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Graphical Abstract

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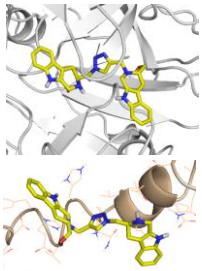
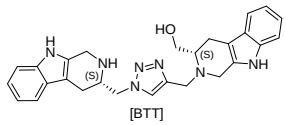
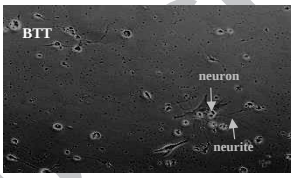
Jutamas Jiaranaikulwanitch[a,b,*], Sarin Tadtong[c], Piyarat Govitrapong[d], Valery V. Fokin[e] and Opa Vajragupta[b]





[BTT]

Bi-functional compound
BACE1 (IC_{50} = 16.73 μM)
Aβ aggregation (IC_{50} = 66.36 μM)



Neurite outgrowth activity

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Neuritogenic Activity of Bi-Functional Bis-Tryptoline Triazole

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ABSTRACT

Alzheimer's disease (AD) is a common neurodegenerative disorder, one of the hallmarks of which is the deposition of aggregated β-amyloid peptides (Aβ40,42) as plaques in the brain. Oligomers of these peptides have been reported to be toxic and to inhibit neurite outgrowth, as evidenced by neurite dystrophy and significant loss of synaptic connectivity of neurons in the AD brain resulting in cognitive decline. These peptides also react with biological metal in the brain to generate free radicals, thereby aggravating neuronal cell injury and death. Herein, multifunctional triazole-based compounds acting on multiple targets, namely β-secretase (BACE1), β-amyloid peptides (Aβ) as well as those possessing metal chelation and antioxidant properties, were developed and evaluated for neuritogenic activity in P19-derived neurons. At the non-cytotoxic concentration (1 nM), all multifunctional compounds significantly enhanced neurite outgrowth. New bis-tryptoline triazole (BTT) increased the neurite length and neurite number, by 93.25% and 136.09% over the control, respectively. This finding demonstrates the ability of multifunctional compounds targeting Aβ to enhance neurite outgrowth in addition to their neuroprotective action.

1. Introduction

Alzheimer's disease (AD) is the most common neurodegenerative disorder characterised by synapse and neuron loss with the accumulation of senile plaques and neurofibrillary tangles.[1] The aetiology of this disease has not been fully understood; several aetiological factors have been implicated in the pathogenesis, such as β-amyloid peptide (Aβ40, 42) accumulation, iron deregulation, oxidative damage and decreasing acetylcholine levels.[2-4] The most interesting target when searching for a new drug for AD treatment was β-amyloid. It was recently found that not only amyloid plaques but also Aβ peptides, as both soluble oligomers and insoluble fibrils, are toxic and the causes of neuronal cell death.[5] The effect of Aβ on reducing neurite outgrowth has been reported in SH-SY5Y human neuroblastoma cell lines[6], mouse neuroblastoma cells[7] and chick sympathetic neurons.[8] Moreover, Aβ oligomers induce the rapid expression of Dickkopf-1 (Dkk1) in the Wnt signalling pathway and Dkk1 causes synapse disassembly and synapse loss.[9] Hence, Aβ in oligomeric forms is more neurotoxic on neurite outgrowth than high-molecular-weight aggregates or fibril forms.[10] Therefore, the inhibition of amyloid production to reduce Aβ-mediated neuronal damage and death is the main target of drug development for AD treatment, resulting in a large number of inhibitors of β-secretase (BACE1) and β-amyloid aggregation.[11,12] Furthermore, other factors that aggravate Aβ-induced neurotoxicity, such as metals[10] and free radicals[13], are the emerging targets of multifunctional drug development for AD. Metals accelerate Aβ-aggregation and produce reactive species or free radicals when interacting with Aβ.[14,15]

In our previous research, the multifunctional compounds of tryptoline and tryptamine triazole derivatives have been discovered to be multifunctional neuroprotective, acting on

