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Discovery of aminoheterocycles as a novel β-secretase inhibitor class: pH dependence on binding activity part 1

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ABSTRACT

We have developed a novel series of heteroaromatic BACE-1 inhibitors. These inhibitors interact with the enzyme in a unique fashion that allows for potent binding in a non-traditional paradigm. In addition to the elucidation of their binding profile, we have discovered a pH dependent effect on the binding affinity as a result of the intrinsic pK_a of these inhibitors and the pH of the BACE-1 enzyme binding assay.

Alzheimer's disease (AD) is the largest unmet medical need in neurobiology and accounts for the majority of dementia diagnosed in the elderly. AD is characterized by a progressively slow decline in cognitive function that leaves the end-stage patient dependent on custodial care with death occurring on the average of 9 years after diagnosis. The lack of an effective treatment for AD has stirred an intense search for novel therapies based on the amyloid hypothesis. This hypothesis states that a gradual and chronic imbalance between the production and clearance of Aβ peptides results in their accumulation in the brain. These secreted peptides polymerize into neurotoxic oligomers that disrupt neuronal function and lead to cell death and memory loss that is phenotypical of AD.[1] β-secretase (BACE-1) is an aspartyl protease representing the rate limiting step in the generation of these Aβ peptide fragments.[2,3] Given the central role of Aβ in AD pathology, inhibition of BACE-1 has become an attractive target for the treatment of Alzheimer's disease.[4]

The identification of small molecule inhibitors of aspartyl proteases is challenging since the enzymes normally recognize 6–10 amino acids of their substrate for binding and selectivity. β-secretase presents an added challenge as it predominately resides in the brain and as such inhibitors of BACE-1 must ultimately possess the appropriate physical properties necessary to cross the blood-brain-barrier. Most of the aspartyl protease inhibitors (HIV-Pr, renin, BACE-1) that have been reported in the literature contain a transition-state isostere as the key binding element.[5,6] However, the peptide-like nature of these compounds typically imparts poor brain penetration properties.[7] Recently there have been reports surfacing in the literature describing novel non-transition state isostere based inhibitors of BACE-1.[8] In an effort to identify leads that differed from the traditional aspartyl protease inhibitor motif, we developed a high concentration high throughput screening (HTS) assay of the Merck sample collection in order to delineate low affinity scaffolds with novel structural paradigms.[9] This endeavor produced a compact 2-aminothiazole structure as a weakly active BACE-1 inhibitor and novel core template for inhibitors in this field.

4-(1-Phenylcyclopentyl)-1,3-thiazol-2-amine (**1**) was identified as a weak inhibitor of BACE-1 (IC_{50} = 217 μM) from the HTS effort (Fig. 1). While only exhibiting meager activity against BACE-1, its compact size represented an alluring ligand efficiency value of 0.3[10] and since **1** comprised a novel structural motif it was hoped that it would provide a foray into a BACE-1 inhibitor series that

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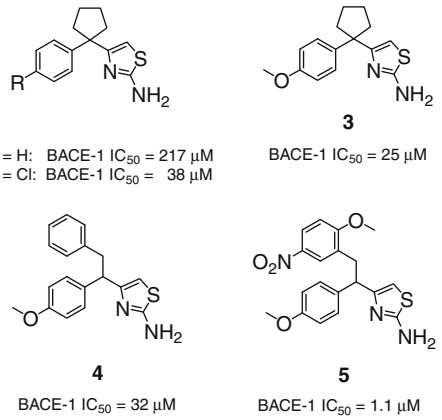


Figure 1. Development of HTS hit **1** into a potent aminothiazole structure.

