitor **34** ($K_i = 14$ pM; $IC_{90} = 1.4$ nM) with remarkable enzyme inhibitory and antiviral activity.[41,49] Inhibitor **35** with the enantiomeric bis-THF was slightly less potent in an antiviral assay ($K_i = 16$ pM; $ID_{50} = 4.1$ nM). Inhibitor **36** with a 3(*S*),3a(*S*),7a(*S*)-hexahydrofuropyranylurethane as the P2-ligand demonstrated significant potency (saquinavir, $K_i = 1.4$ nM and $ID_{50} = 18$ nM, same assay).[41] A preliminary X-ray structure of **34**-bound HIV-1 protease indicated that the inhibitor makes extensive hydrogen bonding throughout the active site. Both oxygen atoms of the bis-THF ligand appear to hydrogen-bond to the backbone NHs of Asp 29 and Asp 30.[50] Furthermore, the 4-methoxy oxygen of the P2′-sulfonamide is within hydrogen-bonding distance of Asp 29′ and Asp 30′ NHs. In comparison, the X-ray structure of **15**-bound HIV-1 protease shows that hydrogen bonding with main chain atoms of the aspartates in the S2′-site is absent. This may explain the robust enzyme inhibitory and antiviral activity of inhibitor **34**.[41,51] Inhibitor **34** was later renamed as TMC-126. The enzyme inhibitory properties of **34** were also assessed against mutant proteases and showed K_i values less than 100 pM, and $K_{i\,mut}/K_{i\,wt}$ was less than 5, thus indicating a low level of resistance against **34** for enzymes with multiple mutations that were shown to be highly resistant to clinically approved first generation PIs.[52] A detailed virological study with **34** was then carried out in Dr. Hiroaki Mitsuya's laboratory at the National Cancer Institute.[53] The inhibitor turned out to be highly potent against a wide

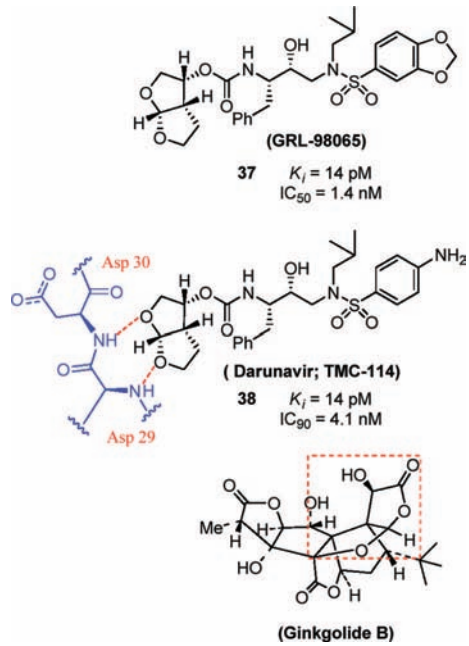


Figure 9. Structures of darunavir and GRL-98065.

spectrum of mutant HIV variants with IC_{50} values ranging from 0.3 to 0.5 nM. A detailed drug-sensitivity study with **34** carried out in Dr. Mitsuya's laboratory demonstrated that **34** conferred significant advantages compared to structurally related sulfonamide isostere-derived amprenavir and other approved PIs in terms of the emergence of drug resistance. As it turned out, viral acquisition of resistance to **34** was substantially delayed and **34**-resistant HIV remained sensitive to all approved PIs except amprenavir. In contrast, the amprenavir-resistant virus was highly cross-resistant to all PIs except saquinavir. Furthermore, **34** was highly potent (IC_{50} = 0.5−5.5 nM) against multi-PI-resistant HIV-1 strains isolated from patients who were harboring drug-resistant HIV-1.[53] This impressive activity against a wide spectrum of drug-resistant HIV variants is presumably due to its robust binding properties in the active site, particularly through its interactions with the backbone aspartates in the S2 to S2′ sites.[50] The combination of the bis-THF ligand with (*R*)-(hydroxyethyl)sulfonamide isostere thus provided an intriguing structural framework for developing a conceptually new generation of PIs to specifically combat drug resistance.

