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Discovery of biphenylacetamide-derived inhibitors of BACE1 using *de novo* structure-based molecular design

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Abstract

Beta-secretase (BACE1), the enzyme responsible for the first and rate-limiting step in the production of amyloid-beta peptides, is an attractive target for the treatment of Alzheimer's disease. In this study, we report the application of the *de novo* fragment-based molecular design program SPROUT to the discovery of a series of non-peptide BACE1 inhibitors based upon a biphenylacetamide scaffold. The binding affinity of molecules based upon this designed molecular scaffold was increased from an initial BACE1 IC_{50} of 323 μM to 27 μM following the synthesis of a library of optimized ligands whose structures were refined using the recently developed SPROUT-HitOpt software. Although a number of inhibitors were found to exhibit cellular toxicity, one compound in the series was found to have useful BACE1 inhibitory activity in a cellular assay with minimal cellular toxicity.

This work demonstrates the power of an *in silico* fragment-based molecular design approach in the discovery of novel BACE1 inhibitors.

Introduction

Alzheimer's disease (AD) is the most common neurodegenerative disease which currently affects more than 30 million people worldwide.[1] The number of AD patients is expected to increase significantly with the ageing human population. While current approved treatments using acetylcholinesterase inhibitors and N-methyl-D-aspartate (NMDA) receptor antagonists provide symptomatic relief for AD patients, there is a lack of effective therapy targeting the underlying pathophysiology of AD.[2]

The accumulation of amyloid-β peptide (Aβ) to form the extracellular senile or amyloid plaques is widely accepted to play a central role in the pathogenesis of AD.[3] According to the amyloid cascade hypothesis,[4] Aβ is generated through the sequential proteolytic cleavage of the amyloid precursor protein (APP) catalyzed by the β- and γ-secretases, respectively.[5] Since β-secretase is responsible for the first and rate-limiting step in the formation of Aβ, it represents an attractive therapeutic target to halt the progression of this debilitating disease.

β-Site APP cleaving enzyme 1 (BACE1) is responsible for β-secretase activity *in vivo*.[6] A type I transmembrane protein, BACE1 belongs to the family of pepsin-like human aspartyl proteases, characterized by a single transmembrane domain. With the catalytic site located on the lumenal side of the membrane, BACE1 processes the substrate APP at Met671-Asp672 (APP_{770} numbering) to release soluble sAPPβ. The membrane-bound fragment C99 is then processed by γ-secretase to generate the amyloidogenic species Aβ.[3, 4, 7] Since the first X-ray crystal structures of BACE1 were reported in 1999,[6, 8, 9] numerous efforts have been made in developing novel and potent inhibitors of BACE1.

In an analogous fashion to the initial development of HIV protease inhibitors in the 1990s,[10] the development of BACE1 inhibitors has primarily focused on substrate-based polypeptides with a non-cleavable transition-state isostere replacing the scissile amide bond. Due to the relatively complex structure of the substrate and the hydrophobic nature of the BACE1 active site, the majority of the early BACE1 inhibitors were characterized by high molecular weights and complex motifs that are lacking in

drug-like properties.[11] More recently, the application of high-throughput screening approaches has led to the identification of non-peptide BACE1 inhibitors.[12-14] A complementary strategy for BACE1 inhibitor discovery is the application of computer-aided methods to design small-molecule non-peptidic inhibitors using X-ray crystallographic structures of BACE1.

Computational structure-based molecular design is a strong research tool in medicinal chemistry and chemical biology, and such methods are gaining popularity both in early-stage hit discovery and in establishing structure-activity relationships (SAR) during lead optimization stages. The utility of one such approach, virtual high-throughput screening, in the discovery of BACE1 inhibitors has recently been demonstrated.[15-18] A powerful alternative ligand identification strategy is the use of *in silico* fragment-based molecular design which, to our knowledge, has not been applied to the discovery of BACE1 inhibitors. In this approach, using an X-ray crystal structure of the target enzyme, new potential ligand structures are constructed *de novo*, via the precise docking of molecular fragments into chosen regions of the targeted site. These fragments are then joined together in ways dictated by the user to yield synthetically approachable ligand scaffolds which are predicted to show good affinity for the target enzyme.[19-21] Here, we present the studies of a new BACE1 inhibitor scaffold designed using the *de novo* molecular design program SPROUT.[22, 23] A library of structural analogs of the initial designed skeleton was synthesized and tested against recombinant BACE1. SAR analysis and ligand optimization of this library of compounds were achieved using hit optimization software SPROUT-HitOpt.

Results and Discussion

De novo ligand design

We have previously described the application of the *de novo* molecular design program SPROUT to the production of a number of enzyme inhibitors and receptor antagonists.[19-21] In order to aid the generation of potent BACE1 inhibitors using SPROUT, the crystal structure of BACE1 co-crystallized with inhibitor OM00-3 reported by Tang and co-workers was used (PDB code 1M4H; 2.1 Å).[24] A simple non-peptidic ligand scaffold was constructed using SPROUT by joining small fragments docked into selected individual target sites with spacer templates consisting of six-membered aromatic rings (**Figure 1**). The proposed mode of inhibitory action of the designed scaffold was to obstruct the binding of the substrate APP by blocking its entry into the binding pocket. During planning for the synthesis of molecules corresponding to the designed ligand scaffold, we decided, for simplicity, to place methylene units at the three points linking the fragments (marked 'X', 'Y', and 'Z' in Figure 1(b)). It should be noted that other substituents at the 'Z' position of the designed ligand scaffold, including C=O (producing an amide) and SO_2 (producing a sulfonamide) were considered as part of the design process and were predicted to bind with similar affinity as for the amino variants (Z = CH_2). However, in light of the rather hydrophobic nature of the designed scaffolds, we decided to concentrate on the amines as these were anticipated to have enhanced aqueous solubility compared to the amide and sulfonamide variants. This revealed our initial target as amine **1** (**Figure 2**, shown bound in BACE1 as the ammonium form as anticipated to predominate under physiological conditions and is located near to the surface of the protein and exposed to solvent). This designed molecular scaffold was synthesized, and tested in an *in vitro* BACE1 activity assay using a quenched fluorescent peptide substrate analog based on the Swedish mutant APP sequence (SEVNL$^{\downarrow}$DAEFK).[25] 25 nM recombinant BACE1 (R&D Systems, Minneapolis, USA) was pre-incubated with inhibitor compounds for 30 mins before addition of substrate analog (FAM-SEVNLDAEFK-TAMRA (5 μM) and kinetic measurement of substrate cleavage by determination of the increase in relative fluorescence units (RFU) compared to controls

(further details in the Supporting Information). Compound **1** was found to display a modest affinity against BACE1, with an IC_{50} of 323 μM (**Table 1**).

