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# Fragment-Based Discovery of Nonpeptidic BACE-1 Inhibitors Using Tethering[‡]

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ABSTRACT: BACE-1 ($\beta$-site amyloid precursor protein cleaving enzyme), a prominent target in Alzheimer's disease drug discovery efforts, was surveyed using Tethering technology to discover small molecule fragment ligands that bind to the enzyme active site. Screens of a library of >15000 thiol-containing fragments versus a panel of BACE-1 active site cysteine mutants under redox-controlled conditions revealed several novel amine-containing fragments that could be selectively captured by subsets of the tethering sites. For one such hit class, defined by a central aminobenzylpiperidine (ABP) moiety, X-ray crystal structures of BACE mutant−disulfide conjugates revealed that the fragment bound by engaging both catalytic aspartates with hydrogen bonds. The affinities of ABP fragments were improved by structure-guided chemistry, first for conjugation as thiol-containing fragments and then for stand-alone, noncovalent inhibition of wild-type (WT) BACE-1 activity. Crystallography confirmed that the inhibitors bound in exactly the same mode as the disulfide-conjugated fragments that were originally selected from the screen. The ABP ligands represent a new type of nonpeptidic BACE-1 inhibitor motif that has not been described in the aspartyl protease literature and may serve as a starting point for the development of BACE-1-directed Alzheimer's disease therapeutics.

Alzheimer's disease is a debilitating world health problem that is marked by the deposition of plaques composed of the amyloid $\beta$ peptide (A$\beta$)[1] in the brain (*1*−*4*). A$\beta$ is a 40−42-residue internal segment of the amyloid precursor protein, which is liberated by the activity of two proteases, $\beta$-secretase and $\gamma$-secretase (*5*). The aspartyl protease BACE-1 ($\beta$ amyloid precursor protein cleaving enzyme) has been shown to be the predominant source of $\beta$-secretase activity in cells (*6*−*9*). The pursuit of BACE-1 inhibitors is thus of great therapeutic interest as an Alzheimer's disease target, based on studies indicating that inhibition of enzyme activity reduces the level of generation of A$\beta$ in the brain and cerebrospinal fluid (*3*, *4*, *10*−*12*).

The vast majority of aspartyl protease inhibitor series that have been reported to date have been derived from peptidic starting points (for reviews, see refs (*13*−*15*)). Typically, the scissile amide bond in a substrate peptide is replaced with a tetrahedral intermediate isostere, most often a secondary alcohol. Notable exceptions include several types of noncanonical renin inhibitors, the most prominent of which being the clinical compound Aliskiren (*16*−*21*). BACE-1 inhibitors reported to date are mostly of the peptidomimetic type (for reviews, see refs (*22*−*24*)). While peptidomimetic inhibitors having high potency in cell-free and cell-based assays can be generated, their relatively large size and the number of hydrogen bond donors and acceptors generally make it challenging to achieve high oral bioavailability, and even more difficult to achieve blood−brain barrier penetration (*25*). For this reason, non-peptidomimetic BACE-1 inhibitors have been of great interest; however, only a few have been reported (*26*−*29*).

Applying fragment-based screening technologies is an emerging and promising approach to the discovery of novel small molecule inhibitor scaffolds (*30*). Using these methods, small, simple active site-engaging fragments that form a small number of high-quality interactions, albeit with weak initial affinity, may be discovered. Such

[‡]X-ray crystal structure coordinates have been deposited in the Protein Data Bank (PDB) as entries 2ZJH for the V332C BACE-1−**1** complex, 2ZJI for the T329C BACE-1−**2** complex, 2ZJL for the V332C BACE-1−**10** complex, 2ZJK for the T72C BACE-1−**3** conjugate, 2ZJJ for the K75A/E77A/T231C BACE-1−**4** complex, 2ZJM for the K75A/E77A BACE-1 complexed with inhibitor **13**, and 2ZJN for WT BACE-1 complexed with inhibitor **14**.

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[1]Abbreviations: BACE-1, $\beta$-site amyloid precursor protein cleaving enzyme, isoform 1; ABP, aminobenzylpiperidine; A$\beta$, amyloid $\beta$ peptide; CBP, 3-carboxamidebenzylpiperazine.

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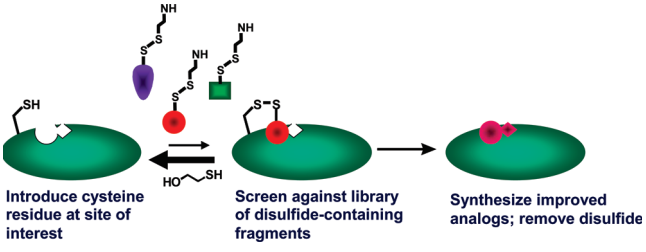
fragments would often be missed in traditional screens demanding initial $IC_{50}$ values in the low micromolar range, even if they are represented as components of some molecules in the library. This is because only a fraction of the ways they might be combined with other pharmacophore elements can be realistically made and tested, and when done in a naïve fashion, these combinations will have a low probability of being arranged in a way that will allow binding with measurable affinity to a target active site (*31*). However, once a fragment has been identified as a specifically bound ligand, and accompanying structural information is gained, it becomes tractable to "grow" the molecule in a structure-guided way and improve affinity to a level qualifying as a starting point for medicinal chemistry efforts. Recent publications by Astex and AstraZeneca researchers highlight the identification of aminopyridine and cyclic amidine classes of nonpeptidic BACE-1 inhibitors using their crystallography, NMR, and modeling-based PYRAMID approaches (*27*, *32*, *33*).

We have shown previously that the fragment-based discovery method, Tethering, can be utilized to identify small molecule fragments that can serve as starting points for the design of potent enzyme and protein−protein interaction inhibitors (Scheme 1) (*34*, *35*). Tethering entails the introduction of individual cysteine mutations surrounding a site of interest on the target protein surface, followed by screening against libraries of thiol linker-containing fragments. The disulfide equilibria that ensue allow identification of weak ligands, since the reversible protein−fragment bonds effectively increase the local concentration of the small molecules at the targeted site. By appropriately controlling redox conditions, fragments in pools compete for disulfide formation with the introduced cysteine. Those having inherent affinity for the protein, apart from the disulfide bond, are predominately conjugated at equilibrium. Such fragment "hits" are detected by mass spectrometry of the mixture, in the form of a peak corresponding to the mass of the specific fragment−protein conjugate. Fragment−protein conjugates can be subjected to SAR analyses and often crystallized for X-ray structural determination, the results of which serve as guides for improvement of affinity and conversion to stand-alone, noncovalent inhibitors by chemical optimization.

In this study, we have employed tethering technology in an effort to discover novel, nonpeptidic small molecule ligands that can serve as starting points for the creation of BACE-1 lead candidates. We report the discovery and structural characterization of a novel fragment that binds to the BACE-1 active site, and its conversion to a moderately potent inhibitor that utilizes a unique binding mode to engage the catalytic aspartates.

