Structure-Based Design: Potent Inhibitors of Human Brain Memapsin 2 (*β*-Secretase)

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Abstract: Memapsin 2 (*β*-secretase) is one of two proteases that cleave the *β*-amyloid precursor protein (APP) to produce the 40−42 residue amyloid-*β* peptide (A*β*) in the human brain, a key event in the progression of Alzheimer's disease. On the basis of the X-ray crystal structure of our lead inhibitor (**2**, OM99-2 with eight residues) bound to memapsin, we have reduced the molecular weight and designed potent memapsin inhibitors. Structure-based design and preliminary structure−activity studies have been presented.

Introduction. The proteolytic enzymes *β*- and *γ*-secretases that cleave the *β*-amyloid precursor protein (APP) to produce the 40−42 residue amyloid-*β* peptide (A*β*) in the human brain are key players in the progression of Alzheimer's disease (AD).[1] While the *γ*-secretase has not been conclusively identified, the recent discovery of *β*-secretase as an aspartyl protease marked the beginning of a significant effort for therapeutic intervention of AD.[2] The *β*-secretase cleaves an easily accessible site at the lumenal side of APP, and its activity is the rate-limiting step in A*β* production in vivo.[3] Recently, we and others cloned the human brain aspartyl protease memapsin 2 (also known as BACE and ASP-2) and demonstrated it to be the long sought *β*-secretase.[4] Memapsin 2 is an excellent target for inhibitor drugs since it occupies the initial step in the pathological cascade of AD. Thus, the in vivo inhibition of memapsin 2 may reduce the production of A*β* and thereby slow or halt the progression of AD. On the basis of knowledge of kinetics and specificity of memapsin 2, we initially designed two seven- and eight-residue peptidomimetic inhibitors incorporating a nonhydrolyzable Leu-Ala hydroxyethylene dipeptide isostere as depicted in Figure 1.[5] The inhibitor based on the sequence Glu-Val-Asn-Leu-Ψ-Ala-Ala-Glu-Phe, where Ψ denotes replacement of CONH by (*S*)-CH(OH)CH_2 (**2**, OM99-2), has exhibited a K_i value of 1.6 nM for human memapsin 2, and it is considerably more potent than inhibitor **1** (OM99-1, K_i of 36 nM). Subsequently, we determined the crystal structure of the protease domain of human memapsin 2 complexed to inhibitor **2** at 1.9 Å resolution.[6] This crystal structure provided invaluable information regarding the specific ligand-binding site interactions in the active site of memapsin **2**. The therapeutic potential of inhibitor **2** may be limited because of its high molecular weight (1100 Da) and numerous peptide bonds. However, the structure of **2** serves as an important molecular template for structure-based design of memapsin inhibitor drugs. Such strategies have been successfully utilized in the design and synthesis of inhibitors of other aspartyl proteases including HIV protease inhibitor drugs.[7] The ability of some HIV inhibitor drugs to cross the blood-brain barrier is particularly encouraging for development of memapsin 2 inhibitor drugs for treatment of AD.[8] Herein, we report preliminary results of our investigation in which X-ray structure-based modification of our lead inhibitors resulted in a series of potent memapsin inhibitors. The molecular size of these inhibitors is substantially reduced while maintaining comparable enzyme inhibitory potencies to **2**.

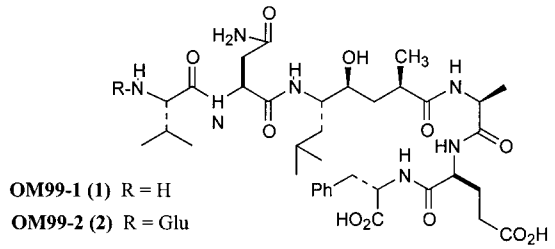

Figure 1.