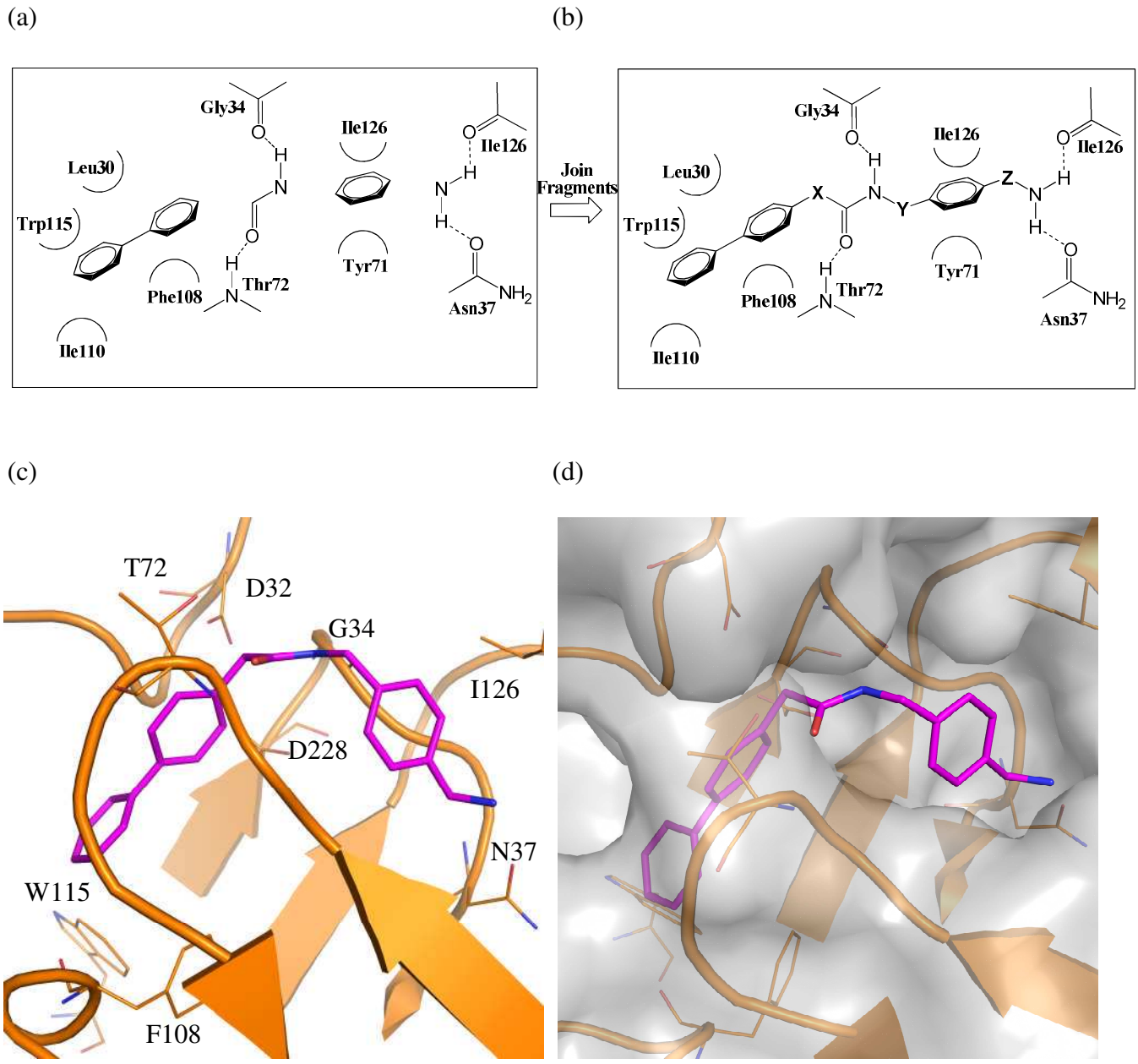


Figure 1. *De novo* design of biphenylacetamide inhibitor **1** in the BACE1 binding pocket (PDB 1M4H). *(a)* Fragments docked into selected individual target sites, *(b)* fragments joined to yield complete ligand scaffold, *(c)* proposed binding mode of **1** (magenta) with key residues labeled (orange); and *(d)* proposed binding mode of **1** with surface of the binding pocket.

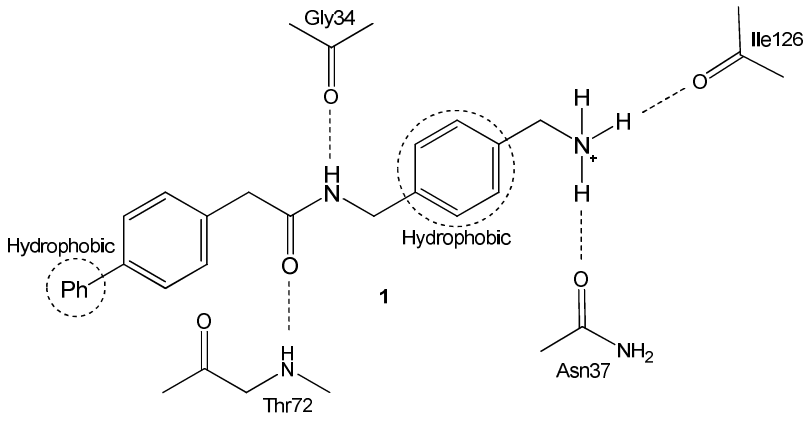
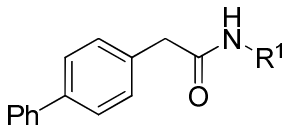


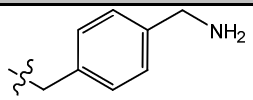
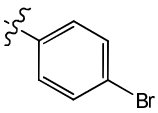
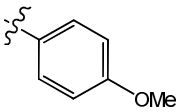
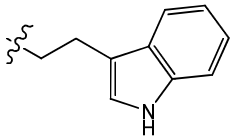
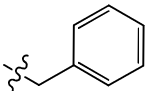
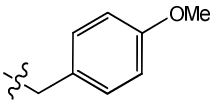
Figure 2. Schematic showing the proposed interactions of inhibitor **1 (shown in ionic form)** with BACE1.

SAR study

To effectively probe the essential features of compound **1** required for BACE1 inhibition, we prepared structural analogs of **1** that possessed different functional groups and flexible chain lengths in an attempt to establish an SAR profile, and to probe the proposed binding mode of the inhibitor. As illustrated in **Figure 2**, the ligand was designed to make important H-bond contacts with residues Asn37 and Ile126, and consequently occupying the deep binding pocket proximal to these residues. The introduction of different flexible chain lengths in the R^1 portion of the inhibitor (**Table 1**, compounds **2**-**4**) would alter the geometry of the designed molecule, and was predicted not to bind according to our model (see Supporting Information for predicted binding scores of selected compounds). We also anticipated that functional group alterations would render the designed scaffold inactive (**Table 1**, compounds **5**-**6**). Biological assay data as listed in **Table 1** agreed well with our model predictions, and support the presence of the amine function in compound **1** as being important for inhibition of BACE1.

Table 1. Analogs of compound **1** synthesized to probe the essential features for BACE1 inhibition



Compound	R^1	Inhibition at 100 μM (%)[b]
1[a]		35.6±1.7
2		n.i.[c]
3		n.i.
4		n.i.
5		15.2±1.5
6		n.i.