Scheme 1: Fragment-Based Lead Discovery by Tethering




## EXPERIMENTAL PROCEDURES

*Tethering Screens against Monophore Libraries.* BACE-1 cysteine mutants were screened against monophore libraries as described previously (*34*, *36*). Briefly, protein was exchanged into reaction buffer [20 mM Tris-HCl (pH 7.5), 30 mM NaCl, and 5 mM $MgCl_2$] using NAP-5 desalting columns (Pharmacia). $\beta$-Mercaptoethanol concentrations for screening were chosen such that 50% of the cysteine mutant protein would become oxidized in the presence of 1 mM cystamine, to allow stringent selection of specifically bound monophores. BACE-1 mutants were adjusted to 10 $\mu$M and incubated with a monophore library pool dissolved in DMSO (up to 10 monophores per pool) such that the final concentration of individual monophores ranged between 160 and 330 $\mu$M and the final concentration of DMSO ranged between 1.5 and 3%, depending on the mutant screened. Solutions were allowed to equilibrate for 5 h and subjected to LC−MS analysis on a Finnigan LCQ instrument as described in the Supporting Information. The strength of monophore thiol−BACE-1 cysteine mutant conjugations was determined by a dose−response assay, in which the monophore concentration was varied from 0.001 to 5 mM at fixed $\beta$-mercaptoethanol and protein concentrations, under conditions described above for the screen. The fraction of protein conjugated to monophore relative to the total was plotted versus the monophore concentration to yield a dose−response curve and an $EC_{50}$ ($[monophore]_{50}$) value when fitted to a sigmoidal four-parameter equation (Prism, Graphpad). The fixed $\beta$-mercaptoethanol concentration in these experiments, ranging from 0.3 to 10 mM, was adjusted to yield a $[monophore]_{50}$ value that was greater than the protein concentration (i.e., not titrating the monophore and thus "bottoming out" the assay), and also not higher than the solubility limits of the monophore (often 1−10 mM). To normalize $[monophore]_{50}$ values determined at different $\beta$-mercaptoethanol concentrations, the following equation was applied:

$$\text{Conjugation Strength} = [\beta\text{-mercaptoethanol}]/[\text{monophore}]_{50} \quad (1)$$

Equation 1 relies on the approximation that $[monophore]_{50}$ will increase linearly with an increasing [$\beta$-mercaptoethanol], which should be accurate when the inherent chemical reduction potential of the monophore thiol group and $\beta$-mercaptoethanol are not significantly different from one another. This assumption is supported by published measurements of various alkyl thiols showing little variation in reduction potential (*37*, *38*), and the fact that all monophores in the study had ethyl or propyl linkers between the thiol and fragment attachment point (typically an amide bond).

*BACE-1 Activity Assays and Inhibitor $IC_{50}$ Assays.* Specific activities of purified mutant BACE-1 enzymes were determined by monitoring the initial rates of hydrolysis of the internally quenched fluorescent peptide substrate, FS-2 (*39*), at two or three enzyme concentrations and normalizing relative to the wild-type (WT) enzyme. Determination of the substrate $K_m$ value was not possible due to the low solubility of the peptide, but at the

concentration utilized (5 $\mu$M), activity was observed to be increasing linearly with an increasing concentration, indicating $< K_m$ conditions. Thus, activities measured should reflect relative $k_{cat}/K_m$ values.

BACE-1 inhibition by compounds was assessed via four independent dose−response measurements using a previously described peptide cleavage assay (*40*). The peptide substrate concentration (125 nM) was well below the $K_m$ value ($> 1$ $\mu$M); under these conditions and assuming competitive inhibition, measured $IC_{50}$ values are equivalent to $K_i$ values (*41*). Reported $K_i$ values are means ± the standard error of the mean.

## RESULTS

*Survey of the BACE-1 Active Site by Tethering*. To design tethering sites for a BACE-1 fragment discovery effort, we modeled the potential trajectories of monophore conjugates to numerous prospective cysteine mutagenesis sites based on the X-ray crystal structures of the enzyme (*42*−*44*). A set of 20 residues was chosen, which yielded thorough spatial coverage of the active site of the enzyme, including the P and P′ subsites as well as the catalytic center (Table 1 and Figure 1A,B). Mutant constructs with introduced cysteines were prepared, and 11 variants were successfully expressed, refolded, and purified in milligram quantities, sufficient for tethering experiments. These mutants retained variable enzymatic activity relative to wild-type BACE-1 (Table 1), but all were able to self-cleave cleanly at their engineered processing sites during purification, to remove their pro domains and yield mature enzymes (see Supporting Information) (*45*). Moderate to strong reductions in catalytic activity were expected (and observed) for many of these active site mutants. However, the retention of measurable activity and the ability to obtain X-ray crystal structures were all strong indicators that these variants were folded properly, with correct formation of the three wild-type disulfide bonds (*42*).

*Tethering Screening and Overview of Results*. We identified numerous hits from tethering screens versus our library of approximately 15000 monophores, assayed in pools of up to 10 compounds per well, via the mass signatures of monophore−protein conjugates (*34*). The relative conjugation strengths of selected hits were then determined by measuring the fraction of protein that remained conjugated to the discrete monophores in the presence of an increasing $\beta$-mercaptoethanol reductant concentration. In these experiments, we did not detect multiple protein labelings with either monophores or $\beta$-mercaptoethanol reductant, indicating that wild-type cystine disulfides were not subject to thiol exchange.

Examination of chemical structures of the hits revealed that many of the fragments fell into a handful of chemotype classes, seven of which are listed in Table 2. With the

