Application of Fragment Screening by X-ray Crystallography to β-Secretase†

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This paper describes an application of fragment screening to the aspartyl protease enzyme, β-secretase (BACE-1), using high throughput X-ray crystallography. Three distinct chemotypes were identified by X-ray crystallography as binding to the catalytic aspartates either via an aminoheterocycle (such as 2-aminoquinoline), a piperidine, or an aliphatic hydroxyl group. The fragment hits were weak inhibitors of BACE-1 in the millimolar range but were of interest because most of them displayed relatively good ligand efficiencies. The aminoheterocycles exhibited a novel recognition motif that has not been seen before with aspartic proteases. Virtual screening around this motif identified an aminopyridine with increased potency and attractive growth points for further elaboration using structure-based drug design. The companion paper illustrates how sub-micromolar inhibitors were developed starting from this fragment.

Introduction

Alzheimer's disease (AD[a]) is a neurodegenerative disorder associated with accumulation of amyloid plaques and neurofibrillary tangles in the brain.[1] The major components of these plaques are β-amyloid peptides (Aβs), which are produced from amyloid precursor protein (APP) by the activity of β- and γ-secretases. β-Secretase cleaves APP to reveal the N-terminus of the Aβ peptides.[2] The β-secretase activity has been identified as the aspartyl protease beta-site APP cleaving enzyme (BACE-1) and this enzyme is a potential therapeutic target for treatment of AD.[3−7] It is therefore of interest to discover inhibitors of BACE-1.

Aspartic proteases have been explored extensively as drug discovery targets and a number of HIV-1 protease inhibitors are used clinically. There has also been considerable drug discovery effort expended on renin, an enzyme more closely related to BACE-1. Renin inhibitors (and HIV-1 protease inhibitors) tend to be large and peptidic, and so obtaining compounds with good pharmacokinetic properties has proved challenging.[8] However, aliskiren[9,10] has shown very promising results in clinical trials recently, and in separate work Oefner et al. have revealed a number of nonpeptidic inhibitors of renin, which while large and lipophilic have a completely different architecture to exisiting inhibitors of aspartic proteases.[11−13] In contrast to renin and HIV-1 protease inhibitors, BACE-1 inhibitors face the additional challenge of crossing the blood−brain barrier where a stricter range of physical properties are required, relative to properties consistent with oral bioavailability (e.g., the Rule of 5).[14] A number of different approaches to the design of BACE-1 inhibitors have recently been reviewed.[15,16] Most work has been concentrated on the design of peptidomimetic inhibitors which usually employ a secondary alcohol as a transition state mimetic to displace the water molecule that sits between the two catalytic aspartic acids of the enzyme.[17−26] A potential problem with peptidomimetic inhibitors is that they tend to be relatively large and possess multiple hydrogen bond donors which means they may fall outside the expected property range required for crossing the blood−brain barrier.[27] Recently, there have been a number of research groups that have disclosed less peptidic[28,29] or nonpeptidic inhibitors,[30−32] although in the latter case there have been no crystal structures to support the binding modes proposed or to help drive the SAR forward. There is therefore considerable interest in the identification of nonpeptidic inhibitors of BACE-1 and in the determination of their associated binding modes.

Fragment-based approaches to lead discovery are gaining momentum in the pharmaceutical industry as a complementary approach to high throughput screening.[33,34] Fragments are compounds of low molecular weight (usually 100−250 Da) and typically would have low binding affinities (>100 μM). Fragment-based screening has a number of advantages over conventional screening of drug-like compounds. First, only small libraries of fragments (between a few hundred and a few thousand) need to be screened because molecules of low complexity have a much higher probability of being complementary to receptors when compared with drug-like molecules of high complexity.[35] The fragment libraries themselves can be chosen to be fragments of drug-like molecules and so in some sense can be thought of as representing the building blocks of drug-like molecules. Additionally, despite their low affinities, fragments possess good ligand efficiency (i.e., high ratios of free energy of binding to molecular size)[36−38] and form a small number of high quality interactions whereas hits from high throughput screening often display low ligand efficiency with potencies derived from a larger number of lower quality interactions.[36] This means that it is often possible to optimize fragments to high quality leads of relatively low molecular weight that possess better drug-like properties. The optimization can be achieved by synthesizing a relatively small number of compounds using structure-based drug design and, due to the simplicity of the initial fragment hits, the synthesis is often straightforward.

