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Identification of a sub-micromolar, non-peptide inhibitor of β-secretase with low neural cytotoxicity through in silico screening

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ABSTRACT

Nowadays identification of novel non-peptide β-secretase (BACE-1, hereinafter) inhibitors with low cytotoxicity and good blood–brain barrier (BBB) property holds common interest of drug discovery for Alzheimer's disease. Twenty SPECS compounds were tested in BACE-1 FRET assays and methylthiazoletetrazolium (MTT) cytotoxicity experiment. Two compounds: **2** and **15** demonstrated IC_{50} values of 0.53 and 9.4 μM. In addition, **2** showed least toxic effect to the neuroblastoma cells. The results from both in silico and in vitro studies provided new pharmacophoric entities for chemical synthesis and optimization on the current discovered BACE-1 small molecule inhibitors.

Alzheimer's disease (AD) is a chronic neurodegenerative disorder of the central nervous system, resulting in severe cognitive deficits along with psychiatric complications. More than 37 million individuals suffer from this condition and the number is expected to continue growing dramatically.[1,2] Five drugs that are currently on the market—the cholinesterase inhibitors donepezil, rivastigmine, galantamine, and tacrine, the *N*-methyl-D-aspartate (NMDA) receptor modulator memantine approved for the treatment of symptoms of AD.[3] However, they are unable to halt or reverse the disease progression. The major pathological hallmark of AD is the deposition and aggregation of amyloid β-peptide (Aβ) in the brain tissues and hence leads to intracellular formation of neurofibrillary tangles. This small peptide consists of 39–43 residues that are endo-proteolytically derived from a transmembrane amyloid precursor glycoprotein (APP).[4] Aβ production involves the sequential actions of two proteases, the BACE-1 and γ-secretase.[5] Inhibition of these enzymes by small molecule interference is a promising strategy to prioritize pharmaceutical candidates and such approach was highly encouraged by the discovery in 2001 that no Aβ were generated in BACE-1 knockout mice which were devoid of BACE-1 activity and displayed a normal phonotype.[6] In the past, the major effort in designing BACE-1 inhibitors was the production of transition state isosteres such as hydroxyethylamines, reduced amides, statine-based peptidomimetic inhibitors countering the catalytic aspartyl groups. Although large number of potent peptidomimetic inhibitors can be generated, their relatively large size and number of hydrogen bond donors and acceptors make it challenging to achieve high oral bioavailability.[7,8] Therefore, identification of novel small molecule non-peptide inhibitors with balanced ADMET properties will make the chemicals more favorable for further development into leads and drugs. Some non-peptide compounds were identified as inhibitors of BACE-1. Astex researchers highlighted their work in discovering aminopyridine and cyclic amidine classes as BACE-1 inhibitors.[9] Barrow et al. reported the identification of spiropiperidine inhibitor template for BACE-1.[10] More recently, researchers from Weyth described their identification of aminohydantoins as potent BACE-1 inhibitors.[11] The most prominent inhibitor was from GSK group which generated GSK188909 as the first orally available BACE-1 inhibitor showing its therapeutic effect in an animal model.[12] More information on non-peptide inhibitors of BACE-1 was reviewed by Huang et al.[13] Despite the painstaking effort put in discovering drug candidates for AD, no truly disease modifying agents are yet available.[14] Hence, investigations on new drug discovery and development for AD draw a great deal of interest to the medicinal chemists.

Herein, we report the discovery of two novel BACE-1 small organic inhibitors via a receptor-based virtual screening followed by compound drug-likeness analysis, cytotoxicity prediction, and bioassays. Scheme 1 shows the overall approach adopted in this study. The preparation of protein structure was done according to our previously reported protocol.[15] The SPECS chemical library

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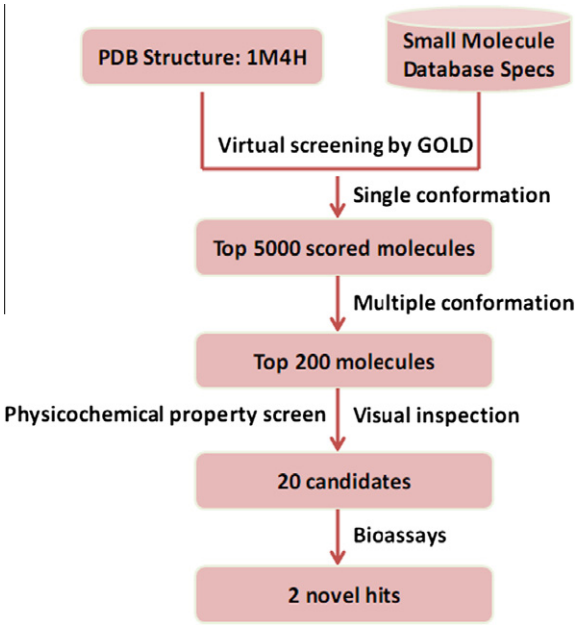


Scheme 1. Flowchart of virtual screening and bioassays for discovering BACE-1 inhibitors.

