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Application of Fragment-Based NMR Screening, X-ray Crystallography, Structure-Based Design, and Focused Chemical Library Design to Identify Novel μM Leads for the Development of nM BACE-1 (β-Site APP Cleaving Enzyme 1) Inhibitors†

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Fragment-based NMR screening, X-ray crystallography, structure-based design, and focused chemical library design were used to identify novel inhibitors for BACE-1. A rapid optimization of an initial NMR hit was achieved by a combination of NMR and a functional assay, resulting in the identification of an isothiourea hit with a K_d of 15 μM for BACE-1. NMR data and the crystal structure revealed that this hit makes H-bond interactions with the two catalytic aspartates, occupies the nonprime side region of the active site of BACE-1, and extends toward the S3 subpocket (S3sp). A focused NMR-based search for heterocyclic isothiourea isosteres resulted in several distinct classes of BACE-1 active site directed compounds with improved chemical stability and physicochemical properties. The strategy for optimization of the 2-aminopyridine lead series to potent inhibitors of BACE-1 was demonstrated. The structure-based design of a cyclic acylguanidine lead series and its optimization into nanomolar BACE-1 inhibitors are the subject of the companion paper (*J. Med. Chem.* **2010**, *53*, DOI:10.1021/jm901408p).

Introduction

Alzheimer's disease (AD[a]) is a dementia-inducing illness which has taken on significant importance as a result of the aging populations worldwide.[1,2] Currently marketed treatments have limited efficacy, leaving a large unmet medical need in terms of true disease-modifying therapies.[3] Neuronal cell death is symptomatic of AD and is a key contributor to cognitive failure in AD patients and is a root cause of AD.[3] Genetic and pathological evidence strongly supports the amyloid cascade hypothesis of AD, which states that the production of β-amyloid (Aβ) peptides in the brain, neuronal cell death, and the subsequent symptoms associated with AD are directly linked.[4]

Aβ is produced by the sequential catalytic cleavage of the amyloid precursor protein (APP) by two proteases, β-secretase (BACE, β-site APP cleaving enzyme) and γ-secretase, respectively.[5,6] BACE is a transmembrane aspartyl protease that exists in two isoforms[7] with BACE-1 located in the central nervous system and BACE-2 expressed mainly in the periphery.[8] BACE-1 is primarily responsible for the cleavage of APP and the subsequent generation of Aβ peptides (e.g., BACE-1 knockout mice are normal, do not produce Aβ peptides and have few overt phenotypes[9−12]), whereas the function of BACE-2 is unknown at present. Therefore, BACE-1 is believed to be the most clinically important isoform to the development of AD.[13] The required selectivity for a clinical compound for BACE-1 versus BACE-2 has yet to be established, but the selectivity for BACE versus other aspartyl proteases such as cathepsin D and E may be more important.[14] The essential role of BACE-1 in Aβ formation, the observation that BACE-1 knockout mice are phenotypically normal, and the availability of a crystal structure of BACE-1[15] to support structure-based drug design together suggested that inhibition of BACE-1 is a very promising approach for the treatment of AD.[16,17] This theoretical enthusiasm was tempered, however, by the known practical difficulties in designing low molecular weight, orally active, brain penetrant aspartic protease inhibitors. The BACE-1 crystal structure also revealed that the BACE-1 active site would present additional challenges to inhibitor design because of its relative hydrophilicity and shallow, elongated topography.

Considerable efforts have been made to develop BACE-1 inhibitors as a therapeutic strategy for the treatment of AD.[18−21] Such efforts have led to the development of peptidomimetic BACE-1 inhibitors with nM affinity based on, for example, the statine,[22,23] hydroxyethylene,[24,25] or hydroxyethylamine[26−28] scaffolds. However, a potential problem with peptidomimetics is that they tend to be relatively

†Coordinates have been deposited in the RCSB Protein Data bank for BACE-1 with compounds **3**, **4**, and **5** with accession codes 3KMX, 3KMY, and 3KNO, respectively.

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[a] Abbreviations: Aβ, β-amyloid peptide; AD, Alzheimer's disease; APP, β-amyloid precursor protein; BACE-1, β-site APP cleaving enzyme 1 (or β-secretase); HSQC, heteronuclear single-quantum correlation; NMR, nuclear magnetic resonance; HTRF, homogeneous time-resolved fluorescence; PDB, protein data bank.

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large and possess multiple hydrogen bond donors and acceptors, which may make them less attractive leads for a therapeutic target that requires oral bioavailability and penetration across the blood−brain barrier.[29,30] Therefore, some of the more recently discovered nonpeptidic small molecule BACE inhibitors are of great interest.[21] Heterocycle-based BACE inhibitors are among the most intriguing inhibitors which are likely to have appropriate pharmacokinetic properties.[31−36]