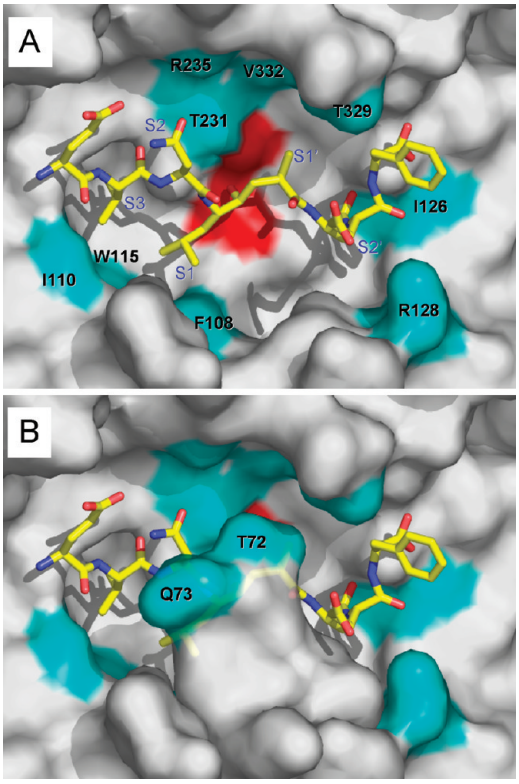


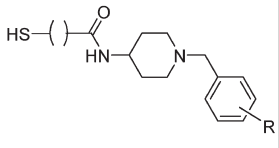
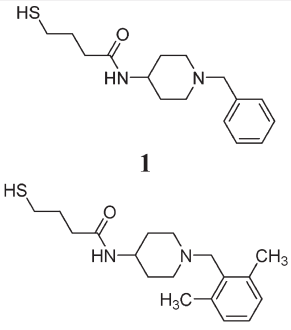
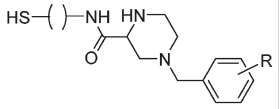
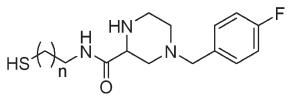
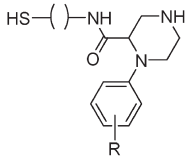
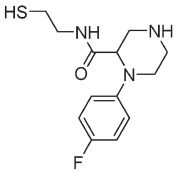
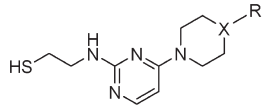
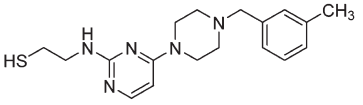
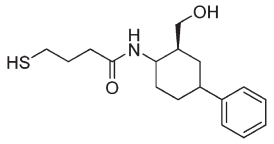
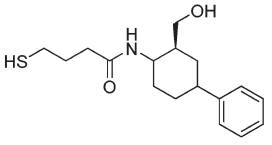

FIGURE 1: Active site of the BACE-1 complex with the hydroxyethylene inhibitor OM-99-2 from Hong et al. (*42*). The 11 residues at which cysteine mutations were introduced individually into protein variants for tethering screens are highlighted in cyan. (A) Flap residues (and other side chain atoms) have been removed for clarity, and the catalytic aspartates are colored red. (B) Flap included to show the T72 and Q73 tethering sites.

Table 1: Tethering Screen Hit Rates and Enzymatic Activities of BACE-1 Protease Domain Variants

| BACE-1 protease domain | specific activity relative to WT[c] | active site areas surveyed |
|---|---|---|
| WT[a] | 1.0 ± 0.17 | NA |
| T72C | ND | catalytic site; S1, S2, S3, S2′, S3′ |
| Q73C | 0.64 ± 0.19 | catalytic site; S1, S2, S3 |
| F108C[b] | 0.03 ± 0.03 | catalytic site; S2, S3, S1′ |
| I110C | 0.28 ± 0.07 | S1, S2, S3 |
| W115C | ND | catalytic site; S2, S3 |
| I126C | 0.35 ± 0.00 | catalytic site; S1′ |
| R128C[b] | 0.41 ± 0.03 | catalytic site; S1′, S2′, S3′ |
| T231C | 0.29 ± 0.03 | S1, S1′, S2′, S3′ |
| R235C | > 0.02 | catalytic site; S1, S2, S3, S1′ |
| T329C | 0.41 ± 0.07 | catalytic site; S1, S2′, S3′ |
| V332C | 0.47 ± 0.07 | catalytic site; S1, S2, S2′, S3′ |

[a] BACE-1 autoprocessing construct yielding residues 32p to 393 in mature form. [b] In K75A/E77A variant background. [c] Averages ± the standard deviation for two or three measurements of initial rates of hydrolysis of a subsaturating concentration (5 $\mu$M) of the internally quenched fluorescent substrate, FS2 (*39*).

Table 2: Hit Chemotypes Selected from BACE-1 Cysteine Mutant Tethering Screens

| Generalized Chemotype | Representative Hit Monophores | Cysteine Sites |
|---|---|---|
|  |  **1** **2** | R235C T329C V332C |
|  |  **3** (n=1); **4** (n=2) | T72C T231C R235C |
|  |  **5** | R235C |
|  |  **6** | Q73C W115C R235C |
|  |  **7** | T329C V332C |

exception of the methylcyclohexyl alcohol **7**, all of these chemotypes were varieties of cyclic amines; for reference, we estimate that less than 5% of the monophore library compounds contained either secondary or tertiary cyclic amines. Also noteworthy was the fact that many of these hit chemotypes were selected at multiple cysteine sites, which in most cases are near one another in the active site. Precedent for the selection of the same fragment at more than one cysteine mutation site, with a common binding mode, was established for the *N*-tosylproline fragment tethering to thymidylate synthase (*34*). This property provided a strong indication that the noncovalent interactions between the fragment itself and the enzyme were primary drivers of the fragment selection. The aminobenzylpiperidine (ABP) and the 3-carboxamidebenzylpiperazine (CBP) classes were examples of fragments that were selected at multiple cysteine sites, having been selected from screens of R235C, T329C, and V332C; and T72C, T231C, and R235C, respectively (Table 2). These classes were intriguing because of their novelty relative to other BACE inhibitors, and the cyclic amine functionalities held promise for specific hydrogen bonding with the enzyme as well as improved solubility in eventual untethered compounds.

The noncovalent forms of initial hit fragments identified from tethering often have affinities for the target protein that are too weak to measure reliably (i.e., millimolar $K_d$ values). Indeed, when the thiol linkers of analogues of representative fragment hits from each class in Table 1 were replaced with methoxy groups, BACE-1 inhibition was not observed at concentrations as high as 600 $\mu$M (data not shown). To guide efforts to improve

affinities by chemical optimization, we determined X-ray crystal structures of several BACE-1 mutant−monophore conjugates.

*Structures of the ABP and CBP Class Fragments Conjugated to BACE-1 Catalytic Domain Cysteine Mutants.* Conjugates between several of the ABP and CBP fragments and the BACE-1 mutants from which they were selected were prepared and crystallized for X-ray structure determination (Figures 2 and 3; see Tables S1 and S2 of the Supporting Information). For the ABP class, structures were generated for two related fragment hits, **1** and **2**, conjugated to two neighboring cysteine mutants, V332C and T329C, at 2.3 and 2.6 Å resolution, respectively (Figure 2A,B). The structures revealed essentially identical binding modes for the fragments beyond the disulfide linker portion of the molecules, despite their differing cysteine tethering sites. Clear electron density for the aminopiperidine moieties was observed, revealing that the ring lies directly over the catalytic aspartates, with its tertiary amine and amide groups engaged in hydrogen bonds to Asp32 (3.0 Å) and Asp228 (2.9 Å), respectively (Figure 2A,B). The active site flaps of the fragment-conjugated enzymes were in an

