

Original Paper

Identification of a BACE1 Binding Peptide Candidate for the Prevention of Amyloid Beta in Alzheimer's Disease

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Key Words

BACE1 • Alzheimer's disease • AD • Amyloid beta (A β) • Phage display • Biopanning

Abstract

Background/Aims: Amyloid plaques, generated during the progression of Alzheimer's disease, cause major neurological deficits due to substantial cell toxicity and death. The underlying cause of plaque generation stems from cleavage of the amyloid precursor protein (APP) by β -secretase (BACE1). A resulting amyloid- β (A β) fragment forms aggregates to produce the main constituent of a plaque. **Methods:** Phage display and biopanning techniques were used to identify a 12-mer peptide that had a natural affinity for the BACE1 enzyme. The peptide was translated from phage DNA and synthetically produced. The peptide, at concentrations of 1nM, 10nM and 100nM, was used to confirm binding by direct assay. Non-specific binding to BACE2, renin and cathepsin D was tested by direct binding assay. A BACE1 activity assay was used to determine the peptide effect on cleavage of an APP substrate. Treatment of SY5Y cells with the peptide was used to determine toxicity and prevention of A β 40 and A β 42 production. **Results:** After identification and synthetic production, the peptide exhibited a strong affinity for BACE1 at nanomolar concentrations in the direct assay. In case of non-specific binding to homologous BACE2, renin and cathepsin D, the peptide showed minor binding but was nullified when in solution with BACE1. The peptide addition to a BACE1 activity assay was able to significantly reduce the amount of substrate cleavage. SY5Y cells, when treated with the peptide, did not show any detrimental morphological changes while being able to reduce the production of natural A β 40 and A β 42. Even under stressed conditions (H₂O₂ treatment) where the A β production was higher, the peptide was still able to significantly reduce the effect of BACE1 while not effecting cell viability. **Conclusion:** The identified peptide exhibited strong binding to BACE1 *in vitro* and was able to reduce production of A β , suggesting a favourable BACE1 inhibitor for future refining and characterisation.

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Introduction

Alzheimer's disease (AD) is a neurodegenerative disease, mainly affecting the elderly generations. Disease progression shows clinical symptoms of, but not limited to, memory impairment, difficulty in performing common tasks, loss of motor functioning and lack of spatial awareness. Vast neuronal cell apoptosis and brain atrophy to both cortical and subcortical brain regions, as a cause of these impairments, eventually leads to death (1, 2). Dissection and histopathological examination of human brains with extensive cerebral amyloidoses classified plaque constituents containing a unique amyloid fibril protein (3). This protein fragment would later be characterised as a protein of approximately 40 residues that can form multimeric aggregates (4). Gene cloning and cDNA analysis of the amyloid monomers determined that they were part of a larger protein and a byproduct of enzymatic modification. That protein was later characterised as the amyloid precursor protein (APP), a 695-amino acid cell surface receptor (5, 6).

The processing of APP occurs by the sequential cleavage by three proteases called secretases, α -, β -, and γ -. The *N*-terminal fragment of APP can be cleaved by either α - or β -secretase creating a non-amyloidogenic (non-pathogenic) or amyloidogenic (pathogenic) product (7-10). The *C*-terminal subsequently gets cleaved by γ -secretase releasing the remnants of APP. Cleavage at the alpha site by α -secretase produces a *C*-terminal fragment of 83 residues (C-83), leaving a longer remaining fragment, which is non-amyloidogenic (11). Instead, cleavage by β -secretase (BACE1), 16 amino acids towards *N*-terminal at the beta site, produces a shorter soluble APP- β (sAPP β) fragment (9). Subsequent processing of the remaining *C*-terminal fragment of 99 residues (C-99) by γ -secretase release an insoluble amyloid fragment (A β).

Additionally, Presenilin 1 and 2 of γ -secretase will then determine the length of A β by producing a 38-43 amino acid fragment. The differing lengths in which sAPP β is processed can determine the way plaques are formed by a higher or lower propensity to aggregate (12-17). Aggregation from these monomers, specifically A β 42, becomes part of a feedback loop that promotes plaque propagation (18). This aggregation around synapses decreases dendritic spine density and increases synaptotoxicity but the effective inhibition of BACE1 could prevent these plaque forming units from being produced (19-21).

Initially, AD was defined by amyloid plaque deposition however, research has expanded to encompass neurofibrillary tangles, brain atrophy, compromised blood brain barrier and genetic factors like apolipoprotein E4 (ApoE4) (22-25). In addition, the inhibition of BACE1 and prevention of amyloid based plaques has a significant effect on disease progression (26, 27). An early detection of disease will undoubtedly increase the chance of remission, as plaque generation and A β is hypothesized to be most effective in the early stages of disease.

Currently, there is no effective treatment for Alzheimer's disease or the deposition of A β , only methods to relieve symptoms. Current attempts to generate viable synthetic and organic inhibitors have been thwarted by poor blood brain barrier permeability, retention in plasma, adverse toxicity, and BACE1 binding specificity.

To address this, we utilised the phage display and biopanning technique to identify a 12 amino acid peptide that could not only bind to BACE1 specifically but prevent the enzymatic ability to cleave APP. Biopanning and phage display has been successful in acquiring positive binding peptides to numerous targets like interleukin-4 receptor, alkaline phosphatase, hepsin, tissue metalloproteinases and tyrosinase (28-32). Defining a construct with effective binding properties is the proactive direction of the current study to refining an effective new candidate for AD.