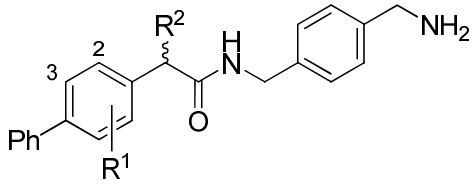
[a] Formulated as the trifluoroacetate salt; [b] Inhibition of BACE1 activity measured as a percentage of the control activity in an *in vitro* BACE1 activity assay (see text). Values shown are mean ± SEM, $n \geq 3$; [c] no inhibition.

Having established one of the important features of **1** required for the inhibition of BACE1, we strived to enhance the binding affinity of the designed skeleton through the introduction of additional functional groups by examining our *in silico* model. First, we observed that while the central aromatic ring of the biaryl system was acting as a spacer, its orientation was in close proximity to the catalytic aspartate residues, Asp32 and Asp228. Introduction of substituents bearing H-bond donor groups on this aromatic ring might form favorable H-bond interactions with the side-chains of these catalytic aspartate residues. Therefore, we synthesized both the *ortho*-hydroxy (**7**) and the *meta*-hydroxy (**8**) analogs to test our hypothesis. However, this proposed alteration did not show significant differences in the inhibition of BACE1 (**Table 2**).

Next, we investigated the availability of additional sub-pockets located near the ligand binding regions proposed. The S1' pocket of the enzyme could be filled with substituents stemming from the

alpha-C position between the biaryl system and the amide scaffold ('X' position in Figure 1(b)). Several analogs with different branch chains at the alpha-C position were synthesized and assayed against BACE1. The trend in the BACE1 IC_{50} values clearly suggested that the larger the branch chain was at this alpha-C position, the higher the affinity was towards BACE1, with compounds containing the isopropyl (**11**) and benzyl (**12**) branch chains achieving a 10- to 12-fold increase in potency compared to **1**, respectively. Compound **12**, in particular, attained a BACE1 IC_{50} of 26.9 μM (**Table 2**).

Table 2. Analogs of compound **1** with enhanced inhibition of BACE1



Compound	R^1	R^2	Inhibition at 100 μM (%)	IC_{50} (μM)
1	H	H	35.6±1.7	323±5
7	2-OH	H	42.5±0.6	>100
8	3-OH	H	28.1±3.3	>100
9	3-F	Me	51.7±2.7	77.1±7.5
10	3-F	Me (pure *R*-isomer)	55.5±1.6	85.7±11.8
11	H	^{i}Pr	66.1±2.2	33.5±4.8
12	H	CH_2Ph	101.5±0.0	26.9±4.7

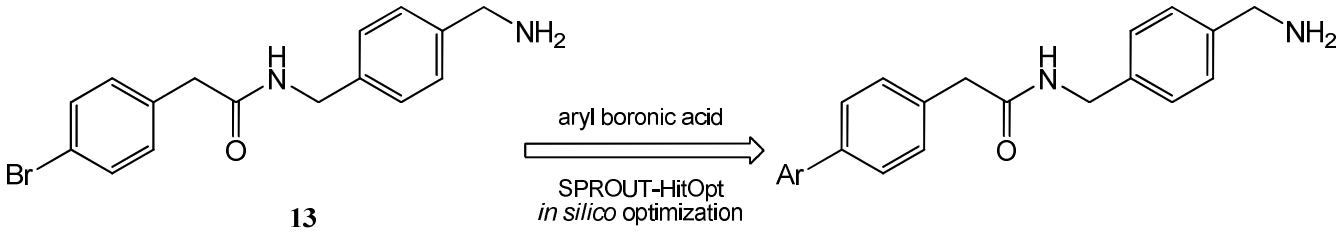
Further optimization using SPROUT-HitOpt

Very recently, we have developed a variant of the *de novo* molecular design software SPROUT, SPROUT-HitOpt, to assist ligand optimization. The aim of this software is to modify the structure of a known ligand and generate variants predicted to display enhanced binding affinity towards the target enzyme, by fulfilling additional H-bond target sites and/or hydrophobic pockets. A particular feature of this software is that the optimization process is performed using part of the known ligand as a core

structure, in which functional groups are identified as points of extension. Extension from this core structure is achieved by connecting the identified functional groups to a library of commercially available starting materials, using connection rules defined in a synthetic chemistry knowledge base. This program was recently reported to have successfully optimized inhibitors of the anti-malarial target *Plasmodium falciparum* dihydroorotate dehydrogenase.[20]

Since the terminal ring of the biaryl system of inhibitor **1** was docked into a hydrophobic site near Phe108 and Trp115, we envisaged that more extensive occupancy of this hydrophobic site by extending deeper into the pocket, or the introduction of additional hydrophobic substituents, would maximize the potential of this binding pocket. Therefore, we decided to utilize SPROUT-HitOpt to perform a thorough exploration of the available binding regions proximal to this hydrophobic site, and to improve the predicted binding affinity of **1**. Using **13** as the core structure for SPROUT-HitOpt extension and an *in silico* library of commercially available aryl boronic acids (**Scheme 1**), compound **14**, with a 10-fold increase in predicted binding affinity compared to inhibitor **1**, was identified as one of the best optimized candidates out of 174 structures generated (**Figure 3**). Biological assay results suggested that compound **14** had an increased binding affinity towards BACE1 compared to inhibitor **1**, attaining an IC_{50} value of 63.9 μM (**Table 3**).

Scheme 1. SPROUT-HitOpt core extension to optimize the terminal aromatic ring of **1**



13

(a) (b)

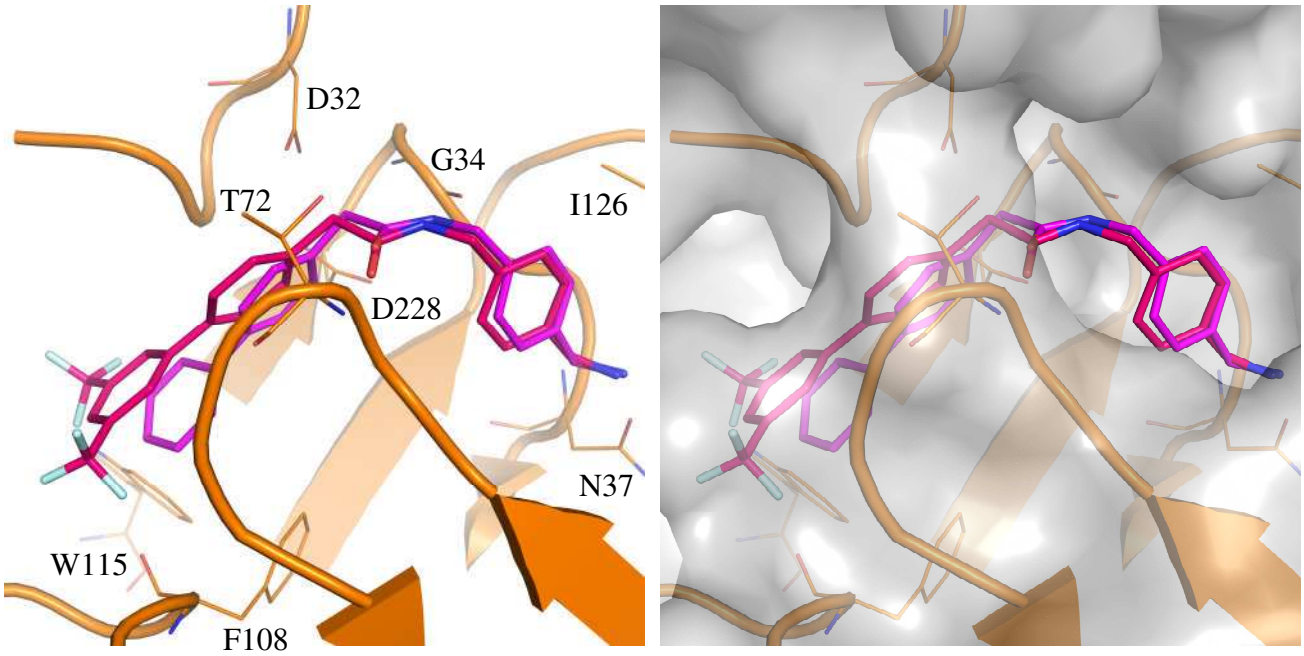
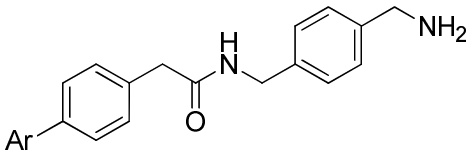