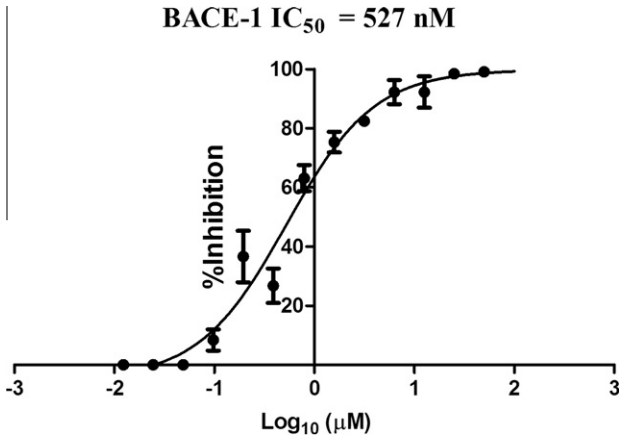
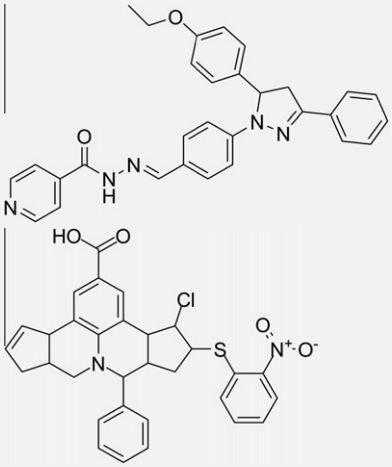


Figure 1. IC_{50} plot of compound **2**. Measurement was done in duplicate and error bar was shown.

Table 1
BACE-1 inhibition results of compound **2** and **15**

Compd	Structure	% inhibition at 100 μM[a]	IC_{50}[b]
2		100	0.53
15		80	9.3

[a] Initial screen were carried out in duplicate and results were presented as the average value.
[b] IC_{50} was done in duplicate. Values were expressed as the average of two assays.

(~280,000 compounds) was screened using GOLD 3.0.1 with default parameters. The active site radius is 10 Å from atom 1869 (OG1) of amino acid Thr232 which was one of the key amino acid residues in BACE-1. The GoldScore fitness function was applied and top 5000 molecules with the highest scores from initial virtual screening were then re-submitted for multiple docking of 10 conformations for each ligand. Finally, the top 200 hits were kept for further selection. To select which of the in silico compounds would be purchased and tested in vitro, several factors were taken account in this step and these included: (1) Information on molecular weight, predicted Log *P*, Log *S* and toxicity values of the compounds, toxicity prediction by Pallas (Compudrug Pty Ltd) software; (2) Our knowledge in the molecular interactions between BACE-1 and its substrate, together with the predicted putative H-bonds formed by the hits and active site residues of BACE-1, potential hydrophobic interaction and aromatic–aromatic interactions. Based on the factors stated above, the consensus judgment from both of the selection criteria led us to purchase 20 compounds for enzymatic assays. These compounds mostly have molecular weights <550 Da, Log *P* <8, hydrogen bond donor <3, and hydrogen bond acceptor <7. The features were considered as favourable physicochemical properties of CNS drugs as highlighted in Pardridge's review.[16] Finally twenty SPECS compounds were purchased and tested for their inhibitory activities against BACE-1 in FRET assays. BACE-1 inhibition assays were carried out using Fluorescense Resonance Energy Transfer (FRET) assay kits according to the protocol described previously.[15] Six compounds exhibited over 50% inhibition of BACE-1 activity at 100 μM. Among them, **2** and **15** had IC_{50} values of 0.53 μM (Fig. 1) and 9.4 μM, respectively. Table 1 shows the structures of the inhibitors and their enzyme assay results. To elucidate the possible interactions between the inhibitors and their active binding site, the docked conformations of **2** and **15** were superimposed in the binding site and the docked poses enabled us to understand the main protein-inhibitor interactions. The top scored conformations were selected and showed from all conformations generated by GOLD. As shown in Figure 2, **2** and **15** fell into two different interaction patterns with BACE-1. Compound **15** was docked into only half of the BACE-1 active pocket (S1′, S1, S2 and S3). Figure 3 depicts the detailed binding modes of the inhibitors in BACE-1. The bulky aromatic groups in **15** made it mainly interact via hydrophobic contacts with the surrounding active residues such as Thr 231, Thr 232, and Tyr71 (Fig. 3b). In contrast, compound **2** spanned the binding with the extension to the prime side of the active site, as seen by the occupancy of the S4′ sub-site (Fig. 3a). In particular (refer to Fig. 4), the ethoxyphenyl occupies the intermediate space between S3 and S4, forming Van der Waals interaction with Leu 30 and Ile 110. The phenyl pyrozol group is more exposed to the solvent but a π-cation interaction could be present between Arg 235 and this group and further stabilizing the binding of **2** entropically. Interestingly, unlike other reported inhibitors which are capable of forming multiple hydrogen bonds with the two aspartic acid residues in BACE-1, **2** does not contain a moiety to form hydrogen bonds with either of the aspartic acid residues (Asp 32 or Asp 228) in the S1 catalytic hole. Instead, a benzene ring occupies the S1 region. The aromatic–aromatic interaction with Tyr 71 and Phe 108 could be the reason for an aromatic group to be localised at the S1 of BACE-1 by GOLD. In addition, due to the hydrophobic nature in the S1–S3 region, it is reasonable that a large hydrophobic or aromatic group