In an effort to discover novel small molecule inhibitors for this challenging drug target, we have applied fragment-based NMR screening[37−39] to identify small molecule hits which bind to the active site of the protein and were used as starting points for the development of validated lead inhibitors of BACE-1. Fragment-based lead discovery is an emerging field in which much lower molecular weight compounds are screened relative to those in high throughput screening campaigns.[40−47] In theory, fragment-based methods offer the possibility of identifying novel leads with improved affinity, selectivity, and pharmaceutical properties, and the rationale behind these fragment-based strategies makes intuitive sense. However, fragment-based hits are typically weak inhibitors/binders ($IC_{50}/K_d \sim \mu M-mM$ range) and therefore need to be screened at higher concentrations using very sensitive detection techniques. Although fragment hits are simpler, less functionalized compounds[48] with correspondingly lower potencies, they typically possess a high "ligand efficiency" (LE)[49−51] and therefore are highly suitable for optimization into clinical candidates with good drug-like properties. Nevertheless, optimization of weak-binding fragments into high-affinity binders can be challenging and fragment-based lead discovery can be difficult in practice. Both the discovery and advancement of fragment hits are areas of intense research. Although there is still much work to be done to fully exploit the potential of this approach, the increasing number of successful applications that have appeared in the literature,[40−47] including the first examples of clinical drug candidates[44,47] originating from this approach, strongly suggest its viability.

Here we report the identification of novel small molecule inhibitors of BACE-1 using fragment-based NMR screening of a highly customized fragment library. A joint approach that involved NMR, bioassay, and X-ray crystal structure determination for hit characterization and focused screens resulted in the identification of more potent isothiourea BACE-1 inhibitor leads. Directed NMR screening of isothiourea isosteres to identify 2-aminopyridines as active site-directed BACE-1 ligands and the structure-assisted synthesis of extended 2-aminopyridines to demonstrate a strategy for further optimization of the 2-aminopyridine lead series to more potent small molecule inhibitors of BACE-1 is also described.

Results and Discussion

Identification of Initial NMR Hits. Two-dimensional ^{15}N-HSQC NMR was used to screen a custom-built fragment library of approximately 10000 compounds against ^{15}N-labeled BACE-1 catalytic domain. About 50% of the compounds in this screening library followed the "Rule-of-Three" concept[48] or similar rules[52,53] consisting of low MW compounds that are highly pure and water-soluble, as well as synthetically tractable, to allow a rapid structural optimization of fragment hits. Hits were identified by comparing two NMR spectra obtained with and without mixtures of small molecule compounds. Clusters which caused chemical shift changes of active site residues of BACE-1 were deconvoluted to identify the active site binding compound(s) within each active cluster. Using this approach, multiple novel hits were identified which bind to the substrate-binding sites of BACE-1 in the $\mu M-mM$ range.

HSQC-based NMR screening is a sensitive and reliable method for the detection of ligand binding over a virtually unlimited affinity range. For weakly bound ligands ($K_d > \sim 10\ \mu M$), where the rate constant for the dissociation of the complex (k_{off}) is fast on the chemical shift time scale, quantitative dissociation constants can be obtained by monitoring the chemical shift changes of the target as a function of ligand concentrations.[54−56] If a small molecule binds to a macromolecular target at a particular site, it causes chemical shift changes of target resonances which in many cases can be analyzed to locate the binding site of the small molecule ligand. HSQC-based methods are not only very robust and reliable to detect ligand binding but may also provide invaluable structural information regarding the binding site location and orientation of a small molecule ligand with respect to the protein surface.[57−59] Therefore, small molecules that bind to the active site(s) of the protein (and nearby sites) can be selected over those that bind to other regions of the protein which are distant from the active site(s). Knowledge of the 3D structure of the target protein (e.g., X-ray crystal structure, NMR solution structure, or homology model) and sequence-specific assignments of at least the active site residues are, however, required to obtain such structural information. At the start of the NMR screening campaign, we did not have NMR resonance assignments of active site residues, which, therefore, were mapped using a known peptidomimetic BACE-1 inhibitor **1** (OM99-2)[15] to identify active-site binders. In the course of the NMR screening campaign, we obtained almost complete backbone NMR resonance assignments of the catalytic domain of human BACE-1,[60] which then allowed a more detailed analysis of ligand binding.

NMR-based fragment screening of the fragment library by ^{15}N-HSQC NMR resulted in the identification of nine chemically distinct classes of BACE-1 active site hits. These active site hits were identified by the chemical shift changes of the active site residues of BACE-1. Representative structures of the nine classes of BACE-1 NMR hits are shown in Figure 1. The characterization and optimization of the isothiourea hit **2** will be described in detail in this paper. Some limited optimization of the other hit classes was conducted by NMR screening of analogues. The number of analogues tested and the dissociation constants (K_d) that were measured for some of the hits by NMR titration experiments are indicated in Figure 1. It is worth mentioning that for the aminodiazobicycloheptene, the endo phenyl stereoisomer induced large chemical shift changes of BACE-1, whereas the exo phenyl stereoisomer showed only small chemical shift changes. For example, a chemical shift change of 0.15 ppm of Gly34 was induced by the endo phenyl compound at 1 mM concentration. A much smaller chemical shift change of 0.03 ppm was observed by the exo phenyl compound under the same conditions. This observation indicated that the stereochemistry of the compounds in that series is important for binding to BACE-1.

