Research Article

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Lead discovery of a guanidinyl tryptophan derivative on amyloid cascade inhibition

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Abstract: Amyloid cascade, one of pathogenic pathways of Alzheimer’s disease (AD), was focused as one of drug discovery targets. In this study, β-secretase (BACE1) inhibitors were designed aiming at the development of multifunctional compounds targeting amyloid pathogenic cascade. Tryptophan was used as a core structure due to its properties of the central nervous system (CNS) penetration and BACE1 inhibition activity. Three amino acid residues and guanidine were selected as linkers to connect the tryptophan core structure and the extended aromatic moieties. The distance between the aromatic systems of the core structure and the extended moieties was kept at the optimal length for amyloid-β (Aβ) peptide binding to inhibit its fibrillation and aggregation. Sixteen designed compounds were evaluated in silico. Eight hit compounds of TSR and TGN series containing serine and guanidine linkers, respectively, were identified and synthesized based on docking results. TSR2 and TGN2 were found to exert strong actions as BACE1 (IC50 24.18 µM and 22.35 µM) and amyloid aggregation inhibitors (IC50 37.06 µM and 36.12 µM). Only TGN2 demonstrated a neuroprotective effect in SH-SY5Y cells by significantly reducing Aβ-induced cell death at a concentration of 2.62 µM. These results support the validity of multifunctional approaches to inhibition of the β-amyloid cascade.

Keywords: tryptophan, multifunction activities, beta-secretase inhibitor, amyloid aggregation inhibition, neuroprotection

1 Introduction

Alzheimer’s disease (AD) is currently the most common neurodegenerative disorder, particularly affecting aged members of society and causing impairments of cognition and memory. Two pathological features, amyloid-β (Aβ) plaques and neurofibrillary tangles, have been found in the brains of patients with AD. The observation of these proteins led to the development of drugs for AD, which obstruct the production of Aβ and neurofibrillary tangles and their neurotoxic cascades [1,2]. In the etiology of Aβ, the β-secretase enzyme (BACE1) is an attractive drug target as it generates Aβ peptide, the main constituent of toxic Aβ oligomers and plaques [3–5]. Decreased Aβ production would obstruct Aβ oligomerization and consequently reduce Aβ aggregation. A number of BACE1 inhibitors and amyloid aggregation inhibitors have been developed as single target drugs but more than half have apparently failed in clinical trials. Only two BACE1 inhibitors are currently in phase III clinical trials and three compounds in phase II clinical trials [6]. The use of a multitarget single ligand is one of the most challenging approaches to AD drug discovery, aiming to reduce the late-stage clinical attrition. This new paradigm has gained popularity because it suits the complex etiology and multiple pathogenic mechanisms involved in AD [7–9].

However, ligand developed to be AD drug should be considered about the possibility to enter the CNS. Three methods of molecule entering to brain are passive diffusion, active transport via solute carrier (SLC) superfamily, and endocytosis [10]. Thus, the objective of this research was to design and synthesize novel BACE1 inhibitors with multifunctional actions including anti-amyloid aggregation and antioxidant properties by using in silico hit screening and finding. Tryptophan was used as a core structure to make a molecule to transport into CNS.
2 Materials and methods

2.1 In silico study

Molecular dockings were performed to determine the binding mode of compounds to BACE1 and Aβ using the AutoDock program suite, version 4.2. The Pymol program was used to visualize the generated docking graphics objects. The 3D-structures of the compounds were generated and optimized with ChemDraw Ultra 12.0 and Chem3D Ultra 12.0.

The protein template preparation and docking parameters using in this research were the same as in previous study [11]. Briefly, the BACE1 template 2IRZ-F constructed from the crystal structures of 2IRZ [12] and 1FKN [13] was used as a BACE1 macromolecule. The docking parameters in the in silico studies for BACE1 were as follows: 100 runs of the genetic algorithm (GA), a population size of 150, 1,50,00,000 energy evaluations per run, and a maximum number of generations of 27,000.

Aβ template, 12OQ-1, was constructed from X-ray crystal structures of inhibitor-bound Aβ (PDB code: 12OQ) [14]. Docking parameters in the in silico studies for Aβ binding were as follows: 100 runs of the GA, a population size of 150, 50,00,000 energy evaluations per run, and a maximum number of generations of 27,000.

2.2 Synthesis part

Chemical reagents were purchased from Acros, Aldrich, or AK Science. Melting points were determined on a Model 9100 melting point apparatus (Electrothermal, UK). IR spectra were recorded using a Nicolet FTIR Instruments machine (ThermoFisher, USA). Nuclear magnetic resonance (NMR) spectra were recorded using Bruker Fourier 300 MHz spectrometers (Bruker, Switzerland) with tetramethylsilane as an internal standard. Mass spectra (MS) were obtained in positive ESI mode (Bruker, Switzerland). The 1H and 13C-NMR spectra of synthesized compounds were shown in the supplemental materials (Figures S1–S20).

2.2.1 General synthesis procedure of TSR1–TSR4

A solution of compound T2 (1 mmol), NH2–R2 (1 mmol), HBTU (1.5 mmol), and DIPEA (3 mmol) in 5 mL of DMF was stirred at room temperature. After 18 h stirring, water was added. The aqueous phase was extracted with ethyl acetate. The organic extracts were combined and dried. The obtained residue was purified by column chromatography on silica gel to yield compound Boc-TSR1 to Boc-TSR4. The protecting group of the obtained compound was removed by 50% of TFA in ethyl acetate at room temperature for 3 h. The reaction mixture was adjusted to pH 7 by saturated sodium bicarbonate solution and extracted with ethyl acetate. The combined organic layers were dried. The residue was purified by column chromatography on silica gel to yield TSR1–TSR4 (Scheme 1).