The low binding affinity of fragments means that they are often difficult to detect with bioassay-based screening. In the case of screening against a target like BACE-1, it was anticipated that particularly sensitive screening methods would be necessary

† Coordinates for the β-secretase complexes with compounds **1**−**5** have been deposited in the Protein Data Bank under accession codes 2OHK, 2OHL, 2OHM, 2OHN, and 2OFO, together with the corresponding structure factor files.

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[a] Abbreviations: AD, Alzheimer's disease; APP, amyloid precursor protein; BACE-1, beta-site APP cleaving enzyme; LE, ligand efficiency.

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because the low ligand efficiency of existing leads for aspartic proteases[8,15,16] implies that fragment-sized molecules are very likely to have affinities in the millimolar range. Crystallography has been shown to be a very sensitive method for detecting weakly binding fragments and has been explored by a number of groups.[34,39−43] We have developed a fragment screening methodology that we have called Pyramid which incorporates high throughput X-ray crystallography as a screening tool.[44−46] We have also described a robust and soakable crystallographic system for obtaining protein−ligand complexes with BACE-1.[47] Here we show that this system is suitable for performing fragment screening using X-ray crystallography and we apply a fragment screening approach to the enzyme. The companion paper describes how medicinal chemistry was used to improve the affinity of some of the hits.

Results and Discussion

Overview of Pyramid Process. In a previous article we have described in detail the Pyramid screening approach and its application to four drug targets (p38 MAP kinase, cdk2, thrombin, and PTP1B).[45] Here we give an overview of the approach and in subsequent sections indicate how some specific steps were carried out for screening against BACE-1.

We first produced a large number of BACE-1 protein crystals which we had previously shown were suitable for soaking.[47] The soaking conditions were demonstrated to be robust for use with a variety of fragments and mixtures of fragments. A library of 347 drug fragments that we had used previously was available for screening in cocktails of six, and several rounds of virtual screening were used to select a further 65 compounds for screening. BACE-1 crystals were soaked with the compound cocktails, and the X-ray structures for the crystals were obtained. AutoSolve[48] was used to fit and score fragments into the Fo-Fc electron density maps that were calculated after initial automatic refinement against an unliganded BACE-1 structure. Protein−ligand structures of bound fragments were then subjected to further refinement steps. The output from the Pyramid process was a set of experimentally determined binding modes for fragments bound to BACE-1 which could then be inspected using a molecular visualization package.

Drug Fragment Library. This library is based on the observation that a relatively small set of drug scaffolds and side chains appear commonly in drugs. A virtual library was produced by enumerating a list of ring scaffolds with a set of side chains according to a defined set of rules. The drug fragment library was made up of the compounds in the virtual library that were commercially available. For BACE-1, 347 drug fragments were screened in cocktails of six. The cocktailing was performed using a proprietary program that optimizes the dissimilarity of any two fragments within a cocktail. The dissimilarity function is specifically designed to reduce the chances of two compounds within the same cocktail having both a similar shape and being able to bind to the same region of a protein. The construction of the drug fragment library and the methodology used to cocktail the molecules have been described in detail elsewhere.[45]

Figure 1 shows the only two clear hits that were generated from screening the drug fragment set against BACE-1 (hit rate around 0.6%). These hits were independently confirmed by single compound soaks. Both compounds will be positively charged at the normal operating pH of the BACE-1 enzyme (around 4.5). The amine group sits in between the side chains of the two catalytic aspartates (Asp32 and Asp228) forming hydrogen bonds, and the protonated pyridine group forms an additional hydrogen bond with the side chain of Asp32. This amidine-like recognition of the catalytic aspartates represents a novel motif for interaction with aspartic proteases. Both compounds bind to an open flap form of the enzyme similar to the apo structure. They differ from each other and the apo form in the precise orientation of Tyr71 which seems to move in order to both accommodate and make favorable contacts with the heterocyclic ligands. The amidine-like pharmacophore was also present in another 14 fragments from the drug fragment set, but none of them produced a clear electron density map which suggested a binding event.

