Communications to the Editor

Design of Potent Inhibitors for Human Brain Memapsin 2 (*β*-Secretase)

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The generation of the 40/42-residue amyloid *β* (A*β*) peptide in human brain by proteolysis of the membrane anchored *β*-amyloid precursor protein (APP) is a key event in the progression of Alzheimer's disease.[1] Proteases involved in the production of A*β* peptide are known as *γ*- and *β*-secretases. *β*-Secretase, which catalyzes the rate-limiting step in A*β* production, hydrolyzes an easily accessible site in the luminal side of APP, and is regarded as the major therapeutic target for the design of inhibitor drugs. Our laboratory recently cloned a human brain aspartic protease called memapsin 2, which we demonstrated to be the long sought *β*-secretase.[2] Several other laboratories also independently discovered the same enzyme.[3] The new knowledge on kinetics and specificity of memapsin 2[2] has enabled us to design and test two potent inhibitors for human memapsin 2.

The *β*-secretase site of APP (SEVKM/DAEFR) is hydrolyzed poorly (k_{cat}/K_m = 40 s^{-1} M^{-1}) by recombinant memapsin 2. However, the same site from the Swedish mutant APP (SEV**NL**/DAEFR) is an excellent substrate (k_{cat}/K_m = 2450 s^{-1} M^{-1}).[2] Thus, for the initial design of memapsin 2 inhibitors, we utilized the template of the *β*-secretase site of Swedish APP with a change of P_1' Asp to Ala. Not only does the specificity at the P_1' site indicate that Ala is a highly preferred residue,[2] such a change also reduces polarity and increases lipophilicity of the inhibitor, factors important for blood−brain barrier penetration.[4] The peptide bond between P_1 and P_1' sites in the inhibitors is replaced by a hydroxyethylene transition-state isostere, which is a highly effective transition-state analogue for the inhibition of aspartic proteases.[5] The structures of two of the initially designed inhibitors, OM99-1 (**1**) and OM99-2 (**2**), are shown in Figure 1.

The strategy for the synthesis of these inhibitors is to first synthesize Leu*Ala dipeptide isostere (the asterisk represents hydroxyethylene isostere) which was then used in solid-state peptide synthesis[6] of the inhibitors. The synthesis of Leu*Ala is outlined in Scheme 1. Commercial Boc-leucine was converted to Weinreb amide **3** by treatment with isobutyl chloroformate and *N*-methylpiperidine followed by treatment of the resulting mixed anhydride with *N*,*O*-dimethylhydroxylamine.[7] Reduction of **3** with lithium aluminum hydride in diethyl ether provided the aldehyde **4** which was reacted with lithium propiolate derived from the treatment of ethyl propiolate and lithium diisopropylamide to

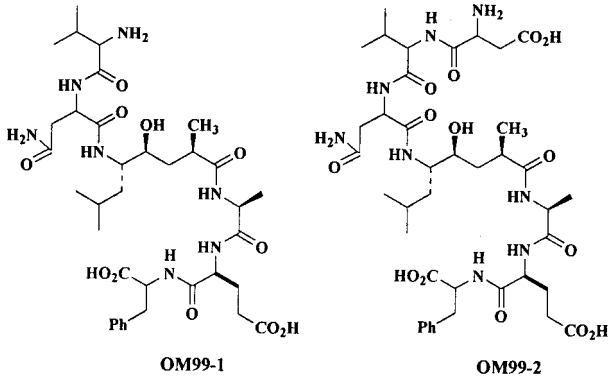
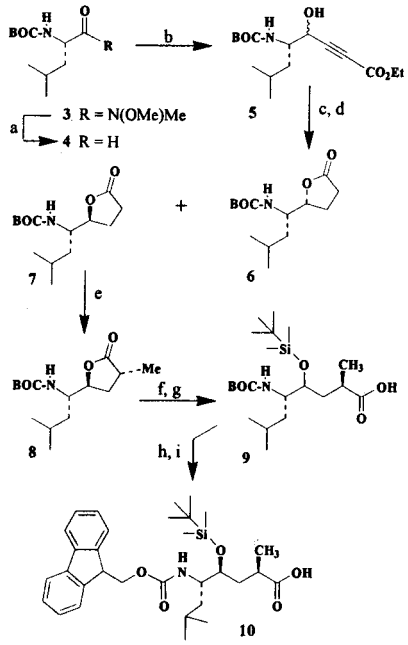


