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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 The 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 dolicolide (**9**)¹² and jasplakinolide (**10**)¹³; novel antibacterial agent platensimycin (**11**)¹⁴; and the 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 upon intermolecular and intramolecular metal chelation.¹⁶ Notable carbon-carbon bond forming synthetic methodologies include highly diastereoselective *syn*- and *anti*-aldol reactions¹⁷; asymmetric inter and intramolecular Diels-Alder and hetero Diels-Alder reactions¹⁸, 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 syntheses 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,

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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 taxol. 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 upon laulimalide and peloruside A. In another instance, using synthetic dolicolide, we have shown that dolicolide 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 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 product synthesis but have expanded to great significance.

Our exploration of the chemistry and biology of natural products brought a unique perspective to our research interest 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, 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. Based upon 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 has been focused on protein-ligand X-ray structure guided design, based upon molecular insights of 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.

Need for Conceptually Novel HIV-1 Protease Inhibitors

The advent of HIV protease inhibitors in late 1995 hailed 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 CD4⁺ 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 US 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-naïve individuals infected with HIV-1 had persistent viral replication despite early HAART, possibly due to the transmission of multi-drug-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 nonpeptidyl PIs and 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 Based upon 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 due to the presence of multiple amide/peptide-like bonds. Based upon 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 towards 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 an ionophore antibiotic monensin²⁹ (**13**, Figure 2) and a 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.³⁰ 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 to incorporate various cyclic ether structural features into our design of 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-quinoline amide carbonyl oxygen forms a hydrogen bond with the Asp 29 NH.³² Based upon this molecular insight, 3 (*R*)-tetrahydrofuran glycine was developed as a novel P₂-ligand for the asparagine side chain of saquinavir.³³ The corresponding inhibitor **16** 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-quinolonic 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*)-tetrahydrofuranyl urethane in the hydroxyethylene isostere-derived inhibitor **18** has shown incredible potency enhancing effect in both enzyme and antiviral assay.³⁴ In contrast, cyclopentyl urethane-derived inhibitor **19** has displayed a significant reduction of potency. Subsequently, researchers at Vertex Laboratories incorporated this 3(*S*)-tetrahydrofuranyl urethane in their structure-based designed (*R*)-hydroxyethyl sulfonamide isostere³⁵ and developed amprenavir³⁶ 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 THF ligand makes weak interactions with Asp 29 and Asp 30 NH's.³⁷

The detailed structure-activity studies, as well as 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-2H-thiopyran-carboxamide provided 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**.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. Based upon 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; $ID_{50} = 1.2$ μ M).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 towards 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*)-tetrahydrofuranyl urethane with the saquinavir-derived isostere.⁴⁰ Incorporation of 3(*S*)-sulfolane in the (*R*)-(hydroxyethyl)sulfonamide isostere with a *p*-methoxy sulfonamide 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 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. Based upon 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-2H-cyclopentafuranyl urethane 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*)-tetrahydrofuranyl urethane 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 in close enough proximity to hydrogen bond with both main chain NHs of aspartates Asp 29 and Asp 30 in the S_2 -subsite. Enzyme inhibitory activity of the corresponding inhibitor with a 3-hexahydro-2*H*-cyclopentafuranyl urethane 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** 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 nonpeptide 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. Based upon this insight, our design hypothesis was to maximize interaction in the active site of the protease with the backbone atoms. Particularly, we desired to design an inhibitor that makes extensive hydrogen bonds throughout the active-site protease backbone.^{47,48} Conceivably, such PIs would retain these hydrogen-bonding interactions with mutant protease and thereby maintain 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 bis-THF ligand in a number of other isosteres including the (*R*)-(hydroxyethyl)sulfonamide isostere with a *p*-methoxy sulfonamide 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*), 3*a*(*S*), 6*a*(*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 antiviral assay ($K_i = 16$ pM; $ID_{50} = 4.1$ nM). Inhibitor **36** with a 3(*S*), 3*a*(*S*), 7*a*(*S*)-hexahydrofuropranyl urethane 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

NH's 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, 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} were less than five, thus indicating a low level of resistance against **34** for enzymes with multiple mutations which 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 spectrum of mutant HIV variants with IC₅₀ values ranging from 0.3 to 0.5 nM. A detailed drug-sensitivity data 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, amprenavir-resistant virus is highly cross-resistant to all PIs except saquinavir. Furthermore, **34** was highly potent (IC₅₀ = 0.5 to 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 profiles.^{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 IC₅₀ values around 0.003 μM and IC₉₀ 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** block 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 protease dimerization at concentrations of 0.01 μM or less and blocked HIV replication in vitro with IC₅₀ values ranged 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). 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 in the combat against drug resistance.

Clinical Development of Darunavir

The full review of clinical studies is beyond the scope of the present article. Darunavir exhibited superior pharmacokinetic properties when co-administered with low doses of ritonavir. The absolute oral bioavailability of a single 600 mg dose by itself and with co-administration 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 non-randomized 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 BID. By week 24, reduction of HIV RNA with an efficacy endpoint 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 upon 'Backbone Binding' Concept to Combat of Drug-resistance

Impressive clinical data and approval of darunavir for the treatment of drug-experienced patients demonstrated the effectiveness of structure-based designs 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 multi-drug-resistant HIV-variants. Figure 11 shows our subsequent design of a number of very potent PIs with novel functionalities based upon our 'backbone binding' design concept.⁴⁸ High resolution X-ray structures of protein-ligand complexes of several PIs revealed extensive hydrogen bonding with 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 multi-drug 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 12, 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 multi-drug-resistant HIV-1 variants.⁶¹ Inhibitor **40** with a 1,3-dioxacycloheptan-5-yl urethane is very potent against multi-drug-resistant variants. The protein-ligand X-ray structure of **40** revealed extensive hydrogen bonding with the protease backbone.⁶² In addition, the P2 ligand form 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 multi-drug-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 methyl-pyrrolidinone as the P1'-

ligand in inhibitor **42** to interact with the Gly-27' backbone and Arg-8'-NH's. Interestingly, it has retained full potency against a range of multi-drug-resistant HIV-1 variants.⁶⁴ X-ray studies and further design of novel PIs based upon our 'backbone binding' concept are currently underway.