Figure 3. The optimized candidate structure **14** (pink), designed using SPROUT-HitOpt, overlaid with the predicted binding pose of **1** (magenta). *(a)* Proposed binding mode of **14** (pink) with key residues labeled (orange); and *(b)* proposed binding mode of **14** with surface of the binding pocket.

Following the improved BACE1 binding affinity of **14** through increasing hydrophobicity of the terminal aromatic ring, we decided to assess the effects of different hydrophobic substituents on BACE1 inhibition. Whereas the pentafluorophenyl analog (**15**) was found to be slightly less potent than **14**, the 3-(trifluoromethyl)phenyl analog (**16**), despite possessing only one CF_3 group, exhibited similar inhibition of BACE1 as compared to **14**. Furthermore, we prepared compound **17** to investigate the importance of hydrophobic substituents on the terminal phenyl ring for binding to BACE1. Compound **17** failed to reach an IC_{50} value below 100 μM, for which we propose the difference was mainly attributed to the presence of the polar phenolic moiety (**Table 3**).

The most potent of our compounds, compound **12** attained an IC_{50} of 26.9 μM under our assay conditions. This is relatively high compared to other published (GSK188909)[25] and commercially available BACE inhibitors (β-secretase inhibitor IV, Calbiochem, Nottingham, UK) that have IC_{50}s in the nM range.

Table 3. Analogs of inhibitor **1** designed to increase hydrophobicity of the terminal phenyl ring of the biaryl scaffold



Compound	Ar	Inhibition at 100 μM (%)	IC_{50} (μM)
1	Ph	35.6±1.7	323±5
14	3,5-$(CF_3)_2C_6H_3$	80.6±2.3	63.9±7.2
15	C_6F_5	51.8±8.4	101±15.7
16	3-$(CF_3)C_6H_4$	60.7±2.2	58.9±9.0
17	4-$(OH)C_6H_4$	41.3±3.5	>100

Compound specificity: BACE2 inhibition

To test the specificity of the compounds against other related proteases, their activity against BACE2 was also measured in the *in vitro* assay. BACE2 is also a type I transmembrane aspartyl protease whose amino acid sequence is 45% identical and 75% similar to BACE1.[26] The assay conditions used were identical to those for BACE1. The results indicated that all of the compounds tested showed at least a two-fold selectivity for BACE1 over BACE2 except for compounds **12** and **14** (**Table 4**). The specificity of our compounds for BACE1 over BACE2 suggests that they will have limited effects on other structurally related proteases.

Table 4. Relative inhibition of BACE1 and BACE2.

Compound	BACE1 IC_{50} (μM)	BACE2 IC_{50} (μM)
1	323±5	>1000

9	77.1±7.5	207.2±31.2
10	85.7±11.8	193.8±34.1
11	33.5±4.8	100.5±4.1
12	26.9±4.7	21.0±2.37
14	63.9±7.2	33.2±12.0
15	101±15.7	245.9±34.9
16	58.9±9.0	319.3±34.9
22	63.3±9.1	>1000

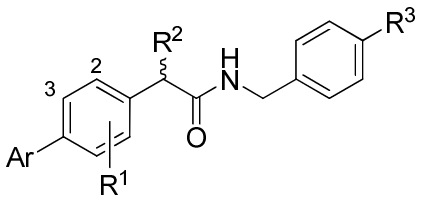
Values shown are mean ± SEM, $n \geq 3$.

Compound toxicity assay and cellular BACE1 inhibition

To assess the potential of this series of *de novo* designed inhibitors for further development, we used mammalian cells to assess compound toxicity and cellular BACE1 inhibition. Compounds were screened for toxicity using the cell viability MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay to determine the percentage of viable cells remaining following compound incubation at a concentration of 100 μM over a 24-hour period. Compounds that induced a loss of greater than 50% in cell viability were not tested further for cellular BACE1 inhibition. Compounds that retained cell viability of 50% or greater at 100 μM were tested on human embryonic kidney (HEK 293) cells stably expressing BACE1 and wild type human APP that was N-terminally tagged with alkaline phosphatase (AP-APP) to determine potential BACE1 inhibitory activity. The release of soluble N-terminal sAP-APP fragments into the media, was determined by measuring the resulting alkaline phosphatase activity of conditioned media samples taken after the incubation. Inhibition of BACE1 would display a decrease in the alkaline phosphatase activity of conditioned media compared to control levels. The alkaline phosphatase activity following compound incubation was expressed as a percentage of the activity in the appropriate control sample.

Many of the changes introduced to the middle aromatic ring of the biaryl scaffold, and also the addition of branch chains at the alpha-C position, rendered the compounds very toxic (compounds **7**-**12**, **Table 5**). In the collection of inhibitors which possessed a terminal phenyl ring with hydrophobic substituents, compound **14**, containing two CF_3 groups, was shown to be highly toxic, whereas the pentafluorophenyl (**15**) and 3-(trifluoromethyl)phenyl (**16**) analogs were less toxic. The primary amine of this series of inhibitors, which, from our earlier studies appeared to be important for BACE1 inhibition, also appeared to contribute to cell toxicity. A comparison between the percentage cell viability of **1** to that of **5** and **6** revealed that compounds without the primary amine, although inactive towards BACE1, were much less toxic at 100 μM. Intrigued by this observation, we decided to further investigate whether the hydrophobic substituents on the terminal phenyl ring of the biaryl scaffold were partially responsible for the toxicity of compounds **14**-**16**. Analogs **18**-**20**, the corresponding analogs of **14**-**16** without the primary amine, were assessed. Consistent with our conclusions from the SAR study, analogs **18**-**20** were shown to be inactive towards BACE1 but compound toxicity was decreased, with the exception of analog **20** (we suspected that this discrepancy might be owing to the low solubility of this compound). It is, however, evident that the hydrophobic substituents on the terminal phenyl ring, including the *bis*-(trifluoromethyl)phenyl and the pentafluorophenyl scaffolds, were accountable for a certain level of toxicity in cells, as the percentage cell viability following incubation with analogs bearing these functionalities was comparatively lower than compound **5** containing only a simple phenyl ring.