Further exploration of a P2′-sulfonamide functionality that can effectively interact with the backbone atoms in the S2′-site led to the identification of a number of PIs including inhibitors **37** and **38** (Figure 9) with marked drug-resistance properties.[54,55] Inhibitor **38** (later named as TMC-114 and then darunavir), however, exhibited the best pharmacokinetic properties and drug-resistance profile.[54,55] A detailed analysis of the antiviral properties of **38** revealed that it is highly potent against laboratory HIV-1 strains and primary clinical isolates with IC_{50} values around 0.003 μM and a IC_{90} value of 0.009 μM.[54] It has also shown minimal toxicity. Furthermore, it effectively blocked the infectivity and replication of each of the HIV-1_{NL4-3} variants exposed and selected for resistance to first generation approved PIs at concentrations up to 5 μM. Inhibitor **38** also exhibited potent activity against highly multi-PI-resistant clinical HIV-1 variants isolated from patients who did not respond to any existing antiviral regimens. Furthermore, in collaboration with Dr. Mitsuya's group, we have shown that **38** blocks dimerization of HIV-1 protease employing an intermolecular fluorescence resonance energy transfer (FRET)-based HIV expression assay consisting of two protease subunits, cyan and yellow fluorescent protein-tagged HIV-1 protease monomers. Inhibitor **38** blocked the protease dimerization at concentrations of 0.01 μM or less and blocked HIV replication in vitro with IC_{50} values ranging from 0.0002 to 0.48 μM.[56] As it appears, our structure-based design effort led to the development of the bis-THF ligand as a nonpeptidic high affinity ligand for the HIV-1 protease substrate binding site. Indeed, the bis-THF is a subunit of ginkgolide B (shown in dotted box, Figure 9). Inhibitor **38** was selected for clinical development and renamed as darunavir. Clinical development of darunavir was carried out by Tibotec-Virco, Belgium.

To obtain molecular insight of the ligand-binding site interactions, we have determined the X-ray structure of **38**-bound wild-type HIV-1 protease in collaboration with Professor Irene Weber at Georgia State University.[57] The structure was refined to an *R* factor of 0.15 and R_{free} of 0.19 at a 1.3 Å resolution. The structure (Figure 10) shows strong hydrogen bonding of the bis-THF ring oxygens with the backbone NHs of Asp 29 and Asp 30 in the S2-site. There is a new polar interaction with the side chain carboxylate of Asp-30. In the S2′-site, hydrogen bonds are evident between the P2′-amine and the carbonyl oxygen and carboxylate of Asp 30′. Our subsequent X-ray studies with a **38**-bound mutant protease indicated that the critical hydrogen bonding pattern of both bis-THF and sulfonamide-amine is retained in the structures.[58] These maximized "backbone binding" interactions may be responsible for the potent activity of darunavir against multi-PI-resistant variants. This concept of "backbone binding" in the enzyme active site may play a key role to combat drug resistance.

Clinical Development of Darunavir

The full review of clinical studies is beyond the scope of the present article. Darunavir (DRV) exhibited superior pharmacokinetic properties when coadministered with low doses of ritonavir. The absolute oral bioavailability of a single 600 mg dose by itself and with coadministration with 100 mg of ritonavir BID was 37% and 82%, respectively.[59] In phase IIB clinical trials (Power 1 and Power 2), ritonavir-boosted DRV was administered to treatment-experienced patients. At week 48, 61% of patients in the DRV/r arm achieved a 90% reduction in viral loads, compared to only 15% patients in the control PI arm. Viral load reduction below 50 copies/mL was attained in 45% of patients as opposed to 10% of the control arm.[59] A nonrandomized open-label Power 3 trial with treatment-experienced patients was subsequently conducted to determine the long-term efficacy and safety of DRV/r 600/100 mg b.i.d.

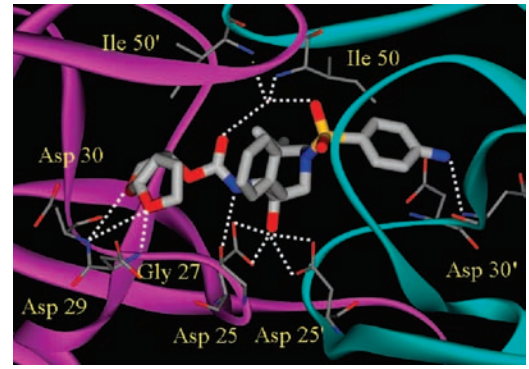


Figure 10. X-ray crystal structure of darunavir-bound HIV-1 protease.

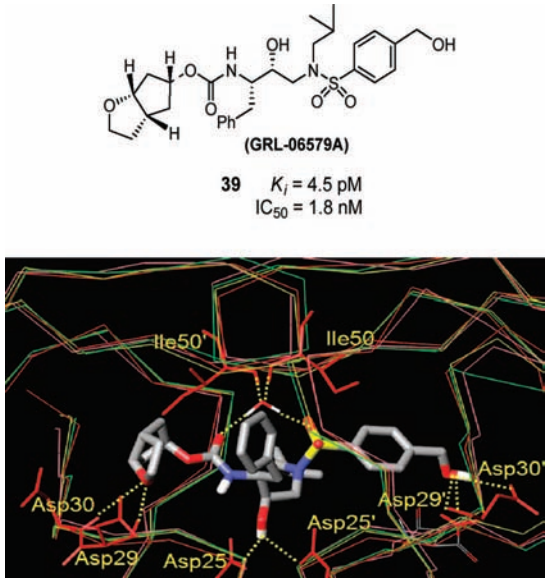


Figure 11. Inhibitor **39** bound to the active site of wild-type HIV-1 protease superimposed on the three most highly mutated drug-resistant proteases. The figure was first published by us in *J. Med. Chem.* **2006**, *49*, 5252−5261.

