Specificity of Memapsin 1 and Its Implications on the Design of Memapsin 2 (β-Secretase) Inhibitor Selectivity†

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ABSTRACT: Memapsin 1 is closely homologous to memapsin 2 (BACE), or β-secretase, whose action on β-amyloid precursor protein (APP) leads to the production of β-amyloid (Aβ**)** peptide and the progression of Alzheimer's disease. Memapsin 2 is a current target for the development of inhibitor drugs to treat Alzheimer's disease. Although memapsin 1 hydrolyzes the β-secretase site of APP, it is not significantly present in the brain, and no direct evidence links it to Alzheimer's disease. We report here the residue specificity of eight memapsin 1 subsites. In substrate positions P_4, P_3, P_2, P_1, P_1', P_2', P_3', and P_4', the most preferred residues are Glu, Leu, Asn, Phe, Met, Ile, Phe, and Trp, respectively, while the second preferred residues are Gln, Ile, Asp, Leu, Leu, Val, Trp, and Phe, respectively. Other less preferred residues can also be accommodated in these subsites of memapsin 1. Despite the broad specificity, these residue preferences are strikingly similar to those of human memapsin 2 [Turner et al. (2001) *Biochemistry 40*, 10001−10006] and thus pose a serious problem to the design of differentially selective inhibitors capable of inhibiting memapsin 2. This difficulty was confirmed by the finding that several potent memapsin 2 inhibitors effectively inhibited memapsin 1 as well. Several possible approaches to overcome this problem are discussed.

The accumulation of β-amyloid peptide (Aβ)[1] in the brain is a central event leading to Alzheimer's disease (*1*). Aβ is a proteolytic fragment of a membrane protein, β-amyloid precursor protein (APP), generated in vivo by two proteases. APP is first cut by β-secretase in its lumenal domain, and the resulting C-terminal fragment is further cut by γ-secretase to release Aβ. Since all forms of familial and sporadic Alzheimer's disease are linked to the accumulation of Aβ in the brain, drug intervention of Aβ production appears to be a logical strategy for clinical treatment of Alzheimer's disease. The pivotal role of β-secretase and its early involvement in the cascade of Alzheimer's disease pathogenesis make it an attractive target for the development of inhibitory drugs.

β-Secretase has been cloned and shown to be an aspartic protease, which we named memapsin 2 (*2*). The enzyme is also known as BACE (*3*), ASP-2 (*4*, *5*), and the "β-site APP-cleaving enzyme" (*6*). Memapsin 2 is a membrane-anchored protease (*2*−*6*) with a broad substrate specificity (*2*). Several aspects of this enzyme important to inhibitor drug design have already been studied. Substrates suitable for kinetic studies of memapsin 2 have been developed (*7*), and the preference for residues of all eight subsites has been kinetically defined (*7*, *8*). Potent transition-state inhibitors with K_i values in the nanomolar range have been designed and studied (*9*, *10*). A crystal structure of the memapsin 2 catalytic domain complexed with an inhibitor has revealed the location of subsites for the binding of substrate or inhibitor side chains (*11*).

Human memapsin 1 (*2*), also known as ASP-1 (*12*) or BACE 2 (*13*), is the closest homologue of memapsin 2, with 50% residue identity. The physiological function of memapsin 1 is unknown. Although memapsin 1 is not significantly present in the brain, its ability to cleave the β-secretase site of APP (*12*, *14*) has prompted the suggestion that it may play a role in Alzheimer's disease (*14*). The memapsin 1 gene is situated on chromosome 21, which also contains the APP gene (*13*). The proximity of these two genes and the fact that trisomy 21 leads to Down's Syndrome and an early onset of Alzheimer's disease suggest a possible role of memapsin 1 in this disease. The knowledge on the specificity of memapsin 1 would provide an in-depth comparison with the activity of memapsin 2 and insight to its biological and pathological roles. It would also assist the design of selectivity into memapsin 2 inhibitors.

