Peptidomimetic modulators of BACE

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ABSTRACT

The β-site APP Cleaving enzyme 1 (BACE1) is a membrane-associated aspartyl protease which mediates the production of amyloid-β (Aβ), a major component of amyloid plaques in the Alzheimer's disease brain. We have synthesised and characterised a series of peptidomimetic analogues of BACE substrates that incorporate two distinct stabilising structures. To demonstrate the potential activity of these compounds, a variety of assaying strategies were used to investigate cleavage susceptibility and inhibition potency under competitive and non-competitive conditions. β-Amino acids and scissile site N-methylation were incorporated into peptide substrate templates as transition state isostere (TSI) substitutes by positional scanning to generate series of non-TSI β-peptidomimetics. The amino acid sequences flanking the β-cleavage site within APP carrying the Swedish double mutation (APP_{SW}), Neuregulin, the synthetic hydroxyethylene-based TSI peptide inhibitor OM99-2 and the high affinity peptide sequence *SEISYEVEFR*, served as the four substrate templates from which over 60 peptides were designed and synthesised by solid phase peptide synthesis. A Quenched Fluorescent Substrate BACE1 assay in conjunction with LC-MS analysis was established to investigate cleavage susceptibility and inhibition potency under competitive and non-competitive conditions. It was determined that β-amino acids substituted at the P^1 scissile site position within known peptide substrates were resistant to proteolysis, and particular substitutions induced a concentration-dependent stimulation of BACE1, indicating a possible modulatory role of native BACE1 substrates.

INTRODUCTION

Beta-site APP Cleaving enzyme (BACE) is a membrane-associated enzyme involved in the production of amyloid-beta which is implicated in the formation of amyloid plaques within the Alzheimer's disease brain. BACE performs the primary cleavage of Amyloid Precursor Protein (APP) and has been investigated as a possible therapeutic target [1-3] although clinical trials have demonstrated off-target effects [4-6]. For over a decade, the BACE drug landscape has grown steadily, from the identification of peptide substrates as functional templates and the rational design of peptidomimetic inhibitors to the acute analysis of BACE structural interactions and the development of potent, selective small molecule BACE inhibitors for the treatment of AD [5, 7-9]. A strict consensus sequence does not appear to be required for BACE substrate recognition or hydrolysis [10]. In addition, BACE is modulated by allosteric interaction and numerous post-translational modifications to the catalytic domain [11-13], and a range of substrates other than APP have been identified to interact with BACE, each with specific functional and modulatory roles in metabolism and signalling [14-16]. Thus, an increased understanding of BACE modulation may be obtained by investigating the effects of the post-translational modifications on BACE activity and specificity.

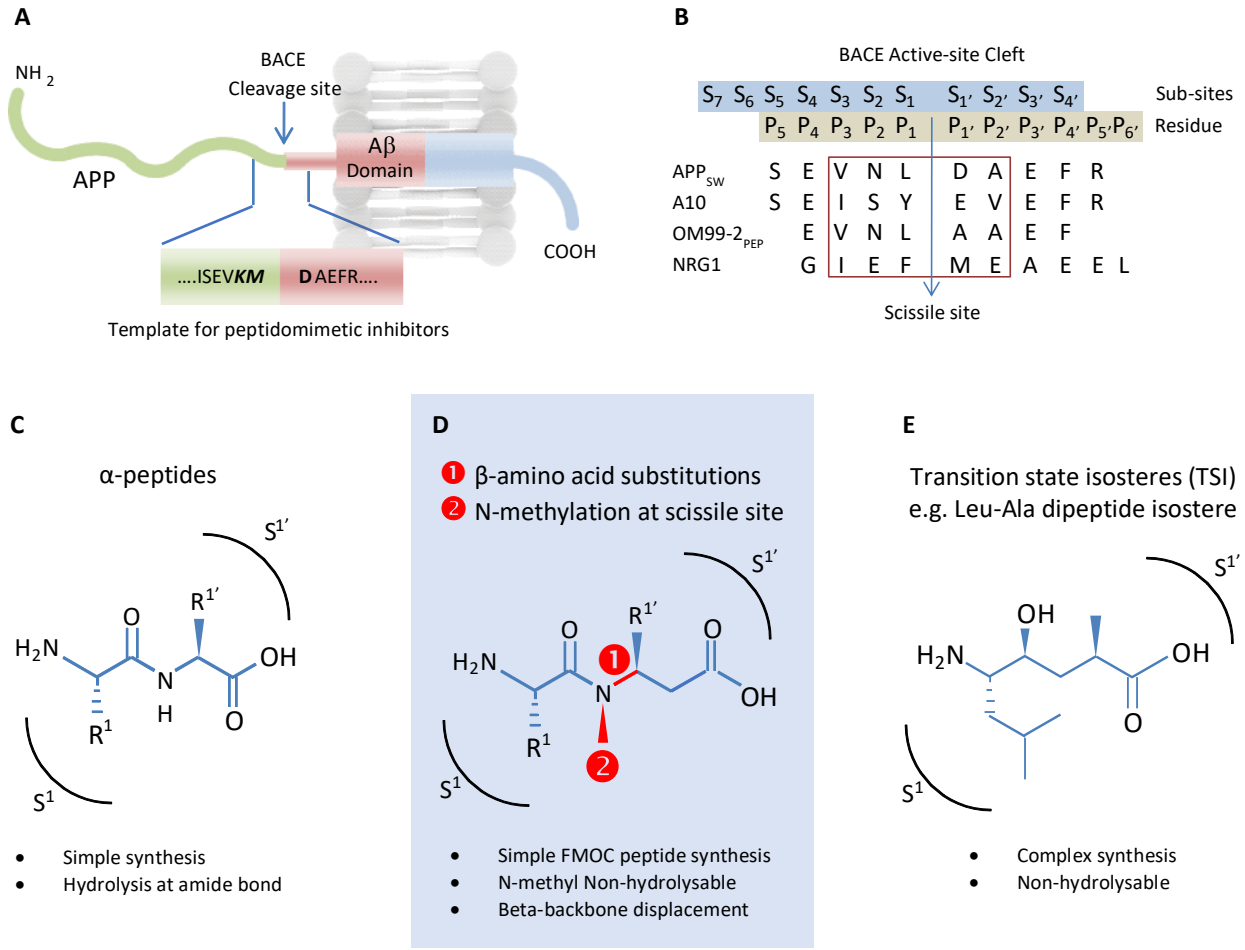
To date, many small molecule and peptidomimetic inhibitors that target the BACE active-site exhibit potent activity *in vitro* but fail to exhibit similar activity in whole cells or *in vivo* [5, 6, 17-19]. Additionally, peptides and polyglycan compounds that do not exclusively engage the transition-state but elicit a variety of modulatory effects have been synthesised [12, 20, 21].