Results and Discussion. Our reported X-ray crystal structure of **2** bound to memapsin 2 provided detailed information relating to specific interaction and orientation of the inhibitor in the active site.[6] In this structure, the inhibitor chain turns at P_2' and the side chains of the P_3'-P_4' residues are not involved in any specific interaction in the S_3'-S_4' sites of memapsin 2. The residues point toward the molecular surface of the protein, suggesting that the P_3'-P_4' residues could be shortened. Further inspection of the crystal structure revealed that the P_2'-carbonyl is involved in hydrogen bonding to the hydroxyl of Tyr-198, forming a rare kink at P_2'. The P_1-P_4 side chains in general are involved in significant interactions in the S_1-S_4 subsites. While P_1-Leu and P_3-Val fill in the hydrophobic pocket effectively, the P_2-Asn is involved in a hydrogen bond with Arg-235. The P_4-Glu site is hydrogen bonded to P_2-Asn, possibly assisting in the formation of the P_2-Asn hydrogen bond with Arg-235. Thus, it appeared that the P_2-ligand binding may be further optimized and the P_4-ligand may be eliminated or simplified. With this molecular insight into the ligand-binding site, we first elected to delete the P_3'-P_4' residues and simplify the ligand using a benzylamide to preserve the critical P_2'-carbonyl interaction and fill in the hydrophobic pocket in the S_3' region of the active site. Second, we planned to probe the new P_2-ligand with a view to delete the P_3 and P_4 residues and further shorten the inhibitor size.

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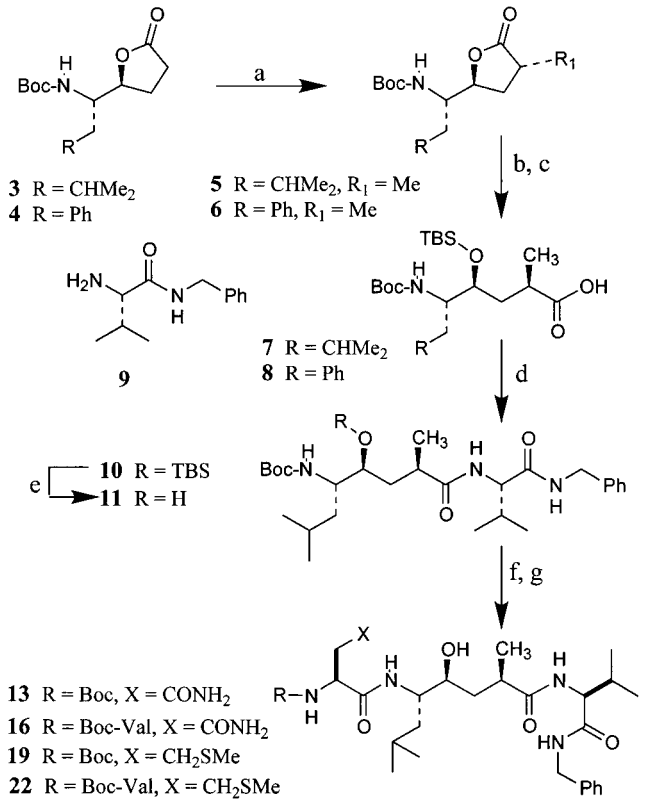
Scheme 1[a]



[a] (a) LiHMDS, MeI, THF, −78 °C, 30 min; (b) aq LiOH, THF−H_2O, 23 °C, 8 h; (c) TBDMSCl, imidazole, DMF, 24 h; (d) EDC, HOBt, amine **9**, *i*Pr_2NEt, DMF−CH_2Cl_2 (1:1), 0−23 °C; (e) *n*$Bu_4N^+F^-$, THF, 23 °C; (f) CF_3CO_2H, CH_2Cl_2, 23 °C; (g) EDC, HOBt, acid, *i*Pr_2NEt, DMF−CH_2Cl_2 (1:1), 0−23°C.