An overall homology with memapsin 2 would predict that the active-site of memapsin 1 also accommodates eight residues. To determine the residue preferences of all eight subsites using the conventional kinetic approach is very laborious. We have developed methods to rapidly determine

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[1] Abbreviations: APP, β-amyloid precursor protein; Aβ, β-amyloid peptide.

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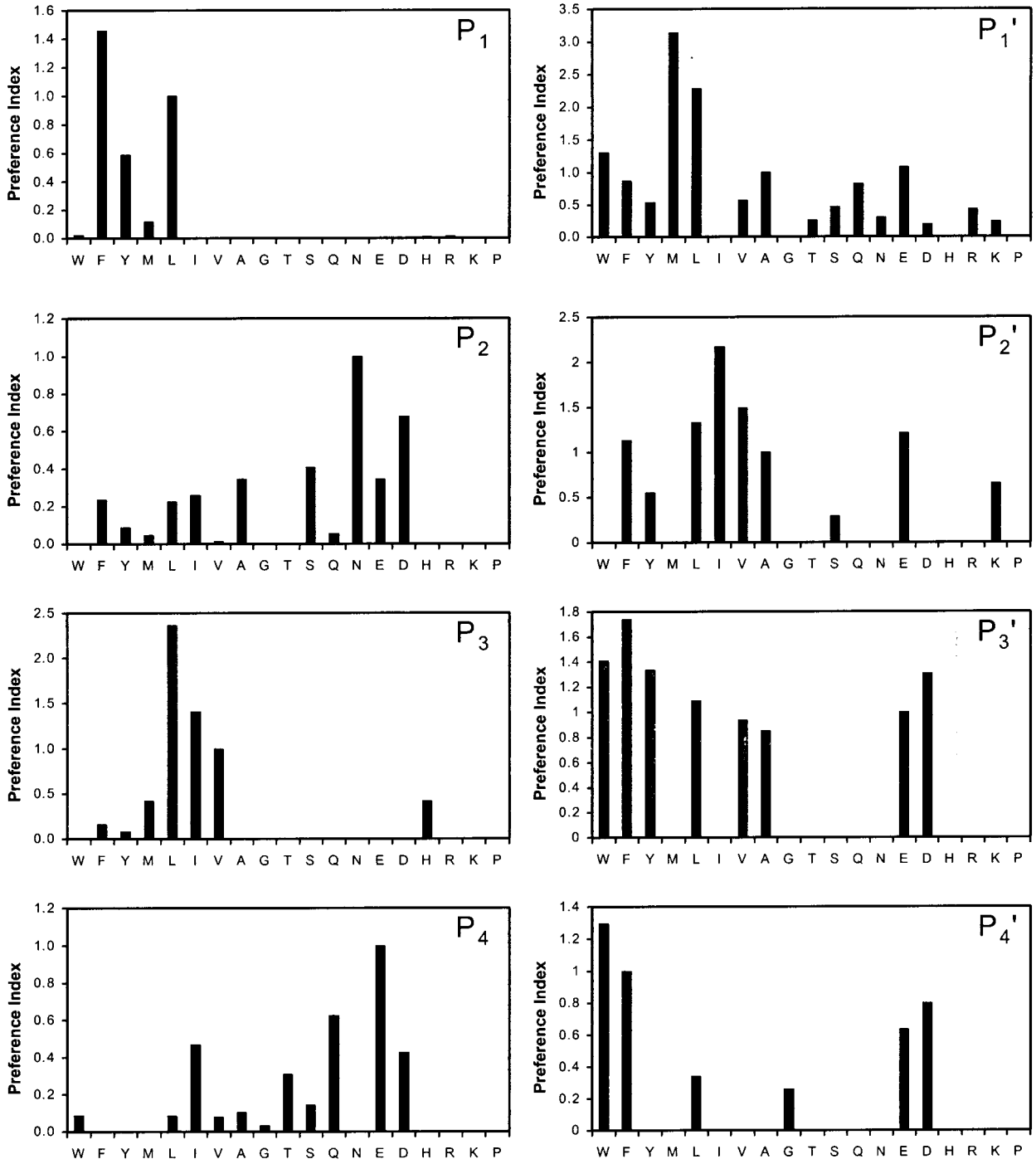