The development of peptidomimetic BACE inhibitors frequently employs a rational design approach whereby compatible peptide substrates act as templates from which lead compounds are developed (**Figure 1**). Within BACE peptidomimetics, scissile-site residues are substituted for non-cleavable extended backbone structures, termed transition state isosteres (**TSI**), designed to mimic the high affinity transition-state as an inhibitory mechanism (**Figure 1E**). Many of the TSIs utilised in BACE inhibitor design are structurally similar to, or are derivatives of, β-amino acids. In addition, SAR-based studies are frequently used to investigate modifications to residues flanking the active-site to improve inhibitor specificity and potency. Due to the focus on the development of small molecule-based BACE inhibitors, there is very little information regarding the effect of peptidomimetic substrates containing an extended peptidomimetic backbone when not engaged in the transition-state. Given the

varied efficacy of current inhibitors, in this study we have investigated peptidomimetic substrates which contain non-transition state β-amino acid analogues that are able to modulate BACE activity toward the APP_{SW} substrate *in vitro*.

The majority of BACE inhibitors implement P^1-$P^{1'}$ transition state mimetics in the form of hydroxyl carrying moieties and enzyme specificity and peptide solubility are modulated by changes in the residues flanking the scissile structure. While the presence of a hydroxyl group at the scissile site is required to mimic the transition state, the orientation and position with respect to the accompanying side chain are significant determinants of inhibitory function and potency. Structural and functional analysis has determined the positional offset provided by the extended β-amino acid-like carbon backbone of the hydroxyethyl- (HEA) and hydroxymethyl- (HMC) mimetics to be a critical determinant of inhibition potency when the transition state is presented [10, 22-24]. Thus, novel peptidomimetic structures which stabilise the scissile bond in a strong binding substrate may represent a viable approach to develop compounds to investigate BACE proteolytic activity.

We therefore aimed to evaluate the effects of backbone modifications using N-methylation and β-amino acid substitutions at the scissile site within known BACE substrates on BACE activity (**Figure 1A, 1B**). Undertaking a rational design approach, peptidomimetics incorporating β-amino acids were synthesised within the following four substrate templates: the β-cleavage site within APP carrying the Swedish double-mutation (APP_{SW}), Neuregulin (NRG1), the synthetic hydroxyethylene-based TSI peptide inhibitor OM99-2 and the high affinity peptide sequence *SEISYEVEFR* (**Figure 1D**). In order to evaluate the peptidomimetics and monitor the effects on BACE activity, a quenched fluorescent substrate (QFS) biochemical competition assay and LC-MS time course analytical methods were developed.



- Simple synthesis
- Hydrolysis at amide bond

- Simple FMOC peptide synthesis
- N-methyl Non-hydrolysable
- Beta-backbone displacement

- Complex synthesis
- Non-hydrolysable

FIGURE 1: BACE1 PEPTIDE SUBSTRATE TEMPLATES FOR PEPTIDOMIMETIC MODULATOR DESIGN

A: APP domains and APP_{SW} BACE cleavage site. **B:** BACE active site subsite alignment with template amino acid sequences utilised in this study. APP_{SW} – 10 residue APP peptide incorporating the *KM→NL* Swedish double mutant [25], A10 – 10 residue synthetic peptide substrate containing the high affinity *ISY-EV* core [26], OM99-2_{PEP} – 8 residue amino acid sequence from the OM99-2 hydroxyethylcarbonyl (statine) TSI [22], NRG1 – 10 residue human Neuregulin 1 Isoform 1 HRG β-1 derived peptide (GenBank NCBI Ref Seq: NP_039250.2). Blue arrow indicates the scissile site. Red box highlights 'core' P^3-$P^{2'}$ template residues. **C:** α-peptide structure scissile site residues in S^1, $S^{1'}$ pocket. **D:** Mimetic approaches employed in this study depicted in red, **(1)** - example of C2 β-amino acid substitution, introduction of β-carbon backbone bond with respect to α-amino acid **(2)** – N-methylation of scissile site amide bond. **E:** Example structure of a transition state isostere, Hydroxyethylene (HE) inhibitor.

RESULTS

Peptide Design

The common structural element of HEA and HMC mimetics that provides the preferred extended carbon backbone is the presence of a scissile site β-amino acid. β-amino acids are both naturally occurring and synthetically engineered arrangements which elicit unique properties on many peptide substrates and proteins [27-29]. β-amino acids differ from their α-amino acid counterparts by the addition of a β-carbon group. The extended backbone allows for additional side chain stereochemistry comprised of four possible isomers for each α-amino acid and two possible R-group conformations on the C2 (Cα) and C3 (Cβ) carbon. Considering the HEA and HMC β-amino acid equivalents, interaction with BACE occurs within the S^1 subsite pocket if incorporated within the substrate at P^1, while the adjacent structure of the HEA at $P^{1'}$ fills the $S^{1'}$ subsite. HMCs which contain a hydroxyl β-amino acid, occupy the P^1 position alone.

A series of peptidomimetics were thus generated from the peptide substrates described in **Figure 1** in order to investigate the effects of non-transition state β-peptidomimetics on BACE activity. The α-peptide templates represent a range of reported substrates of varying sequence and function in order to identify specific effects imposed by each substitution. Firstly, the APP peptide containing the Swedish double mutant (APP_{SW}) was selected to represent endogenous APP as observed in familial AD models [25], and as a control for the APP_{SW} QFS substrate utilised within the rhBACE modulation assay and which also contains this core sequence. The A10 substrate was equivalent to the 10-mer peptide containing the high affinity *ISY-EV* core identified by Tomasselli *et al.* [26]. The A9 and A15 templates were derived from the A10 peptide – the C-terminal Arg was omitted in A9, while A15 contains a 5 amino-acid N-terminal extension respectively. The $OM99\text{-}2_{PEP}$ template represents the 8 amino acid only sequence of the OM99-2 statin transition state inhibitor in which the P^1-$P^{1'}$ statin Leu*Ala isostere is replaced with their corresponding α-amino acids [22]. Finally, the NRG1 template is the 10-mer peptide sequence flanking the BACE cleavage site identified within the Neuregulin 1 Isoform 1 HRG β-1 protein [14, 30](*GenBank NCBI Ref Seq: NP_039250.2*).

Initially, the turnover rate of each substrate template was evaluated and compared to the QFS reference substrate, which included the effects of termini neutralisation by N-

terminal acetylation and C-terminal amidation. Additionally, scissile site N-methylation was also evaluated to determine whether any effects by β-amino acid substitutions would be observed due to the backbone extension or the inability of BACE to cleave the substrate.

SUBSTRATE TEMPLATE ANALYSIS

The peptide templates and peptidomimetics in this study were synthesised by solid phase synthesis followed by reversed-phase HPLC purification. Nine series of peptides were synthesised the peptide sequence, molecular weight, RP-HPLC purification retention time and MS target mass are listed in **Table 1**. The majority of peptides were insoluble at concentrations greater that 50 µM (10 µM final) even when solubilised in 12.5 % DMSO (2.5% final in assay buffer). For this reason, peptide screening was performed at a maximum final concentration of 10 µM. Conversely, at peptide screening concentrations less than 0.1 µM, minimal effects on rhBACE activity were observed. Therefore, for comparative measures all peptides were screened utilising the rhBACE modulation assay at 10, 1 and 0.1 µM concentrations.