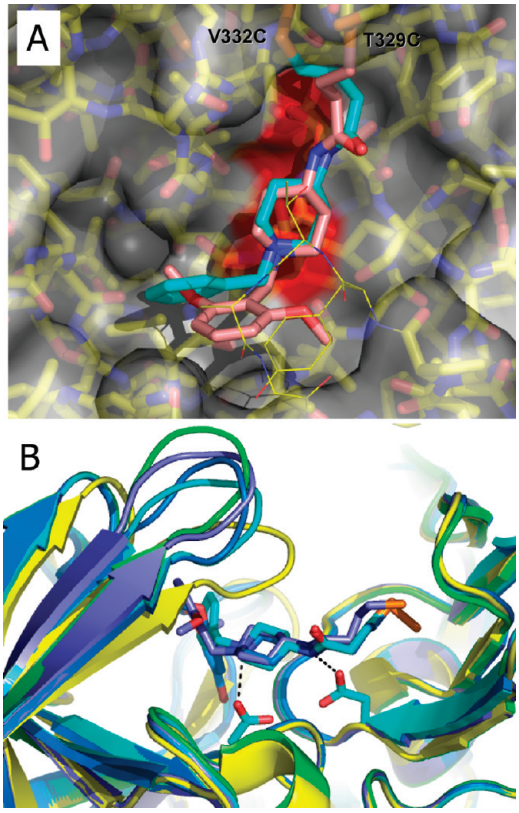



FIGURE 2: Crystal structures of BACE-1 cysteine mutants conjugated to ABP fragment monophores selected from tethering screens. (A) Overlay of ligand structures for V332C BACE-1 conjugated to ABP **1** (cyan) and T329C BACE-1 conjugated to ABP **2** (salmon). The active site flap residues have been removed for clarity, and the catalytic aspartates are colored red. (B) Side view of the same monophore conjugate structures listed above (V332C−**1**, cyan; T329C−**2**, lavender), along with the V332C−**9** complex (blue), the apo-BACE structure from Patel et al. [green; Protein Data Bank (PDB) entry 1W50 (*43*)], and the flap-closed peptidomimetic complex [yellow; PDB entry 1FKN (*42*)]. The fragment hydrogen bonds to the catalytic aspartates, Asp32 and Asp228, and relative flap conformations are highlighted.

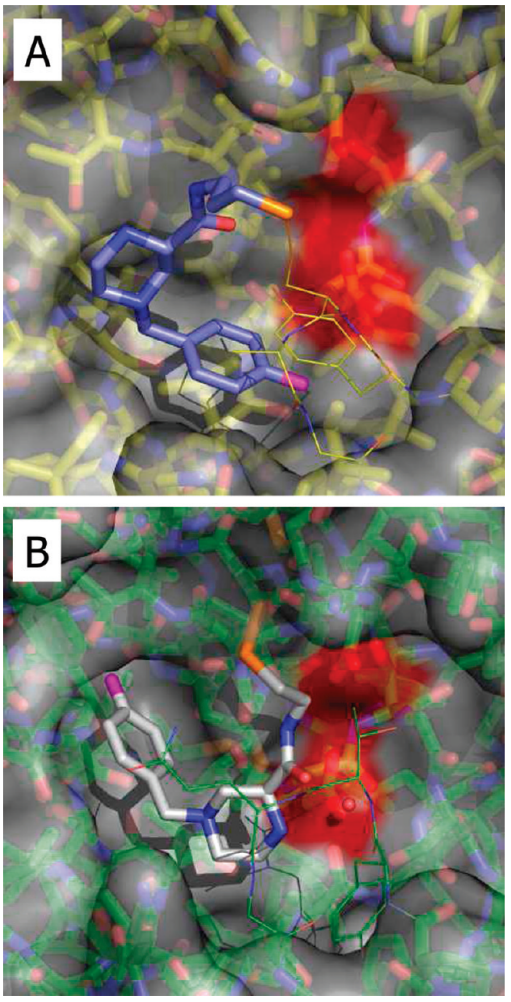
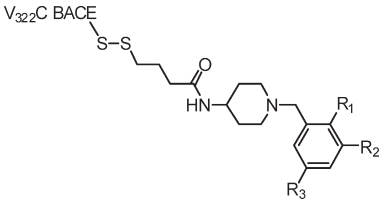



FIGURE 3: Crystal structures of BACE-1 cysteine mutants conjugated to CBP fragment monophores selected from tethering screens. (A) T72C BACE-1 conjugated to **3** (2.8 Å resolution). (B) T231C BACE-1 conjugated to **4** (2.2 Å resolution).

"open" conformation, similar to that observed for the apoenzyme structure (*44*), and other recently reported non-peptidic BACE-1 fragments and inhibitors (*27*, *32*, *46*) (Figure 2B). In contrast, closed conformations are typically seen in structures of BACE-1 complexes with peptidomimetic inhibitors (*42*, *44*). The benzyl fragments bound to the hydrophobic S1 subsite, but at a shallow depth relative to that of benzyl P1 substituents in peptidomimetic inhibitor complexes. The open flap conformation generated more space in the pocket, such that larger P1 substituents could be accommodated.

For the CBP class, structures for two monophores that differed only in their linker lengths (**3** and **4**) in conjugation with T72C and T231C BACE mutants were generated (Figure 3). Although the CBP fragment bound in the S1−S3 region when tethered to either site, the binding modes were completely distinct. Monophore **3** conjugated to T72C BACE placed the piperazine ring in the S2−S3 area, essentially overlapping the site of the P2−P3 amide bond in peptiomimetic inhibitor complexes, and the fluorobenzyl ring occupied the P1 site in a position overlapping P1 benzyl substituents in peptidomimetic inhibitors (Figure 3A). No contacts with the catalytic aspartates were observed. Monophore **4** conjugated to T231C BACE took an opposite path through the S1−S3

Table 3: Tethering Conjugation Strength SAR for ABP Monophore Analogues




| compound | R1 | R2 | R3 | conjugation strength[a] |
|---|---|---|---|---|
| **1** | H | H | H | 1.3 ± 0.16 |
| **8** | OH | $OCH_3$ | Br | 170 ± 100 |
| **9** | $OCH_3$ | $OCH_3$ | Br | 380 ± 80 |
| **10** | $OCH_3$ | $OCH_3$ | Cl | 230 ± 140 |
| **11** | O-Ipr | $OCH_3$ | Cl | 1400 ± 730 |
| **12** | O-Bn | $OCH_3$ | Cl | 350 ± 140 |

[a] Defined as ([$\beta$-mercaptoethanol] in the experiment)/([monophore] required to achieve 50% conjugation).

area, placing the piperazine ring in the S1 trough, and engaged in a water-mediated hydrogen bond to Asp32 (Figure 3B). The benzyl group in this complex occupied the S3 area of the active site.

Since the ABP fragment exhibited a consistent binding mode when tethered to two sites and a novel engagement with the catalytic aspartates via direct hydrogen bonds, this class was further pursued by chemical optimization, in both tethered and untethered mode.

*SAR and Structural Studies of the ABP Fragment and Affinity-Improved Analogues*. To develop an understanding of the structure−activity relationships for the ABP fragment, and find analogues with improved affinity, we first surveyed a set of benzyl group replacements. We chose a reagent set for potentially improved substituents from commercially available aldehydes using a docking score filter (see the Supporting Information), and their effects on the strength of conjugation to V332C BACE-1 were measured as tethered monophores (Table 3).