Materials and Methods

Phage Display Binding to BACE1

Recombinant human BACE1 (Sigma-Aldrich, USA) was diluted to 50 µg/ml in blocking buffer (0.1M NaHCO₃ (pH 8.6), 5mg/ml bovine serum albumin (BSA), 0.02% NaN₃) and added to a single well of a 96-well microtitre plate and incubated overnight at 4°C with shaking. The solution was discarded and replaced with blocking buffer and incubated at 4°C for a further 60 minutes. The well was washed six times with 0.1% TBS-T (50mM Tris-HCl (pH 7.5), 150mM NaCl, 0.1% (v/v) Tween-20) before adding a 100-fold representation of the phage display peptide library (New England Biolabs, USA). The library was incubated with the BACE1 target for 60 minutes at room temperature with gentle rocking. The well was washed a further 10 times with 0.1% TBS-T. The unbound phage in solution were discarded and the bound phage eluted by adding glycine buffer (0.2M glycine-HCl (pH 2.2), 1mg/ml BSA) to the microtitre plate well and gently rocked for 7 minutes. The solution was transferred to a microcentrifuge tube and neutralised with 1M Tris-HCl (pH 9.1), which formed the unamplified eluate. Luria broth (LB) culture was inoculated with a single ER2738 *E. coli* colony picked from a stock plate and incubated at 37°C to early-log phase (OD₆₀₀ 0.01-0.05). 100 µl of unamplified phage was added to the culture and incubated with vigorous shaking at 37°C for 4.5 hours. The culture was transferred to a sterile tube and pelleted by centrifugation at 12,000xg for 10 minutes at 4°C. The upper 80% of the supernatant was precipitated overnight at 4°C with PEG/NaCl (20% (w/v) polyethylene glycol-8000, 2.5M NaCl). The phage were pelleted by centrifugation at 12,000xg for 15 minutes at 4°C. The supernatant was discarded and the pellet re-suspended in TBS (50mM Tris-HCl (pH 7.5), 150mM NaCl). The suspension was pelleted at 17,000xg for 10 minutes. The supernatant was transferred to a new microcentrifuge tube along with 1/6 volume PEG/NaCl and allowed to precipitate on ice for 60 minutes. The phage were pelleted at 17,000xg for 10 minutes with the supernatant being discarded and the pellet re-suspended in TBS. This formed the amplified eluate. An ER2738 culture grown to mid-log phase (OD₆₀₀ ~0.5) was combined with unamplified phage diluted to 10⁻⁴ in LB media and plated in 10-fold increments from 10⁻¹ and amplified phage diluted to 10⁻¹¹ in LB media and plated in 10-fold increments from 10⁻⁸. Dilutions were pre-incubated with the mid-log phase *E. coli* host strain for 10 minutes. Pour plates were constructed using molten top agar (10g/l bacto-tryptone, 5g/l yeast extract, 85mM sodium chloride, 7g/l agar) mixed with the phage/*E. coli* suspension and added to the already prepared IPTG/X-gal plates. Plates were incubated for 18 hours at 37°C and plaque forming units per millilitre (pfu/ml) calculated. All biopanning preparations were maintained in sterile environments of a KS18 Class II safety cabinet (ThermoFisher Scientific) to minimise contamination.

Biopanning Rounds and DNA Sequencing Preparation

Biopanning second round steps were replicated from original phage display protocol but instead of the library being introduced to the immobilised target, the amplified phage from the previous round was added. The pfu/ml of amplified eluate was used to maintain the input of ~1x10¹¹ phage virions into the subsequent rounds. Tween-20 concentrations for wash steps, prior to the phage incubation, were increased by 0.1% (v/v) for every subsequent round. The fifth round of biopanning was not amplified and instead the unamplified eluate was titred as previously described. Within an 18-hour incubation time, twelve individual plaques were picked and transferred to individual ER2738 broth cultures, grown to early-log phase (OD₆₀₀ 0.01-0.05) and incubated for 4.5 hours at 37°C with vigorous shaking. The cultures were transferred to microcentrifuge tubes and pelleted by centrifugation at 14,000xg for 30 seconds. 500 µl of supernatant was mixed with PEG/NaCl and incubated for 20 minutes at room temperature. The PEG precipitation was pelleted by centrifugation at 14,000xg for 10 minutes at 4°C. The pellet was suspended in iodine buffer (10mM Tris-HCl (pH 8.0), 1mM EDTA, 4M sodium iodide (NaI)). 70% (v/v) ethanol was added to the tube and incubated for 15 minutes at room temperature. The precipitate was centrifuged at 14,000xg for 10 minutes at 4°C and the supernatant discarded. The remaining pellet was suspended in TE buffer (1M Tris (pH 8.0), 0.5M EDTA). The precipitated DNA concentration was quantified using a ND-1000 NanoDrop spectrophotometer (ThermoFisher Scientific).

Amplification and Purification of Phage DNA for Sequencing

The purified DNA samples were individually amplified using the BigDye® Terminator v3.1 Cycle Sequencing Kit (ThermoFisher Scientific). The kit was assembled for templates as recommended by the

supplier. Samples were added to an MJ Research PTC-100 thermocycler (Bio-Rad Laboratories) and run for 25 cycles of 96°C for 10 seconds, 50°C for five seconds and 60°C for four minutes. PCR products were transferred to microcentrifuge tubes and mixed with 2µl of 125mM EDTA, 2µl of 3M sodium acetate and 50µl of 100% ethanol and incubated at room temperature for 15 minutes. The tubes were centrifuged at 2500xg for 30 minutes. The supernatant was discarded and 70µl of 70% ethanol added to the pellet and centrifuged at 1650xg for 15 minutes. The bulk of the supernatant was removed with remaining left to evaporate in a class II safety cabinet allowing the pellet to dry on the base of tube, ready for sequencing. The nucleic acids resulting from the sequencing were viewed using Finch TV software (Geospiza Inc.). The synthetic peptide was produced with and without a biotin label by Auspep Pty. Ltd. (Australia).

Direct Assay Determining Binding of Biopanning Candidate to BACE1

Recombinant BACE1, as used in the phage display, was immobilised on wells of a microtitre plate. The peptide, produced from the result of the phage display and biopanning, was biotin labelled and added to BACE1-coated wells at concentrations of 1nM, 10nM and 100nM and incubated for 60 minutes at room temperature with rocking. No-peptide negative controls were also included. The solution was discarded and the wells washed 3 times with 0.3% TBS-T. Then, a 1:500 dilution of streptavidin/HRP conjugate (Amersham Biosciences) in 0.1% TBS-T was added to the wells and incubated for 60 minutes at room temperature with rocking. The solution was discarded and washed three times. For the detection, the o-phenylenediamine dihydrochloride (OPD) substrate was dissolved in phospho-citrate buffer with sodium perborate, added to all wells and incubated for 60 minutes at room temperature in the dark. The reaction was stopped with 4M sulfuric acid before reading the reaction on a HALO MPR-96 plate reader (Dynamica) at wavelength 492nm.

Specificity Test of the Peptide Candidate to BACE1

Solutions of 1µM recombinant BACE1, renin, cathepsin D and BACE2 were prepared in a 0.1M NaHCO₃ (pH 8.6) buffer, added to a 96-well plate and incubated overnight at 4°C with rocking. The solution was discarded and replaced with blocking buffer and incubated for a further hour at 4°C. The blocking buffer was discarded and the wells washed three times with 0.1% TBS-T. Solutions containing 100nM biotinylated peptide were incubated for 60 minutes at room temperature with rocking. The wells were washed again 3 times with 0.3% TBS-T. Streptavidin/HRP conjugate in 0.1% TBS-T was added to the wells and incubated for 60 minutes at room temperature with rocking. The solution was then discarded and washed three times with 0.1% TBS-T. Detection was performed using the same protocol as the direct assay, described above.