Effect of Termini Capping upon Substrate Template Turnover

As a preliminary analysis of the substrate characteristics of the parent peptide templates (**Series 1, Table 1**), each peptide was analysed using the rhBACE LC-MS time-course assay under non-competitive conditions to determine the turnover rates as a percentage relative to that of the QFS_{APP} (**Figure S1**). This assay measured the rate of formation of the N-terminal cleavage product, and of the four peptides that contain the APP_{SW} sequence, only the N- and C-terminally capped peptide (Ac-APP_{SW}10-Am) exhibited a high level of proteolysis (**Figure S2**). In addition, the N- and C-terminally capped analogues Ac-A10-Am, Ac-$OM992_{pep}$-Am and Ac-NRG1-Am were all cleaved to a greater extent than the uncapped parent A10, $OM992_{pep}$ and NRG1 peptides (**Figure S2**), with Ac-A10-Am exhibiting the highest rate of cleavage.

P^1-$P^{1'}$ Scissile Site N-methylation

Peptide Cleavage Analysis

Series 2 peptides were N-methylated at the scissile bond (**Table 1**) and were subject to the rhBACE LC-MS time course assay under non-competitive conditions. All scissile site N-

methylated substrates were resistant to rhBACE proteolysis with a typical LC-MS analysis shown for the A10 peptides shown in **Figure S3** revealing the production of the expected cleavage fragments. In addition, when an equimolar amount of each peptide was incubated with the corresponding N-methyl analogue, there was an approximately 50% decrease in the rate of cleavage (**Figure S3A** and **Figure S3C**). These results indicated that the N-methylated BACE substrates retained the BACE binding characteristics of the un-methylated peptide analogues.

Effects of Capping & Scissile Site N-methylation of Substrates upon rhBACE1 Activity

The Series 1 & 2 template peptides (uncapped, capped, N-methylated and non-N-methylated) were then subjected to the rhBACE modulation assay under competitive conditions (**Figure 2**) followed by peptide cleavage analysis (**Figure S4**). The C-terminal $P^{5'}$ arginine within the A10 substrate does not strictly occupy a sub-site within the BACE active site cleft and therefore an A9 truncated peptide was included to identify any redundancy. The A10 and A9 peptides induced similar effects on rhBACE activity (**Figure 2A**, blue, orange lines). While all peptides were designed to bind to the active site of BACE and act as competitive inhibitors, both inhibitory and stimulatory activity was observed. For example, un-capped A10 and A9 peptides showed little effect at lower concentrations but induced significant stimulation at 10 µM. In contrast, capped A10 and A9 demonstrated moderate inhibition at low concentrations but were ineffective at high concentration (**Figure 2B**). By comparison, the uncapped and capped NRG peptides inhibited at lower concentration, with no observed effect when capped (**Figure 2A & 2B**, purple lines). The OM99-2_{PEP} substrate was ineffective when capped, yet mildly inhibitory QFS processing when capped (**Figure 2A & 2B**, red lines).

N-Methylated un-capped substrates exhibited very little inhibitory activity (**Figure 2 C**), despite competing with their non-methylated analogues (**Figure S4**). Amidation and acetylation of the N-methylated A10 substrate had no effect on QFS turnover (**Figure 2D**, blue line), whereas NRG1 elicited stimulatory activity at high concentrations (**Figure 2D**, purple line) and the OM99-2_{PEP} substrate moderately inhibited QFS processing at 1 and 0.1 µM (**Figure 2D**, red line).

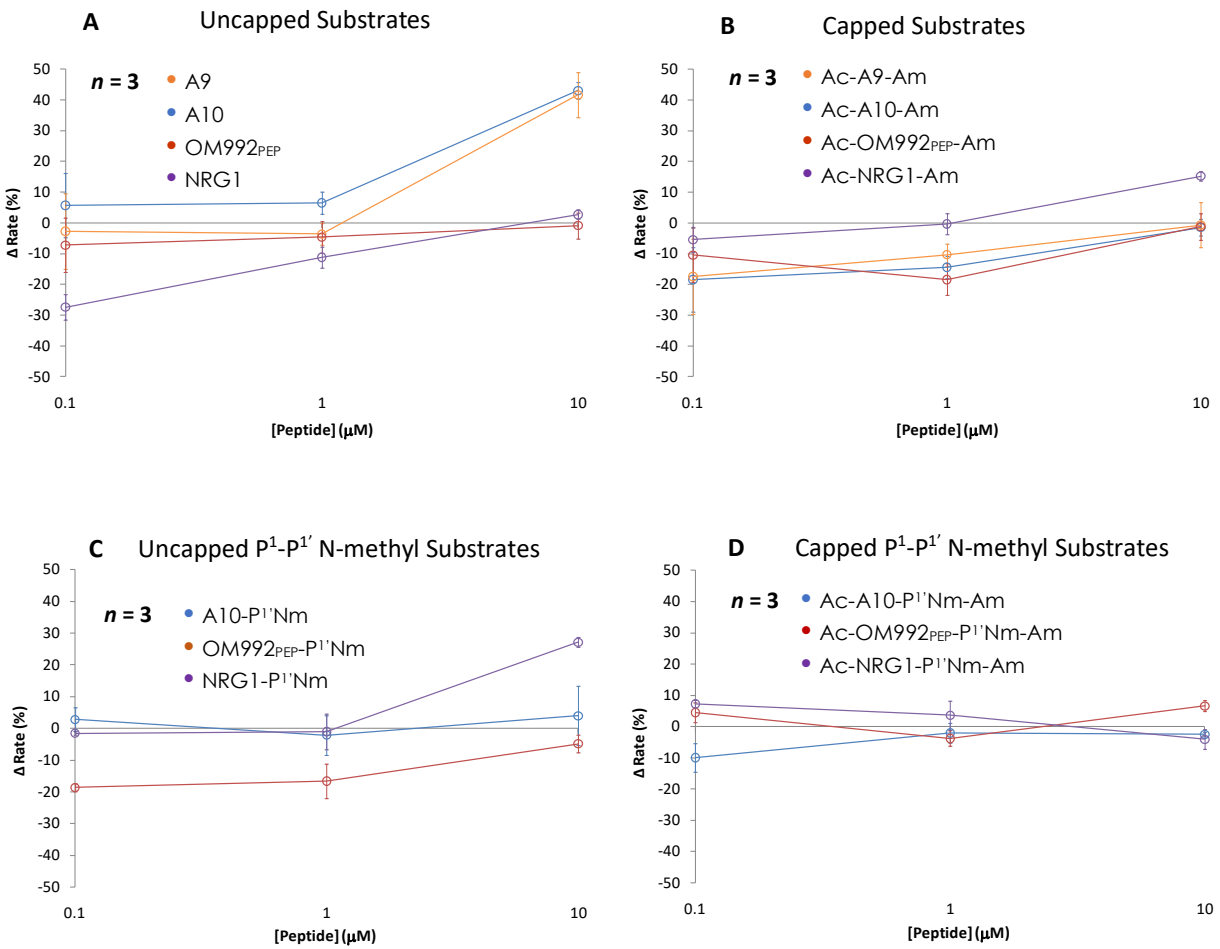


FIGURE 2: EFFECT OF CAPPING & SCISSILE-SITE N-METHYLATION ON RHBACE1 ACTIVITY

Activity analysis by rhBACE QFS_{APP} modulation assay of peptide templates with or without capping and N-methlyation. **A:** Uncapped peptides. **B:** Capped peptides. **C:** Uncapped N-methylated. **D:** Capped N-methylated. Assays performed utilising rhBACE under standard conditions. Activities are expressed as the change in rate of QFS turnover as a percentage of the QFS control. See **Table 1 – Series 1 & 2** for peptide sequences.