Characterization of the Isothiourea NMR Hit 2. One of the most interesting hits from the initial NMR screen contained an isothiourea (compound **2** in Figure 2). This small

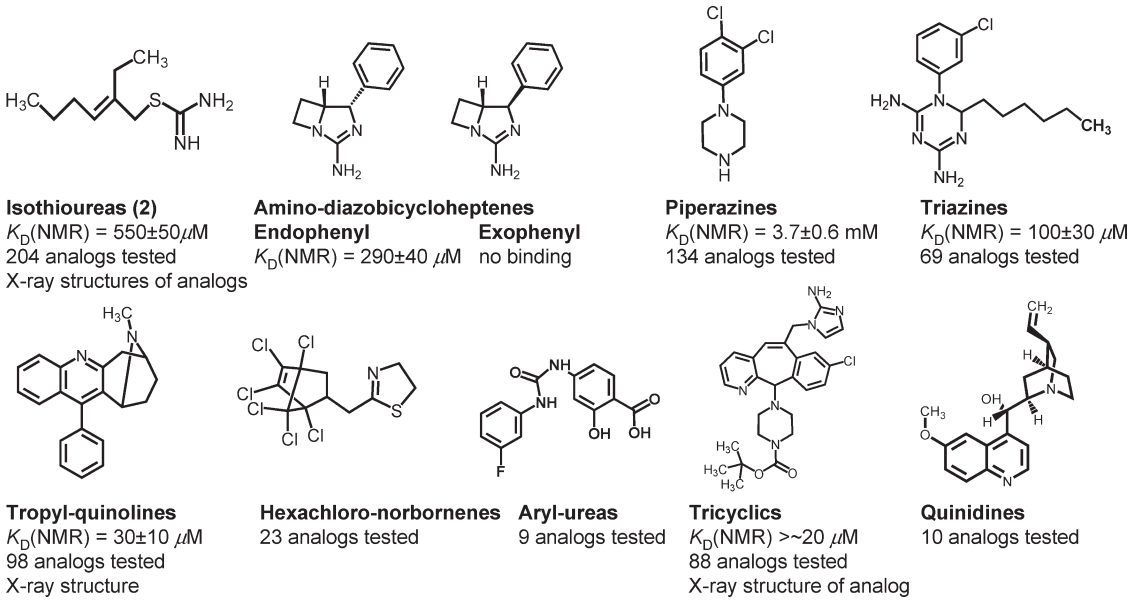


Figure 1. Fragment-based NMR screening hits for BACE-1. Nine classes of BACE-1 active site directed NMR hits were identified by screening 10000 compounds from a customized NMR fragment library by ^{15}N-HSQC NMR.

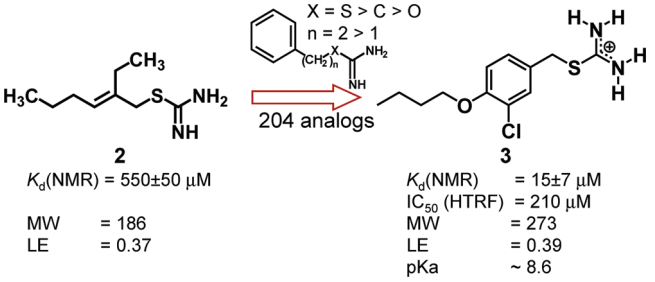


Figure 2. Optimization of the initial isothiourea NMR hit **2** into the improved isothiourea NMR hit **3** for BACE-1. Initial SAR of the starting fragments was evaluated by NMR (see text for details).

fragment hit induced large chemical shift changes of selected peaks of BACE-1 active site residues in the 2D HSQC NMR spectrum of ^{15}N-labeled BACE-1. This is consistent with the observation that many of the same protein peaks were also perturbed by the active site inhibitor **1** when added to the protein sample. The S4 to S2′ peptide substrate pockets of the BACE-1 active site are well-defined in the 1.9 Å resolution crystal structure of the complex between the aspartic protease and the peptidomimetic inhibitor **1**, and the active site of BACE-1 is more open and less hydrophobic than that of other human aspartic proteases in this structure. The NMR hit **2** caused chemical shift changes of the two active site aspartates (Asp32 and Asp228) and chemical shifts changes of Ile118 and Gly230 in the S1 region and Gly34 in the S1′ region. Incremental addition of the NMR hit **2** to ^{15}N-labeled BACE-1 caused dose-dependent protein chemical shift changes of active site residues, which is consistent with weak, site-specific active-site directed binding of **2** to BACE-1 in the micromolar range. An NMR-based titration yielded a dissociation constant (K_d) of 550 μM for **2**. The chemical shift changes of Asp32 and Gly34 as a function of compound concentrations were used for the K_d calculation.

Identification of a More Potent Isothiourea BACE-1 Inhibitor. To accelerate the discovery of higher affinity BACE-1 inhibitors, a tandem approach using NMR and a BACE-1 HTRF assay[61] was employed. Over 200 isothiourea analogues were selected from the corporate compound repository and submitted for a focused screen in a BACE-1 HTRF assay. Fifteen compounds showed significant activity at 50 μg/mL in the HTRF functional assay and were further characterized by NMR. Among those, compound **3** (Figure 2) dose-dependently inhibited BACE-1 with an IC_{50} of about 200 μM in the BACE-1 HTRF assay and was identified as the most potent analogue in this series. A K_d value of 15 μM was determined for **3** from an NMR-based titration experiment. The chemical shift changes of Asp228, Gly34, and Ala39 as a function of compound concentrations were used for the K_d determination. Thus, this combination approach rapidly achieved a 36-fold increase in binding affinity over the initial NMR hit without commitment of synthetic chemistry resources.