FIGURE 1: Preference of amino acids in the eight subsites of memapsin 1 substrates. The preference index was calculated from the relative initial hydrolytic rates of mixed substrates and is proportional to the relative k_{cat}/K_m. Amino acids (single-letter code) appear in the substrate template sequence at the position designated in each panel (P_n or P_n').

the complete subsite preferences of memapsin 2 using mixed substrate kinetics and combinatorial inhibitor library screening (*8*). The application of these methods to memapsin 1 has led to the observation that specificity of the two memapsins are indeed very similar and the inhibitors designed for memapsin 2 significantly cross-inhibit memapsin 1. These observations indicate that new strategies for the design of selective memapsin 2 inhibitors would be needed.

MATERIALS AND METHODS

Expression and Purification of the Catalytic Domain of Memapsin 1. The protease domain of memapsin 1 was expressed in *Escherichia coli* using essentially the same vector construct and procedure as those previously described for memapsin 2 (*2*). The recombinant protease domain starts at Ala^{21} and ends at Pro^{366} (Figure 1 of ref *2*) and cloned into vector pET11. For the convenience of discussion, the produced pro-memapsin 1 and memapsin 1 protease domains will be referred to as pro-memapsin 1 and memapsin 1, respectively. The expressed pro-memapsin 1 inclusion bodies were recovered and washed as previously described (*15*), dissolved in 8M urea, 10mM β-mercaptoethanol, 0.1mM oxidized glutathione, 1mM reduced glutathione, and refolded by dilution into 20-fold volume of 20mM Tris base, 10%

glycerol. The recombinant pro-memapsin 1 was further purified by Sephacryl S-300 and ResourceQ columns as previously described (*15*). Pro-memapsin 1 was converted to memapsin 1 spontaneously (*16*) by acidification to pH 4 with an equal volume of 0.1 M citrate/phosphate buffer for a duration of 30 min.

Determination of Relative k_{cat}/K_m of Substrates in Defined Substrate Mixtures. The relative initial hydrolytic rate of individual peptides in a mixture of substrates was determined. Since these relative rates are proportional to their k_{cat}/K_m values, they are taken as residue preferences when the substrates in the mixture differ only by one residue. The design of substrate mixtures and the condition of experiments are essentially the same as previously described (*8*). Since memapsin 1 hydrolyzes some of the memapsin 2 cleavage sites (*12*, *14*), the substrate mixture successfully used for studying subsite specificity of memapsin 2 (template sequence EVNLAAEF, ref *8*) was adopted for this study. Each substrate mixture contained seven peptides differing by one amino acid at a single position. At each substrate position, the full set of 19 varied amino acids (less cysteine) was accommodated in three substrate mixtures. Each substrate mixture contained a common substrate to serve as an internal standard for normalization of relative initial rates and the calculation of the k_{cat}/K_m values of the other substrates. For the four P′ positions, the template sequence was extended by four residues downstream of the eight APP residues (EVNLAAEFWHDR) to facilitate the detection in MALDI-TOF MS. Likewise, four additional residues were added upstream of the APP residues to characterize the four P positions (RWHHEVNLAAEF). The procedure and conditions for kinetic experiments were the same as those used for memapsin 2 studies (*8*). The amounts of substrate and hydrolytic products were quantitatively determined using MALDI-TOF mass spectrometry as described (*8*). The relative k_{cat}/K_m values are reported as the "preference index".