ANALYSIS OF β-AMINO ACID P^1 & $P^{1'}$ SCISSILE SITE SUBSTITUTIONS

Cleavage Analysis of Peptides with Homologous β-Amino Acid P^1 Scissile Site Substitutions

Series 3, 5 and 6 peptides contained P^1 β-amino acid substitutions at the scissile bond (**Table 1**) and were screened for susceptibility to proteolysis. All scissile site P^1 β-amino acid substituted peptides were resistant to proteolysis by BACE for the duration of the time-course. A typical analysis by LC-MS failed to detect cleavage products apart from those that resulted from the QFS from the A9, OM99-2_{PEP} and NRG1 β-peptides (**Figure S5**).

Effects of β-Amino Acid P^1 and $P^{1'}$ Scissile Site Substitutions upon rhBACE1 Activity

The effects of β-amino acids on BACE inhibition of the **Series 3** peptides were first investigated using the rhBACE modulation assay. These peptides contained scissile bond substitution with either the β-homologues, β-glycine or β-alanine (h-G-A) substitutions at the P^1 scissile site to un-capped A9, OM99-2_{PEP} and NRG1 peptides (**Table 1**), and none of these peptides exhibited inhibitory activity but did show varying degrees of rhBACE stimulation (**Figure 3**). The β-homologue NRG1-P^1βF peptide induced a potent stimulatory effect at 10 µM with a 1.8-fold increase in the rate of fluorescence (***Figure 3A****, purple line*). Interestingly, the parent NRG1 peptide containing the wild-type α-Phe amino acid inhibited QFS cleavage at moderate and low concentrations (***Figure 3A****, purple line*). Stimulation at 10 µM was also observed for the βG and βA heterologues of A9 and OM99-2_{PEP} peptides (***Figure 3A****, blue, red lines*), while homologous substitutions to these scaffolds had no observable effects (***Figure 3A***).

h-G-A β-substitutions were also introduced to the A9 $P^{1'}$ scissile (**Series 4, Table 1**) and the P^1 site within the A5 peptide (a truncated core sequence – *ISY-EV*) (**Series 5, Table 1, Figure 4A & 4C**) to investigate the effect of β-amino acids on scissile site preference and peptide length within the A9 substrate. The results are compared with A9 P^1 site substitutions (**Figure 4B**). The βE and βG $P^{1'}$ substitutions (**Figure 4A,** red, blue) resulted in increased stimulation compared to the corresponding substitutions at the P^1 position (**Figure 4D,** blue & **Figure 4B**, red). The A9-$P^{1'}$βG peptide resulted in a 5-fold and 2.5-fold increase in stimulation at 10 and 1 µM respectively. The mild stimulation that resulted from the A9-P^1βA (**Figure 4B,** purple) was inhibitory to rhBACE activity for A9-$P^{1'}$βA in excess of 70% at 10 µM (**Figures 4A & 4B,** purple lines). rhBACE was inhibited by the truncated A5 core sequence P^1 β-peptides at all

concentrations with the exception of βG at 10 µM (**Figure 4C**). Overall, side-chain removal at the P^1 or $P^{1'}$ site by βG-substitution elicited stimulation at 10 µM (**Figures 4A**,**B**,**C**, red lines).

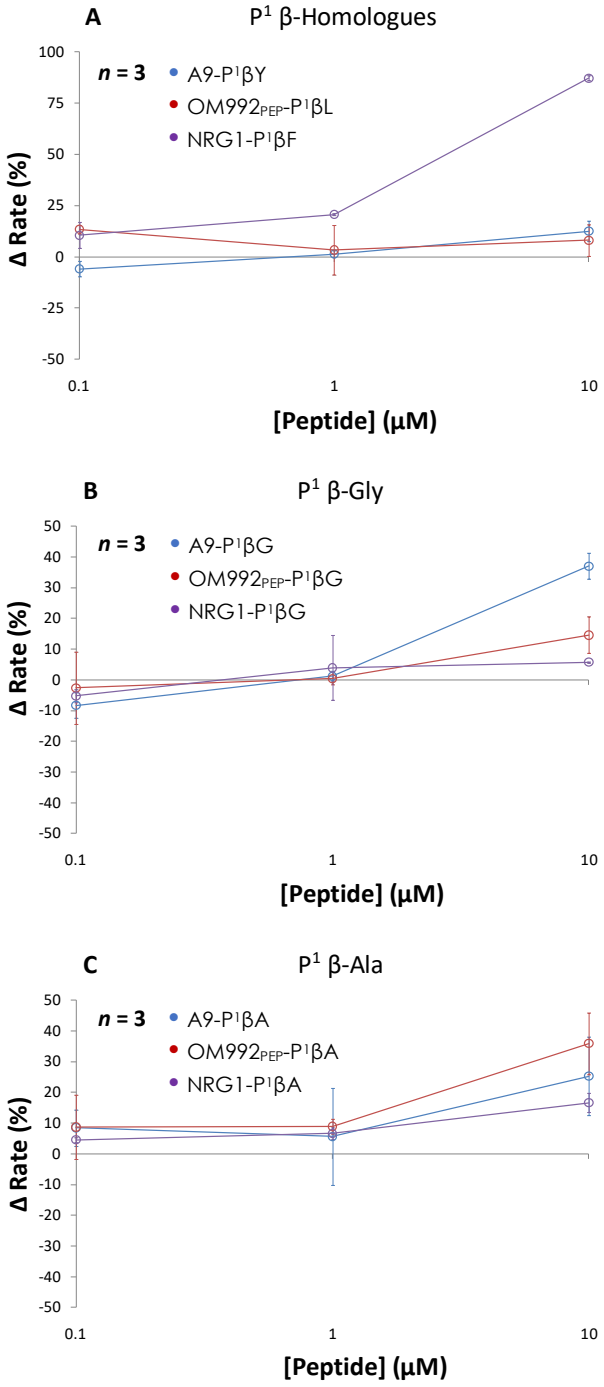


FIGURE 3: EFFECT OF β-AMINO ACID P^1 SCISSILE-SITE SUBSTITUTIONS ON RHBACE1 ACTIVITY

Activity by rhBACE QFS_{APP} modulation assay of P^1 β-amino acid substitutions within peptide templates. **A:** Homologue substitutions. **B:** Glycine substitutions. **C:** Alanine substitutions. Assays were performed utilising rhBACE under standard conditions. Activities are expressed as the change in rate of QFS turnover as a percentage of the QFS control. See **Table 1 – Series 3** for peptide sequences.