2.2.2 (S)-2-Amino-N-((S)-3-hydroxy-1-((4-hydroxyphenyl)amino)-1-oxopropan-2-yl)-3-(1H-indol-3-yl)propanamide (TSR1)

Compound Boc-TSR1 was purified by column chromatography on silica gel (CHCl3/EtOAc/NH4OH 7:3:0.01) to yield white powder. Compound TSR1 was purified by column chromatography on silica gel (CHCl3/EtOAc/MeOH 4:5:1) to yield yellow solid (hygroscopic). Yield 261.45 mg (68.37%). FTIR (ATR, μ, cm−1): 3,392–3,155 (O–H, N–H), 3,110 (sp2 C–H), 2,927 (sp3 C–H), 1,674, 1,514 (C=O, C=C), 1,457, 1,444 (C=C), 1,368 (C=C), 1,201, 1,164 (C–N, C=O). 1H-NMR spectrum in DMSO-d6 (δ, ppm): 10.88 (s, 1H, indole–NH), 9.73 (s, 1H, OH), 8.20 (s, 1H, NHa), 7.38 (s, 1H, NHB), 7.57 (d, 1H, J 8.0 Hz, 1H, H9″), 7.34 (d, 1H, J 8.1 Hz, H6″), 7.38 (d, 2H, J 8.9 Hz, H2″, 6″), 7.20 (s, 1H, H4″), 7.05 (t, 1H, J 8.1 Hz, H7″), 6.96 (t, 1H, J 7.9 Hz, H8″), 6.69 (d, 2H, J 8.8 Hz, H3″, H5″), 3.88 (dd, 1H, J1 6.0 Hz, J2 7.3 Hz, H1″), 3.64 (m, 3H, H2, H4a, H4b), 3.19 (dd, 1H, J1 5.8 Hz, J2 14.6, H2b′), 3.02 (dd, 1H, J1 7.2 Hz, J2 14.6 Hz, H2a′). 13C-NMR spectrum in DMSO-d6 (δ, ppm): 174.9 (C1), 168.7 (C3), 153.9 (C4″), 136.7 (C5″), 130.9 (C1″), 130.9 (C2″, C6″), 127.9 (C10″), 124.3 (C4″), 121.6 (C7″), 118.9 (C9″), 118.7 (C8″), 115.4 (C3″, C5″), 111.8 (C6″), 110.9 (C3″), 62.4 (C1″), 55.6 (C4), 55.6 (C2), 31.1 (C2″). HRMS (ESI) m/z: 405.1532 [M + Na].

2.2.3 (S)-2-Amino-N-((S)-3-hydroxy-1-((4-hydroxybenzyl)amino)-1-oxopropan-2-yl)-3-(1H-indol-3-yl)propanamide (TSR2)

Compound Boc-TSR2 was purified by column chromatography on silica gel (CHCl3/EtOAc/Hex 4:5:1) to yield white powder. Compound TSR2 was purified by column chromatography on silica gel (CHCl3/EtOAc/MeOH/4:4:1) to yield white solid (hygroscopic). Yield 216.25 mg
Scheme 1: The scheme of synthesis of compounds in the TSR and TGN series: (a) Boc$_2$O, NaOH, THF/H$_2$O (1:1), rt, 18 h; (b) i- serine methyl ester hydrochloride, HBTU, DIPEA, DMF, rt, 18 h; (c) NH$_2$-R$_2$, HBTU, DIPEA, DMF, rt, 18 h; (d) N-tert-butoxycarbonyl (Boc) protected 2-methyl-2-thiopseudouridine (1a), HATU, NMM, DMF, rt, 18 h; and (e) i-NH$_2$-R$_2$, HATU, NMM, DMF, rt, 18 h; (ii) TFA:EtOAc (1:1), rt, 1.5 h.

(54.55%). FTIR (ATR, ν, cm$^{-1}$): 3,419–3,184 (O–H, N–H), 1,310 (sp$^2$ C–H), 2,925 (sp$^3$ C–H), 1,667, 1,516 (C=O, C=C) 1,455, 1,435 (C–C), 1,361, 1,337 (C–O), 1,228, 1,192, 1,131 (C–N, C–O). $^1$H-NMR spectrum in DMSO-d$_6$ (δ ppm): 10.96 (s, 1H, indole-NH), 8.45 (s, 1H, OH), 8.26 (t, 1H, J 5.9 Hz, NH), 7.98 (t, 1H, J 5.9 Hz, NH), 7.62 (d, 1H, J 7.8 Hz, H9’), 7.35 (d, 1H, J 7.7 Hz, H6’), 7.20 (s, 1H, H4’), 7.07 (t, 1H, J 7.8 Hz, H7’), 7.05 (d, 2H, J 8.5 Hz, H2”, H6”), 6.97 (t, 1H, J 7.6 Hz, H8’), 6.70 (dd, 2H, J 8.4 Hz, J2 8.4 Hz, H3”, H5”), 4.18 (d, 2H, J 5.6 Hz, H7”), 3.80 (dd, 1H, J 1H, J 5.1 Hz, J 7.6 Hz, H1’), 3.72 (dd, 1H, J 1H, J 4.9 Hz, J 5.6 Hz, H2’), 3.63 (dd, 1H, J 1H, J 5.5 Hz, J 10.9 Hz, H4b), 3.51 (dd, 1H, J 1H, J 5.0 Hz, J 10.9 Hz, H4a), 3.17 (dd, 1H, J 1H, J 5.1 Hz, J 14.1 Hz, H2b’), 2.94 (dd, 1H, J 1H, J 7.7 Hz, J 14.0 Hz, H2a’). $^{13}$C-NMR spectrum in DMSO-d$_6$ (δ ppm): 175.5 (C1), 170.2 (C3), 156.7 (C4”), 136.7 (C5’), 129.7 (C1”), 128.7 (C2”, C6”), 127.8 (C10’), 124.9 (C4’), 121.4 (C7’), 118.9 (C9’), 118.8 (C8’), 115.4 (C3”, C5”), 111.9 (C6’), 109.3 (C3’), 62.1 (C1’), 55.6 (C4), 54.5 (C2), 42.1 (C7’”), 29.6 (C2’). HRMS (ESI) m/z: 419.1687 [M + Na].