NMR was also used to obtain initial SAR regarding the type and length of linker connecting the amidine and the phenyl groups in **3**. Seventeen additional analogues were evaluated which contained different X atoms (X = S, C, O) and linker lengths (n = 1, 2). Careful inspection of the magnitudes of chemical shift changes induced by binding of these compounds to BACE-1 in 2D HSQC spectra of the enzyme revealed that the isothiourea headgroup is preferred over the isourea and amidine groups and a phenethyl extension is preferred over shorter extensions (Figure 2).

Ligand efficiency, defined as the free binding energy per non-hydrogen atom (kcal·mol^{-1} /heavy-atom count), is arguably a better parameter than potency alone in the selection of a lead compound and in the lead optimization process.[49] Compounds with high ligand efficiency are predicted to have an improved probability of binding to the target of interest. Although the NMR hits **2** and **3** have low affinities, because of their low molecular weights they show relatively high ligand efficiency values of 0.37 and 0.39, respectively (Figure 2). These relative high ligand efficiencies are a result of extensive interactions of the hits with the protein active site residues as observed in the NMR binding data and the X-ray crystal structures (see below). Therefore,

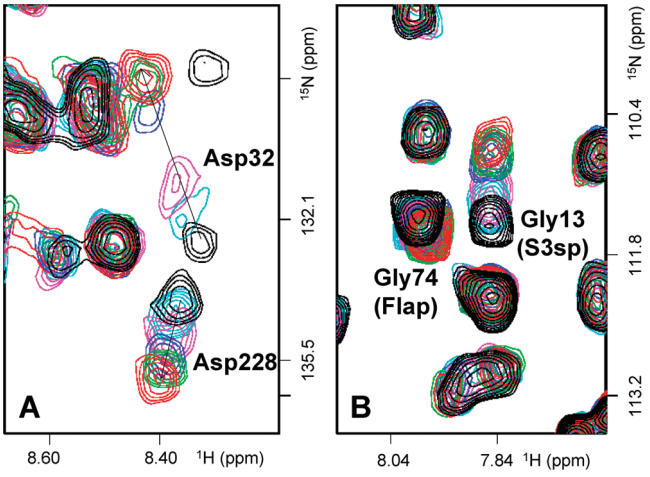


Figure 3. Selected regions of 2D ^{15}N-HSQC spectra measured with a sample of 100 μM uniformly ^{15}N-labeled BACE-1 alone (black), in the presence of 27.5 (cyan), 55 (magenta), 110 (blue), 220 (green), and 440 (red) μM of **3**, suggesting its binding to the two active site aspartates while extending toward S3 and leaving the flap unchanged.

these NMR hits for BACE-1 were considered excellent starting points for further chemical optimization to potent leads.

Structural Characterization of the Isothiourea Hit 3. The more potent isothiourea NMR hit **3** induced significant chemical shift changes in the 2D ^{15}N-HSQC spectrum of BACE-1 as shown in Figure 3. It induced large, dose-dependent chemical shift changes of the two active side aspartates (Asp32 and Asp228) (Figure 3A) and for the residues in the S3sp pocket such as Gly13 (Figure 3B). In addition, it caused significant chemical shift changes of Gly34 in the S1′ subsite, Gly230 in the S1/S2 subsites, and Ile118 and Leu119 in the S1 subsite. In contrast, it induced only minor chemical shift changes of residues within the flap, as can be seen for example in Figure 3B for the flap residue Gly74. The flap of BACE-1 is known to be flexible and plays an important role in the binding of inhibitors.[15] The NMR peak of Gly74 falls into an isolated, well resolved region of the 2D ^{15}N-HSQC spectrum of BACE-1 and can be used as a marker to reveal whether or not ligand binding induces conformation changes in the flap. Overall, the chemical shift perturbation data indicated that **3** interacts with the two active site aspartates and binds to the nonprime side region of the active site of BACE-1, likely extending through the S1 pocket toward S3sp.

These observations from the NMR chemical shift perturbation data were largely confirmed by a 1.8 Å resolution X-ray crystal structure of the complex between the isothiourea NMR hit **3** and BACE-1, revealing in detail the binding of the isothiourea moiety to Asp32 and Asp228 at the active site. As shown in Figure 4, an extensive hydrogen bonding network was observed between the NH groups of the isothiourea moiety and the two catalytic aspartates (Asp32 and Asp228). The crystal structure further revealed that the phenyl group occupies the S1 pocket and the butyl group extends toward the S3sp region. The discovery of aspartyl protease ligands possessing an amidine-type group participating in an H-bond donor−acceptor array with the catalytic Asp residues of the protease, as depicted for **3** in Figure 4, was unprecedented at the time of its discovery.