By week 24, reduction of HIV RNA with an efficacy end point of ≥1 log was observed in 65% of patients and reductions in HIV RNA levels to <400 copies/mL and <50 copies/mL were observed in 57% and 40% patients, respectively. Darunavir received an accelerated approval by the FDA on June 23, 2006, for patients harboring drug-resistant HIV. On October 22, 2008, darunavir received FDA approval for the treatment of all HIV/AIDS patients.[60]

Design of PIs Based on Enhancing "Backbone Binding": Concept to Combat Drug Resistance

Impressive clinical data and approval of darunavir for the treatment of drug-experienced patients demonstrated the effectiveness of structure-based design targeting the protein backbone as a strategy for combating drug resistance.[47,48] Our detailed X-ray crystallographic studies with Professor Irene Weber and Dr. Jordan Tang have provided strong support for our "backbone binding" design concept, as PIs with strong hydrogen bonding capabilities with the backbone atoms in the enzyme active site will be likely to retain these interactions with mutant proteases and thereby effectively maintain potency against multidrug-resistant HIV variants. Figure 11 shows our subsequent design of a number of very potent PIs with novel functionalities based on our "backbone binding" design concept.[48] High resolution X-ray structures of protein−ligand complexes of several PIs revealed extensive hydrogen bonding with the backbone atoms throughout the active site from S2 to S2′ sites. As it turns out, these PIs have shown impressive potency against a panel of multidrug-resistant HIV-1 variants.[61−64] The X-ray structure of Cp-THF derived inhibitor **39**-bound HIV-1 protease was compared with a number of known mutant proteases.[61] A least-sequence fit of the protease α-carbon atoms was carried out by collaborator Professor Eric Walters of Rosalind Franklin University. As can be seen in Figure 11, there is only a small change in the active site backbone position. All key hydrogen bonding interactions, particularly P2- and P2′-ligand interactions, are retained with the mutant proteases.[61] This may explain why inhibitor **39** has maintained marked potency against multidrug-resistant HIV-1 variants.[61] Inhibitor **40** (Figure 12) with a 1,3-dioxacycloheptan-5-ylurethane is very potent against multidrug-resistant variants. The protein−ligand X-ray structure of **40** revealed extensive hydrogen bonding with the protease backbone.[62] In addition, the P2 ligand forms a unique water-mediated interaction with the backbone NH of Gly 48. Inhibitor **41** with a *meso*-bicyclic 1,3-dioxolane as the P2-ligand is very effective against multidrug-resistant HIV-1 variants and appeared to have extensive hydrogen bonding to the backbone atoms in the S2-site.[63] We have designed a stereochemically defined methylpyrrolidinone as the P1′-ligand in inhibitor **42** to interact with the Gly 27′ backbone and Arg 8′-NHs. Interestingly, it has retained full potency against a range of multidrug-resistant HIV-1 variants.[64] X-ray studies and further design of novel PIs based on our "backbone binding" concept are currently underway.

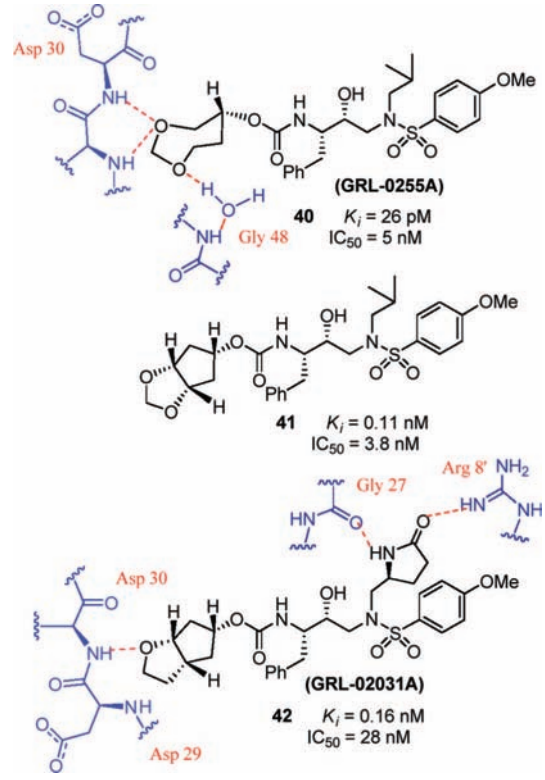


Figure 12. Structures of PIs **40**−**42**.