2.2.4 (S)-2-Amino-N-((S)-3-hydroxy-1-((4-hydroxyphenethyl)amino)-1-oxopropan-2-yl)-3-(1H-indol-3-yl)propanamide (TSR3)

Compound Boc-TSR3 was purified by column chromatography on silica gel (CHCl$_3$/EtOAc/Hex 4:5:1) to yield white powder. Compound TSR3 was purified by column chromatography on silica gel (CHCl$_3$/EtOAc/Hex 4:5:1) to yield white powder.

2.2.5 (S)-N-((2-(1H-Indol-2-yl)ethyl)-2-((S)-2-amino-3-(1H-indol-3-yl)propanamido)-3-hydroxypropanamide (TSR4)

Compound Boc-TSR4 was purified by column chromatography on silica gel (CHCl$_3$/EtOAc/Hex 4:5:1) to yield white powder.
white powder. Compound **TSR4** was purified by column chromatography on silica gel (CHCl₃/EtOAc/MeOH 4:4:1) to yield yellow solid (hygroscopic). Yield 260.36 mg (60.06%). FTIR (ATR, ν, cm⁻¹): 3,392–3,216 (O–H, N–H), 3,113 (sp² C–H), 2,919 (sp³ C–H), 1,659, 1,516 (C=O, C=O), 1,457, 1,433 (C–C), 1,363, 1,336 (C–O), 1,229, 1,202, 1,180 (C–N, C–O). ¹H-NMR spectrum in DMSO-d₆ (δ, ppm): 10.81 (s, 2H, indole-NH), 7.87 (m, 2H, NH, NH), 7.60 (d, 1H, J 7.6 Hz, H7⁺), 7.53 (d, 1H, J 7.7 Hz, H9⁺), 7.32 (d, 1H, J 8.0 Hz, H6¹, H4²), 7.15 (m, 2H, H4¹, H2²), 7.05 (t, 2H, J 7.4 Hz, H8⁺, H6²), 6.97 (t, 2H, J 7.3 Hz, H7⁺, H5⁺), 4.24 (m, 2H, H1¹, H2²), 3.60 (dd, 1H, J 9.9 Hz, H4b), 3.50 (dd, 1H, J 5.7 Hz, J 10.8 Hz, H4a), 3.09 (dd, 1H, J 4.1 Hz, J 14.2 Hz, H2b), 2.94 (m, 1H, H2a'), 2.76 (m, 4H, H9⁺, H10⁺). ¹³C-NMR spectrum in DMSO-d₆ (δ, ppm): 175.1 (C1), 170.3 (C3), 136.9 (C3⁺), 136.7 (C5'), 127.9 (C8'), 127.6 (C10'), 123.2 (C2⁺), 123.0 (C4'), 121.6 (C5'), 121.4 (C7'), 118.9 (C9', C7'), 118.7 (C8', C6'), 112.0 (C4'), 111.8 (C6'), 111.0 (C1'), 110.8 (C3'), 62.3 (C1'), 55.7 (C4'), 55.1 (C2'), 44.9 (C10'), 31.2 (C9'), 26.1 (C2'). HRMS (ESI) m/z 434.2193 [M + Na].

### 2.2.6 General synthesis procedure of TGN1–TGN4

A solution of compound **T3** (1 mmol), NH₂–R₂ (1 mmol), HATU (1.5 mmol), and N-methylmorpholine (NMM) (3 mmol) in 5 mL of DMF was stirred at room temperature. After 18 h stirring, water was added. The aqueous phase was extracted with ethyl acetate. The combined organic extracts were dried. The obtained residue was purified by column chromatography on silica gel to yield compound **Boc-TGN1** to **Boc-TGN4**. The protecting group of the obtained compound was removed by 50% of TFA in ethyl acetate at room temperature for 1.5 h. The reaction mixture was adjusted to pH 7 by saturated sodium bicarbonate solution and extracted with ethyl acetate. The combined organic layers were dried. The residue was purified by column chromatography on silica gel to yield **TGN1–TGN4** (Scheme 1).

### 2.2.7 (S)-2-Amino-N-(N-(4-hydroxyphenyl)carbamimidoyl)-3-(1H-indol-3-yl)propanamide (TGN1)

Compound **Boc-TGN1** was purified by column chromatography on silica gel (EtOAc/CHCl₃/Hex 2:7:1) to yield white powder. Compound **TGN1** was purified by column chromatography on silica gel (CHCl₃/EtOAc/MeOH 6:3:1) to yield white powder. Yield 190.75 mg (56.54%); m.p. = 208 (d) °C. FTIR (ATR, ν, cm⁻¹): 3,417–3,018 (O–H, N–H), 3,080 (sp² C–H), 2,923 (sp³ C–H), 1,678, 1,588, 1,511 (C=O, C=C, C=N), 1,456, 1,432 (C–C), 1,366, 1,341 (C–O st), 1,204, 1,139 (C–N). ¹H-NMR spectrum in DMSO-d₆ (δ, ppm): 10.85 (s, 1H, indole-NH), 9.36 (s, 1H, OH), 8.26 (s, 1H, NH), 7.57 (d, 1H, J 7.6 Hz, H9⁺), 7.52 (s, 1H, NH), 7.34 (s, 1H, NH), 7.34 (d, 1H, J 7.7 Hz, H6⁺), 7.35 (d, 2H, J 8.9 Hz, H2⁺, H6²), 7.21 (d, 1H, J 2.2 Hz, H4⁺), 7.06 (t, 1H, J 7.5 Hz, H7), 6.97 (t, 1H, J 7.4 Hz, H8), 6.70 (dd, 2H, J 8.8 Hz, H3', H5'), 4.05 (m, 1H, H1'), 3.16 (dd, 1H, J 4.1 Hz, J 14.6 Hz, H2b), 2.93 (dd, 1H, J 6.1 Hz, J 14.6 Hz, H2a'). ¹³C-NMR spectrum in DMSO-d₆ (δ, ppm): 171.8 (C1), 158.5 (C2), 154.5 (C4'), 136.4 (C5'), 129.9 (C1'), 128.1 (C2', C6'), 127.9 (C10), 123.9 (C4), 121.2 (C7'), 119.1 (C9'), 118.6 (C8'), 115.8 (C3', C5'), 111.7 (C6'), 110.0 (C3'), 60.8 (C1'), 27.4 (C2'). HRMS (ESI) m/z 374.1590 [M + Na].