Focused NMR-Based Search for Heterocyclic Isosteres. With this X-ray crystal structure in hand, a structure-based

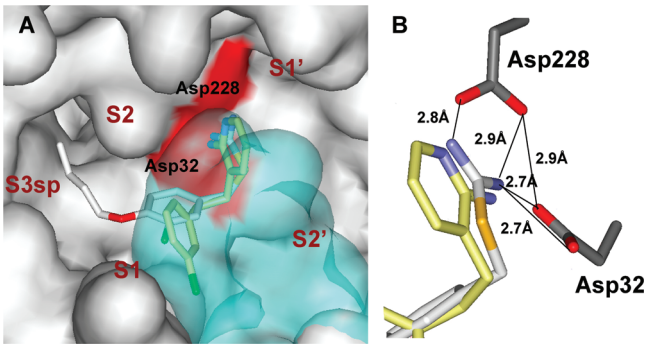


Figure 4. Superposition of the X-ray crystal structures of **3** (white) and **4** (yellow) in the BACE-1 active site (A). BACE-1 flap (light blue) and Asp32, Asp228 (red) are highlighted. The structure of BACE-1 is shown as a solvent-accessible surface in gray. The hydrogen bond networks of **3** and **4** with Asp32 and Asp228 of BACE-1 are shown (B).

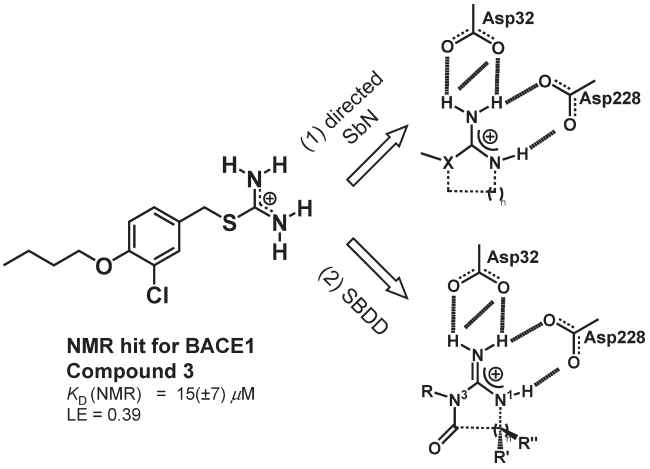


Figure 5. Structure-guided design of the heterocyclic isothiourea isosteres lead to the discovery of the 2-aminopyridine lead series and the iminohydantoin lead series based on the hydrogen bond structure formed between the isothiourea moiety of the NMR hit **3** and the two active site aspartates of BACE-1.

hit-to-lead optimization approach was pursued. Analysis of the X-ray crystal structure of **3** suggested that optimization of binding in the S1 and S3sp pockets was a logical approach to improve affinity. However it was considered that hydrolytic instability of the isothiourea moiety of **3** may render this functional group unsuitable for drug development.[62−64] It was therefore desirable to search for isothiourea isosteres with improved chemical stability. Hence, novel heterocyclic cores that preserved the amidine-like active site binding motif were conceptualized as illustrated in Figure 5. A focused NMR-based search for heterocyclic isothiourea isosteres resulted in the identification of two distinct classes of compounds. Directed NMR screening led to the discovery of a 2-aminopyridine lead series. The characterization and optimization of the 2-aminopyridines will be further illustrated in this paper. The structure-based design of an iminohydantoin lead series, starting from the NMR isothiourea hit **3**, and its optimization into nanomolar BACE-1 inhibitors are the subject of the companion paper.[72]

Characterization of a 2-Aminopyridine BACE-1 Ligand. NMR played an important role in the exploration of the structure−activity relationship in this lead series because the BACE-1 potency of these 2-aminopyridines were initially too

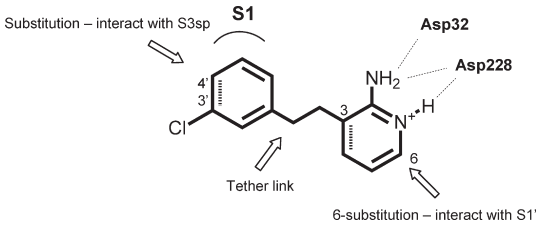


Figure 6. Schematic drawing illustrating the strategies pursued in the optimization of 2-aminopyridine **4** into potent inhibitors of BACE-1.

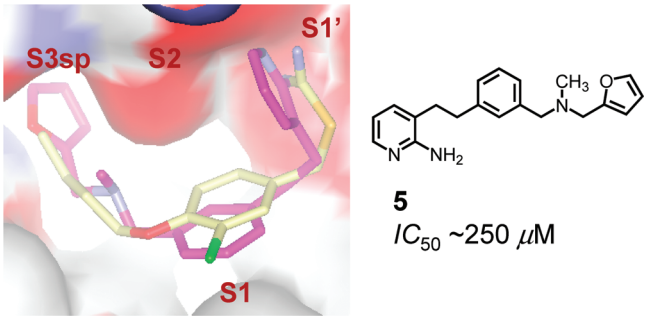


Figure 7. BACE-1 cocrystal structures of **3** (yellow) and **5** (magenta). The substitution at 3′ position of **5** extends to the S3 binding pocket of BACE-1.