Competition Assay to Confirm BACE1 Specificity

A 1µM solution of recombinant BACE1 was immobilised using the same protocol as the direct assay. During well blocking, a solution containing 1µM renin, cathepsin D and BACE2 was incubated with 100nM of peptide in TBS-T for 30 minutes. The plate wells were washed three times with TBS-T, replaced with the pre-treatments and incubated for 60 minutes at room temperature with rocking. The wells were washed again 3 times with 0.3% TBS-T. Then, a 1:500 dilution of streptavidin/HRP conjugate (Amersham Biosciences) in 0.1% TBS-T was added to all wells and incubated for 60 minutes at room temperature with rocking. Detection was performed using the same protocol as the direct assay.

BACE1 Activity Assay with Direct Binding Method

The BACE1 activity was tested using a fluorescent FRET activity assay kit (Millipore Sigma). Instead of placing all kit elements at once into a solution, the inhibitor was immobilized on nitrocellulose. A grid, of 1cm x 1cm squares, was marked out on a nitrocellulose membrane according to the sample numbers and the respective inhibitor concentrations. The squares, used for the BACE1 inhibition, had 10nM, 100nM or 200nM of inhibitor solution pipetted onto the centre of the respective square and allowed to dry, controls were left to dry without inhibitor. All squares were placed into 50ml tubes, according to inhibitor concentration, containing blocking buffer (5% (w/v) instant skim milk powder) and incubated for 30 minutes at room temperature with shaking. The squares were transferred to individual tubes and washed three times for 5 minutes each with Milli-Q water. The squares were then removed and placed in their respective tubes according to reaction number. The tubes contained 78µl of fluorescent assay buffer (FAB) and 20µl of the substrate, diluted 10-folded from the stock. A 40nM BACE1 solution in FAB was added to all tubes and incubated with agitation for 2 hours at 37°C. After incubation, the nitrocellulose squares were removed

and the remaining solution transferred to a 96-well plate and read on a Fluostar Omega fluorometer (BMG Labtech) with excitation of 320nm and emission 405nm.

SY5Y Cell Culture and Treatments with the BACE1 Inhibitor

Cryopreserved SH-SY5Y cells (Millipore Sigma, ATCC CRL-2266) were thawed in a water bath at 37°C, transferred into media (HamsF12:EMEM (1:1), 2mM glutamine, 1% non-essential amino acids and 15% foetal bovine serum (FBS) (v/v)) and placed in an incubator at 37°C with 5% CO₂. The media was discarded and replaced after two days of incubation. Cells were seeded at 80% confluence by removing and discarding the media and washing the cell monolayer twice with PBS containing 137mM NaCl, 2.7mM KCl, 8.1mM Na₂HPO₄ and 1.47mM KH₂PO₄. Cells were harvested by adding 0.25% Trypsin-ethylenediaminetetraacetic acid (EDTA) in PBS for 2 minutes before quenching with fresh media. The cells were diluted 1:10 in a new flask with fresh media.

Cell Viability and Cell Number Calculation

200µl of cells, after trypsinisation and quenching, were transferred to a microcentrifuge tube and pelleted at 1,000xg for 5 minutes to remove any traces of the remaining serum. Media was removed and the pellet washed with PBS. The cells were then suspended in 200µl of HamsF12:EMEM and 20µl transferred to a fresh microcentrifuge tube. 20µl of trypan blue dye solution was added to the 20µl of cells and loaded into the TC10 sample chambers on the counting slide. The slide was added to the TC10 Automated Cell Counter (Bio-Rad Laboratories) and the cell viability and counts analysed.

Cell Treatments

SY5Y cells were grown to 80% confluency and harvested using 3ml of 0.25% trypsin/EDTA/PBS and quenched with 7ml of media. The cells were transferred to a six-well plate and allowed to adhere overnight. Media was pre-incubated for 24 hours with the BACE1 peptide inhibitor to 1nM, 10nM and 100nM. Old media was replaced with treatment media and the cells incubated for 24 hours. Media was removed and the monolayer washed with PBS. Cells were lysed in the wells with CellLytic MT (Millipore Sigma) and protein concentration determined with a Qubit fluorometer (ThermoFisher Scientific). The lysates were used immediately in the Aβ40 and Aβ42 ELISAs.

SY5Y Hydrogen Peroxide Treatment

SY5Y cells were grown to 80% confluency, trypsinised and transferred to 6-well plates, as described above. The media was pre-treated with 200µM hydrogen peroxide (H₂O₂) for 24 hours before adding to the cells. Testing the BACE1 inhibitor, the H₂O₂ pre-treatments contained one of a 1nM, 10nM and 100nM concentrations. After the treated cells were incubated for 24 hours, the monolayer was washed once with PBS and lysed on the plate with CellLytic MT (Millipore Sigma).

Human Aβ40 and Aβ42 ELISA with Treated SY5Y Cell Lysates

The human Aβ40 and Aβ42 ELISAs (Invitrogen) were purchased as kits from ThermoFisher Scientific and performed as recommended by the supplier, following the manufacturer's instructions.

Statistics

Statistical analysis of direct ELISA, specificity test, activity assay and other ELISAs were performed as means (n=3), comparing negative controls to the individual treatments using GraphPad Prism 7.0 software. Cell viability live cell counts were performed by the TC10 Automated Cell Counter (Bio-Rad Laboratories). Statistical significance was determined by unpaired Student's *t*-test (p<0.05 *, p<0.01 **, p<0.001 ***, p<0.0001 ****).

Results

Biopanning and Phage Display

Identification of a peptide with strong binding affinity to BACE1 was determined using the phage display and biopanning techniques. The recombinant BACE1 target was bound to a microtitre plate well while the unbiased library of peptide candidates, expressed on phage capsid, were allowed to freely bind to the large protein. Using the natural affinity to BACE1, the bound phage remained while the unbound was washed away. High affinity binders were tested over five rounds of biopanning to refine the pool down to the best candidates.

Phage titre was determined by dilution of the amplified eluate with infection of the ER2738 host strain. The pooled phage were amplified and used in the subsequent round to test candidate binding affinity. Biopanning rounds were extended from three rounds to five as a stringency requirement to refine the number of BACE1 binders. The increase in stringency represented by the Tween-20 concentration in the wash buffer did not influence the titre produced. Across the five rounds of biopanning, titre increased from 7×10^9 plaque forming units per millilitre (pfu/ml) in the first round to 3×10^{11} by the fourth. The eluate from the fifth round of biopanning was plated with the unamplified eluate to pick the individual plaques for sequencing. Of the 53 plaques produced, 12 were picked and amplified individually. None of the plated phage dilutions contained any contamination with the white, wild-type plaques, suggesting that host transduction was successful and sterile techniques were maintained. Transduction was confirmed with all 12 sequences containing the additional sequence representing the M13KE cloning vector of the phage.