Additional A9 P^1 β-substituted peptides **(Series 6, Table 1)** were evaluated to identify influences of side-chain type and charge (**Figure 4 D,E,F**). Each β-amino acid substitution resulted in a unique modulatory profile. Most notably, β-Pro stimulated the baseline QFS_{APP} cleavage rate by 500% at 10 μM and inhibited the baseline activity by 25% at 0.1 μM (**Figure 4F,** red). The hydrophobic β-Val and β-Ile residues stimulated rhBACE over a 1-10 μM range, however, the β-Leu residue inhibited activity only, indicating a preference for side-chain orientation at the P^1 site (**Figure 4E**).

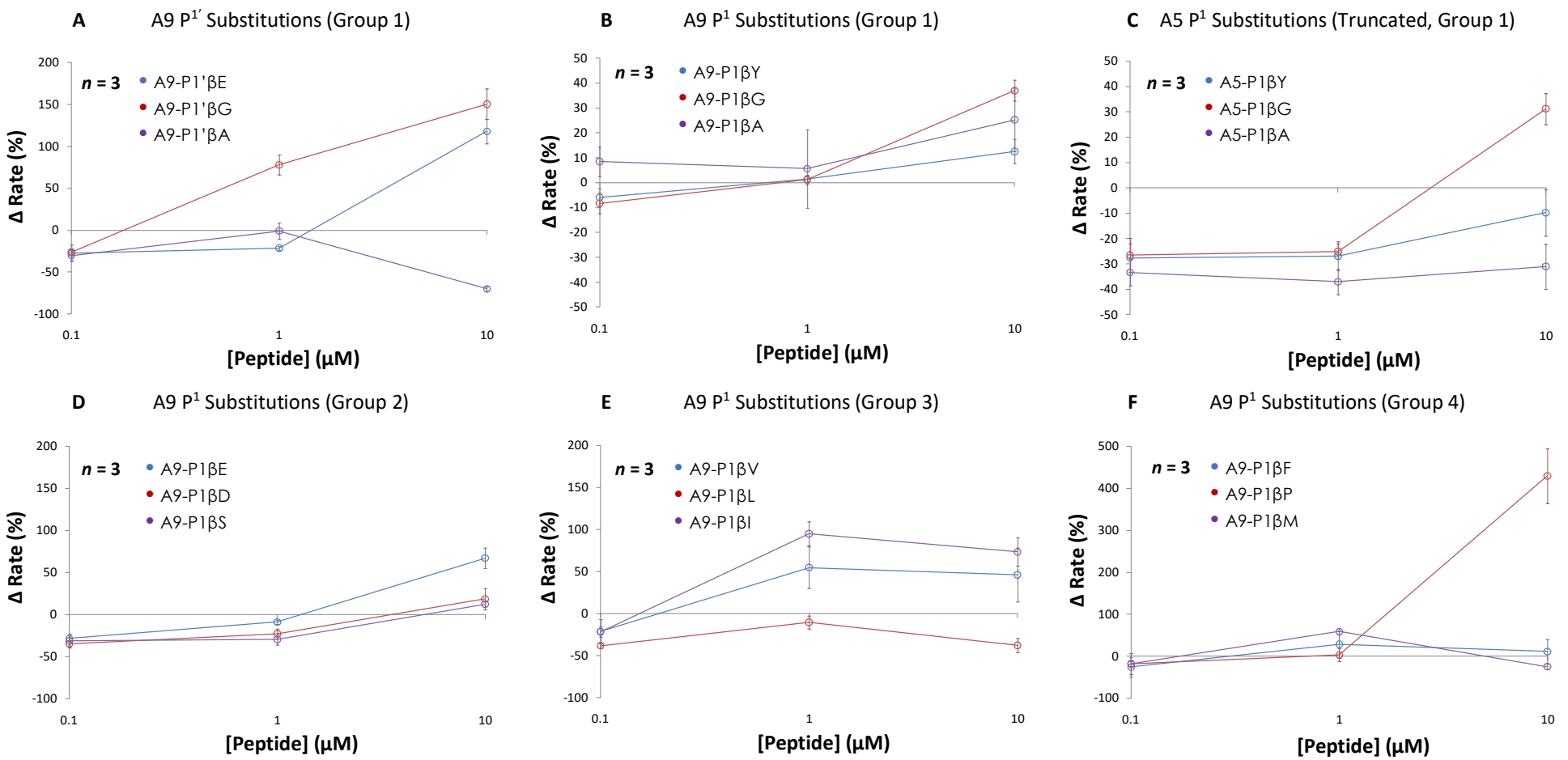


FIGURE 4: EFFECTS OF P^1-$P^{1'}$ β-AMINO ACID SCISSILE-SITE SUBSTITUTIONS IN THE A9 SUBSTRATE ON RHBACE1 ACTIVITY

Activity analysis by rhBACE QFS_{APP} modulation assay of P^1 & $P^{1'}$ β-amino acid substitutions within the A9 peptide template. **A:** $P^{1'}$ substitutions. **B:** P^1 substitutions (Group 1). **C:** P^1 substitutions (Group 1) 5-mer truncated peptide. **D:** P^1 substitutions (Group 2). **E:** P^1 substitutions (Group 3). **F:** P^1 substitutions (Group 4). Assays were performed utilising rhBACE under standard conditions. Activities are expressed as the change in rate of QFS turnover as a percentage of the QFS control. See **Table 1 – Series 5 & 6** for peptide sequences.

ANALYSIS OF AMINO ACID SUBSTITUTIONS N-TERMINAL TO THE SCISSILE SITE

Effects of Upstream α-Alanine Substitutions of A10, A15 Substrates upon rhBACE1 Activity

In order to investigate the high turnover observed within the capped A10 substrate, peptides incorporating an α-alanine substitution upstream of the scissile site were synthesised (**Series 7, Table 1**) and subject to the rhBACE modulation assay. With the exception of P^3αAla, QFS turnover was mildly inhibited within a 10 per cent margin for all of the αAla substituted A10 peptides over the concentration range tested (**Figure 5A**). The P^3αAla substitution was 30 per cent more inhibitory at 0.1 µM compared to 10 µM which had no effect on QFS turnover rates (**Figure 5A**, purple).

The A15 capped peptide, incorporating five additional APP N-terminal sequence amino acids, was also synthesised. In addition, two A15 variants carrying α-alanine substitutions for the P^7 and P^8 glutamate residues were synthesised (**Series 8, Table 1**). Removal of the negatively charged glutamates for the hydrophobic methyl group of the alanine resulted in a similar rhBACE modulation profile compared to the A15 template peptide (**Figure 5B**) and the A10 P^3αAla substitution. The upstream negative charges within the capped peptides did not contribute significantly to modulation of the rhBACE enzyme, although a mild increase in stimulation was noted at 10 µM for each Ala substitution (**Figure 5B,** purple & red) compared to the A15 template (**Figure 5B,** blue).