weak to be reliably detected in the biological assay. Directed NMR screening of 32 2-aminopyridines rapidly identified compound **4**, which binds to the active site of BACE-1. The chemical structure of **4** is shown in Figure 6. Compound **4** induced chemical shift changes of the two active site aspartates (Asp32 and Asp228), Gly34 in the S1′ subsite, and Gly230 and Thr231 in the S1/S2 substrate binding pockets. It also perturbed residues in the flap region, such as Gly74. However, chemical shifts of residues in the S3sp pocket, for instance Gly13, were not perturbed. The 2-aminopyridine **4** bound to BACE-1 with a K_d value of 32 μM (LE = 0.38) as derived from NMR-based titration experiments. Taken together, these NMR data strongly suggested that the 2-aminopyridine **4** binds with high ligand efficiency to the active site of BACE-1 in a binding mode very similar to that of the isothiourea NMR hit **3**. However, **4** did not show significant inhibition in the BACE-1 HTRF assay up to a concentration of 1 mM. Nevertheless, an X-ray crystal structure of **4** complexed to BACE-1 could be determined at a resolution of 2.0 Å, which revealed that **4** bound as suggested from the NMR data, occupying the same position as **3**, showing the same array of hydrogen bonds with the two active site aspartates and placing the phenyl ring into the S1 pocket (Figure 4). However, the phenyl ring of **4** is turned away from the S3sp pocket. The X-ray crystal structure is consistent with the NMR chemical shift perturbation data. The 2-aminopyridine core embodies a novel BACE-1 catalytic aspartate binding motif with improved chemical stability relative to the isothiourea **3** and its chemical accessibility is suited for rapid SAR development. Furthermore the aminopyridine moiety of **4** is weakly basic with a predicted pK_a of ~7.2,[65−67] which is considered to be favorable for brain penetration, and **4** was considered to be an ideal starting point for hit-to-lead optimization of potent BACE-1 inhibitors.

Strategy for Hit-to-Lead Optimization of 2-Aminopyridines. The X-ray crystal structure of **4** suggested possible modifications of the 2-aminopyridine core to increase both the potency against BACE-1 and its selectivity against other aspartic proteases by making favorable interactions with the prime and nonprime binding sites adjacent to the catalytic aspartate residues. The X-ray crystal structure of **4** in complex with BACE-1 suggested that a 3-phenethyl extension off the 2-aminopyridine core could place the phenyl into the S1 pocket and extension at the 3′ and/or 4′ positions in the phenyl could extend toward the S3sp pocket, whereas extensions from the 6-position of the 2-aminopyridine core could probe the prime subsites of the aspartic acid protease. Established chemistry methods could be employed to allow rapid SAR development by introducing substitutions at C6, C3′, and C4′ positions in the 3-phenethyl framework, enabling access to the hydrophobic subsites near the active site (Figure 6). Initial efforts were thus focused on exploiting binding in the hydrophobic subsites (S1, S3sp, and S2′) of BACE-1 and on engaging additional hydrogen bonding interactions with Gly34 (S1′), Gly230 (S1/S2), and Thr232 (S3sp/S4). Focused small compound libraries were designed around this concept and produced for experimental testing. Analysis of ligand-BACE-1 binding by NMR played an important role in this effort because many analogues bound weakly to BACE-1, and their potencies could not be detected accurately in the HTRF assay. NMR data were used to derive accurate K_d values for weak inhibitors in the μM−mM range and infer their binding site locations. X-ray crystal structures were determined for the most promising hits to assist SAR development for chemical optimization. Structure-based design and chemical synthesis resulted in a highly focused and rapid optimization of the initial hits into leads with low-μM potency. As an example of a proof-of-concept, compound **5**, as shown in Figure 7, was designed to interact with S1 and S3sp pocket and synthesized with extension at the 3′ position of the phenyl ring. It showed large chemical shift perturbations of active site residues including residues in the S3sp pocket such as Gly13 and its K_d is about 100 uM by NMR. It inhibited BACE-1 with an IC_{50} value of 250 μM in the BACE-1 HTRF assay. NMR titration is a reliable method to measure K_d's of weakly bound compounds ($\sim\mu$M−mM range). The IC_{50} values of compounds **3** and **5** revealed that these compounds also showed weak dose-dependent activity in a functional assay, as would be expected from their binding mode to BACE-1. The crystal structure of **5** in complex with BACE-1 confirmed that the compound made favorable interactions with the nonprime (S1, S2, and S3sp) subsites as shown in Figure 7. The 2-aminopyridine moiety makes the same hydrogen bond interaction with the two catalytic aspartic acids as the initial hit **4**. The 3-phenethyl group forms hydrophobic interaction in the S1 pocket. The furan moiety indeed reaches the S3sp binding pocket. This structure-assisted synthesis of extended 2-aminopyridines demonstrated a viable strategy for further optimization of the 2-aminopyridine lead series. Given the high ligand efficiency and improved physiochemical properties of **4**, the 2-aminopyridine series promised high potential for further optimization to potent small molecule inhibitors for BACE-1.