### 2.2.8 (S)-2-Amino-N-(N-(4-hydroxybenzyl)carbamimidoyl)-3-(1H-indol-3-yl)propanamide (TGN2)

Compound **Boc-TGN2** was purified by column chromatography on silica gel (EtOAc/CHCl₃/Hex 2:7:1) to yield white powder. Compound **TGN2** was purified by column chromatography on silica gel (CHCl₃/EtOAc/MeOH 2:7:1) to yield brown viscous (hygroscopic). Yield 487.40 mg (53.33%). FTIR (ATR, ν, cm⁻¹): 3,394–3,111 (O–H, N–H), 3,065 (sp² C–H), 2,925 (sp³ C–H), 1,674, 1,611, 1,515 (C=O, C=C, C=N), 1,456, 1,431 (C–C), 1,357, 1,338 (C–O), 1,201, 1,136 (C–N). ¹H-NMR spectrum in DMSO-d₆ (δ, ppm): 10.87 (s, 1H, indole-NH), 9.32 (s, 1H, OH), 8.10 (s, 1H, NH), 7.55 (s, 1H, NH), 7.55 (d, 1H, J 7.7 Hz, H9⁺), 7.34 (s, 1H, NH), 7.34 (d, 1H, J 7.6 Hz, H6⁺), 7.13 (s, 1H, H4'), 6.93 (d, 2H, J 8.4 Hz, H2⁺, H6²), 7.06 (t, 1H, J 7.5 Hz, H7), 6.97 (t, 1H, J 7.5 Hz, H8'), 6.67 (d, 2H, J 8.4 Hz, H3', H5'), 4.24 (m, 2H, H7⁺), 4.06 (m, 1H, H1'), 3.14 (dd, 1H, J 4.0 Hz, J 14.6 Hz, H2b'), 2.83 (dd, 1H, J 6.9 Hz, J 14.6 Hz H2a'). ¹³C-NMR spectrum in DMSO-d₆ (δ, ppm): 171.9 (C1), 156.9 (C4'), 156.8 (C2), 136.5 (C5'), 129.1 (C1'), 128.9 (C2', C6'), 127.9 (C10'), 123.9 (C4'), 121.2 (C7'), 119.0 (C9'), 118.7 (C8'), 115.5 (C3', C5'), 111.7 (C6'), 110.3 (C3'), 61.4 (C1'), 45.0 (C7'), 27.9 (C2'). HRMS (ESI) m/z: 374.1590 [M + Na].

### 2.2.9 (S)-2-Amino-N-(N-(4-hydroxyphenethyl)carbamimidoyl)-3-(1H-indol-3-yl)propanamide (TGN3)

Compound **Boc-TGN3** was purified by column chromatography on silica gel (EtOAc/CHCl₃/Hex 3:6:1) to yield yellow viscous (hygroscopic). Yield 266.68 mg (62.03%). FTIR (ATR, ν, cm⁻¹): 3,387–3,120 (O–H, N–H),
2.2.10 (S)-N-((2-(1H-Indol-2-yl)ethyl)carbamimidoyl)-2-amino-3-(1H-indol-3-yl)propanamide (TGN4)

Compound Boc-TGN4 was purified by column chromatography on silica gel (EtOAc/CHCl₃/EtOAc 2:7:1) to yield white powder. Compound TGN4 was purified by column chromatography on silica gel (CHCl₃/EtOAc/MeOH 3:6:1) to yield white viscous (hygroscopic). Yield 154.22 mg (39.70%). FTIR (ATR, ν, cm⁻¹): 3298.97 (N–H), 3.084 (sp² C–H), 2.924 (sp³ C–H), 1.670, 1.612, 1.493 (C=O, C=C, C=N), 1.455, 1.431 (C–C), 1.359, 1.339 (C–O), 1.201, 1.135 (C–N). ¹H-NMR spectrum in DMSO-d₆ (δ, ppm): 10.98, 10.91 (s, 2H, indole-NH), 7.88 (s, 3H, NH, NH, NH), 7.54 (d, 1H, J 7.6 Hz, H7″), 7.53 (d, 1H, J 7.5 Hz, H9″), 7.36 (d, 1H, J 7.6 Hz, H6″), 7.32 (d, 1H, J 7.6 Hz, H6), 7.23 (d, 1H, J 2.3 Hz, H4″), 7.13 (d, 1H, J 2.3 Hz, H2″), 7.02 (m, 4H, H7″, H8″, H5″, H6″), 4.15 (m, 1H, H1″), 3.14 (dd, 1H, J 4.0 Hz, J2 15.0 Hz H2b″), 3.07 (m, 2H, H10″), 2.98 (m, 2H, H9″), 2.87 (dd, 1H, J1 7.3 Hz, J2 14.9 Hz H2a″). ¹³C-NMR spectrum in DMSO-d₆ (δ, ppm): 185.2 (C1), 168.2 (C2), 136.8 (C3″), 136.5 (C5″), 127.8 (C8″), 127.3 (C10″), 124.1 (C2″), 123.9 (C4″), 121.6 (C5″), 121.3 (C7″), 118.9 (C7″), 118.9 (C8″), 118.5 (C6″), 112.0 (C4″), 111.7 (C6″), 109.8 (C1″), 109.6 (C3″), 61.0 (C1″), 31.1 (C10″), 27.4 (C2″), 23.6 (C9″). HRMS (ESI) m/z: 419.1907 [M + Na].