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 the 40–42 residues amyloid- β peptide (A β) in the human brain. Accumulation of A β results in the formation of amyloid plaques and neurofibrillary tangles. The neurotoxicity of A β 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 of today's drug development today 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 A β 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 analogs through the successful development of HIV-1 protease inhibitor drugs, are well preceded.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-sites 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 7 and 8-residues 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 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.⁷⁰ Based upon 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 both in academic laboratories and the pharmaceutical industry.

Evolution of Potent, Selective, and Peptidomimetic Inhibitors

Our subsequent extensive structural modification aimed at reduction of the molecular size led to the discovery of potent peptidomimetic inhibitors. The X-ray structure of **45**-bound memapsin 2 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 size ranging from 600–700 Da.⁷²

The X-ray structure of the protein-ligand complex revealed the presence of an interesting intramolecular hydrogen bond between the P2-asparagine carboxamide nitrogen and the P4-glutamic acid carbonyl.⁷⁰ Based upon 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 upon this structural motif.⁷³ Interestingly, inhibitor **48** and its analogs have shown some selectivity (2–4 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 1186-fold selectivity over memapsin 1 and 436-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 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 can not be accommodated by cathepsin D or memapsin 1, thus exhibiting marked selectivity.⁷⁶ We should mention here, while these inhibitors are potent and selective, their cellular inhibitory potency in Chinese hamster ovary cells were only in the low micromolar range (IC_{50} 1.4 μ M for **50**).

Evolution to Drug-like 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 IC₅₀ was in the micromolar range. Inhibitor **52** with a (*S*)- α -methyl benzyl as the P3-ligand has displayed much improved cellular IC₅₀ properties. Its molecular size is 648 Da and it is quite potent (K_i = 1.1 nM for memapsin 2) and selective (K_i values of 31 nM and 41 nM vs. memapsin 1 and cathepsin D, respectively). It inhibited A β production in cultured cells with an IC₅₀ of 39 nM. Furthermore, intraperitoneal administration of **52** in Tg2576 mice at 8 mg/kg effected a 30% reduction of plasma A β ₄₀ at 4 h after a single administration.⁷⁷ It should be noted that 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 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 IC₅₀ value of 1 nM. As we have seen in the hydroxyethylene isostere-derived inhibitor series, the choice of ligands and substituents are all critical to inhibitor potency and selectivity and *in vivo* properties. This striking cellular activity of **54** is possibly due to its nice balance of lipophilic and basic amine properties. To obtain molecular insight, a protein-ligand structure of **54**-bound memapsin 2 was determined. 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 bonding with memapsin 2.⁷⁹

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 β ₄₀ production after 3 h. Since A β 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 β 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 multi-step 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 concept 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 available information from Jordan Tang's laboratory played an important role in our involvement and success in the area of memapsin 2 inhibitor 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 at 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 of severe toxicity and drug consumption are likely. 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 BS and MS in chemistry from University of Calcutta and Indian Institute of Technology at Kanpur, India. He obtained his Ph.D. from the University of Pittsburgh and pursued post-doctoral research at Harvard University in Professor E. J. Corey's laboratories. He was a research fellow at Merck Research Laboratories, West Point, PA. He became assistant Professor at the University of Illinois, Chicago in Fall, 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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Figure 1.
Structures of Recent Bioactive Targets

Figure 2.
Structure of Monensin and Ginkgolide B

Figure 3.
Design of Cyclic-ethers as Novel P2-ligands

Figure 4.
Tetrahydrofuranyl Urethane-derived Potent PIs

21 $K_i = 1.4 \text{ nM}$
 $ID_{50} = 17 \text{ nM}$

22 (X = CH₂) $K_i = 3 \text{ nM}$
23 (X = O) $K_i = 4 \text{ nM}$

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

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

Figure 7.
Convenient Synthesis of (-)-bis-THF Ligand

$$K_i = 14 \text{ pM}$$
$$IC_{50} = 1.4 \text{ nM}$$

Figure 8.
Bis-THF and THF-THP-derived Potent Inhibitors

Figure 9.
Structures of Darunavir and GRL-98065

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

39 $K_i = 4.5 \text{ pM}$
 $IC_{50} = 1.8 \text{ nM}$

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

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

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

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

Figure 15.
Structures of Memapsin 2 inhibitors 46–48

X-ray structure of **49**-Memapsin 2

Figure 16.
Structure-based Design of Selective Memapsin 2 Inhibitors

51 M2 $K_i = 1.8$ nM
M1 $K_i = 556$ nM
CD $K_i = 245$ nM
 $IC_{50} = 1.8$ μ M

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 $K_i = 916 \text{ nM}$

54 (GRL-8234)

$K_i = 1.8 \text{ nM}$; $\text{IC}_{50} = 1 \text{ nM}$

Figure 18.
Structure of Memapsin 2 inhibitors **53** and **54**

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