Phage DNA Sequencing

All isolated DNA samples were quantified using an ND-1000 NanoDrop spectrophotometer and analysed for purity. The samples were analysed by comparing the 260/280nm wavelengths, producing results between the expected 1.7 and 2.1 levels. The near pure DNA samples were complemented by chromatograms, which produced clearly identifiable nucleotide peaks. The inserts were identified by the *KpnI/Acc65* and *EagI* restriction enzyme sites, where the DNA insert was ligated, then confirming the presence of the triple glycine leader sequence attached to all 12-residue peptides of the pIII surface proteins. Of the 12 plaques sampled, DNA sequencing revealed the 10^9 varying sequences in the phage library had been reduced to one unanimous candidate. The sequence was analysed using a BLAST search via the National Centre for Biotechnology Information (NCBI) database for possible similarity with other proteins, peptides and inhibitors without success.

Direct ELISA Determining Binding of Biopanning Candidate to BACE1

The phage peptide identified from the sequencing was translated into a synthetic peptide and tested by direct binding assay. The assay followed the same immobilization technique of the BACE1 target as the phage display; recreating the same conditions encountered during the peptide isolation steps. The synthetic peptide was biotinylated at the C-terminus allowing a streptavidin/HRP conjugate to bind, providing a visual representation of the target binding produced with the OPD substrate. The same stringency was used in both the assay and biopanning round 5 to confirm the binding affinity between the peptide and target.

The colorimetric response, as a result of peptide binding to the BACE1 target, increased significantly when compared with the no peptide control. As the peptide concentration increased, so did the response to binding. In comparison to the negative control, containing no peptide, the 1nM test sample showed a significant increase in the amount of response generated (negative control 0.05 ± 0.004 , $n=3$, 1nM 0.158 ± 0.11 , $n=3$, $p=0.001$, $t=8.58$) (Fig. 1). The peptide concentration was increased 10-fold, to 10nM and 100nM, which generated more binding with the BACE1 target (10nM 0.39 ± 0.03 , $n=3$, $p=0.0005$, $t=10.44$, 100nM 0.88 ± 0.01 , $n=3$, $p=0.000001$, $t=76.37$). All experimental variables from 1nM to 100nM concentration, showed strong binding was occurring between the peptide and the immobilised BACE1

target. Blocking buffer and microtitre plate binding controls (data not shown) did not show any attachment by the peptide.

Specificity Test of the Peptide Candidate to BACE1

The BACE1 translated protein shows sequence homology with BACE2 (52%), renin (24%) and cathepsin D (29%), which can be cause for unwanted treatment side effects by means of nonspecific binding (33). To confirm peptide/BACE1 affinity, target specificity was performed on BACE2, renin and cathepsin D by using the same direct binding assay method as used previously for BACE1. All targets were immobilised on corresponding wells of a microtitre plate and introduced to a 100nM solution containing the peptide. As seen in the original binding assay, the peptide showed a strong affinity for BACE1 (negative control 0.057 ± 0.0006 , $n=3$, BACE1 0.997 ± 0.027 , $n=3$, $p=0.000004$, $t=34.45$). However, there was also significant colorimetric response to both cathepsin D (0.08 ± 0.006 , $n=3$, $p=0.013$, $t=4$) and BACE2 (0.221 ± 0.014 ,

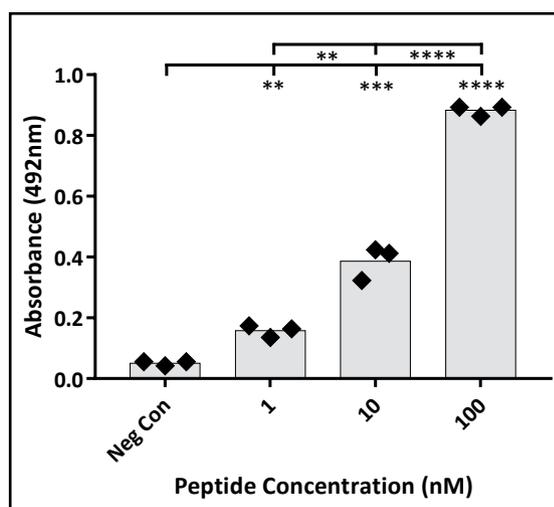


Fig. 1. The identified peptide shows a strong binding affinity for BACE1. The immobilised BACE1 target was introduced to 1nM, 10nM and 100nM solutions containing biotinylated peptide identified from the phage display and biopanning. Negative control (Neg Con) represents the response without a peptide. Total binding between the immobilized target and peptide is represented by a colorimetric response. All values are reported as individual data points ($n=3$) and bars represent mean binding. Statistical significance is determined by unpaired Student's t-test ($p<0.05$ *, $p<0.01$ **, $p<0.001$ ***, $p<0.0001$ ****).

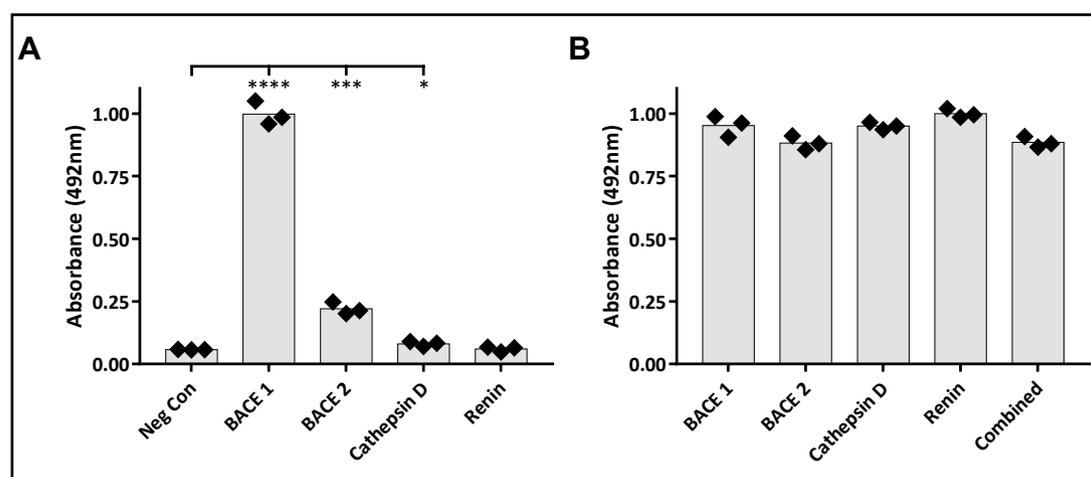


Fig. 2. Identified peptide has a specific affinity to BACE1 when incubated with homologous BACE2, cathepsin D and renin. (a) Immobilised BACE1, BACE2, cathepsin D and renin were incubated with a 100nM solution containing the peptide isolated from the phage display and biopanning experiments. (b) Testing overall peptide affinity for BACE1, individual homologs were preincubated with the peptide then incubated to an immobilized BACE1. A combined solution containing all homologs represents the total non-specific binding potential with these specific proteins. The binding occurring between the immobilized target and peptide is represented by an increase in colorimetric response. All values are reported as individual data points ($n=3$) and the bars represent mean binding. Statistical significance is determined by unpaired Student's t-test ($p<0.001$ ***, $p<0.0001$ ****).

n=3, p=0.0003, t=11.6), albeit much lower than that of BACE1. The only target to not show a significant increase in binding was renin (0.06 ± 0.006 , n=3, p=0.64, t=0.49) (Fig. 2a).