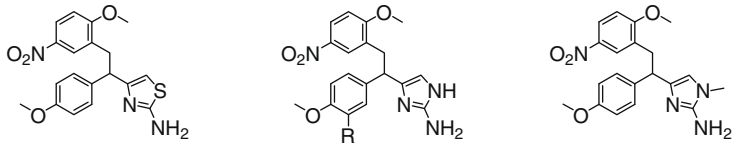
lacked the liabilities of traditional transition-state isosteres. As such, our initial strategy was to increase potency sufficiently to obtain a co-crystal structure with BACE-1. We then envisaged further maximizing activity through rational structure-based design. SAR around the aromatic ring of lead structure **1** provided compound **2**, in which a *para*-chloro substituent was found to impart a 10-fold increase in intrinsic potency (IC_{50} = 38 μM) over the HTS lead. More importantly when compound **2** was administered to mice at a 20 mpk iv dose the brain to plasma ratio was determined to be 3.9 (t = 30 min; [brain] = 15 μM). Throughout the history of BACE-1 inhibitor programs the ability to obtain inhibitors that are both potent and non-substrates for P-gp has hampered progress. These findings provided further impetus to investigate this structural class. Further optimization of the western aryl ring found the *p*-methoxyphenyl substituent, compound **3**, as a local minimum for inhibitor potency in this region. The binding implications of this substituent were found subsequent to this result and will be discussed later. Additional SAR around the lead structure provided compound **4** where the spirocyclopentyl ring was replaced with a benzyl group. While there was a slight loss in potency with this transformation, the opportunity to broaden the SAR in a rapid fashion was viewed as a positive attribute. After screening a multitude of simple benzyl substituents, the 2-methoxy-5-nitro derivative was found to be the most potency enhancing combination, resulting in inhibitor **5** with an IC_{50} = 1.1 μM as a racemic mixture.[11] While we were successful in dramatically improving the potency in the series, we were unable to obtain a co-crystal of the inhibitor in the BACE-1 active site. As such, we synthesized the corresponding aminoimidazole structure **6** (Fig. 2) with the hope that, due to the increased basicity of the aminoimidazole over the aminothiazole, it would be more soluble in the crystallization buffer system. Further implications of the effect of increased inhibitor basicity will be discussed later.

Gratifyingly, this indeed proved successful and we were able to obtain a co-crystal of inhibitor **7** in the BACE-1 enzyme active site at a resolution of 2.7 Å.[12] Compound **7** contained an additional *meta*-fluoro substituent compared with **6**, while this modification had no direct effect on enzymatic potency (IC_{50} pH 6.4 = 3.2 μM) it was fortuitous in co-crystallizing with the enzyme. Analysis of the crystallographic data revealed several interesting elements of the interaction between the inhibitor and the enzyme. First, it was apparent that both the external and one of the internal (N-3) nitrogens of the aminoimidazole are interacting directly with the catalytic dyad through a bidentate interaction with Asp32 and Asp228 (Fig. 3).

The second prominent insight was that the methoxy phenyl portion of inhibitor does not reside in a defined enzyme subsites as described using Schecter and Berger[13] nomenclature and was observed to be involved in an extended face-to-point-to-face π-stack with Phe-108 and Phe-109 (Fig. 4). This extended π-stacking network may contribute to the relatively moderated potency of such a compact inhibitor.

Another interesting feature revealed in the bound conformation was that the inhibitor was found to bind in a 'flap-open' enzyme conformation.[14,8b,c] Further insight on the causative factors of this is displayed in Figure 5. As a reference point, in the normal 'flap-closed' state of the enzyme, depicted as a magenta protein thread, Tyr-71 is involved in a discrete hydrogen bonding interaction with Trp-76. This interaction effectively serves as the 'latch' keeping the flap down. Upon binding with inhibitor **7** it can be seen that the methoxy phenyl supplants Tyr-71 and mimics the closed state binding stabilization as well as the hydrogen bonding interaction with Trp-76. Tyr-71 is thus pushed up and out in the inhibitor bound conformation, resulting in a 'flap-open' enzyme state (green protein thread). Once in the 'flap-open' conformation, Tyr-71 is able to further stabilize the bound conformation by participating in a point-to-face π-stack with inhibitor **7** as seen in Figure 5. The last noteworthy feature identified in the co-crystal structure was that the nitrobenzyl portion of the inhibitor was found to be situated into the large S_1 sub-site of the inhibitor lacking interaction with the S_3 and S_2 sub-sites. Further optimization of this scaffold in this regard will be the subject of a future communication.