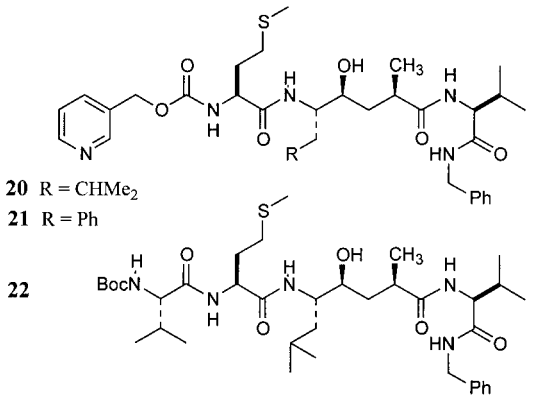
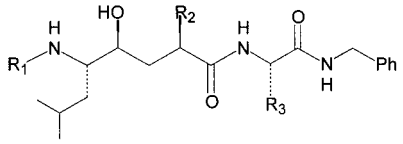
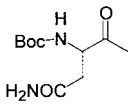
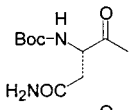
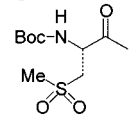
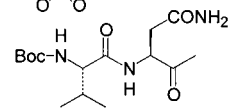
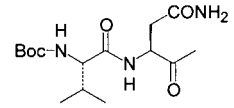
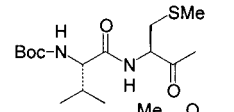
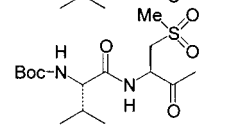
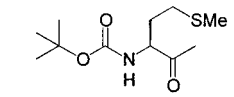
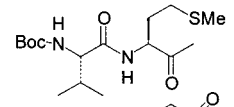
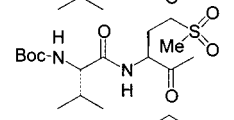
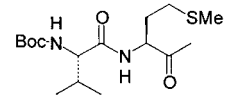


Figure 2.

Table 1. Structure and Potencies of Memapsin Inhibitors



Comp.	R_1	R_2	R_3	K_i (nM)
12.		Me	Me	22,423.0
13.		Me	$CHMe_2$	3134.0
14.		Me	$CHMe_2$	1129.0
15.		Me	Me	61.4
16.		Me	$CHMe_2$	5.9
17.		Me	$CHMe_2$	50.1
18.		Me	$CHMe_2$	9.4
19.		Me	$CHMe_2$	5808.0
22.		Me	$CHMe_2$	2.5
23.		Me	$CHMe_2$	8.0
24.		CH_2CHMe_2	$CHMe_2$	10,491.0

The general synthesis of various inhibitors containing the hydroxyethylene isostere[9] is outlined in Scheme 1. The known[10] γ-lactones **3** and **4** were alkylated stereoselectively by generation of the corresponding dianion with lithium hexamethyldisilazide (2.2 equiv) in tetrahydrofuran at −78 °C (30 min) followed by addition of methyl iodide (1.1 equiv) at −78 °C (30 min). The reaction was quenched with propionic acid (5 equiv) followed by standard workup and provided the desired alkylated lactones **5** and **6** (70−76% yield) along with a small amount (<5%) of the corresponding epimer.[11] The stereochemical assignment of the alkylated lactones was supported by ^1H NMR NOE experiments. Saponification of these lactones followed by protection of the γ-hydroxyl group with *tert*-butyldimethylsilyl chloride in the presence of imidazole and (dimethylamino)pyridine in dimethylformamide afforded the corresponding Leu-Ala **7** and Phe-Ala **8** isosteres (85−90% yield). These hydroxyethylene isosteres were converted to various inhibitors using a standard peptide coupling procedure. Thus, valine derivative **9** was reacted with dipeptide isostere **7** in the presence of *N*-ethyl-*N*′-(dimethylaminopropyl)carbodiimide hydrochloride, diisopropylethylamine, and 1-hydroxybenzotriazole hydrate in a mixture of DMF and CH_2Cl_2 to afford amide derivative **10**. Removal of the silyl protecting group by treatment with tetrabutyl ammonium fluoride in THF afforded memapsin inhibitor **11**. For synthesis of inhibitor **13**, Boc-derivative **11** was first exposed to trifluoroacetic acid (TFA) in CH_2Cl_2 to effect removal of the Boc group. Coupling