To confirm the strong BACE1 specific affinity, BACE2, renin and cathepsin D were pre-treated with peptide prior to the reaction, allowing a possible binding to occur. The pre-treatment was then transferred to wells containing immobilised BACE1 for the direct ELISA to determine the selective nature of the peptide and whether there is an affinity for BACE1 over the homologs. To exacerbate any potential binding, the peptide was also pre-treated in solution with a combination of renin, cathepsin D and BACE2. Pre-treating the peptide with cathepsin D had a minimal effect on subsequent binding to BACE1. This was the same for renin and BACE2 with the responses showing no significant difference to the BACE1 control. The potential binding to BACE2 (0.88 ± 0.02 , n=3, p=0.076, t=2.372), cathepsin D (0.95 ± 0.009 , n=3, p=0.95, t=0.064), or renin (1.0 ± 0.01 , n=3, p=0.14, t=1.82), was also negated as the peptide would bind strongly to BACE1 once it was in contact. Binding occurring between immobilised BACE1 target and peptide was maintained across all experimental wells (Fig. 2b). With all homologs added as a combination experiment, there was no significant decrease in response created by the pre-treatment, and the binding between the peptide and BACE1 remained consistent (BACE1 0.952 ± 0.024 , n=3, combined 0.8847 ± 0.012 , n=3, p=0.071, t=2.44).

BACE1 Activity Assay with Direct Binding Method

Testing the BACE1 specific peptide for inhibitory properties *in vitro* was determined by simulating the *in vivo* pathway. Using a 7-methoxycoumarin labelled APP fragment as a substrate, cleavage by BACE1 was expressed by activation of the fluorophore and a representation of activity. The peptide was immobilised on nitrocellulose before being introduced to the experimental solution containing BACE1 and APP to determine preferential binding. The immobilised peptide was removed, along with any bound BACE1, before the fluorometric analysis was performed. The addition of a BACE1 specific peptide to the reaction reduced the amount substrate cleavage maintaining the quenched fluorophore. Initially, the 1nM concentration sample of immobilised peptide did not significantly reduce to the amount of substrate cleavage (negative control 0nM 117.4 ± 3.64 , n=3, 1nM 117.5 ± 4.6 , n=3, p=0.99, t=0.005). However, 10nM showed a significant 23% reduction (90.64 ± 5.0 , n=3, p=0.012, t=4.33) in the amount of substrate being cleaved. The response was reduced further by 44% (65.86 ± 1.37 , n=3, p=0.0002, t=13.28) with a 10-fold increase in peptide concentration to 100nM (Fig. 3). The increase in peptide concentration was causing a decrease of fluorescent response, which represented cleavage of the substrate, determined at an IC_{50} of 235.2nM.

A β Assays from Treated Cells – SY5Y Cell Viability Test with BACE1 Specific Peptide Treatment

To test the peptide for possible toxicity on neurons, SY5Y cells were treated with 1nM, 10nM and 100nM of BACE1 specific peptide. Cell viability was measured using Trypan blue to confirm live cell numbers. The 1nM (negative control 0nM 91.33 ± 0.33 , n=3, 1nM 92.67 ± 1.2 , n=3, p=0.345, t=1.07), 10nM (91.0 ± 2.08 , n=3, p=0.88, t=0.16) and 100nM (93.67 ± 1.33 , n=3, p=0.164, t=1.7) treatments of BACE1 specific peptide did not exhibit a significant decrease in percentage of live cells when compared to the no treatment, negative control (Fig. 4a).

SY5Y Cell Viability Test for H₂O₂ and BACE1 Inhibitor Treatment

Oxidative stress is one of the earliest events in the initiating phases of AD (34). H₂O₂ induced oxidative stress is an important component of A β deposition and can be an early indicator of neuronal cytotoxicity (35, 36). Simulating this oxidative stress event with H₂O₂ treatments can increase the production of A β in SY5Y cells (37). The increase of oxidative stress on the cell can cause an apoptotic response leading to a decrease in viability. The aim of this study was to treat the cells with H₂O₂ to induce A β release, without triggering the apoptotic response and determine the effect the BACE1 specific peptide could have on A β production. The cells were treated with both H₂O₂ and one of the 1nM (negative control

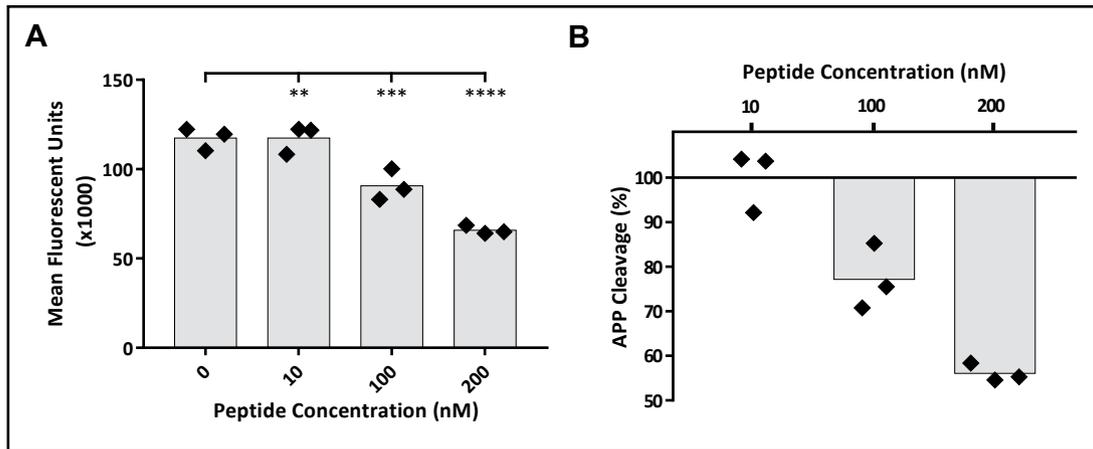


Fig. 3. The BACE1 specific peptide, when introduced to BACE1, can prevent the cleavage of an APP substrate. The BACE1 specific peptide was immobilised on nitrocellulose at concentrations of 10nM, 100nM and 200nM, respectively and introduced to a solution of APP substrate and BACE1. Quantification of mean fluorescence (a) represents the amount of APP cleavage. Percentage of APP cleavage reduction (b) signifies the BACE1 activity after incubation with the immobilised peptide. This data was compared to the negative control as a baseline (n=3). All values are reported as individual data points (n=3) and bars represent mean fluorescence. Statistical significance is determined by unpaired Student's t-test (p<0.01 **, p<0.001 ***, p<0.0001 ****).