While we were able to make dramatic improvements in enzymatic activity of the aminothiazole series (~200-fold over the HTS hit), we were plagued by a complete lack of activity in our cell-based assay. The lack of activity was not attributed to poor cellular penetration since **5** was measured to have an acceptable apparent permeability value ($P_{app} = 20 \times 10^{-6}$cm/s). It is a widely accepted presumption that the BACE-1 enzyme is predominantly localized in acidic intracellular compartments.[15] This supposition and the knowledge that BACE-1 is most active at pH 4.5 in enzymatic assays have provided further evidence to surmise that BACE-1 is most active against APP in these regions. This is depicted graphically in Figure 6 where we plotted BACE-1 activity from three different BACE-1 sources on the Swedish mutant variant of



	5	6 / 7	8
calc. pKa	*5.5*	*8.1*	8.3
pH = 4.5	BACE-1 IC_{50} = 1.1 μM	(**6**) BACE-1 IC_{50} = 5.7 μM	BACE-1 IC_{50} = ND
pH = 6.5	BACE-1 IC_{50} = 0%	(**6**) BACE-1 IC_{50} = 3.4 μM	BACE-1 IC_{50} = 0.47 μM
sAPP_NF	cell IC_{50} = >22000	NA	cell IC_{50} = 1.8 μM

Figure 2. pH dependence on enzymatic and cellular activity.

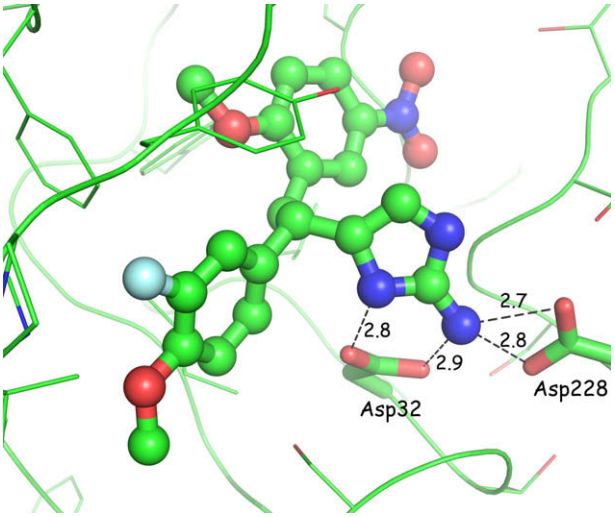


Figure 3. Co-crystal of **7** in BACE-1 active site depicting the bidentate interaction with Asp32 and Asp228.

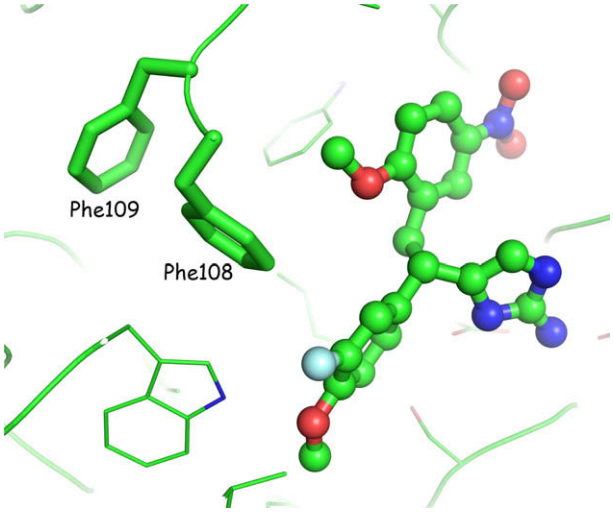


Figure 4. Face-to-point-to-face π-stack with Phe108 and Phe109.

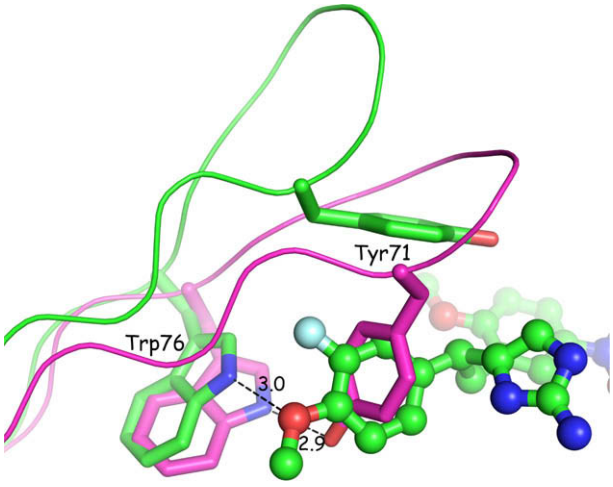


Figure 5. Flap-open binding stabilization of **7** in the BACE-1 active site.