Design of Memapsin 2 (β-Secretase) Inhibitors for the Treatment of Alzheimer's Disease

In late 1998, we became involved in another very intriguing area of biomedical research, that is, the design and synthesis of inhibitors of memapsin 2 (β-secretase), potentially a disease modifying target for the treatment of Alzheimer's disease (AD). In an effort to generate "resistance proof" HIV-1 protease inhibitors, Jordan Tang and I collaborated on the structure-based design of two-isostere-based novel HIV-1 PIs.[65] This work, however, is beyond the scope of this presentation. Amidst our collaboration, Jordan Tang and Martin Citron independently discovered β-secretase in 1999.[66] Memapsin 2 (β-secretase) is one of two proteases that cleave the β-amyloid precursor protein (APP) to produce a 40−42 residues amyloid-β peptide (Aβ) in the human brain. Accumulation of Aβ results in the formation of amyloid plaques and neurofibrillary tangles. The neurotoxicity of Aβ is ultimately responsible for brain inflammation, neuronal death, dementia, and Alzheimer's disease.[67] Therapeutic inhibition of memapsin 2 has emerged as one of the most active areas

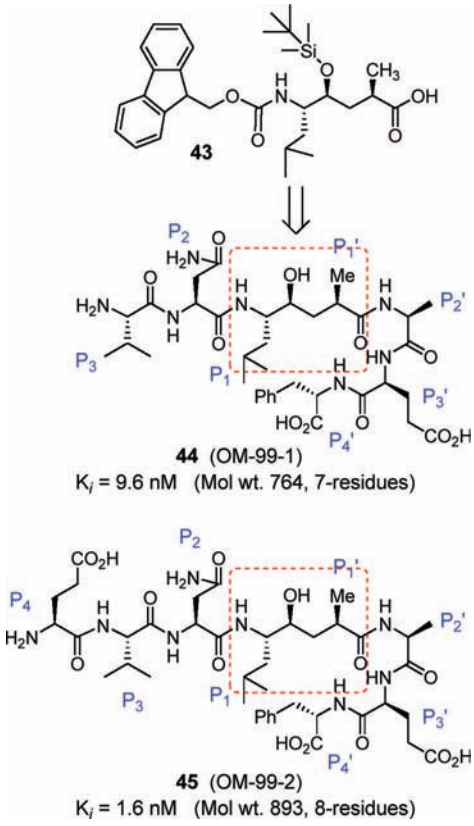


Figure 13. Structures of memapsin 2 inhibitors **43** and **44**.

of today's drug development for the intervention of AD. There are a number of compelling reasons for this. First, memapsin 2 cleavage of APP is the first step in the production of Aβ and inhibition of this step eliminates the subsequent cascade of events leading to AD pathogenesis. Second, memapsin 2 gene deletion in mice does not show serious phenotypic responses, indicating that a clinical application of a memapsin 2 inhibitor appears viable. Third, memapsin 2 is an aspartic protease, for which the inhibition mechanism and the design of transition-state analogues through the successful development of HIV-1 protease inhibitor drugs are well precedented.[68]

Design of the First Substrate-Based Inhibitors and Development of Drug-Design Templates

Immediately following the cloning of β-secretase in Jordan Tang's laboratory, we went on to design and synthesize a number of transition-state inhibitors utilizing β-secretase residue preference in eight subsites and utilizing a Leu-Ala hydroxyethylene isostere. Our strategy for memapsin 2 inhibitors initially focused on the design and synthesis of Leu-Ala dipeptide isostere **43** with appropriate protections of the amine with an Fmoc group and hydroxyl group of the isostere with a silyl group.[69] The Leu-Ala isostere so designed was used for the solid-state synthesis of a random sequence library containing seven- and eight-residue pseudopeptides. Two HPLC purified inhibitors were shown to be highly potent against recombinant memapsin 2 with K_i values of 6.8 nM (**44**, OM99-1) and 1.6 nM (**45**, OM99-2), respectively (Figure 13). This work represents the first case of designed potent inhibitors for this important pharmaceutical target related to Alzheimer's disease.[69] Subsequently, the crystal structure of the protease domain of human memapsin 2 complexed with inhibitor **45** at 1.9 Å resolution was determined in Jordan Tang's laboratory at the Oklahoma Medical Research Foundation.[70] This crystal structure (Figure 14) provided invaluable information regarding the specific ligand-binding site interactions in the active site of memapsin 2 and provided an excellent starting point for structure-based design of drug-like inhibitors.[71] On the basis of this X-ray crystal structure, we reduced the molecular weight and designed potent and selective memapsin inhibitors. Following this work, research and development of β-secretase inhibitors as possible drugs for AD have been intensified in both academic laboratories and the pharmaceutical industry.