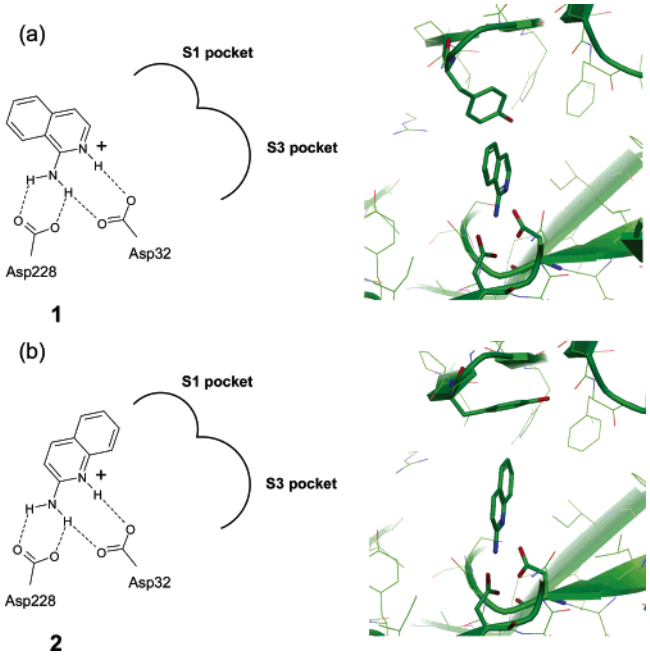

Figure 1. The binding mode of two fragments in the active site of BACE-1. On the left, a schematic representation of the key binding interactions is given. The experimental binding mode appears on the right-hand side with the protein backbone represented as a cartoon and the protein atoms in thin stick. The ligand and the residues Asp32, Tyr71, and Asp228 are represented in thick stick. In this orientation the flap is toward the top of the picture and the S_1 and S_3 pockets are on the right.

These fragment hits, **1** and **2**, display low affinity for the enzyme (approximately 30−40% at 1 mM), but they possess relatively high ligand efficiency. Here we use Hopkins et al.'s definition of ligand efficiency:[37]

$$\mathrm{LE} = -\Delta G/\mathrm{HAC} \approx -RT\ln(\mathrm{IC}_{50})/\mathrm{HAC}$$

where ΔG is the free energy of binding of the ligand for a specific protein, HAC is the number of heavy atoms in the ligand, and the IC_{50} represents the measured potency of the ligand for the protein. A compound of molecular weight 500 Da will have around 36 heavy atoms, and if its activity is 10 nM, then its ligand efficiency will be about 0.3. Assuming the IC_{50}s of **1** and **2** are ∼2 mM then their ligand efficiencies would be 0.33; one way of viewing this is that the initial fragment potency is sufficient to be optimistic of delivering a 10 nM inhibitor within the molecular weight guidelines of the "Rule of Five". We also believe that this ligand efficiency is favorable when compared to known BACE-1 inhibitors and other inhibitors of aspartic proteases, so these fragments represent promising starting points for structure-based optimization.[8,15,16]

Figure 2 shows fragment **2** superimposed onto the BACE-1 crystal structure of an early peptidomimetic inhibitor, OM99-2

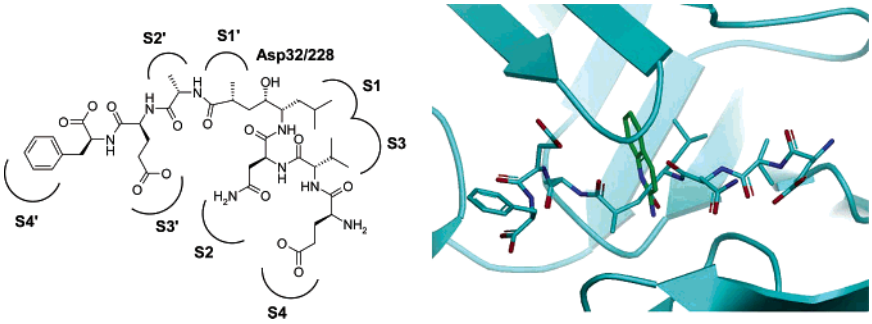

Figure 2. Fragment **2** (in green) superimposed onto a BACE-1 crystal structure (in cyan) with a peptidomimetic inhibitor (PDB Code: 1FKN). The 1FKN structure has a closed flap which is at the top of the figure with the key S_1 and S_3 pockets on the upper right-hand side (note that in contrast to the peptidomimentic inhibitor, fragment **2** binds to an open flap form of the enzyme).

(potency: 1.6 nM, ligand efficiency: 0.19).[49] The growth points off this fragment (and also fragment **1**) are nearly orthogonal to the areas of the active site exploited by the large peptidic ligand. These growth points allow exploration of the region underneath the flap which has been used in some potent ligands of the related aspartic protease, renin.[12] However, one of the initial design goals was to grow fragments into the S_1 and S_3 region of the enzyme because knowledge of the existing SAR on renin and BACE-1 inhibitors would suggest that this was also a useful region of the enzyme to target for improved potency.[10,50] It was therefore interesting to identify other fragments based on the binding motif or pharmacophore exemplified by **1** and **2** that would allow access to the S_1 and S_3 region more easily.