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multiple targets, namely β-secretase (BACE1), Aβ, metal and reactive species. Interestingly, there are three compounds (**6h**, **12c** and **12h**) which not only showed a neuroprotective effect against Aβ-induced neuronal cell death but also increased cell viability more than that of the control.[14] Thus, it would be interesting to explore whether these multifunctional compounds (**6h**, **12c** and **12h**) are able to enhance neurite outgrowth. In addition, the novel bis-tryptoline triazole (**BTT**) was designed focusing on inhibitory action against BACE1 and Aβ aggregation because of the poor inhibitory action of compounds **6h** against BACE1 and **12c** against Aβ aggregation. The newly synthesised bis-tryptoline triazole, **BTT** was evaluated for the multifunctional modes of action neuritogenic activity together with the previously reported compounds **6h**, **12c** and **12h** (Figure 1).

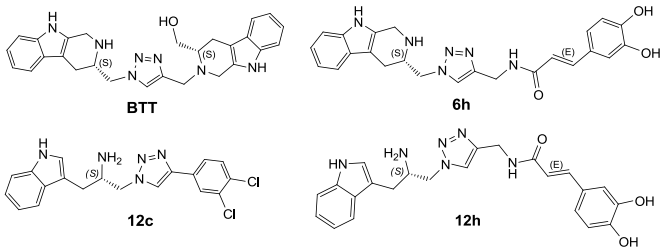

Figure 1. Structures of multifunctional compounds **BTT**, **6h**, **12c** and **12h**

2. Experimental

2.1 General

Molecular dockings were performed to determine the binding mode of **BTT** to BACE1 and Aβ by the AutoDock program suite version 4.2 on the Garibaldi platform at The Scripps Research Institute. **BTT** was generated and optimised with ChemDraw Ultra 9.0 and Chem3D Ultra 9.0. Chemical reagents were purchased form Aldrich, Chem-Impex or AK Science. 1H-NMR and 13C-NMR spectra were acquired on Bruker Avance 300 or 400 MHz instruments. Mass spectra were recorded on either a Thermo Finnigan or LCMS Bruker MicroTof. IR spectra were recorded on Nicolet FTIR 550. BACE1 enzyme and BACE1 substrate were purchased from Sino Biological® and Calbiochem®, respectively. Amyloid-β (1–42) from Anaspec® was used in ThT. Statistics were determined by t-test calculated by OriginPro 8.5.1 and SPSS 17.0.

2.2 *In Silico* Studies

2.2.1 Docking Study of β-Secretase

BACE1 template (2IRZ-F), template was constructed from two crystal structures of BACE1 bound to inhibitors (Protein Data Bank code: 2IRZ[16] and 1FKN[17] as previously described.[15] Docking parameters in the *in silico* studies were 100 run of genetic algorithm (GA), 150 of population size, 15,000,000 per run of energy evaluations and 27,000 of the maximum number of generations.

2.2.2 Docking Study of Amyloid β

The Aβ template (1Z0Q-1) was constructed from crystal structures of the Aβ monomer (Protein Data Bank code: 1Z0Q[18]) as previously described.[14] Docking parameters in the *in silico* studies were 100 run of genetic algorithm (GA), 150 of population size, 5,000,000 per run of energy evaluations and 27,000 of the maximum number of generations.

2.3 Synthesis

Compound **BTT** was synthesised by Cu(I)-catalysed azide alkyne cycloaddition reaction between the azide **5** and alkyne **3i** (Figure 2). Compound **5** was prepared in four steps as previously reported.[15] First, tryptoline-3-carboxylic acid was reacted with methanol using acid catalysis to yield compound **2**. Second, the methyl ester of compound **2** was converted to alcohol by sodium borohydride to obtain compound **3**. Third, the amino group of compound **3** was protected and the hydroxyl group was converted to azide; finally, the amino group of compound **4** was deprotected yielding compound **5**. Alkyne **3i** was prepared by the reaction between compound **3** and propargyl bromide (Figure 2).