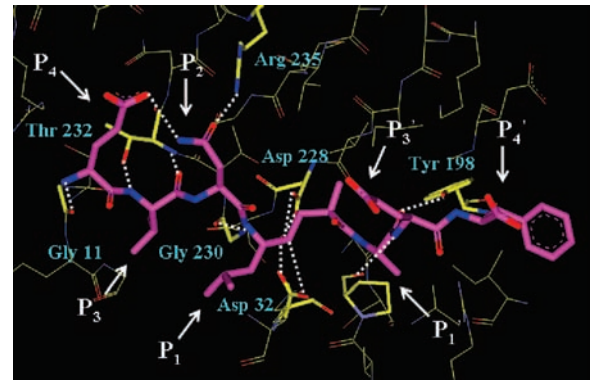


Figure 14. First X-ray structure of **45**-bound memapsin 2.

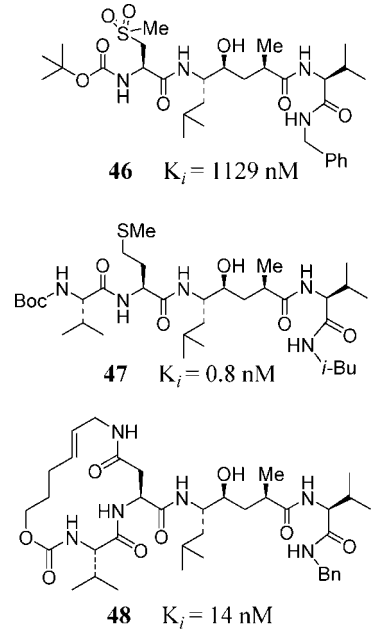


Figure 15. Structures of memapsin 2 inhbitors **46**−**48**.

Evolution of Potent, Selective, and Peptidomimetic Inhibitors

Our subsequent extensive structural modification aimed at the reduction of molecular size led to the discovery of potent peptidomimetic inhibitors. The X-ray structure of **45**-bound memapsin 2 (Figure 14) revealed that both P3′ and P4′ ligands extend beyond the protein surface; therefore, these ligands may not be necessary. The removal of the four outside residues, P4, P3, P3′, and P4′, however, resulted in a large reduction in potency. Inhibitor **46** (Figure 15), with optimized side chains, has shown a greater than 1000-fold higher K_i value compared to inhibitor **45**. Incorporation of a P3 Val and optimization of the P2-ligand improved potency significantly for inhibitor **47**, indicating that highly potent inhibitors can be developed with five subsites, from P3 to P2′, and with molecular sizes ranging from 600−700 Da.[72]

The X-ray structure of the protein−ligand complex with **45** revealed the presence of an interesting intramolecular hydrogen bond between the P2-asparagine carboxamide nitrogen and the P4-glutamic acid carbonyl.[70] On the basis of this molecular insight, we have designed a series of novel macrocyclic amide-urethanes linking the P2 and P4 side chains. We investigated the ring size and substituent effects. Acyclic inhibitors were less potent and cycloamide-urethanes containing a 16-membered ring exhibited low nanomolar inhibitory potency against memapsin 2, suggesting that the introduction of rings to constrain the backbone freedom may be pursued. A number of macrocyclic ligands were subsequently reported based on this structural motif.[73] Interestingly, inhibitor **48** and its analogues have shown some selectivity (2- to 4-fold) against memapsin 1. An X-ray structure of the corresponding saturated inhibitor revealed that both P2- and P3-carbonyls accept hydrogen bonding from the memapsin 2 backbone NHs. The interactions may explain the origin of a slight selectivity for **48** or related inhibitors compared to inhibitors **45** and **47**.