Virtual Screening. Several rounds of virtual screening were used to derive focused sets of fragments to be screened crystallographically against BACE-1. Virtual screening was performed on filtered lists derived either from the Astex Therapeutics Library of Available Substances (ATLAS) or from the Astex company registry. Examples of the properties used for filtering were molecular weight (<300), clogP (<3), the number of hydrogen bond donors (<4),[51] and restriction to chemical suppliers which in our previous experience had proven to be reliable.

In some virtual screens, 2D substructural filtering was also applied. For example, we were keen to find other molecules capable of forming the hydrogen bond interactions shown in Figure 1, and so we specifically searched for molecules containing a 2-aminopyridine substructure; the final selection contained five aminopyridines fragments. Similarly, it was known that cyclic secondary amines are capable of interacting with the catalytic aspartate groups in renin,[12,52] so a virtual screen was performed looking for molecules containing this functionality.

Compounds were docked against different protein conformations of BACE-1 using a proprietary version of the program GOLD.[53,54] The GoldScore[53,54] and ChemScore[55,56] functions were used to score and rank different fragments. Higher scoring solutions were visualized in the protein active site, and compounds which formed good hydrogen bonds with the catalytic aspartic acids and made lipophilic contact with the S_1 pocket of BACE-1 were selected. The virtual screening set consisted of 65 compounds which were then soaked into the crystal structure of BACE-1 yielding eight hits (hit rate around 12%).

Figure 3 gives the X-ray structure of an aminopyridine, **3**, that was selected from this virtual screening approach. This compound shows $IC_{50} = 310\ \mu M$ (LE = 0.32) in an enzymatic assay. The four hydrogen atoms in NH groups on the protonated fragment each form a good quality hydrogen bond with the

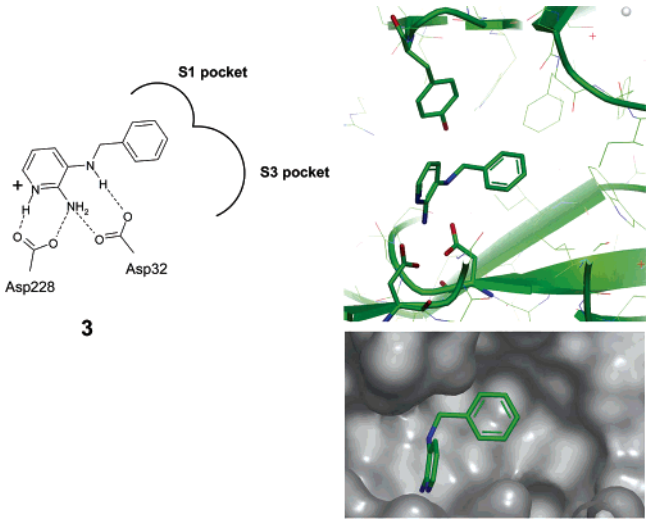

Figure 3. The binding mode of fragment **3** in BACE-1. (a) A schematic representation. (b) The experimental binding mode is given in a similar orientation and color scheme to Figure 1. (c) The binding mode in a surface representation of BACE-1 (in gray) showing the occupancy of the S_1 pocket by the phenyl group and the opportunity to 'grow' into the S_3 region from this phenyl.

catalytic aspartates. The flap is in an open position similar to the observed enzyme conformation with fragment **1**. The phenyl group on fragment **3** is positioned in the S_1 pocket of BACE-1 and provides suitable growth points to allow exploration of the S_3 pocket. It therefore represents an attractive fragment hit against the enzyme.

A virtual screening hit, **4**, that arose from the search for secondary amines is shown in Figure 4. This molecule shows 66% inhibition at 1 mM in an enzymatic assay. The piperidine forms two hydrogen bonds with the catalytic aspartates while the fluorophenyl group makes lipophilic contact in the S_1 pocket. The flap is in an open configuration similar to the one that is observed with fragment **1**. Figure 4(c) shows the crystallographic binding mode in renin of a potent piperazine-based renin inhibitor[52] superimposed onto the crystal structure for **4**. The rings of the two secondary amines superimpose very well, indicating that the key interactions with the aspartates are preserved in the weakly potent fragment. Assuming the IC_{50} of **4** is ~0.5 mM, then its ligand efficiency would be 0.32, indicating that **4** represents an attractive fragment hit for the enzyme.