Figure 1. Structures of OM99-1 (Val-Asn-Leu*Ala-Ala-Glu-Phe) and OM99-2 (Glu-Val-Asn-Leu*Ala-Ala-Glu-Phe) inhibitors. The asterisk in the sequence designates the hydroxyethylene transition-state isostere.

Scheme 1[a]



[a] Reagents and conditions: (a) $LiAlH_4$, Et_2O, −40 °C, 30 min (86%); (b) LDA, HC≡C−CO_2Et, THF, −78 °C, 30 min, then 4, −78 °C, 1 h (42%); (c) H_2, Pd−$BaSO_4$, EtOAc; (d) AcOH, PhMe, reflux, 6 h (74%); (e) LiHMDS, MeI, THF, −78 °C, 20 min (76%); (f) aqueous LiOH, THF−H_2O, 23 °C, 10 h; (g) TBDMSCl, imidazole, DMF, 24 h (90%); (h) CF_3CO_2H, CH_2Cl_2, 0 °C, 1.5 h; (i) Fmoc-OSu, aqueous $NaHCO_3$, dioxane, 23 °C, 8 h (61%).

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(5) (a) Marciniszyn, J., Jr.; Hartsuck, J. A.; Tang, J. *J. Biol. Chem.* **1976**, *251*, 7088−7094. (b) Vacca, J. P. *Methods Enzymol*. **1994**, *241*, 311−334. (c) Greenlee, W. J. *Med. Res. Rev.* **1990**, *10*, 173−236.

(6) The solid-state peptide synthesis of OM99-1 and OM99−2 was carried out at the Molecular Biology Resource Center on the campus of the University of Oklahoma Health Science Center.

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furnish the acetylenic alcohol **5** as an inseparable mixture of diastereomers (5.8:1) in 42% isolated yield.[8] Catalytic hydrogenation of **5** over $Pd/BaSO_4$ followed by acid-catalyzed lactonization of the resulting γ-hydroxy ester with a catalytic amount of acetic acid in toluene at reflux provided the γ-lactones **6** and **7** in 74% yield. The isomers were separated by silica gel chromatography by using 40% ethyl acetate in hexane as the eluent. For introduction of the methyl group at C-2, the lactone **7** was alkylated with methyl iodide stereoselectively. Thus, generation of the dianion of lactone **7** with lithium hexamethyldisilazide (2.2 equiv) in tetrahydrofuran at −78 °C (30 min) and alkylation with methyl iodide (1.1 equiv) for 30 min at −78 °C, followed by quenching with propionic acid (5 equiv), provided the desired alkylated lactone **8** (76% yield) along with a small amount (<5%) of the corresponding epimer.[9] The stereochemical assignment of alkylated lactone **8** was made based on extensive ^{1}H NMR NOE experiments.

Aqueous lithium hydroxide promoted hydrolysis of the lactone **8** followed by protection of the γ-hydroxyl group with *tert*-butyldimethylsilyl chloride in the presence of imidazole and (dimethylamino)pyridine in dimethylformamide afforded the acid **9** in 90% yield. Selective removal of the BOC-group was affected by treatment with trifluoroacetic acid in dichloromethane at 0 °C for 1 h. Treatment of the resulting amine salt with commercial Fmoc-succinimide derivative in dioxane in the presence of aqueous $NaHCO_3$ provided the Fmoc-protected L*A isostere **10** in 65% yield after chromatography. Protected isostere **10** was utilized in the solid-state peptide synthesis of inhibitors **1** and **2**. In this synthesis, **10** was inserted in a coupling step as for other amino acid residues. After the last residue coupling step, the peptide was cleaved from the solid-state resin using 95% trifluoroacetic acid, which also removed all the side-chain protecting groups including the silyl group of **10**. Inhibitors OM99-1 and OM99-2 were separately purified in reverse-phase HPLC and the mass was verified by mass spectrometry.

