Discovery and X-ray Crystallographic Analysis of a Spiropiperidine Iminohydantoin Inhibitor of β-Secretase‡

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Abstract: A high-throughput screen at 100 μM inhibitor concentration for the BACE-1 enzyme revealed a novel spiropiperidine iminohydantoin aspartyl protease inhibitor template. An X-ray cocrystal structure with BACE-1 revealed a novel mode of binding whereby the inhibitor interacts with the catalytic aspartates via bridging water molecules. Using the crystal structure as a guide, potent compounds with good brain penetration were designed.

Treatments for Alzheimer's disease, especially those that can slow or reverse neurodegeneration, represent a significant unmet medical need. Incidence of the disease increases with age and is thus growing in prevalence due to the aging population. As a consequence, Alzheimer's disease is forecasted to have a significant increased healthcare burden.[1] Abnormal production and/or clearance of a small peptide termed Aβ has been implicated in the pathophysiology of Alzheimer's disease from genetic and other studies, and agents that modulate Aβ production represent an attractive strategy in the search for novel therapeutic agents.[2] The Aβ peptide arises from processing of the amyloid precursor protein (APP[a]) by several proteases, first by β-secretase followed by γ-secretase. On the basis of these observations, β-secretase (BACE-1) is a promising drug target for disease-modifying therapy and has attracted significant attention from the medicinal chemistry community.[3]

BACE-1 is a challenging target because it is an aspartyl protease with a site of action inside the central nervous system. Experience from other aspartyl protease drug targets such as renin and HIV protease led to transition-state isosteres as first-generation inhibitors.[4] Despite good potency, these initial compounds had poor brain penetration and there are few reports of central Aβ lowering following peripheral dosing of compounds.[5] Given the high value of this target, many approaches to finding new leads with better brain-penetration prospects have been investigated. Modification of the traditional transition-state isosteres has proved successful in several instances, and fragment-based approaches are now beginning to bear new leads.[6] High-throughput screening (HTS) of large collections of diverse compounds is often successful at identifying leads for medicinal chemistry programs; however, for practical reasons, the screens are often done at 10 μM or lower concentration of test compound, thereby missing weak hits. Given the low hit-rate of our initial BACE-1 HTS screen, a second screen was performed at higher test compound concentration (100 μM) whereby the substrate was immobilized to allow for extensive washing to minimize artifacts.[7]

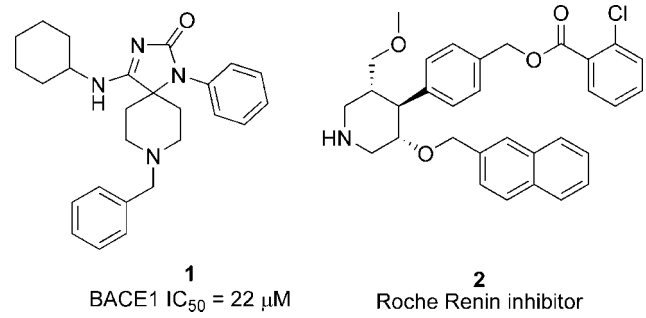
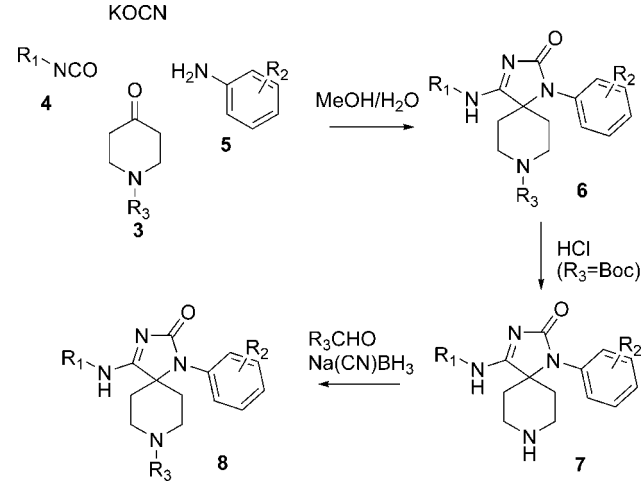