Probing Random Sequence Inhibitor Library. The combinatorial inhibitor library was based on the sequence of OM99-2:EVNLψAAEF, where letters represent amino acids in single letter code and ψ represents a hydroxyethylene transition-state isostere, as previously described (*9*). Four substrate positions, P_2, P_3, P_2', and P_3', incorporated random amino acids (less cysteine). Positions P_1 and P_1' were fixed due to the use of diisostere LeuψAla derivative in a single step of solid-phase peptide synthesis of inhibitors (*9*). By using the "split-synthesis" procedure (*17*), each of the resin beads contained peptides of only one sequence, while the sequence was random between different beads. The overall library sequence was Gly−Xx1−Xx2−LeuψAla−Xx3−Xx4−Phe−Arg−Met−Gly−Gly−[Resin bead]. The immunochemical procedure for probing the binding of memapsin 1 to the combinatorial library and the sequence determination of the inhibitors were essentially the same as previously described (*8*). Affinity purified polyclonal antibodies against memapsin 2 were used since we had shown that the two memapsins had considerable immunochemical cross recognition (data not shown).

Inhibitor Synthesis. Inhibitors GT-1831 (Asn−Val−Met−LeuψAla−Ala−Ile−Phe) and GT-1832 (Glu−Glu−Asn−LeuψAla−Met−Glu−Phe) were synthesized as described previously (*8*, *9*) by SynPep (Dublin, California).

Table 1: Amino Acid Residues[a] Preferred by the Subsites of Memapsins 1 and 2

	Memapsin 1			Memapsin 2[b]		
position	best	2nd	3rd and others	best	2nd	3rd and others
P_1	F	L	Y	L	F	M, Y, T
P_2	N	D	S, A, E	D	N	M, F, Y, S
P_3	L	I	V	I	V	L, E, H
P_4	E	Q	D, I	E	Q	D, N, G
P_1'	M	L	W, E, A, F	M	E	Q, A, D, S
P_2'	I	V	L, E, F, A, K	V	I	T, L, F, M, Y
P_3'	F	W, Y, D	L, V, A, E	L, W, V	I, T	D, E
P_4'	W	F	D, E, L	D, E	W	F, Y, M

[a] Amino acid residues are shown in one-letter code. [b] Memapsin 2 data taken from Turner et al. (*8*).

Table 2: Observed Residues from Binding of Memapsin 1 to Combinatorial Inhibitor Library[a]

bead no.	P_3	P_2	P_2'	P_3'
1	Leu	Asp	Val	Met
2	Leu	ND[b]	Ala	Leu
3	Leu	Glu	Val	Gln
4	Leu	Asp	Val	Trp
5	Ile	Asp	Val	Val
6	Ile	Phe	Val	Glu
7	Ile	Asp	Val	Asn
8	Ile	Asn	Val	Leu
9	Leu	Asp	Val	Lys
10	Leu	Asp	Val	Thr
11	Leu	Glu	Val	Trp
12	Leu	Gln	Val	Ile
13	Leu	Asn	Val	Glu
14	Leu	Asp	Val	Leu
consensus	Leu > Ile	Asp > Asn/Glu	Val	none
negative controls[c]	Gly	Phe	Gly	Gln
	Asp	Leu	Pro	Val

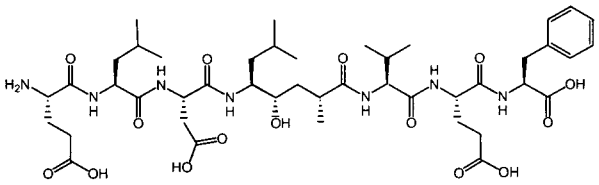
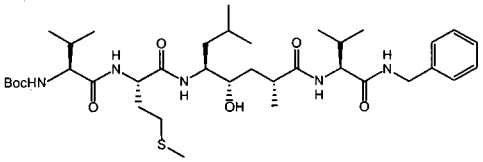
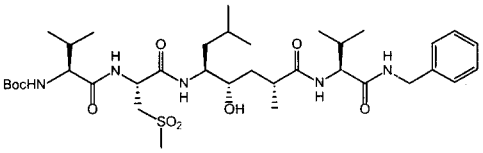
[a] Library template: Gly−P_3−P_2−LeuψAla−P_2'−P_3'−Phe−Arg−Met−Gly−Gly resin. [b] ND, not determinable. [c] Negative controls are randomly selected beads with no memapsin 1 binding capacity.