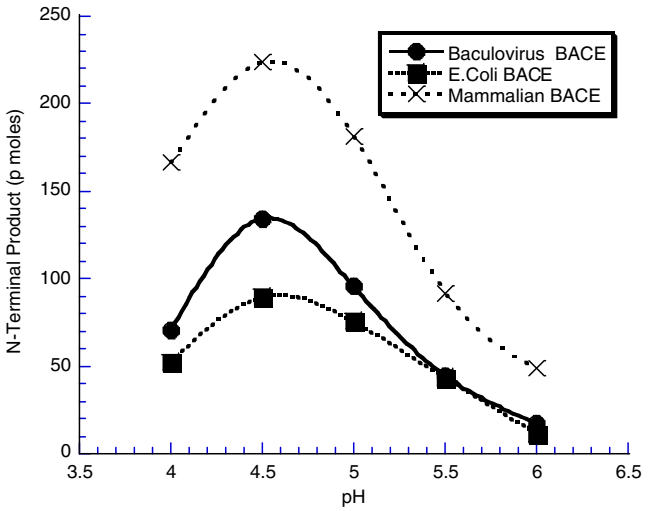


Figure 6. Plot of BACE-1 activity versus pH.

APP versus pH. As such most BACE-1 enzymatic assays are conducted at pH 4.5 to conserve isolated enzyme due to the highest activity at this pH. The calculated pK_a value for aminothiazole **5** is 5.5 and as such, would be expected to be protonated under the pH 4.5 assay conditions but not abundantly protonated under the higher pH of the cell-based assay.

Since other compounds from parallel series were found to have intrinsic potency dependent on the pH under which the enzymatic assay was conducted we next examined the activity of compound **5** at pH 6.5 and found it to be completely inactive. Owing to the success that the synthesis of the corresponding aminoimidazole had on co-crystallization we further investigated the ramifications of increasing the basicity of the inhibitor in our enzymatic assay. This subtle change from an aminothiazole to an aminoimidazole served to effectively increase the intrinsic calculated pK_a of the amine to 8.1 from 5.5 and as such aminoimidazole **6** would be expected to be protonated at both pH 4.5 and 6.5 assay conditions. *This was indeed the case as the activity of inhibitor* **6** *was similar at either pH* (Fig. 2).[16] While compound **6** effectively demonstrated the pH versus pK_a differences in enzymatic activity, the potency was not sufficient to obtain an accurate activity readout in our cell-based assay. Serendipitously, a methyl group was added to the internal aminoimidazole nitrogen (compound **8**) which resulted in a 7-fold enhancement in enzyme activity. While compound **8** was active in the pH 6.5 enzymatic assay it more importantly demonstrated low micromolar activity in our cell-based assay. To conclude this section we have delineated subtleties in pH dependent binding and, at least in our modified HEK-293 cell line,[17] there is a pronounced pH dependence demonstrating that if the enzymatic BACE-1 assay is conducted at pH 6.5 rather than pH 4.5 it is more predictive of the measured cellular potency.[18] Taken together, the enzymatic and cellular data tends to support the notion that APP cleavage is occurring in less acidic cellular components than previously theorized. The subtlety of this pH dependence versus the activity in relation to the pK_a of an inhibitor, from a different structural series, is delineated further in an future manuscript.

In summary, we have developed a series of aromatic heterocycles as BACE-1 inhibitors. The potency of the weak initial lead structure was dramatically enhanced using traditional medicinal chemistry. These inhibitors were shown to bind in a unique fashion that allowed for potent binding in a non-traditional fashion. In addition to their binding profile we have also discovered a pH dependence on binding potency between the intrinsic pK_a of the inhibitor and the pH of the binding assay. This finding may be

reflective of BACE-1 compartmentalization within the cell that suggests BACE-1 activity is occurring in less acidic regions of the cell than is currently believed.

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