Figure 1. Piperidine aspartyl protease inhibitors

Scheme 1. General Synthesis of Inhibitors



From this effort, compound **1** emerged as a weak BACE-1 inhibitor with a potency of 22 μM in the isolated enzyme assay but inactive in a cell-based assay (Figure 1).[8] Despite the weak activity, this spiropiperidine structure looked promising as a lead. The overall molecular scaffold is quite different than traditional transition-state isosteres and more similar to piperidine-based renin inhibitors (such as **2**) discovered by Roche more than a decade ago.[9] The binding mode of **1** was not clear from docking the compound into known crystal structures of BACE-1 or models generated from renin complexed with **2**. Furthermore, attempts to soak or cocrystallize the compound with BACE-1 were unsuccessful, thus it was deemed that a more potent and soluble compound would be needed.

‡ A PDB file for the BACE-1/inhibitor **16** complex (PDB identifier 3E3W) has been deposited with the Protein Data Bank.

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[a] Abbreviations: APP, amyloid precursor protein; BACE-1, β-site amyloid precursor protein cleaving enzyme or β-secretase; HTS, high-throughput screening; PGP, P-glycoprotein.

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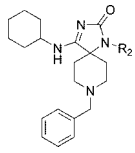
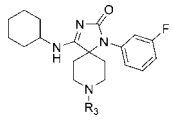
Table 1. *N*-Aryl Variations



no.	R_2	BACE-1 IC_{50} (μM) [a]	sAPPβ (μM) [b]
9	H	>100	NT
10	$CH_2CH_2CH(CH_3)_3$	>100	NT
11	benzyl	>100	NT
12	3-fluorophenyl	11 ± 0.98	32 [c]
13	4-acetamidophenyl	8 ± 0.22	21 ± 8.7

[a] IC_{50} determinations were performed as described in ref 8. [b] Cell-based assay as described in ref 8. NT = not tested. [c] Average of two determinations.

Table 2. Piperidine Modifications



no.	R_3	BACE-1 IC_{50} (μM) [a]	sAPPβ (μM) [b]	PGP[d]
14	H	>100	NT	NT
12	benzyl	11 ± 0.98	32 [c]	3.4
15	4-fluorophenyl	>100	NT	NT
16	4-acetamidobenzyl	2.8 ± 0.12	8.2 ± 1.7	19

[a] IC_{50} determinations were performed as described in ref 8. [b] Cell-based assay as described in ref 8. NT = not tested. [c] Average of two determinations. [d] B:A/A:B ratio in MDR1 transfected cells (see ref 16).

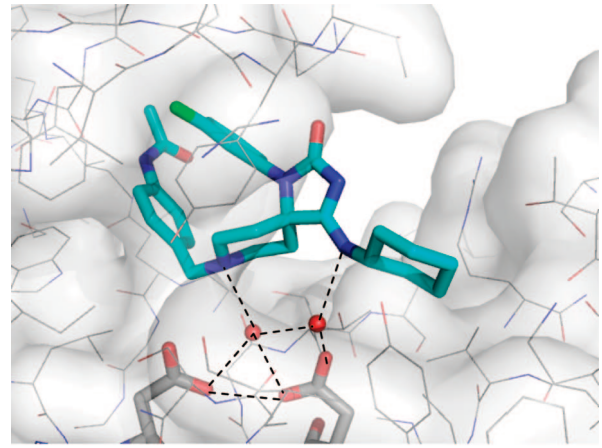
Synthesis of these compounds can be achieved by an Ugi four component coupling with piperidone **3**, an isonitrile **4**, amines **5**, and KOCN in modest yield as shown in Scheme 1.[10] Use of the Boc-protected piperidone **3** allows for subsequent Boc removal and alkylation and acylation of the piperidine to give inhibitors **9**−**18**.

Initial studies with a variety of commercially available isonitriles to explore the exocyclic amine substituent revealed that the original cyclohexyl was superior to benzyl or smaller alkyl groups (data not shown) and thus the cyclohexyl was used for subsequent studies.

Significant variations of the *N*-aryl substituent were not tolerated as shown in Table 1.[8] Alkyl and benzyl were inactive; however, some substitutions of the phenyl resulted in improved potency such as 3-fluoro and 4-acetamido. Importantly, these changes brought measurable cell-based activity and the shift between enzyme potency and cell-based activity was only 3-fold. With other scaffolds, large discrepancies between enzyme potency and cell-based activity are common, and the results in Table 1 gave further encouragement to pursue this lead.