General virtual screening also identified fragment **5**. Although the molecule shows no inhibition at 1 mM in an enzymatic assay, it was possible to obtain its crystal structure with BACE-1 (Figure 5). This molecule contains an aliphatic secondary alcohol that acts as a transition state mimetic forming multiple hydrogen

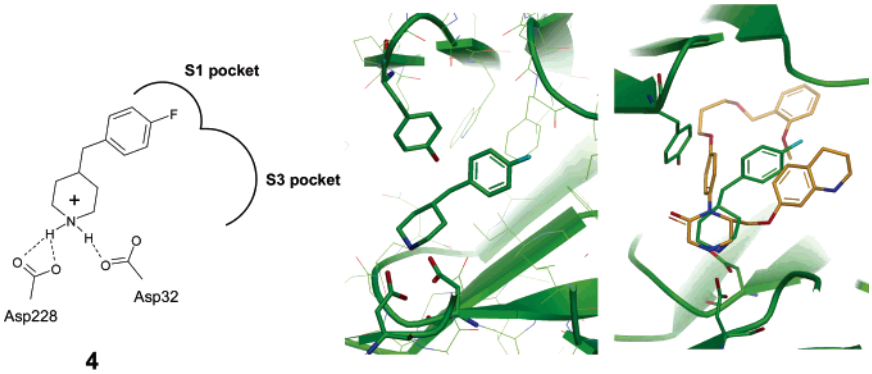

Figure 4. The binding mode of fragment **4** in BACE-1. (a) A schematic representation. (b) The experimental binding mode is given in a similar orientation and color scheme to Figure 1. (c) A crystal structure (PDB Code: 2BKT) of a potent piperazine-based renin inhibitor (orange) has been superimposed onto the crystal structure of **4**, illustrating the conserved nature of the interactions of the cyclic amine with the catalytic aspartate groups.

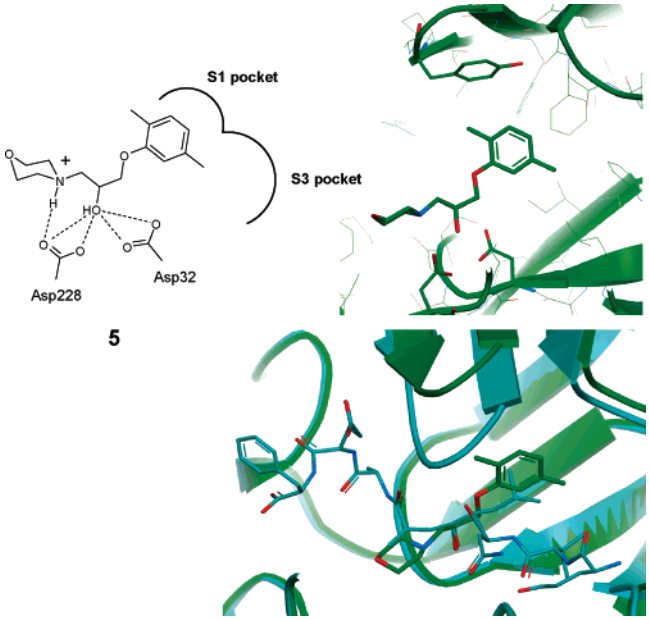

Figure 5. The binding mode of fragment **5** in BACE-1. (a) A schematic representation. (b) The experimental binding mode is given in a similar orientation and color scheme to Figure 1. (c) A crystal structure (PDB Code: 1FKN) of a peptidomimetic (cyan) has been superimposed onto the crystal structure of **5** (green) with the protein represented as a cartoon to illustrate the conserved nature of the hydroxyl interaction with the catalytic aspartates.

bonds with the catalytic aspartates. Such secondary hydroxyl groups are a common feature of aspartic protease inhibitors, and the position of this group is identical to that observed with peptidomimetic inhibitors (see Figure 5c).[49] The dimethylphenoxy group occupies the S_1 pocket and pushes up against the flap residue Tyr71 which is in a similar position to that observed with fragment **2**. The fragment is interesting from a design point of view because it is possible to grow off the phenyl group into the S3 region and/or into the region underneath the open flap. Additionally, the central portion of the molecule is reminiscent of a number of 'beta blockers' such as propanolol and is therefore a well precedented substructure in drugs. However, assuming this fragment has an $IC_{50} > 2$ mM, then its ligand efficiency is <0.19 which is considerably lower than the other fragments and perhaps indicates that this fragment would prove more difficult to optimize.