Fragment conjugation strength was quantitated by titrating monophores into fixed concentrations of BACE-1 cysteine mutants and $\beta$-mercaptoethanol reductant and monitoring the fraction of the protein conjugated by mass spectroscopy. The $EC_{50}$ ("monophore$_{50}$") values from the resulting curves, normalized to the concentration of $\beta$-mercaptoethanol in the reaction mixture, were used to calculate conjugation strengths of mutant−monophore disulfide pairs (see Experimental Procedures). Direct calculation of $\Delta G$ values of binding for the noncovalent interactions between monophores and protein is complicated by significant entropic contributions of the linker, which are difficult to estimate (*35*). However, for a monophore series that has identical tethering sites and linkers, as well as a conserved binding mode, measured conjugation strengths should have similar linker contributions and thus reflect relative noncovalent interaction affinities.

From this survey, the methoxybromophenol substituent (compound **8**) was found to significantly improve (180-fold) conjugation strength relative to the initially

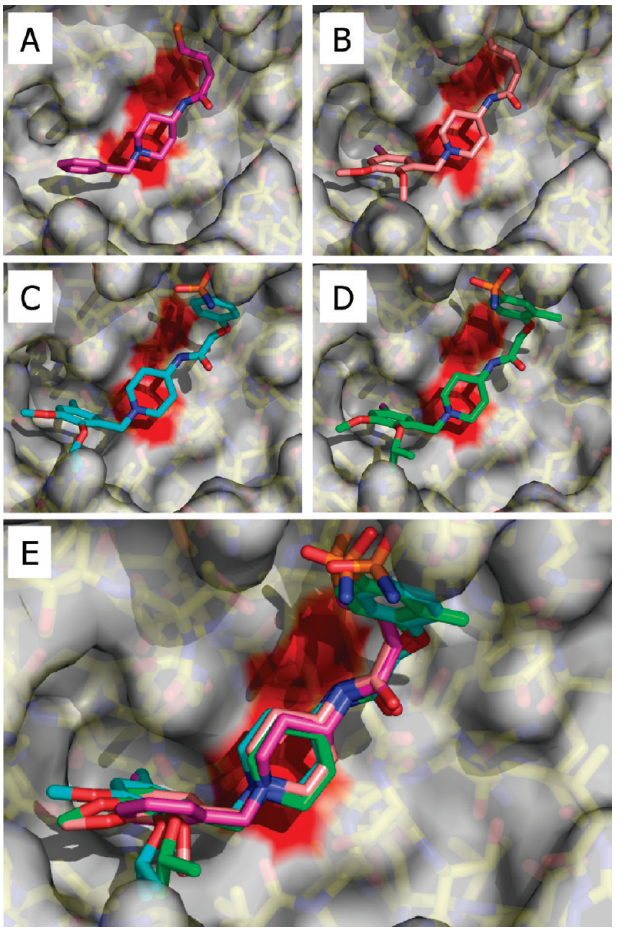


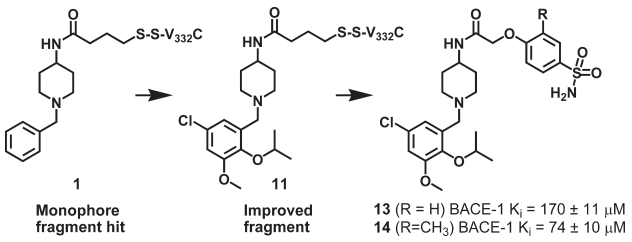

FIGURE 4: Crystal structures of ABP ligands in complex with BACE-1 variants, in both thiol-conjugated and noncovalent form. The ligand binding mode, including hydrogen bonding to the catalytic aspartates, is conserved throughout the evolution of the fragment. (A) V332C BACE-1 conjugated to ABP monophore **1** (2.3 Å resolution). (B) V332C BACE-1 conjugated to ABP monophore **9** (2.1 Å resolution). (C) BACE-1 complexed with inhibitor **13** (1.9 Å resolution). (D) BACE-1 complexed with inhibitor **14** (2.7 Å resolution). (E) Overlay of the four structures, demonstrating conservation of binding mode.

selected monophore **1** (Table 3). Further exploration revealed that replacement of the 2-hydroxyl group with a methoxy substituent yielded improved affinity (**9**). At the 4 position, chloro and bromo substituents were similar (**9** vs. **10**). When a 5-isopropoxy substituent was introduced (**11**), an 1100-fold improvement in conjugation strength relative to the parent benzyl group was achieved.

Determination of the crystal structure of the V332C BACE-1−**9** conjugate revealed that the aminopiperidine fragment tethered into a position virtually identical to that observed for the original fragment, with hydrogen bonds to the catalytic aspartates preserved (Figure 4, panel B vs panel A). The added bulk from the benzyl group substituents resulted in better occupancy in the large S1 pocket of the open flap conformation, with clearer density for this portion of the molecule. Most prominently, the 3-bromo group yielded a snug fit into the back of the S1 pocket, causing slight displacement of the Leu30 side chain.

*Conversion of Tethered Ligands to Noncovalent BACE-1 Inhibitors*. After improving the benzyl P1 element, we sought to create noncovalent ABP inhibitors of WT BACE-1 by replacing the thiol-containing linker

Scheme 2: Aminobenzylpiperidine Fragment Evolution




fragment with a variety of substituents, filtered on the basis of constrained docking. These surveys and limited followup analogues led to inhibitors **13** and **14** (Scheme 2), having BACE-1 $K_i$ values of 170 ± 11 and 74 ± 10 $\mu$M, respectively. We determined the crystal structures of in **13** and **14** complex with BACE-1 at 1.9 and 2.7 Å resolution, respectively (Figure 4C,D). The aminopiperidine hydrogen bonds and substituted benzyl group were found in positions essentially identical relative to the disulfide-conjugated monophore fragments (Figure 4E). The 3-sulfonamide phenoxy substituents in **13** and **14** were directed toward the S1′ subsite, causing the Thr329 and Ser328 main chains and side chains to fold back into the enzyme relative to the peptidomimetic-complexed and apo BACE-1 structures. The sulfonamide group formed hydrogen bonds to the main chain Ser328 amide (2.9 Å), the Arg235 guanidine group (2.7 Å), and potentially to the Ser328 hydroxyl group as well (2.9 Å), although the angle for the latter was oblique. The aryl group of the P1′ fragment packed up against the Val332 and Ile226 side chains, but did not show a high degree of burial in this shallow pocket, perhaps explaining the modest inhibition potencies for this series.

## DISCUSSION

Tethering is one of several technologies that have been proven to be successful in generating fragment-based ligands for a variety of targets (*35*, *47*). Using this method, simple, weak, and novel core fragment ligands may be identified, which may be further elaborated into hit starting points for lead optimization efforts. When we applied this technology against BACE-1, we were able to identify several novel fragments that could be selectively captured by one or more cysteines introduced by mutagenesis in and around the active site. Investigation of the ABP fragment class revealed a novel catalytic aspartate binding motif that was preserved across two different tethering sites and throughout the evolution of the molecule in both conjugated and noncovalent mode.