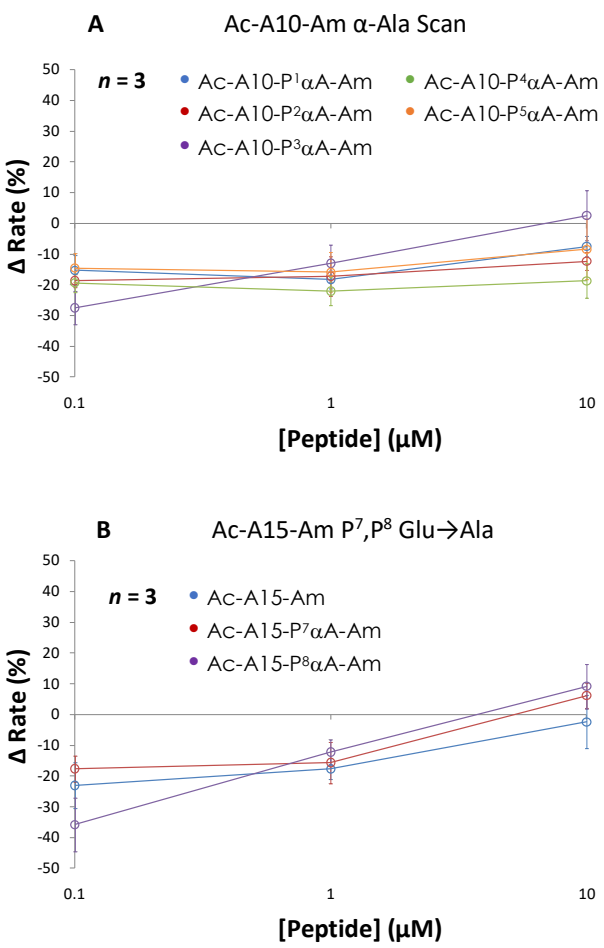


FIGURE 5: EFFECT OF UPSTREAM α-ALA SUBSTITUTIONS ON RHBACE1 ACTIVITY

Activity analysis of upstream α-Ala substitutions within the A10 & A15 peptide template by rhBACE QFSAPP modulation assay. A: P5-P1 ala substitutions within the capped A10 template. B: P7,P8 ala substitutions within the capped A15 extended template. Assays performed utilising rhBACE under standard conditions. Activities are expressed as the change in rate of QFS turnover as a percentage of the QFS control. See Table 1 – Series 7 & 8 for peptide sequences.

Cleavage Analysis of Upstream β-Amino Acid Substitutions within the A9 Substrate

The susceptibility to BACE cleavage of the A9 peptides containing upstream β-amino acid substitutions (**Series 8, Table 1**) was analysed using the rhBACE1 LC-MS time-course assay under QFS_{APP} competitive conditions (***Figure 6***). All A9 β-peptides carrying h-G-A substitutions upstream of the P^2 site were cleaved by rhBACE and the product turnover cleavage rates increased with distance away from the scissile site at which the β-substitutions were placed (***Figure 6***). Homologous substitutions were cleaved more readily than the corresponding βG and βA substitutions. Of note, the A9-P^4βE peptide exhibited the highest rate of turnover - a

rate marginally greater than the parent α-A9 template. Substitutions made to the P^2 site retained the proteolytic resistance observed in the P^1 and $P^{1'}$ substituted peptides, regardless of the β-amino acid type (***Figure 6***).

Effects of Upstream β-Amino Acid Substitutions of A9 Substrates upon rhBACE1 Activity

h-G-A β-amino acid substituted peptides at the P^5-P^2 position induced stimulation at high concentrations and inhibition at low concentrations (***Figure 7***), as observed previously in P^1 β-substituted peptides (***Figure 4A***). While both A9 P^1 and P^2 β-substituted peptides resisted proteolysis, substitutions at the P^2 position elicited a greater stimulatory effect on QFS turnover (***Figure 7 D***). Interestingly, proteolysis rates of both the A9-P^4βE peptide (***Figure 7 B,*** *blue*) and the competing QFS_{APP} substrate were greater than that of the corresponding turnover rates of wild-type A9 peptide (***Figure 2B,*** *orange*) and QFS_{APP} under competitive conditions.

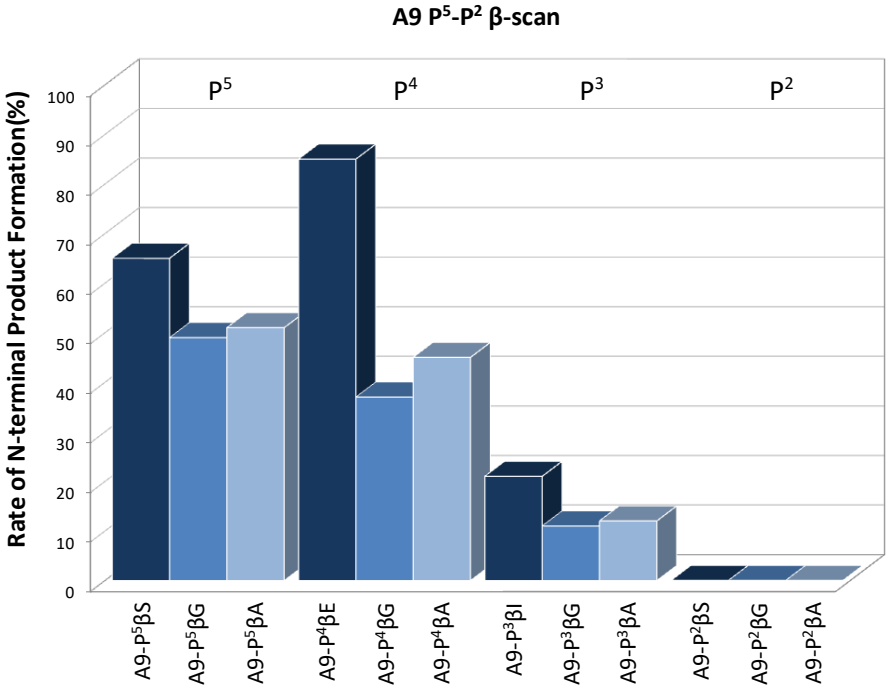


FIGURE 6: EFFECT OF UPSTREAM P^5-P^2 β-AMINO ACID SUBSTITUTIONS ON SUBSTRATE TURNOVER

Product turnover analysis of P^5-P^2 homologous, Gly & Ala β-amino acid substitutions within the A9 peptide template. Rates of N-terminal product turnover were determined by LC-MS time-course. Error between replicates was negligible. All assays were performed utilising rhBACE with 10 μM peptide. Product turnover is expressed as a percentage of the determined QFS_{APP} rate (100%). See **Table 1 – Series 9** for peptide sequences.