The purified OM99-1 and OM99-2 were tested for inhibition of recombinant human memapsin 2 prepared from *E. coli* expression.[2] Figure 2 shows the inhibition profile of OM99-1 and OM99-2 against memapsin 2.[10] Both of these inhibition curves are characteristic of tight-binding inhibitors. The K_i values calculated[11] were 6.84×10^{-8} M $\pm$ 2.72×10^{-9} M for OM99-1 and 9.58×10^{-9} M $\pm$ 2.86×10^{-10} M for OM99-2.

The above results demonstrated that highly potent inhibitors of human memapsin 2 have been designed and synthesized from the current available specificity information. Since memapsin 2 cut APP to produce the Aβ peptide involved in Alzheimer's disease, its inhibition is of significance pharmacologically. Pepstatin A has been shown to be a weak inhibitor of memapsin 2.[2] One of the two synthetic decatetrapeptides with statine at the C-terminus was reported to inhibit β-secretase with an IC_{50} near 30 nM.[3c] The pharmaceutical potential of having the transition-state isostere (statine) at the C-terminus remains to be seen. Therefore, the inhibitors reported here represent the first designed potent inhibitors for memapsin 2. To evolve the current compound into a new generation of memapsin 2 inhibitors with drug potentials will require a reduction of molecular size and optimization of lipophilicity to penetrate the blood−brain barrier. We have also in preliminary studies considered the selectivity question. The most serious selectivity demand for memapsin 2 inhibitors is to be distinguished from human intracellular aspartic proteases such as cathepsin D. The K_i of cathepsin D against OM99-2 is 48 nM, about 5-fold higher than that for memapsin 2. It is expected that as the molecular sizes are reduced in the new generation of inhibitors, the selectivity will be enhenced since the subsites P_1, P_2, P_1', and P_2' are the most important specificity determinants in aspartic proteases. It is also noteworthy that memapsin 2 inhibitor drugs may not need to reach the potency of the HIV protease inhibitors (<1 nM), which is required to completely abolish HIV replication. Since memapsin 2 may have important physiological functions unrelated to APP hydrolysis,[2] potency at which some of these functions are preserved may be a desirable feature of inhibitor design. Further investigations aimed at improvement of potency, reduction of molecular size, and structure-based design of specific ligands are underway in our laboratories.

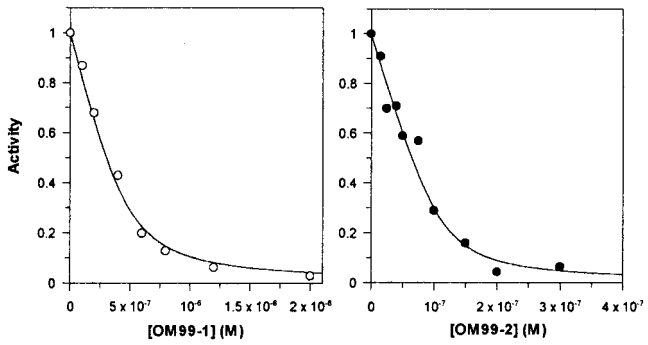

Figure 2. Inhibition of memapsin 2 activity toward fluorogenic substrate 10. K_i values are described in the text.

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Supporting Information Available: Experimental procedures for the synthesis of Leu-Ala dipeptide isostere and solid-phase synthesis of OM99-1 and OM99-2 (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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(10) Memapsin 2 inhibition was measured using recombinant enzyme produced from *E. coli* expression as described in ref 2. 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% dimethyl sulfoxide, 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 elsewhere.

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