Recently, several publications and patents have emerged which also report promising nonpeptidic small molecule BACE inhibitors which are of great interest to the field.[21] For example, Murray, et al. reported an application of fragment-based screening by X-ray crystallography to

BACE-1 in which three distinct chemotypes were identified as binding to the catalytic aspartates in the millimolar range either via an aminoheterocycle, a piperidine, or an aliphatic hydroxyl group.[32] Virtual screening around the 2-aminoquinoline motif identified an aminopyridine with increased potency, which was used as a starting point for the development of sub-μM 3-substituted 2-aminopyridine BACE-1 inhibitors which show an extensive H-bonding network with the two catalytic aspartates and extend into the adjacent S2′ and S1−S3sp binding pockets.[33] Another recent report by Cole, et al., describes the structure-based optimization of an initial low μM acylguanidine inhibitor, which was discovered in a high-throughput screen using a FRET assay into sub-μM acylguanidine inhibitors which interact with the two catalytic aspartates and extend into the adjacent S1−S3sp, S2′, and S1′ binding pockets.[68]

Conclusions

Fragment-based drug discovery provides exciting new opportunities to tackle "difficult" drug targets such as BACE-1. For this CNS aspartic acid protease, structurally novel, highly brain penetrant inhibitors needed to be developed while restricting the molecular weight and the number of H-bond donors and acceptors to impart oral absorption and blood−brain barrier permeability.[29,30] Fragment-based drug discovery approaches might also be suitable for new target classes such as protein−protein interactions which historically have not been considered "druggable".[69]

In this work, we employed a highly integrated approach of NMR-based fragment screening, X-ray crystallography, structure-based design, and focused chemical library design to identify novel μM leads for the development of nM BACE-1 inhibitors. HSQC-based NMR screening of the fragment library yielded multiple, active-site directed fragment hits in the μM−mM range, which could be categorized into nine distinct chemical classes. A rapid optimization of an initial NMR hit **2** was achieved by a combination of NMR and a functional assay, leading to the identification of an isothiourea hit **3** with a dissociation constant (K_d) of 15 μM for BACE-1, reflecting a 36-fold increase in binding affinity over the starting fragment **2**. NMR structural data and the crystal structure of the isothiourea **3** complexed with BACE-1 revealed that the small molecule makes hydrogen bond interactions with the two catalytic aspartic acid residues, occupies the nonprime side region of the active site of BACE-1, and extends toward the S3sp binding pocket. Structure-based design and directed NMR screening led to the discovery of the 2-aminopyridine lead series as a novel isostere for the isothiourea NMR hits with improved stability, physiochemical properties, and chemical accessibility. The crystal structure along with NMR data suggested that the 2-aminopyridine **4** binds to the BACE-1 active site. The X-ray structures of compound **4** suggested distinct opportunities for the optimization of potency and selectivity. Focused small compound libraries were synthesized to exploit binding in the hydrophobic S1, S3sp, and S2′ subsites. For example, structure-guided design and chemical synthesis resulted in compound **5**, which extends through S1 toward the S3sp binding pocket as designed, thereby validating the 2-aminopyridine core as a novel aspartyl protease active site binding motif for the development of small molecule inhibitors for BACE-1.

The work presented here and in the companion paper[72] describe several attractive, ligand efficient, active site directed low molecular weight cores for BACE-1 as a result of this highly focused fragment-based approach, which present unique opportunities for the development of sub-μM BACE-1 inhibitors.

Experimental Section

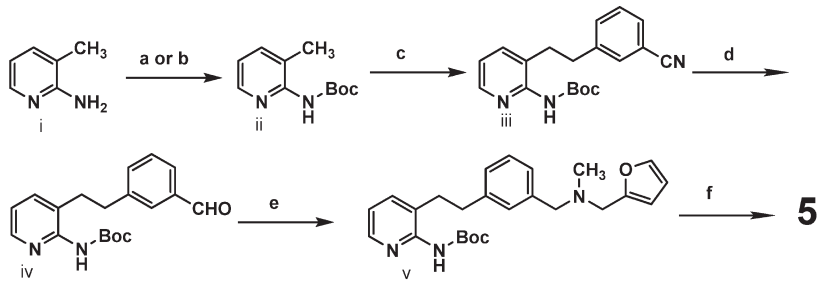
Protein Expression and Purification. The expression of the human BACE-1 protein (residues 14−454) was described in detail previously.[70] Briefly, the human BACE-1 protein (residue 1−454) was expressed in *E. coli* BL21 (DE3) strain as a precursor protein containing the Pre, Pro, and protease domains. The precursor protein was insoluble and refolded from inclusion bodies that were treated with denaturant and detergent and subsequently subjected to a Superdex-200 gel filtration column. The fractions with BACE catalytic activity were collected and combined. The protein solution was first applied to a Resource-Q column and subjected to a Superdex-200 column for further purification. The Pre and Pro peptides of BACE-1 were autocatalytically removed to yield residues 46−454 of the catalytic domain with a molecular weight of 45341 Da (BACE-1). Uniform ^{15}N-labeling of BACE-1 was performed in ^{15}N labeled minimal medium using $^{15}NH_4Cl$ as a sole nitrogen source. The efficiency of refolding BACE is roughly 40%, and the amount of starting material recovered from *E. coli* prior to refolding was 100 mg/L of cell paste (grown in minimal media with ^{15}N labeling). Therefore, each liter of *E. coli* that was grown yielded 40 mgs of refolded material for NMR analysis after refolding and protein purification. Uniformly ^{15}N labeled BACE-1 protein was exchanged into a phosphate buffer containing 75 mM K_iPO_4, pH 7.5, 150 mM NaCl, and 5% D_2O for NMR studies. The BACE-1 inhibitor **1** was purchased from Bachem Bioscience Inc. (King of Prussia, PA).