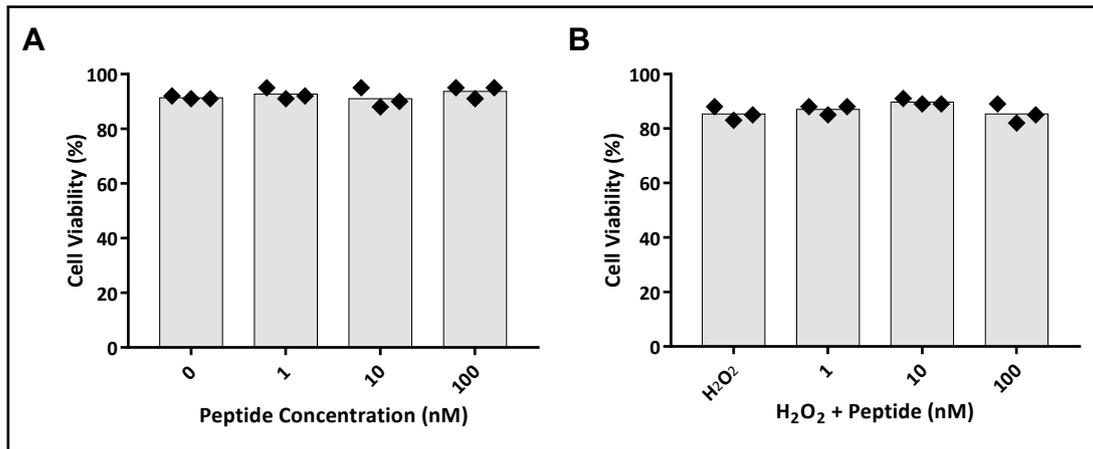


Fig. 4. Treatments with the BACE1 specific peptide does not cause a decrease in SY5Y cell viability. (a) Confluent SY5Y cells were tested for potential toxic effects by apoptosis due to treatments of 1nM, 10nM and 100nM peptide in the media. (b) Co-treatment effects of and H₂O₂ induced oxidative stress with 1nM, 10nM and 100nM concentrations of peptide determined by cell viability percentage of live cells. Percentage data was generated by cell counts of live and dead cells (n=3). All values are reported as individual data points (n=3) and bars represent the mean percentage of live cells from individual flasks.

0nM 85.33±1.45, n=3, 1nM 87.0±1, n=3, p=0.398, t=0.94), 10nM (89.67±0.67, n=3, p=0.054, t=2.71) and 100nM (85.33±2.03, n=3, p>0.99, t=0) concentrations of BACE1 specific peptide for 24 hours. Viability, as a result of the BACE1 inhibitor treatments, was not compromised as there was no significant increase or decrease in the number of live cells (Fig. 4b).

Aβ Production by SY5Y Cells are Reduced with BACE1 Inhibitor Treatment

SY5Y cells naturally produce Aβ in culture, which can be identified by ELISA. Capture antibodies bind to free Aβ released when the cells are lysed. The fluctuations in APP processing by BACE1 are determined by the increase or decrease of Aβ production. The cells