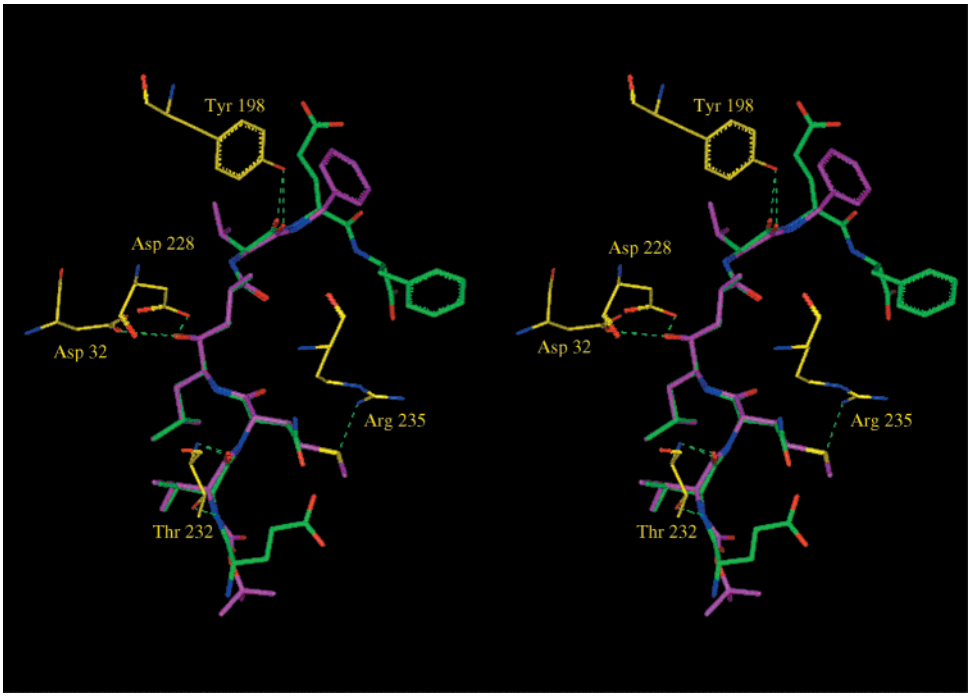


Figure 3. Energy minimized model structure of inhibitor **22** (magenta) overlayed with OM99-2 (green) bound X-ray crystal structure of memapsin 2.

of the resulting amine with Boc-asparagine provided inhibitor **13**. Treatment of **13** with TFA and subsequent coupling of the resulting amine with Boc-valine afforded inhibitor **16**. The synthesis of inhibitor **19** with P_2-methionine was carried out by exposure of **11** to TFA followed by coupling of the resulting amine with Boc-methionine. Removal of the Boc group in **19** and coupling of the resulting amine with Boc-valine provided inhibitor **22**. Oxidation of **22** with oxone in a mixture (1:1) of methanol and water at 23 °C for 12 h furnished inhibitor **23**. Other inhibitors in Table 1 were prepared by analogous procedure as described above. Inhibitor **20** with pyridylmethyl urethane was prepared by removal of the Boc group in **19** followed by coupling of the resulting amine with pyridylmethylsuccinimidyl carbonate in CH_2Cl_2 as described previously (Figure 2).[12] By following similar procedures, the corresponding inhibitor **21** with a phenylalanine side chain at P_1 was prepared from isostere **8**.

Structure and inhibitory potencies of various peptidomimetic inhibitors against recombinant memapsin 2 are shown in Table 1.[13] Our initial inhibitor target had been inhibitor **12** with Leu-Ala isostere, P_2-Asn, and P_2'-Ala-benzylamide; however, it is a weak inhibitor with a K_i of 22.4 μM against memapsin 2. Because the **2**-bound crystal structure revealed that the S_2'-site could possibly accommodate a side chain larger than P_2'-Ala, we have examined the effect of Val at P_2'. Indeed, the resulting inhibitor **13** has exhibited at least a 7-fold potency enhancement. As mentioned earlier, the P_2-Asn weakly hydrogen bonds to Arg-235 (bonding distance 3.86 Å). We therefore, incorporated a methyl sulfone derivative at P_2, assuming that one of the sulfone oxygens could conceivably hydrogen bond effectively with the Arg-235. Such hydrogen bonding capabilities of sulfone derivatives have been previously demonstrated.[14] The corresponding inhibitor **14** improved its K_i to 1.1 μM. However, it appeared that the extension of P_3-Val is necessary for further improvement of inhibitor potency.