Discussion

We have applied the Pyramid screening technique to BACE-1 and identified a number of different fragments binding to the

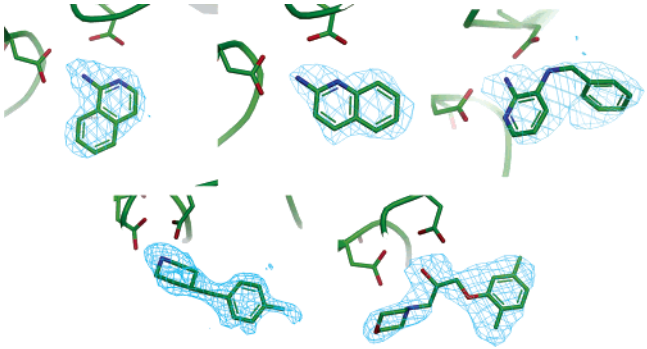

Figure 6. The ($F_o - F_c$) electron density OMIT maps in blue mesh for the weakest binding fragment of each chemotype: fragment **1** (left, contoured at 2σ), fragment **2** (middle, contoured at 2σ), fragment **3** (right, contoured at 1.7σ), fragment **4** (second row, left contoured at 2σ), and fragment **5** (second row, right contoured at 1.7σ). The maps have been clipped to aid visualization. Despite their low affinity, the fragments can be unambiguously positioned and can be seen to form key interactions with the two catalytic aspartates (top left of each figure).

enzyme that represent potential starting points for medicinal chemistry. The fragments form multiple hydrogen bonds with the catalytic aspartate groups and often form additional contact with the lipophilic S_1 pocket of BACE-1. The fragments can be classified according to the chemical motif that interacts with the catalytic aspartates. The first of these motifs is a secondary alcohol which is extremely well precedented in aspartic protease inhibition.[57] The second observed fragment motif is a secondary amine, and such a moiety (i.e., a piperidine) has recently been reported to bind renin,[11−13] prompting a considerable amount of interest in the design of related nonpeptidic aspartic protease inhibitors.[8,52] Much more recently *N*-phenylpiperazines have been reported as binding to BACE-1 although no crystal structure for these compounds has been reported so far.[30] Here, it has been demonstrated that fragment screening could straightforwardly identify this alternative chemotype without needing relatively potent and complex examples to be present in a high throughput screening collection. Additionally, the observed binding mode of these key recognition motifs in the fragments is the same as the observed binding mode of these motifs in potent compounds taken from the literature (Figure 4(c) and Figure 5(c)), indicating that the fragment binding modes are suitable starting points for structure-based drug design.

The third motif could be described as a planar delocalized positively charged group incorporating an NH_2 and another NH, exemplified here as a charged aminopyridine or aminoheterocycle. To the best of our knowledge this chemotype has not previously been observed to bind to the catalytic groups of

Table 1. Crystallographic Data Collection and Refinement Statistics[a]

compound	1	2	3	4	5
		Data Collection			
X-ray source	ESRF ID14.1, $\lambda = 0.934$ Å	SRS 14.2, $\lambda = 0.978$ Å	SRS 14.2, $\lambda = 0.978$ Å	ESRF ID14.2, $\lambda = 0.933$ Å	in house, $\lambda = 1.54$ Å
resolution (Å)	2.2	2.6	2.7	2.1	2.2
no. unique reflections	27344	15979	14335	29243	23.631
completeness (%)[b]	99.8(100)	100(100)	97.6(97.1)	99.6(99.1)	97.6(87.4)
average multiplicity	2.6	2.5	2.4	2.5	2.4
R_{merge}[b]	8.6(35.0)	13.2(42.2)	13.9(42.3)	8.5(36.1)	6.9(35.0)
		Refinement			
R_{free}	27.9	28.2	27.9	28.5	28.0
R_{cryst}	22.1	20.2	21.1	22.8	22.0
rmsd bond lengths (Å)	0.013	0.013	0.012	0.012	0.013
rmsd bond angles (deg)	1.4	1.5	1.4	1.4	1.4
average *B*-factor protein (Å^2)	37.3	37.4	44.9	44.4	45.1
average *B*-factor ligand (Å^2)	42.0	49.4	60.3	50.6	61.3
average *B*-factor solvent (Å^2)	37.2	31.4	33.9	44.3	41.2