The piperidine amide motif found in this study displayed a hydrogen bonding scheme distinct from those of previously identified BACE-1 inhibitors. Nonpeptidic cyclic amine binding motifs have been identified previously for aspartyl proteases, most notably several high-potency renin inhibitors (*18*−*20*). In the renin inhibitors having piperidine as a catalytic aspartate-engaging element, the piperidine rings are unsubstituted and exhibit a binding mode completely distinct from the ABP series, in which the piperidine NH group forms hydrogen bonds to both catalytic aspartates. Recently reported non-canonical amine-containing BACE-1 inhibitors include aminopyridines (*27*, *32*, *48*), acylguanidines (*46*), *N*-phenylpiperazines (*49*, *50*), cyclic amidines (*33*), and spiropiperidines (*29*). In Astex studies, piperidine-containing fragments were also identified as initial weak-binding crystallography hits, which also showed hydrogen bonding to the catalytic aspartates, albeit in arrangements different from that of the ABP class reported here (*32*). ABP class molecules are relatively small, have only two hydrogen bond donors in their core motif, and thus share chemical properties common to CNS active agents (*51*, *52*).

The initial ABP fragment hits displayed weak binding affinities but nevertheless a specific and consistent binding mode. The central aminopiperidine moiety does not appear to have significant affinity on its own, but it does serve as a specific hydrogen bonding element that orients the inhibitor in a precise conformation, while providing solubility to the molecule. The primary drivers of binding energy appear to be the hydrophobic contacts, which we were able to improve primarily in the P1 element. At S1′, suitable disulfide linker replacement groups that occupied the S1′ pocket were found, but this pocket is relatively shallow and is not likely to serve as an efficient source of ligand binding energy. Refinement of the P1 and P1′ substituents resulted in molecules with potencies similar to starting points derived from high-throughput screening campaigns. For most other reported BACE-l inhibitors, the flap is closed, and occupancy of the S2−S3 subsite area is essential for attaining high potency (i.e., $K_i$ < 100 nM). To develop high-affinity binding from the ABP series, additional contacts in these regions and perhaps induction of flap closure may be required.

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## SUPPORTING INFORMATION AVAILABLE

Chemical synthesis of monophores and inhibitors, computational chemistry methods, BACE-1 variant cloning, expression, and purification, tethering screening method details, and X-ray crystallographic methods and refinement data. This material is available free of charge via the Internet at http://pubs.acs.org.

## REFERENCES

1. Fratiglioni, L., De Ronchi, D., and Aguero-Torres, H. (1999) Worldwide prevalence and incidence of dementia. *Drugs Aging 15*, 365–375.
2. Hardy, J., and Selkoe, D. J. (2002) The amyloid hypothesis of Alzheimer's disease: Progress and problems on the road to therapeutics. *Science 297*, 353–356.
3. Sinha, S. (2002) The role of $\beta$-amyloid in Alzheimer's disease. *Med. Clin. North Am. 86*, 629–639.
4. Selkoe, D. J., and Schenk, D. (2003) Alzheimer's disease: Molecular understanding predicts amyloid-based therapeutics. *Annu. Rev. Pharmacol. Toxicol. 43*, 545–584.
5. Walter, J., Kaether, C., Steiner, H., and Haass, C. (2001) The cell biology of Alzheimer's disease: Uncovering the secrets of secretases. *Curr. Opin. Neurobiol. 11*, 585–590.
6. Hussain, I., Powell, D., Howlett, D. R., Tew, D. G., Meek, T. D., Chapman, C., Gloger, I. S., Murphy, K. E., Southan, C. D., Ryan, D. M., Smith, T. S., Simmons, D. L., Walsh, F. S., Dingwall, C., and