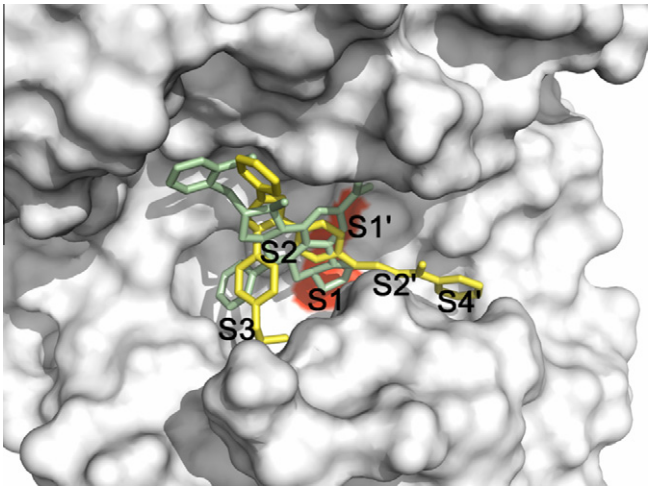


Figure 2. Superimposed binding of **2** and **15** derived from docking by GOLD. Compound **2** is colored by yellow and compound **15** is indicated using green. Protein surface is colored white. Surfaces of catalytic aspartic acids 32 and 228 are colored in red. Two residues, Thr 72 and Gln 73, were deleted for a whole view of active site. Sub-sites of BACE-1 are labeled in black. The picture was generated by Pymol (DeLano, W. L. The PyMOL Molecular Graphics System (2002) DeLano Scientific, San Carlos, CA, USA).

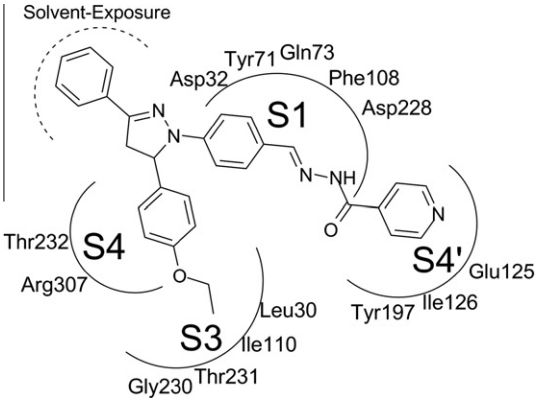


Figure 4. Docked binding mechanism of **2** in BACE-1 active site.

should be well tolerated. The overall comparison in the binding modes of compounds **2** and **15** derived from docking were hence in good agreement with the difference in their inhibitory potencies from enzyme assays.