2.2.11 (S)-2-((tert-Butoxy carbonylamino)-3-(1H-indol-3-yl)propanoic acid (T1)

A solution mixture of l-tryptophan (20.45 g, 0.10 mol), sodium hydroxide pellets (8.80 g, 0.22 mol), and di-tert-butyl dicarbonate (24.01 g, 0.11 mol) in THF/H₂O (1:1) 100 mL was stirred at room temperature for 18 h. After the reaction was complete, water was added to dissolve the precipitate. Then, THF was removed under reduced pressure, and aqueous layer was extracted with dichloromethane. The aqueous layer was acidified by 1 N HCl to pH 4 and extracted with ethyl acetate. The organic phase was dried to yield white solid of compound T1. Yield 24.05 g (79%); m.p. 141–143 °C. ¹H-NMR spectrum in DMSO-d₆ (δ, ppm): 12.55 (s, 1H, COOH), 10.84 (s, 1H, indole NH), 7.53 (d, 1H, J 7.7 Hz, H7″), 7.34 (d, 1H, J 8.0 Hz, H6″), 7.15 (d, 1H, J1 1.5 Hz, H4″), 7.07 (t, 1H, J 7.4 Hz, H7″), 6.99 (t, 1H, J 7.14 Hz, H8″), 4.16 (m, 1H, H1′″), 3.14 (dd, 1H, J1 4.5 Hz, J2 14.5 Hz, H2b″), 2.98 (dd, 1H, J1 9.4 Hz, J2 14.5 Hz, H2a″), 1.33 (s, 9H, CH₃).
of DMF was stirred at room temperature for 18 h. After the reaction was complete, water was added and extracted with ethyl acetate. The organic solution was dried. The obtained residue was purified by column chromatography on silica gel (EtOAc/Hex 1:4) to yield compound 5 as white powder. Yield 324.77 mg (68.20%); m.p. 80–81 °C. 1H-NMR spectrum in DMSO-d6 (δ, ppm): 12.17 (s, 1H, NH), 10.85 (s, 1H, indole-NH), 7.49 (s, 1H, NH), 7.57 (d, 1H, J 7.75 Hz, H9′), 7.33 (d, 1H, J 8.0 Hz, H6′), 7.19 (d, 1H, J 1.7 Hz, H4′), 7.07 (t, 2H, J 7.0 Hz, H2′), 6.99 (t, 1H, J 7.0 Hz, H8), 4.27 (m, 1H, H1′), 3.19 (dd, 1H, J1, 4.8 Hz, J2 14.7 Hz, H2b′), 2.97 (dd, 1H, J1, 9.8 Hz, J2 14.6 Hz, H2a′), 2.30 (s, 3H, CH3), 1.45 (s, 9H, CH3), 1.33 (s, 9H, CH3).

### 2.2.14 N-tert-Butoxycarbonyl (Boc)-protected 2-methyl-2-thiopseudourea (1a)

A solution of methylisothiourea hemisulfate salt (198 mg, 1 mmol) in THF/H2O (1:1) 30 mL was added with sodium bicarbonate (168 mg, 1 mmol), di-tert-butyl dicarbonate (218 mg, 1 mmol), and stirred at room temperature for 18 h. After the reaction was complete, THF was removed under reduced pressure, and aqueous layer was extracted with dichloromethane. The organic phase was dried to yield compound 1a as colorless viscous liquid. Yield 195.71 mg (76.62%). 1H-NMR spectrum in DMSO-d6 (δ, ppm): 8.54 (s, 1H, NH), 2.31 (s, 3H, CH3), 1.40 (s, 9H, CH3).

### 2.3 Biological experiment part

BACE1 enzyme and the BACE1 substrate were purchased from Sino Biological® and Calbiochem®, respectively. β-amyloid (1–42) from Anaspec® was used in the ThT assay. IC50 values were calculated using GraphPad Prism 4 with nonlinear regression curve fit. Statistics were determined by ANOVA, calculated using IBM SPSS statistics version 24.

#### 2.3.1 BACE1 inhibition assay

The test compound, prepared at various concentrations in 5% DMSO, was added to black 96 well plates followed by 30 µL of enzyme working solution (0.01 unit/µL) and 20 µL of substrate solution (125 µM). The final volume was adjusted to 100 µL with 100 mM sodium acetate buffer (pH 4.5), and the reaction plate was incubated at 37°C for 30 min. The emitted fluorescence (Eem) was measured at 510 nm after excitation (Eex) at 380 nm [15]. Compound 12c, previously synthesized and described in [16], was used as an in house BACE1 inhibitor positive control at a final concentration of 100 µM. The resulting data were analyzed, and compounds showing greater than 70% inhibition were evaluated at concentrations of 5–25 µM to determine their IC50 values. The IC50 values were calculated using GraphPad Prism 4 with a nonlinear regression curve fit.

#### 2.3.2 Aβ aggregation inhibition assay

Amyloid solution in 50 mM Tris buffer (pH 7.4) at a concentration of 25 µM (9 µL) was added to transparent 96 well plate followed by test compounds (1 µL) at various concentrations prepared in DMSO. The reaction was mixed gently by tapping and incubated in dark at 37°C for 48 h. After incubation, 200 µL of 5 µM thioflavin-T (Sigma®) in 50 mM Tris buffer (pH 7.4) was added to each well. The emitted fluorescence (Eem) was measured at 490 nm after excitation (Eex) at 446 nm [17]. Curcumin was included in the assay as a positive control. The resulting data were analyzed and compounds showing more than 70% inhibition were evaluated to determine their IC50 values. The data were analyzed using GraphPad Prism 4 with a nonlinear regression curve fit.