Determination of Kinetic Parameters. The K_i values of free inhibitors against memapsins 1 and 2 were determined as previously described (*7*).

RESULTS AND DISCUSSION

Determination of Side-chain Preference in Memapsin 1 Subsites. The relative hydrolytic preference of memapsin 1 at all eight positions of the substrate is presented in Figure 1. As in the case of memapsin 2 (*8*), multiple residues can be accommodated in each of the subsites. The subsites on the P side are in general more stringent in specificity than those in the P′ side. P_1 is by far the most stringent position, where only three residues, Phe, Leu, and Tyr, are strongly preferred. All other subsites allowed more residues (Figure 1). The most preferred residues are summarized in Table 1 alongside those of memapsin 2 (*8*). Farzan et al. (*14*) reported that memapsin 1 hydrolyzes APP preferentially at two bonds following phenylalanine residues in the sequence −Lys−Leu−Val−Phe−Phe−Ala−Glu−Asp−. Cleavage at either site conforms with our specificity data, especially where the most favored residue at P_1 was Phe, and the other residues were highly preferred at P_2, P_1', P_2' and P_3'. However, P_2, P_4, and one of the P_4' residues were clearly unfavorable (Figure 1). The discrepancy may be due to the fact that we measured only the initial hydrolysis rates, whereas in the

Table 3: Potency of Memapsin 2 Inhibitors against Memapsin 1

Inhibitor	Structure[a]	Memapsin 2 K_i (nM)	Memapsin 1 K_i^{app} (nM)[b]
OM00-3		0.13[c]	0.18
GT-1017		2.5[d]	1.2
GT-1026		9.4[d]	44.7

[a] Boc, *tert*-butoxycarbonyl. [b] Apparent K_i measured at [S] = 3 μM. [c] Ref *8*. [d] Ref *10*.

Table 4: Selectivity of Memapsin 2 and Memapsin 1 against Inhibitors

inhibitor	structure[a]	Memapsin 2	Memapsin 1
		IC_{90} (μM)	
GT-1831	*Asn*−Val−*Met*−LeuψAla−Ala−*Ile*−Phe	800	65
GT-1832	Glu−*Glu*−Asn−LeuψAla−*Met*−Glu−Phe	1600	55

[a] Residues unfavorable for memapsin 1 (Figure 1) are shown in italics. The ψ symbol denotes hydroxyethylene transition-state isostere.

Farzan study of APP hydrolysis (*14*), the time of hydrolysis was much longer. These observations do suggest that memapsin 1 can tolerate substrates with some amino acid residues unfavorable for hydrolysis.

To substantiate the substrate specificity findings for memapsin 1, we employed a combinatorial inhibitor library to ascertain amino acid residue preferences in the context of an inhibitor with a transition-state isostere moiety (*8*). Immunochemical screening of memapsin 1 bound to the library produced about 30 darkly stained beads. The sequences of 14 of the darkest produced consensus residues in three of the four randomized positions: P_3, Leu > Ile; P_2, Asp > Asn/Glu; P_2' Val (Table 2). Subsite preference at the P_3' position did not produce a clear consensus. Leu and Trp and Glu, which appeared more than once here, were also preferred in the substrate hydrolysis determinations (Figure 1). However, other residues unfavorable for substrates are also present. The lack of consensus at the P_3' position in the inhibitor library (Table 2) differs with memapsin 2 kinetic results which clearly prefers Glu and Gln (*8*). This discrepancy suggests that given the templates used, the nature of the P_3' residue is more important for effective substrate hydrolysis than for inhibitor binding.