Christie, G. (1999) Identification of a novel aspartic protease (Asp 2) as β-secretase. *Mol. Cell. Neurosci. 14*, 419–427.
7. Sinha, S., Anderson, J. P., Barbour, R., Basi, G. S., Caccavello, R., Davis, D., Doan, M., Dovey, H. F., Frigon, N., Hong, J., Jacobson-Croak, K., Jewett, N., Keim, P., Knops, J., Lieberburg, I., Power, M., Tan, H., Tatsuno, G., Tung, J., Schenk, D., Seubert, P., Suomensaari, S. M., Wang, S., Walker, D., and John, V.; et al. (1999) Purification and cloning of amyloid precursor protein β-secretase from human brain. *Nature 402*, 537–540.
8. Vassar, R., Bennett, B. D., Babu-Khan, S., Kahn, S., Mendiaz, E. A., Denis, P., Teplow, D. B., Ross, S., Amarante, P., Loeloff, R., Luo, Y., Fisher, S., Fuller, J., Edenson, S., Lile, J., Jarosinski, M. A., Biere, A. L., Curran, E., Burgess, T., Louis, J. C., Collins, F., Treanor, J., Rogers, G., and Citron, M. (1999) β-Secretase cleavage of Alzheimer's amyloid precursor protein by the transmembrane aspartic protease BACE. *Science 286*, 735–741.
9. Lin, X., Koelsch, G., Wu, S., Downs, D., Dashti, A., and Tang, J. (2000) Human aspartic protease memapsin 2 cleaves the β-secretase site of β-amyloid precursor protein. *Proc. Natl. Acad. Sci. U.S.A. 97*, 1456–1460.
10. Luo, Y., Bolon, B., Kahn, S., Bennett, B. D., Babu-Khan, S., Denis, P., Fan, W., Kha, H., Zhang, J., Gong, Y., Martin, L., Louis, J. C., Yan, Q., Richards, W. G., Citron, M., and Vassar, R. (2001) Mice deficient in BACE1, the Alzheimer's β-secretase, have normal phenotype and abolished β-amyloid generation. *Nat. Neurosci. 4*, 231–232.
11. Sankaranarayanan, S., Price, E. A., Wu, G., Crouthamel, M. C., Shi, X. P., Tugusheva, K., Tyler, K. X., Kahana, J., Ellis, J., Jin, L., Steele, T., Stachel, S., Coburn, C., and Simon, A. J. (2008) In vivo β-secretase 1 inhibition leads to brain Aβ lowering and increased α-secretase processing of amyloid precursor protein without effect on neuregulin-1. *J. Pharmacol. Exp. Ther. 324*, 957–969.
12. Sankaranarayanan, S., Holahan, M. A., Colussi, D., Crouthamel, M. C., Devanarayan, V., Ellis, J., Espeseth, A., Gates, A. T., Graham, S. L., Gregro, A. R., Hazuda, D., Hochman, J. H., Holloway, K., Jin, L., Kahana, J., Lai, M. T., Lineberger, J., McGaughey, G., Moore, K. P., Nantermet, P., Pietrak, B., Price, E. A., Rajapakse, H., Stauffer, S., Steinbeiser, M. A., Seabrook, G., Selnick, H. G., Shi, X. P., Stanton, M. G., Swestock, J., Tugusheva, K., Tyler, K. X., Vacca, J. P., Wong, J., Wu, G., Xu, M., Cook, J. J., and Simon, A. J. (2009) First demonstration of cerebrospinal fluid and plasma Aβ lowering with oral administration of a β-site amyloid precursor protein-cleaving enzyme 1 inhibitor in nonhuman primates. *J. Pharmacol. Exp. Ther. 328*, 131–140.
13. Erickson, J. W., and Eissenstat, M. A. (1999) HIV Protease as a Target for the Design of Antiviral Agents for AIDS. In *Proteases of Infectious Agents* (Dunn, B., Ed.) pp 1−60, Academic Press, San Diego.
14. Bursavich, M. G., and Rich, D. H. (2002) Designing non-peptide peptidomimetics in the 21st century: Inhibitors targeting conformational ensembles. *J. Med. Chem. 45*, 541–558.
15. Cooper, J. B. (2002) Aspartic proteinases in disease: A structural perspective. *Curr. Drug Targets 3*, 155–173.
16. Vieira, E., Binggeli, A., Breu, V., Bur, D., Fischli, W., Guller, R., Hirth, G., Marki, H. P., Muller, M., Oefner, C., Scalone, M., Stadler, H., Wilhelm, M., and Wostl, W. (1999) Substituted piperidines: Highly potent renin inhibitors due to induced fit adaptation of the active site. *Bioorg. Med. Chem. Lett. 9*, 1397–1402.
17. Wood, J. M., Maibaum, J., Rahuel, J., Grutter, M. G., Cohen, N. C., Rasetti, V., Ruger, H., Goschke, R., Stutz, S., Fuhrer, W., Schilling, W., Rigollier, P., Yamaguchi, Y., Cumin, F., Baum, H. P., Schnell, C. R., Herold, P., Mah, R., Jensen, C., O'Brien, E., Stanton, A., and Bedigian, M. P. (2003) Structure-based design of aliskiren, a novel orally effective renin inhibitor. *Biochem. Biophys. Res. Commun. 308*, 698–705.
18. Cody, W. L., Holsworth, D. D., Powell, N. A., Jalaie, M., Zhang, E., Wang, W., Samas, B., Bryant, J., Ostroski, R., Ryan, M. J., and Edmunds, J. J. (2005) The discovery and preparation of disubstituted novel amino-aryl-piperidine-based renin inhibitors. *Bioorg. Med. Chem. 13*, 59–68.
19. Holsworth, D. D., Powell, N. A., Downing, D. M., Cai, C., Cody, W. L., Ryan, J. M., Ostroski, R., Jalaie, M., Bryant, J. W., and Edmunds, J. J. (2005) Discovery of novel non-peptidic ketopiperazine-based renin inhibitors. *Bioorg. Med. Chem. 13*, 2657–2664.
20. Powell, N. A., Clay, E. H., Holsworth, D. D., Bryant, J. W., Ryan, M. J., Jalaie, M., and Edmunds, J. J. (2005) Benzyl ether structure-activity relationships in a series of ketopiperazine-based renin inhibitors. *Bioorg. Med. Chem. Lett. 15*, 4713–4716.
21. Holsworth, D. D., Jalaie, M., Belliotti, T., Cai, C., Collard, W., Ferreira, S., Powell, N. A., Stier, M., Zhang, E., McConnell, P., Mochalkin, I., Ryan, M. J., Bryant, J., Li, T., Kasani, A., Subedi, R., Maiti, S. N., and Edmunds, J. J. (2007) Discovery of 6-ethyl-2,4-diaminopyrimidine-based small molecule renin inhibitors. *Bioorg. Med. Chem. Lett. 17*, 3575–3580.
22. Cumming, J. N., Iserloh, U., and Kennedy, M. E. (2004) Design and development of BACE-1 inhibitors. *Curr. Opin. Drug Discovery Dev. 7*, 536–556.
23. Hills, I. D., and Vacca, J. P. (2007) Progress toward a practical BACE-1 inhibitor. *Curr. Opin. Drug Discovery Dev. 10*, 383–391.
24. Ghosh, A. K., Kumaragurubaran, N., Hong, L., Koelsh, G., and Tang, J. (2008) Memapsin 2 (β-secretase) inhibitors: Drug development. *Curr. Alzheimer Res. 5*, 121–131.
25. Gao, J., Winslow, S. L., Vander Velde, D., Aube, J., and Borchardt, R. T. (2001) Transport characteristics of peptides and peptidomimetics: II. Hydroxyethylamine bioisostere-containing peptidomimetics as substrates for the oligopeptide transporter and P-glycoprotein in the intestinal mucosa. *J. Pept. Res. 57*, 361–373.
26. Coburn, C. A., Stachel, S. J., Li, Y. M., Rush, D. M., Steele, T. G., Chen-Dodson, E., Holloway, M. K., Xu, M., Huang, Q., Lai, M. T., DiMuzio, J., Crouthamel, M. C., Shi, X. P., Sardana, V., Chen, Z., Munshi, S., Kuo, L., Makara, G. M., Annis, D. A., Tadikonda, P. K., Nash, H. M., Vacca, J. P., and Wang, T. (2004) Identification of a small molecule nonpeptide active site β-secretase inhibitor that displays a nontraditional binding mode for aspartyl proteases. *J. Med. Chem. 47*, 6117–6119.
27. Congreve, M., Aharony, D., Albert, J., Callaghan, O., Campbell, J., Carr, R. A., Chessari, G., Cowan, S., Edwards, P. D., Frederickson, M., McMenamin, R., Murray, C. W., Patel, S., and Wallis, N. (2007) Application of fragment screening by X-ray crystallography to the discovery of aminopyridines as inhibitors of β-secretase. *J. Med. Chem. 50*, 1124–1132.
28. Hussain, I., Hawkins, J., Harrison, D., Hille, C., Wayne, G., Cutler, L., Buck, T., Walter, D., Demont, E., Howes, C., Naylor, A., Jeffrey, P., Gonzalez, M. I., Dingwall, C., Michel, A., Redshaw, S., and Davis, J. B. (2007) Oral administration of a potent and selective non-peptidic BACE-1 inhibitor decreases β-cleavage of amyloid precursor protein and amyloid-β production in vivo. *J. Neurochem. 100*, 802–809.
29. Barrow, J. C., Stauffer, S. R., Rittle, K. E., Ngo, P. L., Yang, Z., Selnick, H. G., Graham, S. L., Munshi, S., McGaughey, G. B., Holloway, M. K., Simon, A. J., Price, E. A., Sankaranarayanan, S., Colussi, D., Tugusheva, K., Lai, M. T., Espeseth, A. S., Xu, M., Huang, Q., Wolfe, A., Pietrak, B., Zuck, P., Levorse, D. A., Hazuda, D., and Vacca, J. P. (2008) Discovery and X-ray crystallographic analysis of a spiropiperidine iminohydantoin inhibitor of β-secretase. *J. Med. Chem. 51*, 6259–6262.
30. Erlanson, D. A., and Jahnke, W. (2006) *Fragment-based Approaches in Drug Discovery*, Wiley-VCH, Weinheim, Germany.
31. Hann, M. M., Leach, A. R., and Harper, G. (2001) Molecular complexity and its impact on the probability of finding leads for drug discovery. *J. Chem. Inf. Comput. Sci. 41*, 856–864.
32. Murray, C. W., Callaghan, O., Chessari, G., Cleasby, A., Congreve, M., Frederickson, M., Hartshorn, M. J., McMenamin, R., Patel, S., and Wallis, N. (2007) Application of fragment screening by X-ray crystallography to β-secretase. *J. Med. Chem. 50*, 1116–1123.
33. Edwards, P. D., Albert, J. S., Sylvester, M., Aharony, D., Andisik, D., Callaghan, O., Campbell, J. B., Carr, R. A., Chessari, G., Congreve, M., Frederickson, M., Folmer, R. H., Geschwindner, S., Koether, G., Kolmodin, K., Krumrine, J., Mauger, R. C., Murray, C. W., Olsson, L. L., Patel, S., Spear, N., and Tian, G. (2007) Application of fragment-based lead generation to the discovery of novel, cyclic amidine β-secretase inhibitors with nanomolar potency, cellular activity, and high ligand efficiency. *J. Med. Chem. 50*, 5912–5925.
34. Erlanson, D. A., Braisted, A. C., Raphael, D. R., Randal, M., Stroud, R. M., Gordon, E. M., and Wells, J. A. (2000) Site-directed ligand discovery. *Proc. Natl. Acad. Sci. U.S.A. 97*, 9367–9372.
35. Erlanson, D. A., Ballinger, M. D., and Wells, J. A. (2006) Tethering. In *Fragment-based approaches in Drug Discovery* (Jahnke, W., and Erlanson, D. A., Eds.) pp 285−310, Wiley-VCH, Weinheim, Germany.
36. Choong, I. C., Lew, W., Lee, D., Pham, P., Burdett, M. T., Lam, J. W., Wiesmann, C., Luong, T. N., Fahr, B., DeLano, W. L., McDowell, R. S., Allen, D. A., Erlanson, D. A., Gordon, E. M., and O'Brien, T. (2002) Identification of potent and selective small-molecule inhibitors of caspase-3 through the use of extended