#### 2.3.3 Antioxidant assay

The test compounds were prepared at a concentration of 500 µM in 50% DMSO. DPPH, prepared at a concentration of 500 µM in methanol, was added to transparent 96 well plates in a volume of 70 µL per well. Methanol was used to adjust the volume to 80 µL. After adjusting the volume, 20 µL of test compound was added to each well. The reaction plate was incubated at room temperature in dark for 30 min. The absorption was measured at wavelength 517 nm [18]. Ascorbic acid was used as a positive control. The percent inhibition was calculated. Compounds showing inhibition greater than 70% were evaluated to determine their IC50 values.

#### 2.3.4 Neuroprotective effect on β-amyloid-induced cell damage

Protective effect on β-amyloid-induced cell damage was evaluated in human neuroblastoma cell (SH-SY5Y) cells.
SH-SY5Y cells were cultured in Ham’s F12:Dulbecco’s modified Eagle’s medium (DMEM) containing 50 IU/mL penicillin, 50 g/mL streptomycin, 2 mM L-glutamine, and 10% fetal bovine serum. Cell cultures were maintained at 37°C in an atmosphere of 95% humidified air and 5% CO₂. For the assays, SH-SY5Y cells were sub-cultured into a 96-well plate for 24 h. The cells were then incubated with Aβ1–42 (25 µM) with or without various concentrations of the test compounds for 24 h. Curcumin at a concentration of 10 µM was used as positive control. Cell viability was determined by staining the cells using water-soluble tetrazolium salt (WST-8) assay. The absorption was measured using a well plate reader at 450 nm [19].

**Ethical approval:** The conducted research is not related to either human or animal use.

### 3 Results and discussion

#### 3.1 Design and docking study

Tryptophan was designed to be the core structure due to its properties in blood brain penetration via the large neutral amino acid transporter 1 or LAT1 transporter [20,21] and the BACE1 inhibitory activity [11]. The modification of the tryptophan was designed by including aromatic systems substituted with nucleophilic groups, such as hydroxyl groups, into the moieties based on reported pharmacophore models of anti-amyloid aggregation, antioxidant and metal chelating properties [22,23]. The proposed of extended part is to enhance the binding affinity for BACE1 in order to access the S3 pocket and also to contribute to multifunctional properties.

The expanded moiety was composed of two parts: R1 and R2. The amino acid (R1) was designed as a linker which contained a hydrogen bond acceptor to enable additional hydrogen bonding with Asp32 or Asp228, the key catalytic residues in BACE1 (Figure 1). Thus, amino acids with side chains bearing hydrogen bond donor such as –OH and –NH₂, serine, tyrosine, threonine, and guanidine were selected as R1 linkers. The R2 terminal of the expanded moiety was designed to be an aromatic system with a nucleophilic motif to provide antioxidant activity. Moreover, the number of carbon atoms between the amino acid linker, R1, and the aromatic terminal R2 was varied between 7 and 10 Å, considering the optimal 8–9 Å distance when binding to Aβ (Figure 1) [22]. All 16 designed compounds were screened in silico with the main target BACE1, 2IRZ-F template, using the AutoDock program suite, version 4.2, to identify hit compounds for synthesis. The docking results were showed in Table 1.

The docking results showed that compounds in the TSR and TGN series generally gave better binding results than those in TYR and TTH series. This was because their binding mode allowed the linker R1 (serine or guanidine) to form extra H-bonds with the catalytic residues, Asp32 or Asp228. Moreover, ligand efficiency (LE) values and free binding energy of the four compounds in the TYR series with tyrosine as the linker (LE: −0.26 to −0.30 and ΔG: −9.1 to −10.0 kcal/mol) were not as favorable as those of the compounds in the TSR (LE: −0.38 to −0.43 and ΔG: −11.5 to −12.5 kcal/mol) or TGN series (LE: −0.44 to −0.48 and ΔG: −11.7 to −14.0 kcal/mol). The inferior binding affinity of the compounds in the TYR series was possibly due to their steric properties and greater flexibility of the tyrosine side chain. Compounds in the TTH series also presented good LE values and binding affinities, however, the linker R1 could not form an extra H-bond with the key residues Asp32 or Asp228. Therefore, compounds in the TYR and TTH series were not included in the synthesis part of the study.

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**Figure 1:** The proposed designed compounds (X = O or N atom).
Table 1: Docking results of the designed compounds against the BACE1 template