[a] $R_{merge} = \Sigma_h \Sigma_i |I(h,i) - (h)| \Sigma hSi(h)$; $I(h,i)$ is the scaled intensity of the ith observation of reflection h and (h) is the mean value. Summation is over all measurements. $R_{cryst} = \Sigma_{hkl,work}||F_{obs}| - k|F_{calc}||/\Sigma hkl|F_{obs}I$, where F_{obs} and F_{calc} are the observed and calculated structure factors, k is a weighting factor, and work denotes the working set of 95% of the reflections used in the refinement. $R_{free} = \Sigma_{hkl,test}||F_{obs}| - k|F_{calc}||/\Sigma_{hkl}|F_{obs}|$, where F_{obs} and F_{calc} are the observed and calculated structure factors, k is a weighting factor, and test denotes the test set of 5% of the reflections used in cross validation of the refinement. λ refers to wavelength, rmsd to root-mean-square deviations. All ligands have been refined with occupancies of 1.0. [b] Numbers in parentheses indicate the highest shell values.

aspartic proteases and therefore represents a particularly interesting moiety on which to base a campaign of structure-based drug design. It is unclear why this motif was unprecedented against this class of enzymes and perhaps reflects that fragment screening offers superior sampling of chemical space relative to high throughput screening of drug-like molecules (for this system). In the companion paper we describe our experience in optimizing the potency of aminopyridine fragments.

The fragment hits identified in this work have low affinity for BACE-1, generally >1 mM, and yet as illustrated in Figure 6, they can be unambiguously identified in the unbiased electron density maps produced during fragment screening. As with other examples in the literature,[45,58] there is no indication that such weak inhibitors yield uninterpretable electron density maps, as might have been expected if fragment binding was driven by nonspecific interactions. On the contrary, weak inhibitors with high ligand efficiencies generally form high quality, specific interactions that are readily identified using crystallography.

BACE-1 crystallography entailed soaking into an apo structure in which the flap adopts an open conformation, and it had previously been demonstrated that under these soaking conditions peptidomimetic inhibitors can cause flap closure.[47] All the fragments identified here bind to an open flap form of the enzyme although the exact positioning of the flap and the Tyr71 side chain differs from fragment to fragment and from the apo structure; this is consistent with our previous experience that weakly bound fragments are capable of driving changes in protein conformation.[45] These observations also suggest that the flap is very mobile, and its closure is driven by the formation of hydrogen bonds with the ligand and by the precise occupancy of pockets that contact flap residues. It is interesting to note that none of fragments revealed here contain a single peptide bond, so it is perhaps not surprising that they bind to the open flap form of the enzyme.

Fragment screening typically utilizes biophysical techniques such as high throughput crystallography to identify fragments because of the concern that enzymatic assays may not be reliable at the very high concentrations required for fragment screening. It was our experience that the BACE-1 assay used to measure affinity in this work exhibited a high false positive rate, suggesting that further biophysical characterization of hits may be essential for this system. The use of an assay as a prescreen for the crystallography would have been problematic in this case because the false hits were often significantly more potent than the true binders and prioritization using assay results may easily have overlooked them. A future publication will report on the results of using NMR and Biacore methodologies to prioritize molecules for BACE-1 crystallography.

Conclusions

We have applied a fragment screening approach to BACE-1, successfully identifying and crystallizing three classes of novel nonpeptidic fragments that might form the basis of a medicinal chemistry program. One of these classes included a recognition motif that has not previously been observed to bind to aspartic proteases.[59] The companion paper shows how sub-micromolar inhibitors of BACE-1 have been generated starting from fragments that exhibit this novel recognition motif.

Experimental Section

Crystallography. Recombinant human beta secretase 1 (BACE-1) residues 14−453 was produced in bacteria as inclusion bodies and refolded using a method described previously.[47] Crystals of apo BACE-1 suitable for ligand soaking were obtained by a procedure described previously.[47] In brief, BACE-1 was buffer exchanged into 20 mM Tris (pH 8.2), 150 mM NaCl, 1 mM DTT, and concentrated to 8 mg/mL. DMSO (3% v/v) was added to the protein prior to crystallization. Apo BACE-1 crystals were grown using the hanging drop vapor diffusion method at 20 °C. Protein was mixed with an equal volume of mother liquor containing 20−22.5% (w/v) PEG 5000 monomethylethyl (MME), 200 mM sodium citrate (pH 6.6), and 200 mM ammonium iodide. Crystals were cryoprotected for data collection by brief immersion in 30% PEG 5000 MME, 100 mM sodium citrate (pH 6.6), 200 mM ammonium iodide, and 20% (v/v) glycerol. For inhibitor soaking, 0.5−0.1 M DMSO stock solutions were made. A tenfold dilution of the compound stock solution in a stabilization solution (33% (w/v) PEG 5000 monomethylethyl (MME), 110 mM sodium citrate (pH 6.6), and 220 mM ammonium iodide) was made. Crystals were added to the soaking solution for up to 6 h prior to flash freezing and data collection. The soaking was performed at pH of 6.6 and lower pHs are not tolerated by the crystals; this compares with the pH of 5.0 used in the assay. Data were collected at a number of synchrotron beam lines at the European Synchrotron Radiation Facility, Grenoble, France. All data sets were processed with MOSFLM[60] and scaled using SCALA.[61] Structure solution and