The selectivity of inhibitors over other human aspartic proteases may be very important, particularly against memapsin 1 and cathepsin D. Memapsin 1 has specificity similarity with memapsin 2 and appears to have an independent physiological function.[74] Cathepsin D is abundant in all cells and is involved in cellular protein catabolism.[75] The absence of selectivity would likely consume inhibitor drugs as well as lead to toxicity. As mentioned, our initial inhibitors, such as **45** and **47**, did not exhibit selectivity against memapsin 1 or cathepsin D. It seemed clear that a successful drug candidate must possess significant selectivity. Through our structure-based design efforts, we have designed very potent and highly selective β-secretase inhibitors.[76] As shown in Figure 16, inhibitor **49** was very potent against β-secretase and displayed 1185-fold selectivity over memapsin 1 and 435-fold selectivity over cathepsin D. The X-ray structure of **49**-bound memapsin 2 revealed the structural basis of its selectivity. Subsequently, we designed inhibitor **50**, which is exceedingly potent against β-secretase and remarkably selective over memapsin 1 (>3800-fold) and cathepsin D (>2500-fold).[76] As it turned out, both the P2-sulfone group and the P3-heterocycle in inhibitors **49** and **50** are involved in extensive hydrogen bonding with the Arg 235 and Thr 232 of the β-secretase active site and these interactions cannot be accommodated by cathepsin D or memapsin 1, thus exhibiting marked selectivity.[76] We mention here that while these inhibitors are potent and selective, their cellular inhibitory potency in Chinese hamster ovary cells was only in the low micromolar range (IC_{50} = 1.4 μM for **50**).

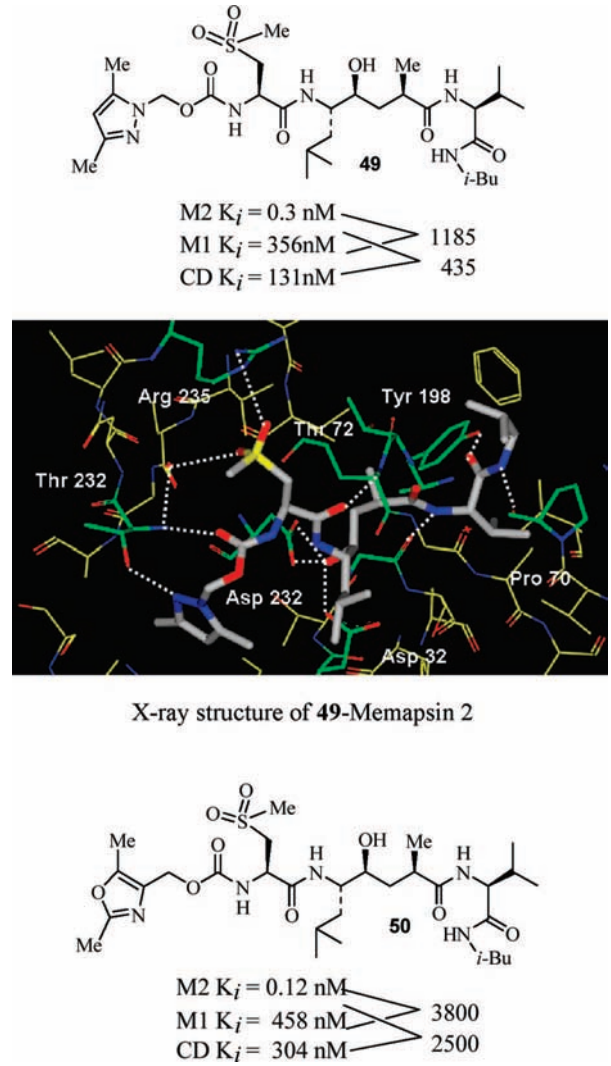
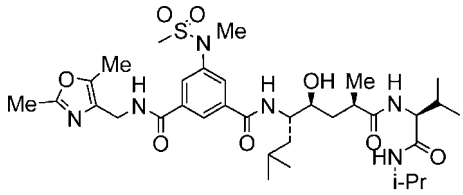


Figure 16. Structure-based design of selective memapsin 2 inhibitors.

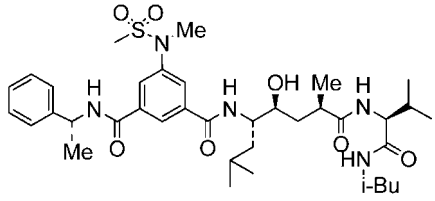
Evolution to Druglike Inhibitor Structures