Thus, we have appended the P_3-Val in **12**, and the resulting inhibitor **15** has shown a marked improvement in its potency to 61.4 nM (K_i value). Consistent with earlier observation, the corresponding P_2'-Val derivative **16** has exhibited K_i of 5.9 nM, a 10-fold potency enhancement over inhibitor **15**. Inhibitor with P_2-methyl sulfone derivative (inhibitor **18**) showed comparable memapsin inhibitory potency to inhibitor **16**, indicating that one of the sulfone oxygens may be involved in hydrogen bonding to Arg-235. This speculation is supported by the fact that the corresponding inhibitor with P_2-methylcysteine is much less potent than **18**. Molecular modeling studies support this possible hydrogen bonding interaction. Interestingly, incorporation of P_2-Met resulted in the potent inhibitor **22** (K_i 2.5 nM) with comparable inhibitory potency to **2** which has eight residues. Contrary to inhibitors **17** and **18**, oxidation of the methyl sulfide to sulfone derivative **23** resulted in a 3-fold attenuation in potency compared to **22**. To gain insight into specific ligand-binding site interactions, an energy minimized model structure of **22** was created in the active site of memapsin (the active site based on the crystal structure of memapsin bound to **2**). On the basis of this model, it seems that the methyl sulfide moiety of P_2-Met hydrogen bonds to Arg-235 in the S_2-region of the active site. This interaction may simply be a hydrophobic interaction as well. Generation of a crystal structure of this complex is currently being attempted in order to further understand this interaction. Furthermore, it appears that the P_2'-Val has substantial hydrophobic contact with the enzyme. The key hydrogen bonding interaction with Tyr-198 is also quite apparent as shown in Figure 3.[15] Preliminary structure−activity studies indicated that Leu-Ala isostere is preferred over Leu-Leu isostere (compound **24**) by the enzyme active site. Comparison of inhibitory

properties of compounds **20** (K_i 1.4 mM) and **21** (K_i 2.1 mM) also suggested preference for Leu-Ala over Phe-Ala isostere by the memapsin active site. Inhibitor **22** also potently inhibited memapsin 1, the closest homologous protease to memapsin 2,[4a] with a K_i value of 1.2 nM. Since memapsin 1 is also known to cleave β-amyloid precursor protein at the β-secretase site, this cross-inhibition is not entirely surprising.[16] Furthermore, it has been suggested that memapsin 1 may be involved in the Down's syndrome form of Alzheimer's disease.[17] If this is confirmed, the cross-inhibition of compound **22** to both enzymes may be of some medicinal importance.

Conclusion. Structure-based modification of our lead inhibitor (**2**) led to the discovery of a series of potent and considerably low molecular weight peptidomimetic memapsin 2 inhibitors. The P_3'-, P_4'-, and P_4-peptidic ligands in **2** have been deleted, and the P_2-ligand has been replaced with a designed ligand to allow effective hydrogen bonding with specific residues in the S_2-active site. The size of the modified inhibitors (molecular weight around 722) are comparable to a number of approved peptidomimetic HIV protease inhibitor drugs. Further investigation of peptidomimetic approaches including specific nonpeptidal ligand design will follow from these preliminary studies. Our efforts are now directed toward the incorporation of features in the inhibitor to address issues relating to cell penetration, pharmacokinetic properties, and crossing of the blood-brain barrier.

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Supporting Information Available: Experimental procedures for compounds **5**−**11**, **13**, **16**, **19**−**22** along with purity (HPLC) and mass spectroscopy data. This material is available free of charge via the Internet at http://pubs.acs.org.

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(13) Memapsin 2 inhibition was measured using recombinant enzyme produced from *E. coli* expression as described in ref 4a. A fluorogenic substrate Arg-Glu(EDANS)-Glu-Val-Asn-Leu-Asp-Ala-Glu-Phe-Lys (Dabcyl)-Arg was used with 0.47 μM of the enzyme in 0.1 M Na-acetate + 5% dimethylsuloxide, pH 4.5 at 37 °C. The excitation wavelength was 350 nm, and the emission wavelength was 490 nm. Details of this assay will be described shortly.

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(15) To understand the actual interaction in the active site, determination of crystal structure protein−ligand complex of **22** is in progress.

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