Table 5. MTT compound toxicity assay data



Compound	Ar	R^1	R^2	R^3	IC_{50} (μM)	Cell viability[a] (%)

1	Ph	H	H	CH_2NH_2	323±5	42±3
5	Ph	H	H	H	*n.a.*[b]	93±1
6	Ph	H	H	OMe	*n.a.*	82±1
7	Ph	2-OH	H	CH_2NH_2	>100	18±1
8	Ph	3-OH	H	CH_2NH_2	>100	50±7
9	Ph	3-F	Me	CH_2NH_2	77.1±7.5	11±0
10	Ph	3-F	*R*-Me	CH_2NH_2	85.7±11.8	18±1
11	Ph	H	^{i}Pr	CH_2NH_2	33.5±4.8	50±4
12	Ph	H	CH_2Ph	CH_2NH_2	26.9±4.7	15±1
14	3,5-$(CF_3)_2C_6H_3$	H	H	CH_2NH_2	63.9±7.2	8±2
15	C_6F_5	H	H	CH_2NH_2	101±5.7	68±5
16	3-$(CF_3)C_6H_4$	H	H	CH_2NH_2	58.9±9.0	46±5
18	3,5-$(CF_3)_2C_6H_3$	H	H	H	*n.a.*	76±2
19	C_6F_5	H	H	H	*n.a.*	61±3
20	3-$(CF_3)C_6H_4$	H	H	H	*n.a.*	27±3

Values shown are mean ± SEM, $n \geq 3$. [a] Data measured at an inhibitor concentration of 100 μM, percentage inhibition relative to control; [b] not active.

The cell viability assay results suggested that only compound **15** could be tested further to assess whether it resulted in cellular BACE1 inhibition; all other compounds were either considered too toxic, or showed no significant inhibition of BACE1 and were therefore not suitable for further cell-based testing. Incubation of HEK cells expressing AP-APP and BACE1 with 100 μM of compound **15**, resulted in a 39.4±2.7% inhibition of sAP-APP production, indicating a potentially cell-active compound. To determine the effects of compound **15** on BACE1 activity specifically, we analyzed the effect of the compound on HEK cells over-expressing BACE1. The cells were incubated with the compound, then the conditioned media from these cells was western blotted using a specific sAPPβ

antibody, 1A9 (see Supporting Information for further details).[27] Compound **15** significantly decreased sAPPβ levels in these cells, while sAPPα levels were not significantly altered (**Figure 4**). These results suggested that compound **15** significantly inhibited cellular BACE1 activity with little effect on α-secretase activity, and indicated that compound **15** was a cell-active BACE1 inhibitor.

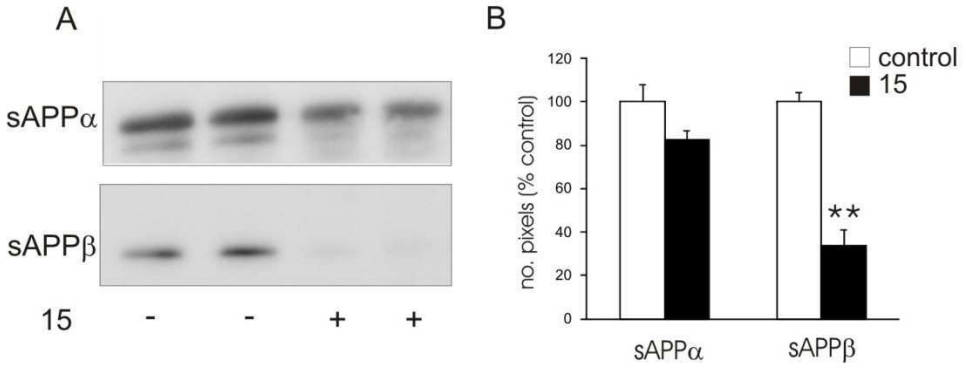


Figure 4. A) Representative western blot of sAPPα and sAPPβ fragments in conditioned media from HEK 293 cells over-expressing BACE1 incubated for 24 hours with compound **15** at 100 μM. B) Densitometric analysis of A). Data shown as mean ± SEM, $n = 4$, **$p<0.01$.

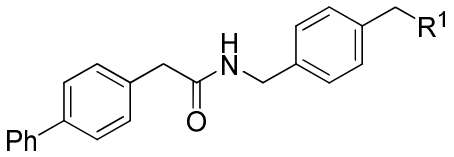
Tackling compound toxicity

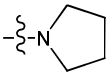
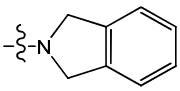
Whilst the presence of the primary amine appeared to be an important feature for the binding of this series of inhibitors within BACE1, the presence of this functionality resulted in the compounds exhibiting cell toxicity. We reasoned that this might be owing to the presence of the benzylamine moiety which is particularly prone to cytochrome P450 metabolism at the methylene position to produce toxic metabolites such as benzaldehydes in cell media.[28, 29] As indicated previously, although the amide and sulfonamide variants of compound **1** (Figure 1(b), Z = CO and SO_2) were predicted to show favorable binding to BACE1, we were concerned about the solubility of such variants. As an alternative, we reasoned that conversion of the primary amine into a tertiary amine moiety might reduce the toxicity associated with this inhibitor series. Interestingly, modeling of the tertiary amine variants of compound **1** within BACE1 indicated that these derivatives may adopt a significantly different binding pose to that

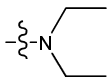
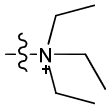
predicted for compound **1**. The tertiary amine moiety (presumably protonated at physiological pH) would not be involved in H-bonding contacts to the protein but would instead re-orientate in order to allow hydrophobic interaction between the N-alkyl groups and the side-chain of Ile126. Additionally, considerable movement of the core section of the molecule was predicted to result in the formation of new H-bond contacts involving Thr231 (see Supporting Information).

Four analogs containing tertiary or quaternary amines were synthesized. All these analogs were shown to be active against BACE1, with compound **22**, in particular, attaining an IC_{50} against BACE1 of 63.3 μM (**Table 6**). Cell viability of compound **22** was also improved as anticipated (80% cell viability at 100 μM compound concentration) and in the cell-based assay, compound **22** (100 μM) resulted in a 21.7±9.9% inhibition of sAP-APP production. Compound **22** was therefore assessed for cellular BACE1 inhibition in HEK cells over-expressing BACE1 by western blotting (as described for compound **15**). Compound **22** (100 μM) decreased sAPPβ production by 33% compared to control levels, but this was not statistically significant (p=0.064); sAPPα levels were decreased by 10% (**Figure 5**).