To validate the toxicity prediction from Pallas, MTT assays[17] were conducted on the neuroblastoma cell line SH-SY5Y. MTT reacted with the mitochondrial succinic dehydrogenase in the living cells to produce a dark-purple formazan and the reaction was indicative of the presence of viable cells.[17] Neuroblastoma cell lines, SH-SY5Y, were purchased from ATCC through local vendor. The base medium mixture was created using F12 medium and ATCC-formulated Eagles's minimum Essential medium with a ratio of 1:1. The complete medium was formed by adding FBS, fetal bovine serum into the base medium with the final concentration of 10%. Cells were cultured in T25 flasks containing 7 ml of complete medium. The flask was incubated for 5–7 days at 37 °C in 5% CO_2 incubator. The SH-SY5Y cells were sub-cultured and fed with fresh medium every 5–7 days. Briefly, the test compounds were serially diluted by complete medium to achieve final concentrations ranging from 12.5 to 1.56 μM in the 96-well plate. One hundred microlitre of test compound was added into the neuroblastoma cells pre-seeded at the concentration of 3×10^5 cells/ml and incubated for 4 days. Total DMSO concentration in the well is less than 2%. Next, 100 μl of MTT solution was introduced to each well (final MTT concentration = 0.5 mg/ml) and incubated for 4 h at 37 °C + 5% CO_2. Finally, 100 μl of detergent (sodium dodecyl sulfate + isobutanol + 36.5% hydrochloric acid) was added into each well and the absorbance values of 570 and 630 nm (reference) were taken after overnight incubation. The culture medium was used as control in the MTT experiment. The cell viability percentage was calculated using the formula $(A_{570} - A_{630})_{sample}/(A_{570} - A_{630})_{control} \times 100\%$.

Compound **2** was tolerable by the cells at a concentration of up to 25 μM whereas compound **15** exhibited a 50% reduction in cell viability at 12.5 μM. Hence, compound **2** showed a 50-fold selectivity in inhibitory potency over toxic effect. Meanwhile, **2** has a molecular weight of 489, predicted Log *P* of 4.93, Log *S* of −8.4, six hydrogen bond acceptors and one hydrogen bond donor. These features make it nearly fit the criteria for being CNS drugs especially for the BBB permeation.[16] The overall 'portfolio' of **2** suggested it is an attractive hit and worth taking downstream steps to make it into lead compounds against AD.

Albeit **2** showed BACE-1 inhibition at sub-micromolar, there is still no enough information whether it can reduce the Aβ from the cells. Future work on the measurement of the secreted amount of Aβ from the current cell line will help us understand more on the medicinal potential of compound **2**.

In conclusion, an in silico virtual screening of commercial database SPECS together with enzyme assays and cell-based toxicity testings resulted in the identification of two novel non-peptide inhibitors of human BACE-1. Compound **2** emerged as a sub-micromolar inhibitor (IC_{50} 0.53 μM) with low cytotoxicity. Docking of compound **2** in the BACE-1 active site revealed that a central

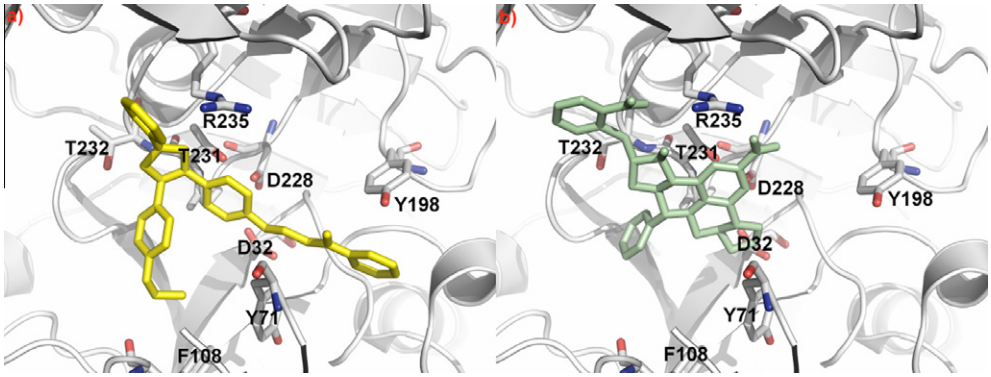


Figure 3. Cartoon representation of BACE-1 active site. Key residues are shown in stick and colored by atom type. (a) Molecular docking pose of compound **2**. (b) Molecular docking pose of compound **15**. The binding mode was derived from GOLD and the picture was generated by Pymol software.

hydrophobic benzene ring occupied the aspartic acid catalytic holes and also suggested the putative binding and interactions with other sub-sites. MTT assays indicated compound **2** was well tolerated by the brain cell line SH-SY5Y. Therefore, we believe that future effort in optimizing present sub-micromolar hit into more potent nanomolar leads based on the molecular clues from this research will lead the drug discovery for Alzheimer's disease to another luminous milestone.

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