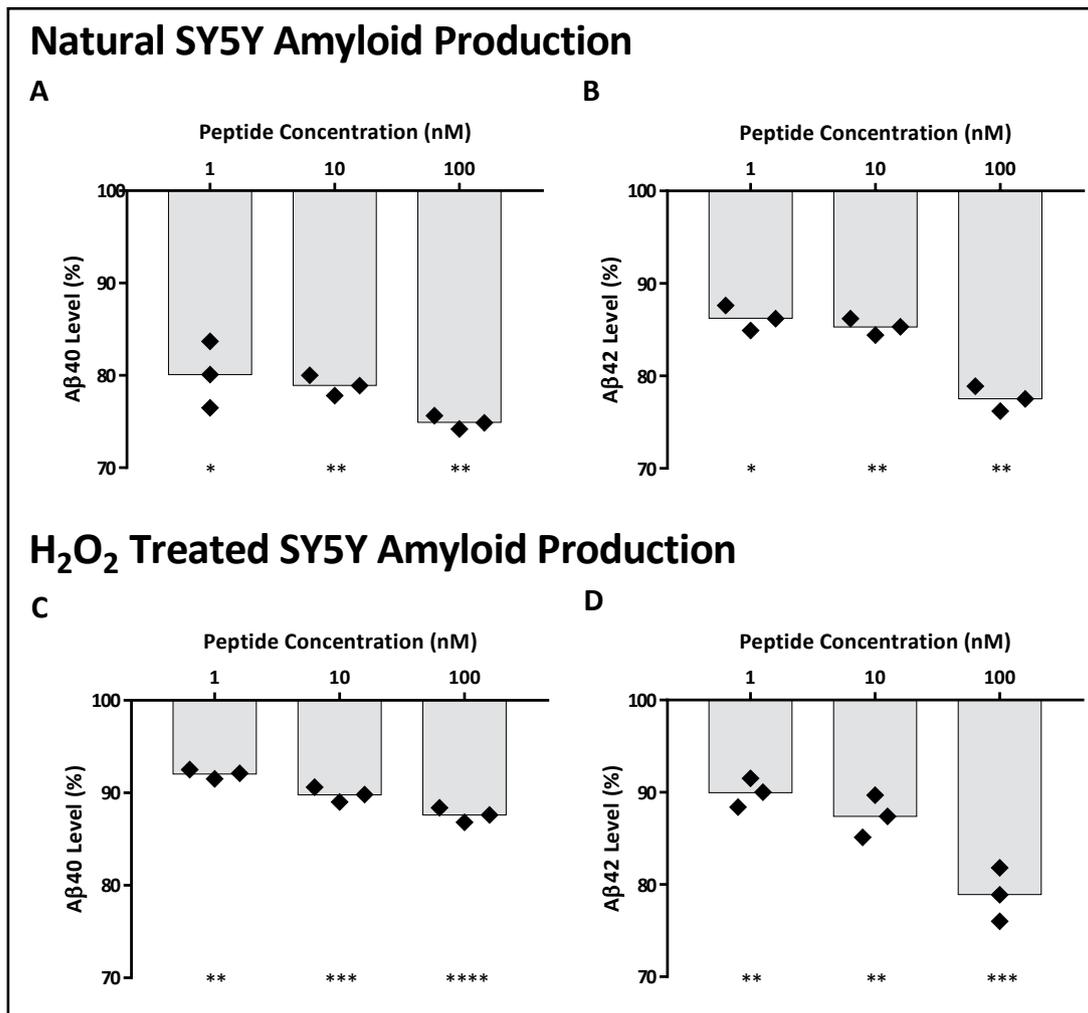


Fig. 5. BACE1 specific peptide treatment of SY5Y cells reduces the amount of Aβ40 and Aβ42 production. SY5Y cells grown to confluence were treated with concentrations of BACE1 specific peptide at 1nM, 10nM and 100nM in media. Naturally grown cells were tested with inhibitor and analysed by ELISA for (a) Aβ40 and (b) Aβ42. Testing for oxidative stress on SY5Y cells and potential for amyloid control by BACE1 specific peptide treatment at concentrations of 1nM, 10nM and 100nM. H₂O₂ induced stress on treated cells were tested by ELISA for the production of (c) Aβ40 and (d) Aβ42 and calculated for the percentage reduction as a result of the treatment. All values are reported as individual data points (n=3) and bars represent mean percentage reduction. Statistical significance on raw data where treatments are compared to the negative control is determined by unpaired Student's t-test (p<0.05 *, p<0.01 **, p<0.001 ***, p<0.0001 ****).

were treated with the BACE1 specific peptide to influence these fluctuations and to reduce the Aβ loading. Both the Aβ40 and Aβ42 produced by the cells were significantly reduced after the treatment with BACE1 inhibitor. Cellular Aβ40 levels were decreased by 20% with a 1nM treatment (negative control 0nM 0.595±0.009, n=3, 1nM 0.477±0.01, n=3, p=0.001, t=7.83), 21% for 10nM (0.47±0.004, n=3, p=0.0001, t=13.3) and 25% for 100nM (0.446±0.003, n=3, p=0.00008, t=16.53)(Fig. 5a). The 10-fold increase in peptide treatment, from 10nM to 100nM, significantly reduced the amount of Aβ40 (p=0.006, t=5.26). The cellular Aβ42 also showed a similar trend with significant reductions of 14% for 1nM (negative control 0nM 0.218±0.005, n=3, 1nM 0.188±0.002, n=3, p=0.004, t=6.082), 15% for 10nM (0.186±0.001, n=3, p=0.003, t=6.72) and 22% for 100nM (0.169±0.002, n=3, p=0.0006, t=9.93), albeit with a very low signal produced by the ELISA (Fig. 5b).

H₂O₂ Treatment of SY5Y Cells Increase A β Production but is Reduced by BACE1 Inhibitor

Oxidative stress placed on the SY5Y cells produces an increase in the amount of A β monomers. Cell treatments with H₂O₂ to induce oxidative stress, increased the amount A β , which is an indication of more BACE1 cleavage of APP. Treatment of SY5Y cells with H₂O₂ increased the amount of A β 40 by 11% and A β 42 51%, when compared to the untreated groups. The BACE1 specific peptide was added to the reaction aimed at controlling the amount of A β produced under stress. A β 40 levels decreased by 8% with 1nM of the BACE1 specific peptide (negative control 0nM 0.657 \pm 0.001, n=3, 1nM 0.605 \pm 0.002, n=3, p=0.00004, t=19.73), 10% with 10nM (0.59 \pm 0.003, n=3, p=0.00004, t=19.9) and 12% with 100nM (0.576 \pm 0.003, n=3, p=0.00002, t=22.53)(Fig. 5c). A β 42 levels decreased by 10% with 1nM of BACE1 specific peptide (negative control 0nM 0.329 \pm 0.008, n=3, 1nM 0.296 \pm 0.003, n=3, p=0.01, t=4.104), 13% with 10nM (0.287 \pm 0.004, n=3, p=0.009, t=4.79) and 21% with 100nM (0.26 \pm 0.005, n=3, p=0.002, t=7.48)(Fig. 5d). The more peptide added to the reaction, the less A β monomers were being produced.

Discussion

Successful identification of a peptide with natural binding affinity for BACE1 was obtained using the phage display and biopanning techniques. Utilizing the M13 bacteriophage PIII surface protein, normally fundamental for F-pilus binding to host bacterial cells, gene modification allows for the expression of a small peptide sequence to be added without the loss of function (38). Refining the library of 1.1x10⁹ different peptide sequences expressed on the PIII protein allowed for natural binding affinity to occur and identification of that specific sequence. Performing the higher stringency of the 5 biopanning rounds, instead of the usual 3, removed any non-specific candidates. Performing the amplified eluate consistently increased titre, as the biopanning rounds became more stringent, suggesting the pool of phage was being enriched. The transduction of phage DNA to the ER2738 *E. coli* host strain transferred the LacZ gene represented by the production of blue plaques, denoting the metabolism of X-gal. The metabolism of X-gal, included in the LB agar plates, was generated by the expression of the β -galactosidase enzyme, which caused plaques to develop a blue colour. The fifth round eluate did not contain any white plaques indicating that aseptic techniques were maintained throughout the experiments. The 12 blue plaques, from the biopanning round 5 unamplified eluate, produced a consistent sequence motif via DNA sequencing. The possibility of picking the wild-type phage was rejected as the plaques picked were blue in colour and maintained the insert, consistent with the M13KE cloning vector and triple glycine leader sequence.

The phage DNA insert sequence was translated and produced into a more pharmacologically friendly free synthetic peptide structure, without the connected phage, and reaffirmed for the binding shown in the biopanning rounds. Using a biotin tag on the peptide, the amount of binding was recorded by colorimetric assay, allowing for the natural binding affinity to BACE1 to still occur. Using peptide concentrations of 1nM, 10nM and 100nM, each 10-fold increase was significantly higher than the previous, even when using the higher stringency wash conditions in the final round of biopanning. The direct assay is highly specific as the peptide can only bind to one of the protein target or blocking buffer, but the negative, no BACE1, control provided no colorimetric response which explains that specific binding was confirmed.