initial refinement of the protein−ligand complexes were carried out by our automated scripts using a modified apo BACE-1 structure (PDB code: 1W50) as a starting model. Bound ligands were automatically identified and fitted into $F_o - F_c$ electron density using AutoSolve[48] and further refined using automated scripts followed by rounds of rebuilding in AstexViewer v2[62] and refinement using Refmac.[63] Data collection and refinement statistics for crystal structures are presented in Table 1.

Modeling. One of the key challenges in docking and virtual screening against BACE-1 is the problem of protein flexibility. As described above, crystal structures showed that the size and the shape of the active site vary because the flap region is mobile and it can adopt different conformations.

In order to take into account protein flexibility during docking experiments it was decided to dock against distinct, experimentally determined protein conformations and choose the best ligand solution obtained against this ensemble. Four structures were selected for dockings and virtual screens. The PDB structure 1FKN was used to target the binding site with the flap in the closed conformation. The apo, aminoquinoline, and aminoisoquinoline structures were used to target different conformations of the binding site with the flap in the open state. For each structure a number of binding sites were constructed; each of them contained the catalytic region (Asp32 and Asp228), the flap residues potentially involved in the binding of the ligands (Val69, Pro70, Tyr71, Thr72, and Trp76), and finally one of the following combinations of adjacent pockets: S_1, S_1 plus S_3, S_2' plus S_1 and a large binding site constituted by S_1, S_1', S_2', and S_3. Two different protonation states were adopted for the catalytic aspartic dyad. Neutral compounds (e.g., secondary alcohols) were mainly docked against protein structures with neutral Asp32 and ionized Asp228 (in line with previous work[64,65]), whereas charged compounds (e.g., aminopyrimidines or piperidines) were mainly docked against protein structures with both aspartic acids in the ionized state.

All dockings and virtual screens were run on a Linux cluster using the Astex web-based virtual screening platform[66] with methods and settings previously described by Verdonk et al.[56] A number of filters (heavy atoms count, molecular weight, number of donors, number of acceptors, ClogP, and polar surface area) combined with the scoring functions Goldscore[53,54] and Chemscore[55] were applied to score and to rank the docked libraries. Of the two scoring functions, Goldscore performed generally better in terms of reproducing the correct binding modes of the different chemotypes and in scoring and ranking the ligands. Finally, virtual screens were visually analyzed, and fragments were selected based on the binding mode, the chemical tractability, and the score.

BACE-1 Assays. Activity of BACE-1 was measured using the peptide R-E(EDANS)-E-V-N-L-*D-A-E-F-K(DABCYL)-R-OH from Bachem. Assays were carried out in 50 mM sodium acetate, pH 5, 10% DMSO in 96-well black, flat-bottomed Cliniplates in a final assay volume of 100 μL. Compounds were preincubated with in-house produced BACE-1, and the reaction was initiated by adding 10 μM peptide substrate. The reaction rate was monitored at room temperature on a Fluoroskan Ascent platereader with excitation and emission wavelengths of 355 and 530 nm, respectively. Initial reaction rates were measured and IC_{50}s were calculated from replicate curves using GraphPad Prizm software.

Compounds that fluoresced under the conditions described above were assayed in an alternative assay. BACE-1 activity was measured using a FRET-based substrate supplied by PanVera (kit P2985). Assays were carried out in 50 mM sodium acetate, pH 4.5 (provided with kit), 5% DMSO in 96-well black, flat-bottomed 1/2 area Costar plates in a final assay volume of 50 μL. Compounds were preincubated as above for 5 min with in-house produced BACE-1, and the reaction was initiated by adding 0.25 μM peptide substrate. The reaction rate was monitored at room temperature on a SpectraMax Gemini XS platereader (Molecular Devices) with excitation and emission wavelengths of 545 and 595 nm, respectively. Initial reaction rates were measured and IC_{50}s were calculated as described above.

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