Table 6. Tertiary and quaternary amine analogs of inhibitor **1** designed to tackle the toxicity associated with this series of inhibitors.



Compound	R^1	Inhibition at 100 μM (%)	IC_{50} (μM)	Cell viability[a] (%)
1	NH_2	35.6±1.7	323±5	42±3
21		24.4±7.6	>100	24±1
22		70.1±1.2	63.3±9.1	80±6

23		34.4±3.3	>100	38±2
24		44.1±8.7	>100	40±1

Values shown are mean ± SEM, $n \geq 3$. [a] Data measured at an inhibitor concentration of 100 μM, percentage inhibition relative to control.

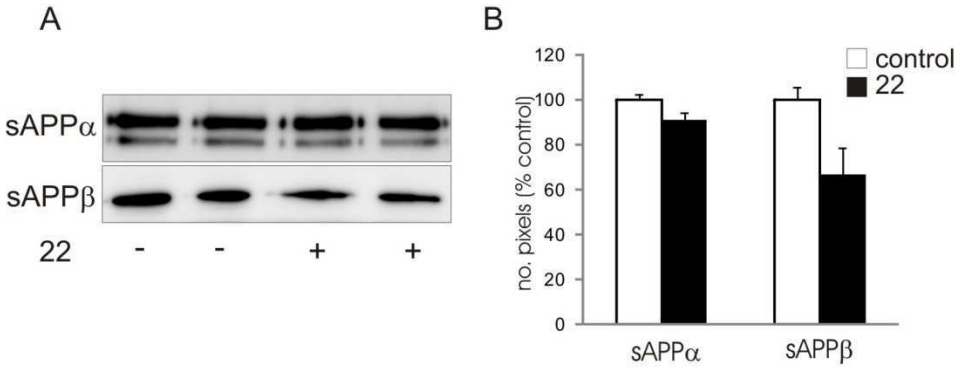
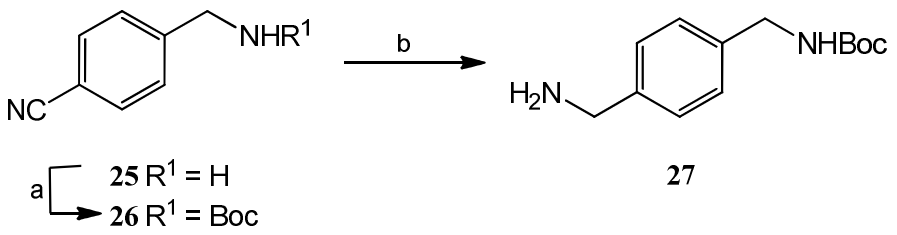


Figure 5. A) Representative western blot of sAPPα and sAPPβ fragments in conditioned media from HEK 293 cells over-expressing BACE1 incubated for 24 hours with compound **22** at 100 μM. B) Densitometric analysis of A). Data shown as mean ± SEM, $n = 3$.

Chemistry

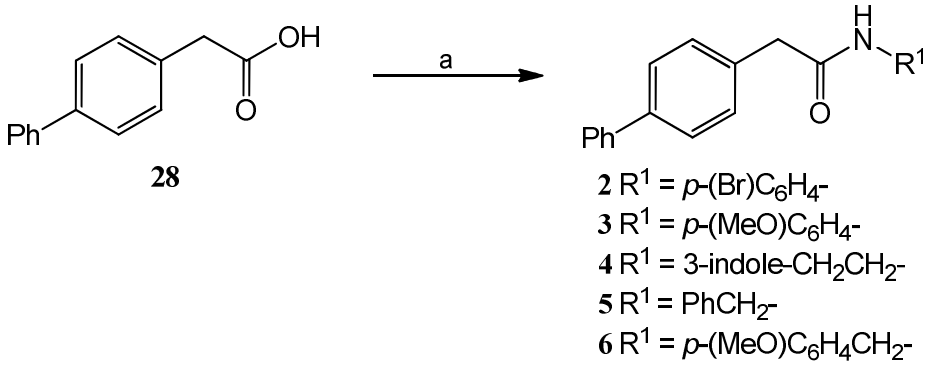
Preparation of **1** was achieved by firstly protecting 4-(aminomethyl)benzonitrile **25** following the procedure described by Goodyer *et al.* to give the Boc-protected amine **26**,[30] which was reduced using $LiAlH_4$ to obtain the intermediate **27** in 52% yield over two steps (**Scheme 2**). The coupling of 4-biphenylacetic acid **28** with **27** delivered **1** in 65% yield using the peptide coupling reagents HOBt and EDAC. Similarly, compounds **2**-**6** were readily synthesized by coupling 4-biphenylacetic acid **28** with the respective amine (**Scheme 3**).

Scheme 2.



Reaction conditions: a) $(Boc)_2O$, NEt_3, CH_2Cl_2, 0°C-rt, 16 h, 95%; b) $LiAlH_4$, THF, 0°C-rt, 48 h, 55%.

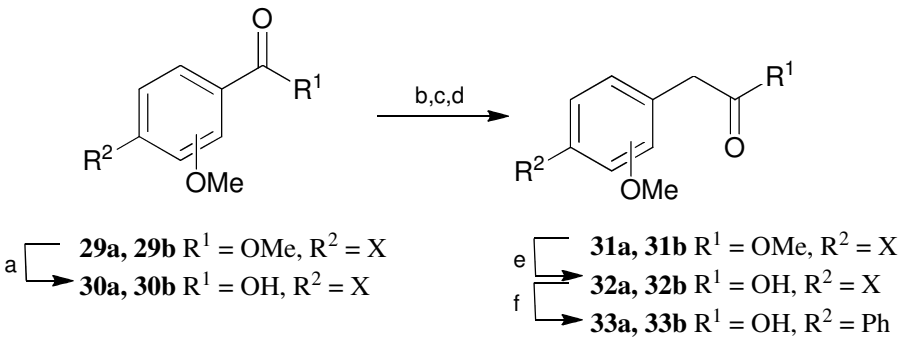
Scheme 3.



Reaction conditions: a) R^1-NH_2, HOBt, EDAC, NMM, CH_2Cl_2, 0°C-rt, 20 h, 42-70%.

The appropriately substituted biaryl acetic acid **33a** and **33b**, intermediates for compounds **7** and **8** respectively, were prepared according to the general scheme shown in **Scheme 4**. Methyl 4-bromo-2-methoxybenzoate **29a** was first hydrolyzed to the benzoic acid intermediate **30a**, which the homologation was then achieved following literature procedures described by Aoyama and Cesar to give methyl 4-bromo-2-methoxyphenylacetate **31a** in 62% yield over four steps.[31, 32] Subsequent hydrolysis of **31a**, followed by Suzuki coupling of the resultant phenylacetic acid **32a** with phenylboronic acid using tetrabutylammonium bromide (TBAB),[33] attained the biaryl scaffold **33a** in 50% yield. Similarly, the 3-methoxy-4-phenyl substituted scaffold **33b** was obtained following the same procedure using methyl 4-iodo-3-methoxybenzoate **29b,** which the preparation followed the synthetic route described by Baret *et al*.[34]

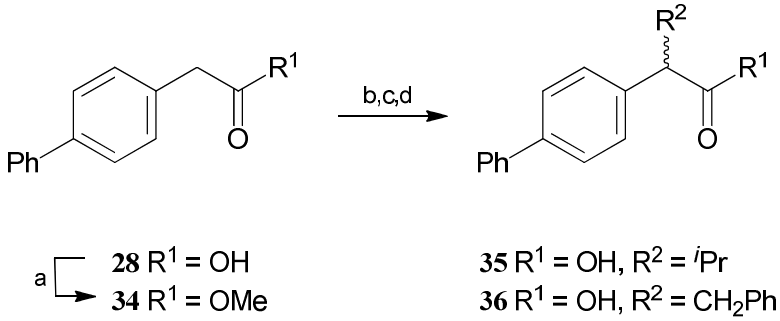
Scheme 4.