Using the potency-enhancing 3-fluorophenyl, changes to the piperidine alkyl group were examined as shown in Table 2. Deviations from the original benzyl substitution resulted in a loss in potency, and acylation also diminished activity. Extensive empirical exploration of substituted benzyl groups failed to deliver significant gains in potency; however, the 4-acetamido substituted benzyl compound **16** was one of the few that offered an improvement in solubility without compromising activity, and cell-based activity was now 8.2 μM.

This combination of potency and solubility allowed for successful determination of the X-ray cocrystal structure of **16** with the BACE-1 enzyme at 1.8 Å resolution,[11] revealing a new mode of aspartyl protease inhibition. Proteases in this class use two interacting aspartic acid residues to activate a water molecule that attacks the scissile amide bond.[12] Most classes



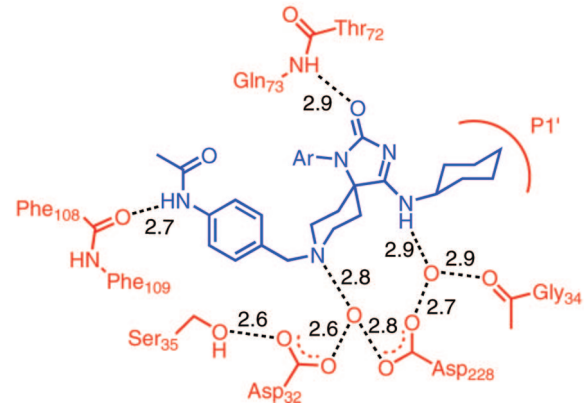


Figure 2. Two views of the key hydrogen bond interactions between **16** and BACE-1, a three-dimensional view of the protein with the catalytic aspartates and bridging water molecules highlighted, and a two-dimensional representation. Heavy-atom distances are shown in Å.

of aspartyl protease inhibitors displace the catalytic water molecule upon binding,[13] although there are recent reports of a novel inhibitor from these laboratories[14] and a weak fragment[15] interacting with BACE-1 via a water molecule with the catalytic aspartates.

The most notable feature of the **16**−BACE-1 complex is the bridging water molecules between the catalytic aspartates and the basic piperidine and exocyclic NH of the inhibitor (Figure 2), in contrast to the Roche renin inhibitor **2**, where the piperidine interacts directly with the aspartates. This unanticipated mode of binding for **16** explains the lack of success with initial modeling using previously defined structures and defines a new mode of aspartyl protease inhibition. The catalytic water molecule bridges the aspartates to the basic nitrogen of the piperidine. A second water is within hydrogen-bonding distance of Asp228, Gly34, and the exocyclic nitrogen of the inhibitor. Because the structure is at 1.8 Å resolution, the locations of the hydrogen atoms cannot be specified. The exocyclic NH is likely a hydrogen bond donor because pK_a measurements of **16** determined by potentiometric titration indicate pK_a values of 2.41 for the iminohydantoin and 6.79 for the piperidine.

As a result of the intervening water molecule, the inhibitor sits higher in the active site and the flexible hairpin loop of residues from Val69 to Trp76 called the "flap" cannot fully close down as it does in traditional structures (Figure 3). There is a hydrogen bond formed between the iminohydantoin carbonyl and the flap backbone NH of Gln73, and the raised flap allows the Tyr71 side chain to shift down into the traditional P1 pocket and form new hydrogen bonds with the structural water network

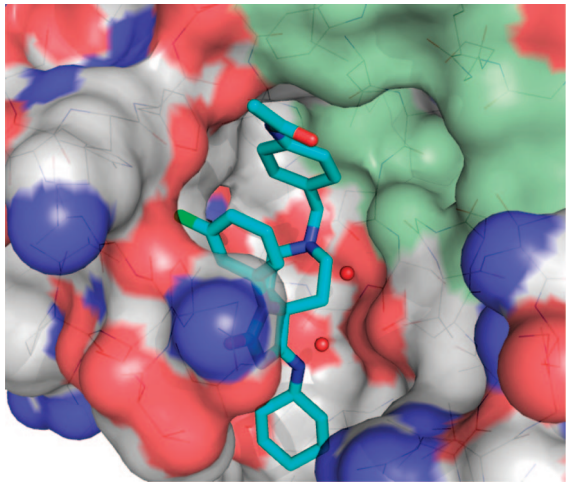
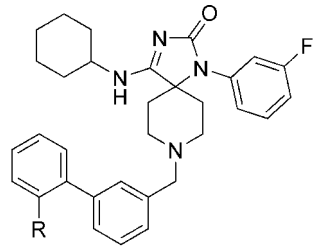