The cross reactivity of a BACE1 inhibitor to the closely related BACE2, renin and cathepsin D can cause potentially harmful adverse reactions as part of a treatment. The significant binding and inhibition of renin could alter cardiac output and systemic blood flow while alteration of cathepsin D activity can influence cell signalling and apoptosis (39, 40). The specificity assay was designed to test for potential cross reactivity by immobilizing one of the homologs, the same as for BACE1, and allowed to freely bind the peptide candidate. The peptide did not show any affinity to renin but cathepsin D and BACE2 produced a significant

increase in binding when compared with the negative control. However, when the peptide was pre-incubated in solution with a combination of homologs then added to a plate-well-immobilised BACE1, the peptide no longer exhibited the cross-reactive effects. The binding to BACE1 was consistent across all experimental groups. These results give confidence when exposing the peptide to an *in vivo* environment that binding may not occur to these homologous proteins.

The peptide showed a binding affinity for BACE1 specifically, even in the presence of similar enzymes, but required the ability to inhibit the cleavage of APP to prevent production of the plaque forming A β biproducts. The peptide was immobilised on nitrocellulose before the BACE1 activity reaction could take place to prevent unwanted background responses. The BACE1 substrate and nitrocellulose bound peptide were incubated together to determine the amount fluorometric response, which is an indicator of enzymatic activity. The APP fragment, maintained between the 7-methoxycoumarin-4-acetyl fluorophore and the 2, 4-dinitrophenyl quencher, was cleaved by the active BACE1, which triggered the fluorometric response. The negative control did not contain any BACE1 specific peptide and produced the highest amount of substrate cleavage. The amount of inhibition increased significantly with the 10-fold addition of peptide. The peptide was able to bind to BACE1 and, as a consequence, APP cleavage was reduced.

Common inhibitor complications involved with using small permeable chemicals and antibodies are centred around specificity, binding affinity and toxicity. In an aim to identify a potential acute toxic affect as a result of the treatment, cell viability was determined. Using the SY5Y human neuroblast cell line in culture, treatments were given in media to identify any cell death that may be a consequence. The effective direct binding seen in the assays gave a precedent for the treatment dose, with the expectation that it could bind to membrane bound BACE1. As a result, there was no significant cell death caused by the treatments and visual physiology did not show dysfunction. This gives a confidence that the peptide is well accepted by this human neuronal cell type. In the interest of usage and the effective concentrations exhibited in both the direct binding and activity assays, LD50 was not determined however, it would be more appropriate when used in an *in vivo* model. The use of a viability test gives an understanding that there is no acute toxicity associated with the treatment. However, this could be better understood using proteomics and RNASeq techniques to ascertain potential changes occurring. In terms of peptide specific BACE1 inhibition and the possible repercussions, knockout studies have shown that there is minimal cellular dysfunction with the lack of the gene, regardless of the potential for incomplete inhibition (41, 42).

Confident in the treatment concentrations from the direct assays, BACE1 activity, and the A β produced as a consequence, was tested with addition of the BACE1 specific peptide as an intervention strategy. As there was no observable toxicity generated by the viability test, the same concentrations were used. Using this result as the background for treatment on normally functioning healthy cells, the peptide was able to significantly reduce A β production at nanomolar concentrations. While the response levels were viable but low, especially for A β 42, there was still a significant reduction suggesting that in a non-diseased state there is an effectiveness for the peptide. Samples treated with a 100nM concentration of BACE1 specific peptide incorporated into the media was able to reduce A β 40 by 25% and A β 42 by 22%.

The onset of AD is met by the accumulation of neurofibrillary tangles and amyloid plaques with the underlying pressure of oxidative stress (43). The deposition of plaques and tangles cause significant damage to surrounding cells by triggering a feedback loop. Under conditions of oxidative stress, the SY5Y cells have been reported to influence APP processing by increasing the amount of cytosolic A β (44). In this study, SY5Y cells were treated with H₂O₂ to simulate stress to increase the cleavage of APP. The cells were tested both with and without H₂O₂ and both produced a significant amount of A β 40 and A β 42. The treatment of H₂O₂ did not affect cell viability or observable physiology, even with the extra stress, but did significantly increase the amount of A β produced above that of the untreated cells. The possibility for toxic effects while already stressed would have been shown with a decrease

in the number of live cells, however it was not observed with this model. Adding 100nM of BACE1 specific peptide to the media treatment elicited a 12% reduction in A β 40 and 21% of A β 42. Even with A β 42 being produced in less amounts, it is the more amyloidogenic of the two, maintaining a higher binding affinity for other A β fragments (38-43) causing acute plaque generation (45-47). These reductions in BACE1 activity may be further reduced with higher peptide inhibitor concentration treatments.

For the peptide to be successful in preventing this pathway, it would need to remain unchanged within the media for the 24-hour time period. To apply maximum effectiveness, the peptide needs to bind to BACE1 and inhibit the cleavage of APP while being able to transport across the membrane into the cell. The BACE1 specific peptide was able to reduce BACE1 activity within the SY5Y neuronal cell line while under oxidative stress, thus generating less of the oligomer producing A β . The reduction of cellular A β could therefore, reduce the potential for plaque generation but would need to be tested in an *in vivo* model.

While showing effectiveness in a cellular model, inhibitor focus has been directed at chemicals with blood brain barrier permeability and oral availability. However, the requirement to develop a strong binding candidate is still an important direction. Using the phage display technique to identify a 12-residue peptide with significant binding properties at a high potency, we have successfully identified a new candidate for future investigation into a pharmaceutical intervention for the production of AD plaques, which may form as the backbone for a new treatment option. This method may not follow Lipinski's rule of 5 in terms of having a predictably viable and physiochemically ready backbone but further refining using alanine scanning and truncated peptide analysis in our future studies will help identify the most important residues and reduce the molecular weight (48). As it is a peptidic structure, there are many options for peptidomimetic modification without diminishing effectiveness.

Conclusion

In summary, with application of the phage display and biopanning techniques, a BACE1 specific peptide was successfully identified. Sequencing of phage DNA determined the expressed peptide on the capsid. The peptide was synthetically produced and tested successfully for a direct binding affinity to BACE1. A potential cross reactivity with the peptide and closely related BACE2, cathepsin D and renin was refuted using competition assays. The peptide also reduced the enzymatic cleavage of an APP-like substrate showing an ability to prevent BACE1 activity. Taking the peptide to a human neuronal cell culture did not cause any detriment to cell growth while being able to reduce the amount of A β 40 and A β 42. Inducing cells with oxidative stress via treatment with H₂O₂ challenged the normal cellular growth patterns without causing death, and when co-treated with inhibitor did not challenge viability. While the H₂O₂ treatments increased the amount of A β 40 and A β 42, the BACE1 specific peptide was able to significantly reduce their production. With the ability to reduce A β in culture, the BACE1 specific peptide shows early promise in the treatment of plaque generation in AD.

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Disclosure Statement

The authors have no conflicts of interest.

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