Our research efforts then focused on further optimization of structural features, particularly, design of small molecule, non-peptide, drug-like inhibitors with improved cellular inhibitory and in vivo properties. Toward these objectives, we have explored a variety of isosteres, ligands (P2 and P2′), and scaffolds. Structural changes along this line gave rise to inhibitor **51** (Figure 17), which contains a substituted isophthalamide ring at P2 and an oxazolylmethyl at P3.[77] It has shown very good potency for memapsin 2 and good selectivity over memapsin 1 (>300-fold) and cathepsin D (>130-fold); however, its cellular IC_{50} was in the micromolar range. Inhibitor **52** with a (*S*)-α-methylbenzyl as the P3-ligand has displayed much improved cellular IC_{50} properties. Its molecular size is 648 Da, and it is quite potent (K_i = 1.1 nM for memapsin 2) and selective (K_i of 31 and 41 nM vs memapsin 1 and cathepsin D, respectively). It inhibited Aβ production in cultured cells with an IC_{50} of 39 nM. Furthermore, intraperitoneal administration of **52** in Tg2576 mice at 8 mg/kg effected a 30% reduction of plasma Aβ_{40} at 4 h after a single administration.[77] Of particular note, we and others have optimized and reported a variety of substituted isophthalamide-derived memapsin 2 inhibitors.[78]

We have investigated inhibitors incorporating a diverse range of hydroxyethylamine and other designed isosteres. As shown in Figure 18, inhibitors with a P1-leucine containing hydroxyethylamine isostere resulted in inhibitor **53** with a K_i value of 916 nM.[79] Incorporation of a P1-phenylmethyl side chain resulted in inhibitor **54** with a memapsin 2 K_i value of 1.8 nM and a remarkable cellular IC_{50} value of 1 nM. As we have seen in the hydroxyethylene isostere-derived inhibitor series, the choice of ligands and substituents is critical to inhibitor potency and selectivity and in vivo properties. This striking cellular activity of **54** is possibly due to its nice balance of liphophilic and basic amine properties. To obtain molecular insight, a protein−ligand structure of **54**-bound memapsin 2 was determined (Figure 19). As shown in Figure 18, the structure shows that both the hydroxyl group and the secondary amine group form a network of tight hydrogen bonding with the active site aspartic acid residues. Furthermore, P2-sulfonamide derivative fits well into the S2-site and makes extensive hydrogen bonds with memapsin 2.[79]

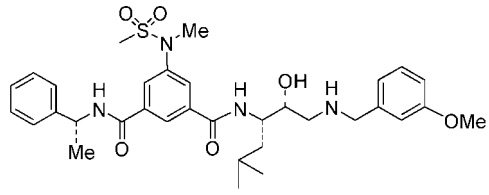


51 M2 K_i = 1.8 nM
M1 K_i = 556 nM
CD K_i = 245 nM
IC_{50} = 1.8 μM

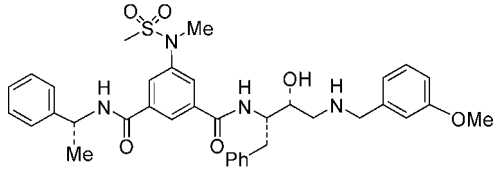


52 M2 K_i = 1.1 nM
M1 K_i = 31 nM
CD K_i = 41 nM
IC_{50} = 39 nM

Figure 17. Structures of memapsin 2 inhibitors **51** and **52**.



53 K_i = 916 nM



54 (GRL-8234)
K_i = 1.8 nM; IC_{50} = 1 nM

Figure 18. Stucture of memapsin 2 inhibitors **53** and **54**.

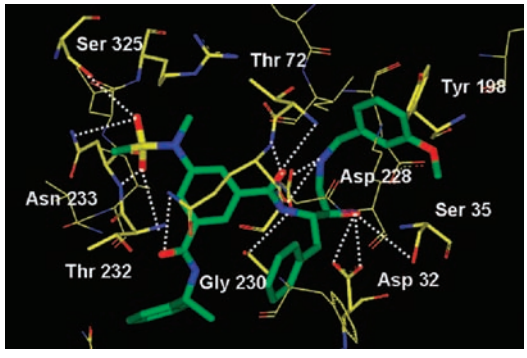


Figure 19. X-ray structure of **54**-bound memapsin 2.

Inhibitor **54** is selective over other aspartyl proteases (39-fold over memapsin 1 and 23-fold over cathepsin D). It has shown very impressive in vivo properties in transgenic mice. A single intraperitoneal administration of **54** to young Tg2576 mice at 8 mg/kg resulted in up to 65% reduction of $A\beta_{40}$ production after 3 h. Since $A\beta$ production in young Tg2576 mice occurs almost exclusively in the brain, this in vivo result suggests that at least part of the inhibition of $A\beta$ production in these mice is likely due to the inhibition of β-secretase in the brain by inhibitor **54**.[79] Our extensive structure-based design, synthesis, development of numerous in vivo markers and tools have provided the basis for further structural modification and optimization of a range of inhibitors with novel ligands and scaffolds. We have optimized a β-secretase inhibitor drug candidate CTS-21166 (**55**, structure has not yet been disclosed) in collaboration with Jordan Tang and CoMentis,[80] a biopharmaceutical company. This inhibitor has now completed a phase I clinical trial.[81]