Reaction conditions: a) 10% aq. KOH/MeOH, 90°C, 3 h, 96-98%; b) $SOCl_2$, 60°C, 3 h; c) $TMSCHN_2$, NEt_3, THF/MeCN, 0°C, 24 h; d) $PhCO_2Ag$, NEt_3, MeOH, 30 mins; e) 10% aq. KOH/MeOH, 90°C, 3 h, 16-49%; f) $PhB(OH)_2$, $Pd(OAc)_2$, Na_2CO_3, TBAB, H_2O, 120°C, microwave, 30-45 mins, 64-89%.

Intermediates **35** and **36** were synthesized from the conversion of 4-biphenylacetic acid **28** to the methyl ester **34** followed by alkylation at the alpha-C position using procedures reported by Robichaud *et al.*[35] The alkylated intermediates were subsequently hydrolyzed to give the corresponding biphenylacetic acid intermediates **35** and **36** (**Scheme 5**).

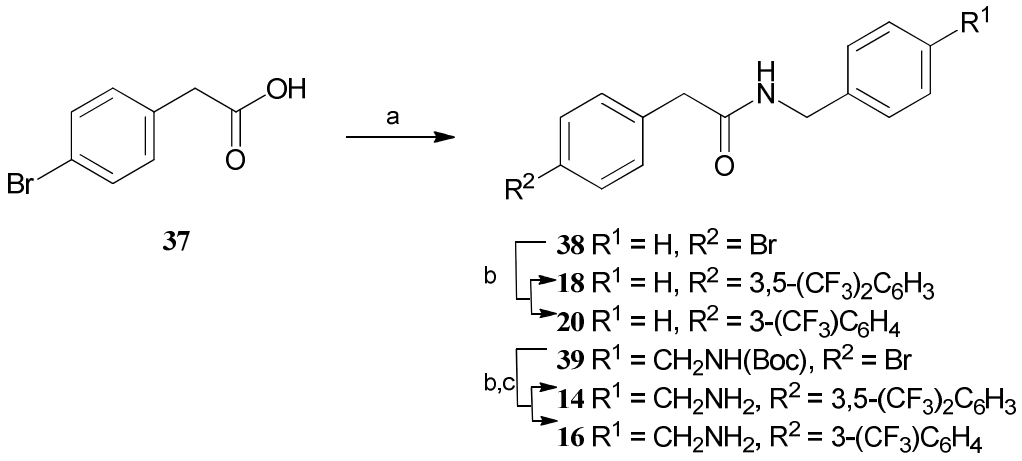
Scheme 5.



Reaction conditions: a) MeOH, H_2SO_4, 65°C, 16 h, 83%; b) KHMDS, THF, 0°C, 20 mins; c) R^2X, THF, 0°C, 16 h; d) 10% aq. KOH/MeOH, 90°C, 3 h, 36-94%.

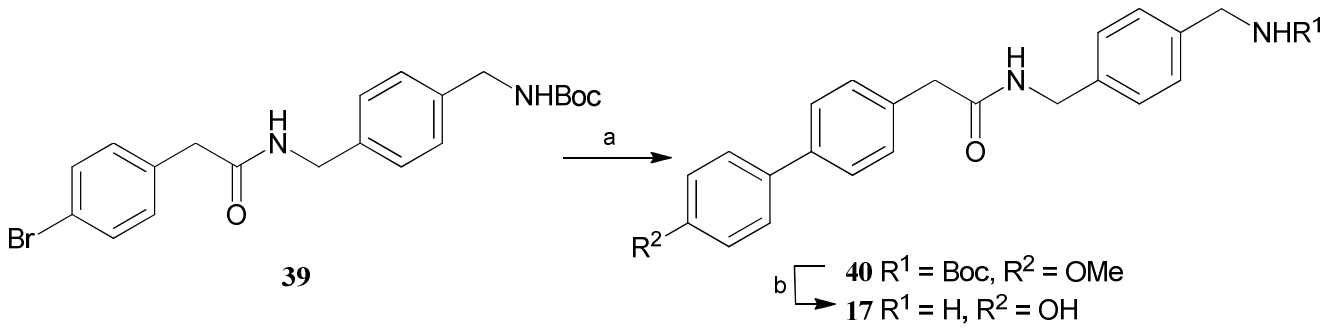
Aryl halide intermediates **38** and **39** were prepared by coupling 4-bromophenylacetic acid **37** with benzylamine and **27** respectively, which the products were coupled to the appropriately substituted aryl boronic acid under Suzuki coupling conditions to furnish the corresponding biaryl scaffolds. Compounds **18** and **20** were obtained in 39-41% yields, whereas compounds **14** and **16** were obtained in 46-52% yields after deprotection using TFA (**Scheme 6**). Similarly, compound **17** was obtained from the deprotection of **40** using BBr_3 (**Scheme 7**).

Scheme 6.



Reaction conditions: a) $PhCH_2NH_2$ or 27, HOBt, EDAC, NMM, CH_2Cl_2, 0°C-rt, 20 h, 65-81%; b) $R^2B(OH)_2$, $Pd(OAc)_2$, PPh_3, K_2CO_3, toluene, 120°C, microwave, 30-45 mins, 49-95%; c) TFA, CH_2Cl_2, 0°C, 2-3 h, 75-95%.

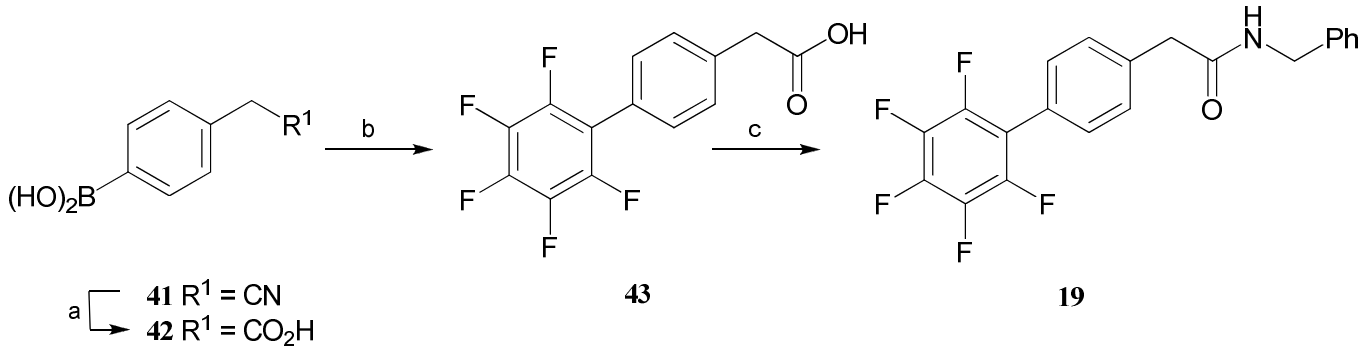
Scheme 7.