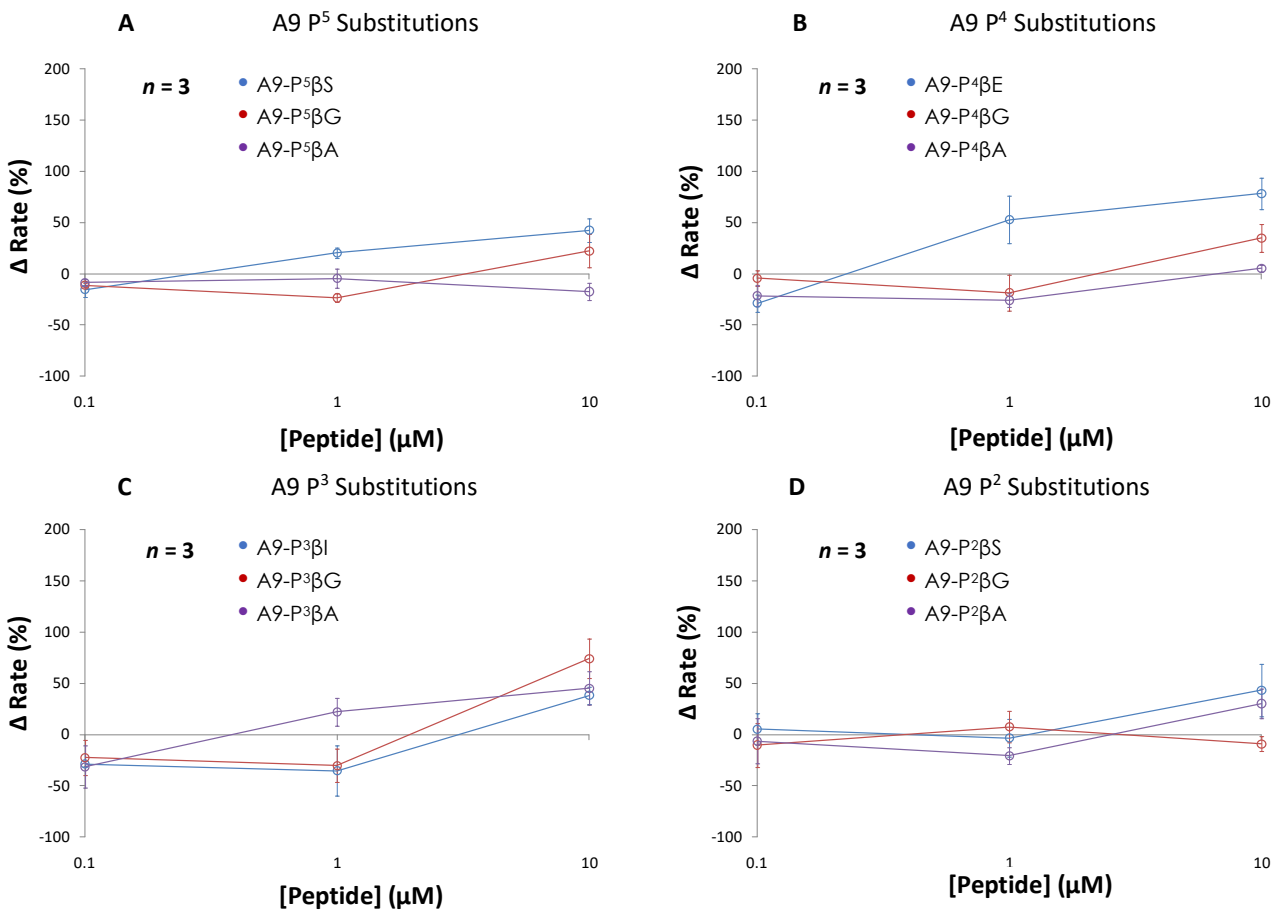


FIGURE 7: EFFECT OF UPSTREAM P^5-P^2 β-AMINO ACID SUBSTITUTIONS ON RHBACE1 ACTIVITY

Activity analysis by rhBACE QFS_{APP} modulation assay of P^5-P^2 homologous, Gly & Ala β-amino acid substitutions within the A9 peptide template. **A:** P^5 substitutions. **B:** P^4 substitutions. **C:** P^3 substitutions. **D:** P^2 substitutions. All assays were performed utilising rhBACE under standard conditions. Activities are expressed as the change in rate of QFS turnover as a percentage of the QFS control. See **Table 1 – Series 9** for peptide sequences.

DISCUSSION

β-Amino acids are mimetic structures of their α-amino acid counterparts and have been used as a mimetic to stabilise a range of peptide scissile bonds. [27-29]. The use of β-peptides to inhibit enzymatic proteolysis has been previously demonstrated by studies using β-hydroxy amino acids in the form of transition state HEA and HMC peptidomimetics against and BACE [31, 32]. Additionally, β-peptides which do not contain a transition state mimetic are also effective inhibitors of a range of enzymatic targets [33-35]. One notable advantage of utilising β-amino acids is that they may be incorporated into peptide-based structures by standard peptide synthesis techniques. The aim of this study was to evaluate the use of β-amino acids in place of currently utilised TSI mimetics for BACE inhibition. It was hypothesised that a β-amino acid substitution at the scissile site would provide the extended carbon backbone of the HEA and HMC moieties and would compete with the reference QFS substrate for the BACE active site. Thus, a kinetic model typical of competitive inhibition was expected, ie: cleavage of one substrate would be inhibited by an increase in concentration of the other, and conversely, turnover of a competing substrate would be increased with a decrease in concentration of the other as competition for the active site is reduced. Thus far, BACE active-site inhibitors reported in the current literature compete for the active site in this manner to inhibit proteolysis.

The results presented here indicate that the substrate templates and peptidomimetic structures within this study may not exclusively interact with the active-site. Instead, modulatory kinetics profiles were observed whereby the rate of QFS APP_{SW} substrate cleavage was stimulated or inhibited by BACE, dependant on the concentration and mimetic substitution of the competing test peptides. In order to quantify the apparent bimodal kinetics, a time course study was performed and the products analysed by LC-MS. In this manner, the products of cleavage were quantified in which the extent of QFS turnover was used as a relative measurement of BACE inhibition or stimulation to establish standardised modulatory profiles. Modulators of BACE activity were identified based on stimulatory and inhibitory effects monitored by the change in rate of QFS APP_{SW} turnover. When coupled with LC-MS analysis, substrate cleavage products were quantified which provided further insight into the mechanism of peptidomimetic induced BACE modulation.

The A10 and NRG1 displayed inconsistent stimulatory and inhibitory effects upon QFS turnover which was dependent on the type of modification. This was in contrast to the inhibitory effects observed on substrate turnover under competitive conditions by N-methyl and parent peptide mixtures. QFS turnover was not stimulated by the OM99-2_{PEP} substrate, which acted as a mild inhibitor when capped, irrespective of termini capping and N-methylation.

Stimulation was mitigated by the capped A10/A9 peptides despite their higher turnover rate compared to the uncapped variants. With the exception of NRG1, mild BACE inhibition at mid- to low-range concentrations by capped peptides suggests an increased affinity for the BACE active site in agreement with the noted increased turnover. In contrast, QFS turnover was neither stimulated nor inhibited by N-methylation at the scissile site within uncapped peptides, however in capped peptides, sequence dependant inhibition and stimulation was observed even though cleavage analysis indicated N-methylation of the scissile site rendered these peptides uncleavable. Unlike N-methylation, the structure of the peptide bond is maintained by substituted β-amino acids. As observed in HEA and HMC TSIs, the displacement imposed by the β-carbon within the P^1 backbone is accommodated by the active-site. Regardless of side chain, P^1 substituted β-peptides were resistant to proteolysis and displayed significant concentration dependant modulatory profiles which indicated the potent inhibition observed by TSI peptidomimetics was heavily reliant upon the presence of the hydroxyl intermediate and its coordination between the aspartates and the flap region during interaction with the active site S^1 and $S^{1'}$ sub-sites.