Figure 3. View of the X-ray structure of inhibitor **16** in the BACE-1 active site. The S3 site of the enzyme is highlighted in green.



17 R= H BACE1 IC_{50} = 4.6 **± 0.41** μM
18 R=Me BACE1IC_{50} = 0.11 **± 0.04** μM
sAPPβ IC_{50}= 5.2 **± 2.4** μM

Figure 4. Designed BACE-1 inhibitors.

rather than Trp76. This Tyr71 motion and the raised flap forms a new pocket just under the flap, and the fluorophenyl ring of the inhibitor tucks into this space, thereby explaining the tight SAR in this region. The cyclohexyl group fills the S1′ pocket, and the acetamido NH makes a hydrogen bond to the Phe108 backbone carbonyl. Most notably, **16** does not fill either the S2 or S3 sites of the enzyme.

Further profiling of **16** revealed no selectivity for BACE-2 (IC_{50} = 1.5 μM) but excellent selectivity versus other aspartyl proteases (IC_{50} > 100 μM versus renin and cathepsin D). Despite the basic nitrogen, significant hERG binding was not observed (IC_{50} > 10 μM).

Given the promising cellular potency and overall selectivity versus other enzymes, further optimization was undertaken with focus toward occupying the S2 or S3 site of BACE-1. Traditionally, appropriate substituents in these sites contribute significantly to potency; therefore, initial efforts were aimed at growing the molecule in these directions.

We chose to focus on filling the S3 site by substituting the benzyl group. Despite the extra hydrogen bond interaction with the backbone carbonyl of Phe108, the 4-acetamido group did not give a significant boost in potency (**16** vs **12**, Table 2) and was considered a liability in terms of brain penetration as compound **16** showed a high P-glycoprotein (PGP) transport ratio (B:A/A:B ratio of 19) with low permeability.[16] Therefore, this substituent was removed and S3 access was attempted from the 3-position of the benzyl ring. While an unsubstituted 3-phenyl group did not offer any benefit on its own (compound **17**, Figure 4), it served as a good scaffold to orient groups in the S3 pocket from the meta position. Addition of a methyl group (Figure 4, compound **18**) resulted in a 10-fold increase in BACE-1 potency. This can be rationalized based on the X-ray structure of compound **16** in that a model of the *ortho*-methyl substituted phenyl group demonstrated that it more effectively filled the S3 pocket compared to the unsubstituted phenyl compound **17**. Despite the modest potency in the cell-based assay, this was a key compound because it was not a PGP substrate (B:A/A:B transport ratio 1.8) and had good brain levels (brain concentration 1.7 μM, brain/plasma ratio 0.44) after a 30 mg/kg ip dose to mice. After a 100 mg/kg dose of **18** to transgenic mice expressing human APP under the control of a yeast artificial chromosome vector,[17] a statistically nonsignificant reduction in brain Aβ40 of 8% was observed. Plasma and brain levels of **18** were high at 16 and 14 uM, respectively; however, when adjusted for mouse plasma protein binding (9% unbound), free compound concentration is well below the 5.2 μM cellular IC_{50}, which may explain the less than robust result. Nevertheless, the good brain penetration observed in this study with this new class of BACE-1 inhibitors is encouraging.

In summary, a high-throughput screening campaign has identified a novel, nonpeptidic template for aspartyl protease inhibition with good potential for brain penetration. While cellular potency needs improvement for in vivo efficacy, this class of compounds represent a good starting point for further development.

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Supporting Information Available: Experimental procedures, characterization of new compounds, and X-ray structure coordinates. This material is available free of charge via the Internet at http://pubs.acs.org.

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