Reaction conditions: a) 4-$(MeO)C_6H_4B(OH)_2$, $Pd(OAc)_2$, PPh_3, K_2CO_3, toluene, 120°C, microwave, 30-45 mins, 84%; b) BBr_3, CH_2Cl_2, rt, 16 h, 66%.

Synthesis of the pentafluorophenyl analog **19** involved initially the hydrolysis of 4-cyanomethylphenylboronic acid **41**, following the procedure described by Serafin and Makosza.[36] Subsequent Suzuki coupling of **42** with pentafluorophenylbromide furnished **43**, which was then coupled with benzylamine to give compound **19** in 14% yield over three steps (**Scheme 8**).

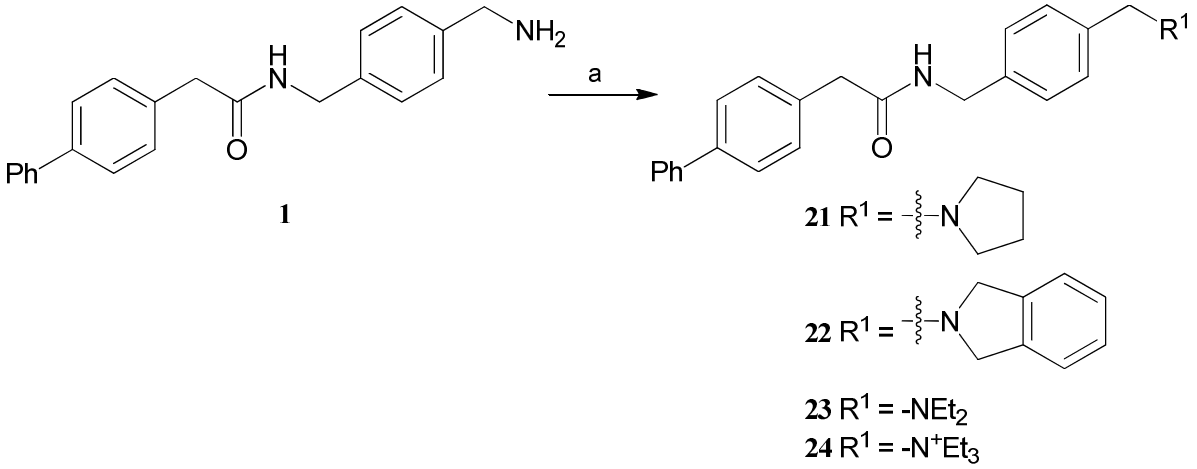
Scheme 8.



Reaction conditions: a) 20% aq. KOH, 100°C, 2 h, 87%; b) C_6F_5Br, $Pd(OAc)_2$, Na_2CO_3, TBAB, H_2O, 120°C, microwave, 30-45 mins, 39%; c) $PhCH_2NH_2$, HOBt, EDAC, NMM, CH_2Cl_2, 0°C-rt, 20 h, 40%.

Alkylation of **1** using the conditions reported by Ju and Varma[37] afforded compounds **21**-**24** in moderate yields (**Scheme 9**).

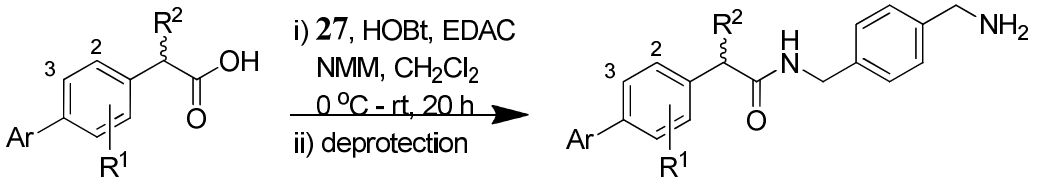
Scheme 9.



Reaction conditions: a) K_2CO_3, H_2O, 120°C, microwave, 20 mins, 34-53%.

Table 7 summarizes the synthesis of compounds **1**, **7**-**12**, and **15** from the respective intermediates described above using a two-step coupling-deprotection method.

Table 7 Synthesis of compounds **1**, **7**-**12**, and **15**.



28, 33a, 33b
35, 36, 43

1, 7-12, 15

Compound	Intermediate	Ar	R^1	R^2	Deprotection method[b]	Yield (%)
1	**28**	Ph	H	H	1	65
7	**33a**	Ph	2-OH	H	2	83
8	**33b**	Ph	3-OH	H	2	59
9	c.s.[a]	Ph	3-F	Me	1	71
10	c.s.[a]	Ph	3-F	*R*-Me	1	79
11	**35**	Ph	H	^{i}Pr	1	75
12	**36**	Ph	H	CH_2Ph	1	68
15	**43**	C_6F_5	H	H	1	77

[a] commercial source; [b] Reaction conditions: Method 1) TFA, CH_2Cl_2, 0°C, 2-3 h; Method 2) BBr_3, CH_2Cl_2, rt, 16 h.

Conclusions

We have successfully used the structure-based molecular design program SPROUT to develop a new BACE1 inhibitor scaffold using a *de novo* fragment-based ligand design approach. Based upon a simple biphenylacetamide core structure, an SAR study around the designed ligand was conducted which confirmed that the amine functionality was essential for BACE1 inhibition. The binding affinity of the initial designed skeleton was enhanced, from an initial IC_{50} against BACE1 of 323 μM to 26.9 μM for one of the most potent inhibitors, using a recently developed *in silico* optimization software SPROUT-HitOpt. Subsequent cell viability assays revealed significant toxicity associated with the majority of compounds within this series of inhibitors. However, we were able to demonstrate that compound **15**, with a relatively low toxicity, was a cell-active BACE1 inhibitor which selectively inhibited cellular BACE1 activity with little effect on α-secretase activity. Although the toxicity associated with this series of compounds has rendered them unsuitable for further studies in *in vivo* experiments, we have

demonstrated the successful application of SPROUT and SPROUT-HitOpt in the development of a small-molecule non-peptidic inhibitor against an enzyme that is a challenging therapeutic target.

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Abbreviations: AD, Alzheimer's disease; APP, amyloid precursor protein; Aβ, amyloid-β peptide; BACE1, β-site APP cleaving enzyme 1; SAR, structure-activity relationships

Supporting Information Available: Tables listing the degree of purity for all target compounds (area percent and retention time), elemental analyses, details of the experimental procedures and spectroscopic data for each compound, details of the biological assays, and modeling data for selected key compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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