| Cpd | R₁ | R₂ | Δ\(\Delta G\) (kcal/mol) | LE\(^a\) | H-bond number | H-bond interacting residues (distance, Å) |
|-----|----|----|--------------------------|---------|---------------|------------------------------------------|
| TSR1 | Ser |  | -11.5 | -0.41 | 6 | Asp32 (1.8), Gln73 (2.0), Asp228 (1.9, 2.0), Thr231 (1.9, 1.9) |
| TSR2 | Ser |  | -12.5 | -0.43 | 6 | Asp32 (1.7), Ser36 (1.9), Asn37 (1.9), Thr73 (2.1), Tyr198 (2.2), Asp228 (2.0) |
| TSR3 | Ser |  | -11.8 | -0.39 | 7 | Asp32 (1.7), Ser35 (2.0), Asn37 (1.9), Gln73 (2.3), Asp228 (1.9, 1.9), Thr231 (2.2) |
| TSR4 | Ser |  | -11.8 | -0.38 | 4 | Asp32 (1.9), Asp228 (1.8, 1.9), Thr231 (2.1) |
| TYR1 | Tyr |  | -9.1 | -0.26 | 5 | Gly34 (2.1), Gln73 (2.1), Phe108 (2.5), Thr231 (2.0), Thr232 (2.0) |
| TYR2 | Tyr |  | -10.5 | -0.30 | 4 | Asp32 (1.8), Ser229 (2.1), Gly230 (2.0), Thr232 (2.1) |
| TYR3 | Tyr |  | -10.3 | -0.28 | 3 | Phe108 (2.0), Gly230 (2.0), Thr231 (2.5) |
| TYR4 | Tyr |  | -10.0 | -0.26 | 4 | Gln73 (1.9), Gly230 (2.0), Thr231 (2.2), Thr232 (2.0) |
| TTH1 | Thr |  | -11.6 | -0.40 | 4 | Asp32 (1.9), Gln73 (1.9), Asp228 (1.8), Thr231 (2.1) |
| TTH2 | Thr |  | -12.5 | -0.41 | 5 | Asp32 (1.6), Ser35 (2.0), Thr72 (2.3), Asn73 (2.1), Asp228 (1.9) |
| TTH3 | Thr |  | -12.7 | -0.40 | 6 | Asp32 (1.6), Ser35 (1.7), Thr72 (2.4), Asn73 (2.0), Asp228 (1.8), Thr231 (2.0) |
| TTH4 | Thr |  | -13.0 | -0.39 | 5 | Asp32 (1.7), Thr72 (2.1), Gln73 (2.0), Asp228 (1.8), Gly230 (2.0) |
| TGN1 | Gnd |  | -11.7 | -0.47 | 5 | Asp32 (1.9), Asp228 (1.7, 2.2), Thr72 (2.1), Thr232 (2.1) |
| TGN2 | Gnd |  | -12.3 | -0.47 | 5 | Asp32 (1.7, 2.0), Thr72 (2.2), Asp228 (2.0), Thr232 (1.9) |
| TGN3 | Gnd |  | -12.0 | -0.44 | 6 | Asp32 (1.8), Thr72 (2.1), Asp228 (1.7, 2.1), Thr231 (1.9), Ser325 (2.0) |
| TGN4 | Gnd |  | -14.0 | -0.48 | 3 | Asp32 (1.7), Asp228 (1.7), Gly230 (1.9) |

\(^a\) LE = Δ\(\Delta G\)/\(N\), \(N\) is number of nonhydrogen atom.
3.2 Multifunctional assay

The compounds in the TSR and TGN series were initially screened for their inhibitory actions against BACE1, anti-amyloid aggregation effects, and antioxidant properties at a concentration of 100 µM, and the screening results are shown in Table 2. Compounds that demonstrated a percentage inhibition greater than 70% were further evaluated to determine their IC50 values. Positive controls for each assay were performed at the same concentration.

3.2.1 BACE1 inhibition assay

TSR2, TSR4, and TGN2 were found to be the potent inhibitors of BACE1. The IC50 values of these compounds were in the range of 21.38–24.18 µM. The binding modes of the three compounds are shown in Figure 2. The amino group in the tryptophan core structure of these three compounds provided H-bonds with the catalytic residues Asp32 and Asp228, and the compounds were well accommodating in binding pockets to form 5–6 hydrogen bonds. The OH of the serine and the NH of the guanidine linker did not form hydrogen bonds with the catalytic amino acids as expected, instead the NH of the amide backbone of both TSR4 and TGN2 formed additional H-bonds with Asp32 and Asp228. Moreover, the OH of the serine linker in compound TSR4 formed an H-bond with Thr231. The indole in the tryptophan core of compounds TSR2 and TSR4 was anchored to the S1 binding pocket whereas the middle part of TSR2 binding pose was aligned in the same orientation as TGN2. However, the tryptophan cores of TSR2 and TGN2 flipped into the opposite direction, the phenolic end (R1) of TSR2 directed to the S2′/S3′ pocket, providing H-bonding and hydrophobic interactions with residues in these pockets. The access to the S2′/S3′ pocket accommodated the phenolic OH and the NH between the middle serine and the phenolic group of TSR2 to form three hydrogen bonds with Ser36, Asn37, and Tyr198 (Figure 2b). Moreover, the insertion of the phenolic moiety of TSR2 contributed to interactions with the residues in the S3′ pocket, especially with Arg128, the amino acid reported to affect the binding conformation of a potent inhibitor (Figure 2b) [13]. These interactions enabled TSR2 to achieve the same level of inhibition as TSR4 and TGN2, although it was inaccessible to the S3 pocket.

3.2.2 Aβ aggregation inhibition assay

The anti-Aβ aggregation screening results obtained at a concentration of 100 µM showed that compounds TSR1, TSR2, and TGN2 inhibited amyloid aggregation by more than 70% (Table 2), and the IC50 values of these compounds were 38.55 µM, 37.06 µM, and 36.12 µM, respectively. The distances between aromatic terminals in the docked poses of compounds TSR1, TSR2, and TGN2 were measured and found to be 8.50 Å, 9.88 Å, and 8.00 Å, respectively, which are within the theoretical optimal distance (8–9 Å) [22]. The binding mode of the lead compounds with Aβ template revealed that TSR1-formed H-bonds with Glu11 (1.78 Å), Gln15

Table 2: Multifunctional activity of TSR and TGN compounds

| Compounds | BACE1 inhibition | Anti-amyloid aggregation | Antioxidant |
|-----------|------------------|--------------------------|-------------|
|           | % inhibition at 100 µM (± SD) | IC50 (µM) (± SD) | % inhibition at 100 µM (± SD) | IC50 (µM) (± SD) |
| TSR1      | 50.64 (±0.72)* | — | 88.06 (±1.22)* | 38.55 |
| TSR2      | 88.07 (±2.87) | 24.18 | 73.15 (±0.50)* | 37.06 |
| TSR3      | 46.72 (±2.12)* | — | 53.33 (±1.85)* | — |
| TSR4      | 96.98 (±1.62)* | 23.18 | 46.32 (±1.23)* | — |
| TGN1      | 49.60 (±2.33)* | — | 36.12 (±0.51)* | — |
| TGN2      | 94.35 (±1.86) | 22.35 | 86.96 (±0.82) | 36.12 |
| TGN3      | 99.68 (±1.02) | 31.03 | 54.32 (±2.35)* | — |
| TGN4      | 85.65 (±0.15) | 35.35 | 49.78 (±1.54)* | — |
| BACE1 inhibitor (12c) | 92.64 (±1.72) | 20.89 | — | — |
| Curcumin  | — | — | 84.02 (±1.07) | 0.63 |
| Ascorbic acid | — | — | — | 54.16 (±0.77) |