tethering and structure-based drug design. *J. Med. Chem. 45*, 5005–5022.
37. Keire, D. A., Strauss, E., Guo, W., Noszal, B., and Rabenstein, D. L. (1992) Kinetics and equilibria of thiol/disulfide interchange reactions of selected biological thiols and related molecules with oxidized glutathione. *J. Org. Chem. 57*, 123–127.
38. Millis, K. K., Weaver, K. H., and Rabenstein, D. L. (1993) Oxidation/reduction potential of glutathione. *J. Org. Chem. 58* 4144–4146.
39. Ermolieff, J., Loy, J. A., Koelsch, G., and Tang, J. (2000) Proteolytic activation of recombinant pro-memapsin 2 (pro-$\beta$-secretase) studied with new fluorogenic substrates. *Biochemistry 39*, 12450–12456.
40. Pietrak, B. L., Crouthamel, M. C., Tugusheva, K., Lineberger, J. E., Xu, M., Dimuzio, J. M., Steele, T., Espeseth, A. S., Stachel, S. J., Coburn, C. A., Graham, S. L., Vacca, J. P., Shi, X. P., Simon, A. J., Hazuda, D. J., and Lai, M. T. (2005) Biochemical and cell-based assays for characterization of BACE-1 inhibitors. *Anal. Biochem. 342*, 144–151.
41. Cheng, Y., and Prusoff, W. H. (1973) Relationship between the inhibition constant (K1) and the concentration of inhibitor which causes 50% inhibition (I50) of an enzymatic reaction. *Biochem. Pharmacol. 22*, 3099–3108.
42. Hong, L., Koelsch, G., Lin, X., Wu, S., Terzyan, S., Ghosh, A. K., Zhang, X. C., and Tang, J. (2000) Structure of the protease domain of memapsin 2 ($\beta$-secretase) complexed with inhibitor. *Science 290*, 150–153.
43. Patel, S., Vuillard, L., Cleasby, A., Murray, C. W., and Yon, J. (2004) Apo and inhibitor complex structures of BACE ($\beta$-secretase). *J. Mol. Biol. 343*, 407–416.
44. Yang, W., Lu, W., Lu, Y., Zhong, M., Sun, J., Thomas, A. E., Wilkinson, J. M., Fucini, R. V., Lam, M., Randal, M., Shi, X. P., Jacobs, J. W., McDowell, R. S., Gordon, E. M., and Ballinger, M. D. (2006) Aminoethylenes: A tetrahedral intermediate isostere yielding potent inhibitors of the aspartyl protease BACE-1. *J. Med. Chem. 49*, 839–842.
45. Ballinger, M. D., and Randal, M. (2005) Constructs for homogeneously processed preparations of $\beta$ site APP-cleaving enzyme. PCT/US2004/021816.
46. Cole, D. C., Manas, E. S., Stock, J. R., Condon, J. S., Jennings, L. D., Aulabaugh, A., Chopra, R., Cowling, R., Ellingboe, J. W., Fan, K. Y., Harrison, B. L., Hu, Y., Jacobsen, S., Jin, G., Lin, L., Lovering, F. E., Malamas, M. S., Stahl, M. L., Strand, J., Sukhdeo, M. N., Svenson, K., Turner, M. J., Wagner, E., Wu, J., Zhou, P., and Bard, J. (2006) Acylguanidines as small-molecule $\beta$-secretase inhibitors. *J. Med. Chem. 49*, 6158–6161.
47. Erlanson, D. A., Wells, J. A., and Braisted, A. C. (2004) Tethering: Fragment-based drug discovery. *Annu. Rev. Biophys. Biomol. Struct. 33*, 199–223.
48. Coburn, C. A., Holloway, M. K., and Stachel, S. J. (2006) 2-Aminopyridine compounds useful as $\beta$-secretase inhibitors for the treatment of Alzheimer's disease. *PCT International Application.*
49. Garino, C., Pietrancosta, N., Laras, Y., Moret, V., Rolland, A., Quelever, G., and Kraus, J. L. (2006) BACE-1 inhibitory activities of new substituted phenyl-piperazine coupled to various heterocycles: Chromene, coumarin and quinoline. *Bioorg. Med. Chem. Lett. 16*, 1995–1999.
50. Garino, C., Tomita, T., Pietrancosta, N., Laras, Y., Rosas, R., Herbette, G., Maigret, B., Quelever, G., Iwatsubo, T., and Kraus, J. L. (2006) Naphthyl and coumarinyl biarylpiperazine derivatives as highly potent human $\beta$-secretase inhibitors. Design, synthesis, and enzymatic BACE-1 and cell assays. *J. Med. Chem. 49*, 4275–4285.
51. Fischer, H., Gottschlich, R., and Seelig, A. (1998) Blood-brain barrier permeation: Molecular parameters governing passive diffusion. *J. Membr. Biol. 165*, 201–211.
52. Habgood, M. D., Begley, D. J., and Abbott, N. J. (2000) Determinants of passive drug entry into the central nervous system. *Cell. Mol. Neurobiol. 20*, 231–253.