BACE-1 HTRF Assay. The BACE-1 homogeneous time-resolved fluorescence (HTRF) assays were conducted according to the procedures described previously.[61] The HTRF assay utilizes the fluorescence resonance energy transfer (FRET) pair europium and allophycocyanin for measuring BACE-1 activity in a high-throughput manner.

NMR Spectroscopy. NMR experiments were performed at 25 °C on a Varian INOVA 600 MHz NMR spectrometer equipped with a 5 mM triple resonance probe head. The fast ^{1}H-^{15}N-HSQC experiment was used to improve sensitivity and to avoid water saturation.[71] All HSQC experiments were acquired with 96 scans per t_1 increment, and a recycle delay of 0.7 s. The ^{1}H and ^{15}N spectral widths were 8000 Hz with 1024 (t_2) complex points and 2200 Hz with 48 indirect complex points, respectively. Linear prediction to 96 complex points was performed in the indirect dimension before Fourier transformation. NMR data were processed and analyzed with the Felix program (Accelrys, CA) on a Silicon Graphics workstation.

Compounds in the custom-built fragment library were screened in clusters of 10 compounds each against BACE-1. The 2D HSQC spectra were acquired on a sample of 500 μL of 100 μM uniformly ^{15}N-labeled BACE-1 in the absence or presence of compounds. In the initial screening, the compound concentration was 500 μM. Clusters which showed chemical shift changes in the 2D HSQC spectra of ^{15}N-BACE-1 were deconvoluted to identify those compounds that caused the chemical shift changes. Active site directed hits were identified by chemical shift changes of residues within the substrate binding pockets and the two active site aspartates of BACE-1. Identified hits were further confirmed by repeating the NMR experiments with freshly prepared compound from solid samples. Using this procedure, multiple weak hits with affinities in the tens of μM to low mM range were discovered. These novel chemically distinct classes of hits bind to the active site region of BACE-1 based on the chemical shift changes of ^{15}N-BACE-1.

NMR titration experiments were performed to determine the dissociation constants (K_d) of ligands. The chemical shift



a. $(Boc)_2O$, Hexane : EtOAc 5:1, Δ (53%); b. $((CH_3)_3Si)_2Na$ in THF, $(Boc)_2O$, O° C (82%); c.1. n-BuLi, THF, -5° C, 2. 3-$NCC_6H_4CH_2Br$, -78° C 2 h.,3. Preparative TLC hexane:EtOAc 6:4 (55%); d. 1. 1M DIBAL, CH_2Cl_2, -5° C, 2. Stir with silica gel (76%); e. 1-(Furan-2-yl)-*N*-methylmethanamine, $NaBH(OAc)_3$, (3 equiv.), 4A molecular sieves, dichloroethane; f. 4M HCl in dioxane (35 % 2 steps).

Figure 8. Synthesis of the 2-aminopyridine **5**.

changes were monitored as a function of the compound concentration and the dissociation constants were obtained by nonlinear fits of the NMR titration data.

X-ray Crystallography. X-ray crystal structure of the complex of BACE-1 and compound **3** was obtained by cocrystallization. BACE-1 (16 mg/mL, 150 mM NaCl, and 20 mM Hepes at pH 7.5) was complexed with 1 mM of compound **3** and mixed 1:1 with reservoir (15% PEG3350 and 0.2 M Na/K Tartrate). The hanging drops were incubated at 4 °C for several weeks until diffraction quality crystals grow. Crystals were cryoprotected (15% PEG3350, 0.2 M Na/K tartrate and 15% PEG400) and flash frozen prior to data collection. Data was collected with an Raxis IV detector using a Raxis RU-H2R generator. The final data set was 99% complete to 1.8 Å resolution with an *R* merge of 5.7%. Crystal structures of the complexes for **4** and **5** were obtained by soaking the cocrystals of compound **3**. The data for compound **4** extend to 1.9 Å resolution and was collected similar to compound **3**. The data for compound **5** extend to 1.85 Å resolution and was collected at IMCA-CAT 17-BM (Advanced Photon Source).

Chemistry. LC-Electrospray-Mass spectroscopy with a C-18 column using a gradient of 5% to 95% MeCN in water as the mobile phase was used to determine the molecular mass and retention time. Compounds described in this work were ≥95% purity, with the exception of compound **5**, which was produced from a parallel synthesis experiment and the purity was determined to be 74% by LCMS with no single contaminant present in an amount greater than 5%. X-ray structure determination of compound **5** confirmed the structural assignment.

The amine **5** was prepared starting from 2-amino-3-methylpyridine by reaction with BOC anhydride and subsequent alkylation with 3-cyanobenzylbromide. Reduction of the nitrile to the aldehyde with DIBAL, followed by reductive alkylation with 2-*N*-methyl methylaminofuran and removal of the BOC group provided the amine **5** (Figure 8).

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