Conclusions

Over the years, our longstanding interest in the chemistry and biology of natural products brought a unique perspective to our work in the area of biomedical research involving the power of organic synthesis. It all started as a seemingly academic pursuit in which we intended to design bioactive natural-product-derived scaffolds or ligands to mimic the biological mode of action of peptide bonds. This would provide a means to alleviate some of the inherent problems associated with peptide-based drugs. The concept of this strategy evolved from our deep interest in the biology of natural products as well as our awareness of possible implications to the improvement of therapy. The significant challenges presented by the stereochemical complexity of such design and the necessary multistep synthesis portrayed the strategy as a purely academic endeavor. Our in-depth work in the design of HIV-1 protease inhibitors began to demonstrate the practicality of this strategy. As we have mentioned, there continues to be major challenges with the currently approved therapy for HIV/AIDS. In an effort to address these challenges, our research objective was to design stereochemically defined cyclic ether or polyether-like templates to replace peptide bonds where an ether oxygen can mimic the biological action of a peptide carbonyl.

Our experience and accomplishments in the synthesis of many complex natural products provided the requisite synthetic power and motivation, which in turn also provided our seemingly unlimited design capability. Another important academic objective is to teach and train students in my laboratories. Students in my laboratory have brought tremendous motivation and determination to these synthetically challenging projects. We have examined many X-ray protein−ligand structures of HIV-1 protease and critically analyzed our design with respect to our molecular insight and structure−activity information. Our concept of targeting the backbone of HIV-1 protease emerged from our superimposition of wild-type and mutant protease X-ray structures. Darunavir thus evolved by combining our ligand-design concept, inspired by polyether natural products as well as our design concepts to target the protease-backbone to combat drug resistance. Our concept of maximizing "backbone binding" is a vital key and may well have important implications in its application to other systems. The combination of our in-depth antiviral studies, our collaborations with Dr. Hiroaki Mitsuya, and X-ray crystallographic studies with Professor Irene Weber has been extremely critical to further improve our design and synthesis. The design of darunavir, culminating in its FDA approval, has been a very gratifying experience. However, nothing is more gratifying than the knowledge that darunavir is making a positive difference in the lives of many who are living with HIV/AIDS.

Our involvement in the design and synthesis of memapsin 2 inhibitors for treatment of Alzheimer's disease grew out of our broad experience in structure-based design. We were in the right place at the right time. Because of our ongoing collaboration in the HIV-1 protease arena with Jordan Tang, we were perfectly positioned to follow up on the events of his successful cloning of memapsin 2 and the subsequent kinetic and specificity studies in his laboratory. Our success with HIV-1 protease inhibitor design and the available information from Jordan Tang's laboratory played an important role in our involvement and success in the area of memapsin 2 inhibitors work. We made rapid progress in designing the key Leu-Ala isostere which led to the first potent transition-state inhibitor and allowed us to gain important molecular insight into the ligand-binding site interactions. This led us to quickly develop one of the first small molecule peptidomimetic inhibitors. The advent of β-secretase projects in a number of pharmaceutical laboratories rapidly followed. Our subsequent transition to the design of clinical drugs met with many challenges. The design of selectivity over other aspartyl proteases is extremely important. Without a high degree of selectivity, problems such as severe toxicity and drug consumption are likely to emerge. The successful inhibitor must also possess the ability to cross the blood−brain barrier in order to exert its effects on the human brain memapsin 2. In addition, we must design inhibitors with good in vivo pharmacokinetic properties. We have made excellent progress in this area. My collaboration with Jordan Tang led to the formation of a startup company to further develop memapsin 2 inhibitor-based therapy. This has been a very interesting and gratifying experience, as we have taken an academic lead structure and developed clinical agents. Inhibitor **55** has completed phase I clinical trials with excellent results. This compound represents the first disease-modifying therapy for Alzheimer's disease and brings hope for improved treatment for this debilitating disorder. It has been personally very satisfying to teach and train students through this challenging work. We plan to continue to draw inspiration from nature as we enlist the power of organic synthesis to meet the challenges of today's medicine.

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Biography

Arun K. Ghosh received his B.S. and M.S. in Chemistry from the University of Calcutta and the Indian Institute of Technology at Kanpur, India. He obtained his Ph.D. from the University of Pittsburgh and pursued postdoctoral research at Harvard University. He was a Research Fellow at Merck Research Laboratories, West Point, PA. He became Assistant Professor at the University of Illinois, Chicago, in the fall of 1994, and rose to full Professor in 1998. From 2005 to present, he is a Professor in the Department of Chemistry and Department of Medicinal Chemistry at Purdue University.

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