Comparison of Subsite Preferences for Memapsin 1 and Memapsin 2. The overall substrate specificity of memapsin 1 subsites is strikingly similar to that for memapsin 2 (*8*). As shown in Table 1, the top subsite preferences are either identical (for P_4) or differ only in the order of preference (for P_1, P_2, P_3, and P_2'). The two memapsins do differ in residue preferences at the least specific P_3' and P_4' sites. The close similarity in consensus inhibitor residues at positions P_3, P_2, and P_2' are also seen for the inhibitor library (Table 2). In contrast to the preference of Glu and Gln in the memapsin 2 subsite S_3', memapsin 1 failed to show a preference in this subsite. This indifference, consistent with the lack of substrate residue preference, suggests that the P_3' side chain interacts poorly with memapsin 1 S_3' site. Poor binding of both P_3' and P_4' has also been observed for the binding of inhibitor OM99-2 to memapsin 2 (*11*).

Implications on the Design of Selectivity for Memapsin 2 Inhibiitors. The involvement of memapsin 2 in the pathogenesis of the Alzheimer's disease provides an opportunity to develop inhibitors for clinical treatment of the disease. The difference in tissue distribution of memapsins 1 and 2 suggests that they have independent physiological functions. Recent reports on the lack of phenotype in mice devoid of the memapsin 2 gene (*18*−*20*) suggests that the inhibition of its activity may be physiologically tolerated. No gene deletion study on memapsin 1 has been reported thus far. There is, however, no direct evidence linking memapsin 1

to Alzheimer's disease. Thus, the design of highly selective memapsin 2 inhibitory drugs without significant cross inhibition of memapsin 1 seems desirable. The closeness in subsite specificity of the two proteases described above poses a severe challenge to the design of high selectivity in memapsin 2 inhibitory drugs. This is illustrated by the observation that memapsin 1 was inhibited almost as well by three memapsin 2 inhibitors we have produced: OM00-3 (*8*) and inhibitors GT-1017 and GT-1026 (compounds 22 and 18, respectively, in ref *10*) (Table 3). In view of the side chains present in these inhibitors, we could see little overall difference in the preference indexes between memapsin 1 (Figure 1) and memapsin 2 (*8*). However, inhibitor GT-1026, which exhibited nearly 5-fold selectivity for memapsin 2, possesses a dimethyl sulfone side chain in the P_2 position. This observation suggests that more success in inhibitor selectivity may be achieved by exploring the use of non-natural side chains.

We have explored the specificity differences to attempt to design selective transition-state analogue inhibitors by incorporating residues that were strongly unfavorable for memapsin 1 substrates (Figure 1) but still moderately favorable for memapsin 2. Such residues include P_4 Asn, P_3 Glu, P_2 Met, P_2' Met, and P_3' Ile. Two inhibitors, GT-1831 and GT-1832, which contained three and two residues unfavorable to memapsin 1, respectively, were found to actually inhibit memapsin 1 better than memapsin 2 (Table 4). Although the combination of optimal residues at each subsite resulted in the most active substrate for memapsin 2 (*8*), the presence of a few residues unfavorable for memapsin 1 was, however, not sufficient to induce inhibitor selectivity for memapsin 2. One possible explanation of the disparity is that the relative preferences measured here are the ratio of k_{cat}/K_m while transition-state inhibition relates primarily to k_{cat}. Therefore, the use of residues unfavorable to memapsin 1 due to high K_m values would not help to create selectivity. However, measuring steady-state kinetic parameters to examine this possibility is precluded by the fact that these individual substrates are poorly hydrolyzed by memapsin 1 (Figure 1). It is also possible that multiple unfavorable residues may have a compensatory binding effect, depending upon the substrate side chain structure binding to other subsites. Such an intersubsite influence has been observed in HIV protease (*21*). Preliminary inspection of a binding-site model of memapsin 1 based on the memapsin 2 structure (*11*), however, reveal little apparent reason for the enhanced binding of GT-1831 and GT-1832 to memapsin 1 (results not shown). The current observations suggest that the use of residues unfavorable for memapsin 1 for designing inhibitor selectivity may be assisted by further information on kinetics of substrates for both memapsins and the structure of memapsin 1- inhibitor complexes.

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