N-terminal to the scissile-site, the proteolytic resistance conferred by β-substitution was dependent on engaging the S^1 sub-site. β-Substitution upstream of P^1 or $P^{1'}$ resulted in concomitant increase in β-peptide turnover, yet all β-substitutions modulated QFS turnover differentially from the α-alanine substituted peptides which were largely inhibitory. Overall, these results demonstrate the ability of β-peptides to both stimulate and inhibit BACE activity which suggests different binding sites may exist within BACE. It has also been observed that that some compounds can stimulate BACE by relieving steric hindrance at the active site caused by the pro-domain and commercial preparations of BACE1 contain a large proportion of proenzyme [11, 12, 36, 37]. Since it is likely that inhibition occurs via interaction within the defined active site, while stimulation occurs allosterically, the introduction of β-amino acids

at defined positions within the template sequences and the local concentration clearly affect the binding mode type and modulation potency.

In summary, a series of peptidomimetic BACE substrates were synthesised and their effects as modulators of BACE1 activity were investigated. Unexpectedly, the substitution of scissile site residues with β-amino acids identified unique modulatory profiles. From these results, a dual binding model can be considered whereby at low concentrations, the peptidomimetics engage the active site, and at high concentrations, they interact with BACE1 either allosterically or via effects on the pro-domain. Though preliminary, these observations represent primary evidence to suggest BACE activity may be regulated allosterically by its own active-site substrates.

CONCLUSION

The use of β-amino acids has played a diverse role in peptidomimetic drug design for numerous targets, particularly for BACE1, in the form of TSI analogues which have been readily developed as high affinity active site inhibitors. P^1 β-peptidomimetics in this study which did not present a transition state analogue structure were found to be uncleavable, yet induced significant BACE modulation. Besides concentration, the magnitude of modulation was influenced by the particular β-amino acid side chain charge and structure, differentially from active site subsite preferences at the corresponding P^1 position. These results identified a novel influence upon BACE1 modulation, in addition to known structural and environmental factors.

EXPERIMENTAL

Peptide Synthesis

Peptides were synthesised utilising established SPPS methods [Supplementary Information]. Wang and Rink-amide AM resins were used for C-terminal carboxylic acid and amide moieties respectively. Fmoc-deprotection, amino acid activation and coupling steps were cycled to N-terminally extend the peptide sequence. N-methylation was performed following deprotection of the required residue C-terminal to the N-methylation site, followed by subsequent coupling. The required peptides were N-terminally acetylated following the final deprotection of a completed peptide sequence. Kaiser tests and acetyl-capping procedures were performed at various stages to troubleshoot specific syntheses and maximise target peptide yield. Following synthesis, peptides were cleaved from the resin and side chains deprotected. Crude peptides were then isolated from the resin, lyophylised and the target peptide identified by MS. Crude peptide containing minimal deletion products and a sufficient yield of target were deemed fit for RP-HPLC purification.

RHBACE1 Modulation Assays

QFS Cleavage As a Relative Measure of rhBACE Activity

Peptides (10 µM) were subject to the QFS_{APP} rhBACE1 modulation assay under standard conditions. Reaction rate was determined for each assay triplicate and an average rate and standard deviation was calculated. Rate of QFS cleavage was then expressed as a change in rate, as a %, with respect to the control QFS cleavage rate as follows:

$$\Delta\ Rate\ (\%) = \left(\frac{rate_{(QFS+peptide)}\ (FU/min)}{rate_{QFS}\ (FU/min)} \times 100\right) - 100$$

The QFS control rate is observed as the zero baseline. Values less than the QFS control rate - a reduction in the rate of QFS_{APP} turnover - observed as negative values, were deemed as inhibition. Rates in excess of the baseline rate - an increase in the rate of QFS_{APP} turnover - observed as positive values, were deemed as stimulatory.

Cleavage Product Analysis by Gradient LC-MS

Peptides (10 µM) were subject to the QFS_{APP} rhBACE modulation assay under standard conditions. Following incubation, an equal volume (100 µL) of 10 % TFA in assay buffer was immediately administered to quench the remaining rhBACE activity. The sample was analysed by microflow-capillary gradient LC-MS utilising a Zorbax SD C-18 reverse-phase column. Prior to injection, samples were diluted 2-fold in 50 % MeOH / 0.1 % FA / H_2O (10 µL).

Samples were introduced to the column at a constant 5 % Buffer B for 20 min to minimise DMSO detector saturation/tailing effects. Following sample loading, separation was initiated and performed over a 5-85 % Buffer B gradient over 60 mins at a 15 µL/min flow-rate with detection wavelengths set to 215/280 nm. Mass spectra were acquired via ESI-MS over the entire 90 min gradient over a 100-2,000 mass/charge (m/z) scan range in positive ion mode with a capillary voltage of 3,000 V. Target mass/charge ratio (500 m/z), accumulation time (200 ms), trap-drive level (70 %) and compound stability (30 %) settings were maintained for all data acquisition. The chromatogram absorbance peaks were identified by TIC-MS analysis.

Cleavage Product Rate Determination by Gradient LC-MS Time-Course

Initially, peptide turnover was determined by rhBACE assay over a time-course (0, 1, 10, 15, 30, 60 and 180 min) for the A10 peptide. Assays were performed in the absence of the QFS_{APP} to enable relative turnover measurements to be determined. As the majority of C-terminal products eluted within the tailing DMSO injection peak and were difficult to quantify, the kinetics derived from N-terminal product formation were a more reliable relative indicator for the rate of substrate cleavage. LC-MS was then performed for each time-point.

The identified chromatographic absorbance peaks (Abs: 215 nm) were integrated (mAU^2) and plotted. As the turnover of N-terminal product remained within the steady state within the first 30 mins, all subsequent time courses were performed for 0, 15 and 30 mins time-points only and linearly extrapolated. In addition, a reference peak area absorbance was determined by complete cleavage of the same peptide achieved by overnight incubation. The rate at which product was formed over time was determined from the integrated peak

absorbance at 30 mins during the steady-state (linear) phase expressed as a percentage of the total integrated peak absorbance following complete cleavage of the peptide as follows:

$$Rate\ (\%) = \frac{abs_{steady-state\ (30\ min)}\ (mAU^2)}{30\ x\ abs_{complete\ cleavage}\ (mAU^2)} \times 100$$

This value was converted to a relative percentage with respect to the QFS turnover reference rate normalised to 100 % according to:

$$\mathbf{B}\quad Relative\ Rate\ (\%) = \frac{rate_{peptide}\ (\%)}{rate_{QFS}\ (\%)} \times 100$$

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CONFLICT OF INTEREST DECLARATION

The authors declare no conflicts of interest.

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