Data are mean ± SD (n = 3), *p value < 0.001 compared with the positive control of each group (paired t-test statistic from Microsoft Excel). Structure of positive control compounds was shown in the supplemental information (Figures S21 and S22).
(2.09 Å), Glu22 (1.86 Å) and Asp23 (2.01 Å), while **TSR2** provided H-bonds with Glu22 (1.92, 1.94 and 2.11 Å) and Asp23 (1.86 Å) (Figure 3a). The potent inhibitory activity of **TSR1** and **TSR2** against amyloid aggregation was possibly a result of two crucial types of interactions, H-bond interactions and hydrophobic interactions, with the key residues responsible for self-binding and the oligomer formation leading to aggregation.

H-bond interactions have been detected, particularly with Asp23, which is the key amino acid for intermolecular salt bridge formation of Aβ peptides in protein aggregation [24,25]. Hydrophobic interactions have been

![Figure 2](image2.png)

**Figure 2:** The binding modes of **TSR2** (yellow), **TSR4** (green), and **TGN2** (magenta) in the active binding site of BACE1, overlay of docked poses (a); and the interacting residues, showing hydrogen bonds as green dotted lines (b–d).

![Figure 3](image3.png)

**Figure 3:** The binding modes of the lead compounds with Aβ peptide: (a) **TSR1** (cyan) and **TSR2** (yellow); (b) **TGN2** (magenta).
found with the key residue Phe19, the amino acid in the hydrophobic interaction region [25] that connects two β-strands of Aβ monomers, in the initiation of oligomer and fibril formation [26]. Thus, ligand binding with these amino acid residues (Phe19 and Asp23) was a contributing factor to the blocking of Aβ oligomer formation and hence to anti-aggregation activity. Besides Phe19 and Asp23, TSR1 and TSR2 were found to interact with His13 and His14, in the metal-binding site [27]. The metal-binding site (His6, His13, and His14) has been reported as the part of two monomers that coordinate with a metal ion (Cu²⁺ or Zn²⁺) [28,29]. This metal binding stabilizes and increases amyloid oligomerization [28,30]. Compound TGN2 formed H-bond interactions with Ala2 (1.74 Å), Glu3 (1.94 Å), and Glu11 (1.85 Å) (Figure 3b). Hydrophobic interactions of TGN2 were also found with all key amino acid residues in the metal-binding site, His6, His13, and His14 [31]. Moreover, all three compounds also had hydrophobic interactions with Gln15 and Val18, which are self-recognition residues in the aggregation process [32]. Taken together, binding at the important regions responsible for Aβ aggregation, namely, the hydrophobic region, the intermolecular salt bridge, and the metal-binding site resulted in the enhancement of anti-amyloid aggregation activity in the tested compounds.

3.2.3 Antioxidant assay

The antioxidant activity of the hit compounds was evaluated at concentrations of 100 µM using the DPPH scavenging method [18]. TGN1 was the only compound that showed high inhibition, with IC₅₀ values of 36.14 µM, approximately three times better than that of ascorbic acid (IC₅₀ 94.92 µM). The direct guanidine substitution at the para-position of the phenol group in TGN1 might be the reason for its antioxidant properties, because of its strong electron-donating group via the π-bond and increased number of conjugated double bonds in the TGN1 molecule [33,34].

3.2.4 Neuroprotective effect on β-amyloid-induced cell damage

Among the eight compounds, two compounds, TSR2 and TGN2, possessed dual activities. TSR2 and TGN2 were evaluated for their neuroprotective effects on Aβ-induced cytotoxicity in SH-SY5Y neuroblastoma cells. The cytotoxicity of TSR2 and TGN2 was initially tested at 1, 10, and 100 µg/mL by the MTT assay. No significant reduction in cell viability was found at any of the tested concentrations, which indicated nontoxicity at the highest concentration (100 µg/mL). TSR2 and TGN2 were then screened at the highest concentration (100 µg/mL) for neuroprotective effects with the WST-8 assay, in which Aβ1-42 (25 µM) was used to induce cytotoxicity in SH-SY5Y cells. Cell viability was determined by measuring the absorption of the WST-8 at 450 nm [19]. The screening results are shown in Figure 4a. Neither compound showed neuroprotection at 100 µg/mL as anticipated, instead they presented lower percentage viability than the Aβ-treated group.

Figure 4: Neuroprotective effects of dual activity compound against Aβ induced cytotoxicity: (a) the screening results at the nontoxic concentration of 100 µg/mL; (b) neuroprotective effects of TGN2. Data are mean ± SD (n = 3), #p < 0.01 compared with the control group and *p < 0.01 compared with the Aβ-treated group (paired t-test statistic from Microsoft Excel).
Although **TSR2** and **TGN2** alone were nontoxic at 100 µM, it is likely that a synergistic effect occurred with the combination of Aβ and the test compounds at this concentration. As the percentage viability of TGN2 treated cells was slightly lower than that of Aβ treated cells, TGN2 was tested at lower doses (1 µg/mL and 10 µg/mL) to avoid the synergistic effect. TGN2 at lower concentrations appeared to reduce the cytotoxicity of Aβ (Figure 4b). However, a significant neuroprotective effect was observed only at 10 µg/mL or 2.62 µM.

### 4 Conclusion

Among the developed compounds, **TGN2** was found to exhibit multifunctionality, with potent actions as a BACE1 inhibitor and against amyloid aggregation. **TGN1** was found as a potent antioxidant with greater activity than ascorbic acid. Moreover, based on its multifunctional activity on the Aβ cascade, **TGN2** at a concentration of 2.62 µM showed a significant neuroprotective effect against Aβ-induced cytotoxicity.

**Conflict of interest:** The authors have declared no conflict of interest.

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