2.3.1 (*S*)-(2-(Prop-2-ynyl)-2,3,4,9-tetrahydro-1*H*-pyrido[3,4-b]indol -3-yl) methanol (3i)

Compound **3** (0.3526 g, 1.74 mmol), sodium bicarbonate (0.1754 g, 2.01 mmol) and propargyl bromide (0.22 mL, 2.02 mmol) were stirred in 10 mL DMF at 70°C for 3 hours. Then, 30 mL water was added to the reaction and the reaction was extracted with ethyl acetate (20 mL x 3). The combined organic phase was washed with brine, dried over sodium sulphate and evaporated under reduced pressure. The obtained residue was purified by column chromatography on silica gel ($EtOAc/CHCl_3/NH_4OH$ 2:8:0.1) to yield yellow oil (0.1415 g, 34%); R_f = 0.33 ($CH_2Cl_2/EtOAc/NH_4OH$ 3:7:0.1); FTIR (ATR) (cm^{-1}): 3545 (O-H, st), 3394 (indole N-H, st), 3282 (alkyne C-H, st), 3051 (aromatic C-H, st), 2922, 2845 (aliphatic C-H, st), 2103 (C≡C, st), 1660 (C-N, bending), 1451 (aromatic C=C, st), 1327 (O-H, bending), 1223 (C-O, st), 1155 (C-N, st), 1068 (C-O, st), 742 (aromatic C-H, bending); ^{1}H-NMR (400 MHz, $CDCl_3$) 7.82 (s, 1H, H9): δ, 7.44 (d, *J* = 7.63 Hz, 1H, H5), 7.30 (d, *J* = 7.95 Hz, 1H, H8), 7.15 (t, *J* = 7.47, 7.47 Hz, 1H, H7), 7.09 (t, *J* = 7.22, 7.22 Hz, 1H, H6), 4.08 (d, *J* = 16.61 Hz, 1H, H1b), 4.01 (d, *J* = 16.36 Hz, 1H, H1a), 3.71 (dd, *J* = 6.57, 1.86 Hz, 2H, H10), 3.64 (dd, *J* = 16.70, 2.39 Hz, 1H, H11a), 3.43 (dd, *J* = 16.69, 2.33 Hz, 1H, H11b), 3.38-3.32 (m, 1H, H3), 2.86 (dd, *J* = 16.07, 5.47 Hz, 1H, H4b), 2.59 (dd, *J* = 16.08, 6.01 Hz, 1H, H4a), 2.26 (t, *J* = 2.33, 2.33 Hz, 1H, H13), 2.25-2.03 (br, 1H, OH); LRMS (ESI) m/z 241.25 [M+H].

2.3.2 (*S*)-3-((4-((*S*)-(3-Hydroxymethyl-2,3,4,9-tetrahydro-1*H*-pyrido[3,4-b]indol-2-yl)methyl)-1*H*-1,2,3-triazol-1-yl)methyl)-2, 3,4,9-tetrahydro-1*H*-pyrido[3,4-b]indole, (BTT)

A mixture of compound **5** (0.0983 g, 0.43 mmol), compound **3i** (0.1250 g, 0.52 mmol), 5% mol $CuSO_4$ and 20% mol sodium ascorbate in 10 mL of *t*-BuOH/H_2O/EtOH (2:2:1) was stirred at room temperature for 24 hours. After ethanol was evaporated out, water (10 mL) was added to the reaction. The aqueous solution was extracted with ethyl acetate (10 mL x 3). The organic phase was dried over sodium sulphate, concentrated and purified by column chromatography ($EtOAc/MeOH/NH_4OH$; 9.5:0.5:0.1). A light brown powder of **BTT** was obtained (0.0746 g, 37%); R_f = 0.27 ($EtOAc/MeOH/NH_4OH$; 9:1:0.1); m.p. 178-180 ºC; FTIR (KBr) (cm^{-1}): 3398 (O-H, s), 3056 (aromatic C-H, st), 2917, 2844 (aliphatic C-H, st), 1630 (N-H, bending), 1451 (aromatic C=C,

st), 1330 (O-H, bending), 1229 (C-O, st), 1150 (C-N, st), 1049 (C-O, st), 748 (aromatic C-H, bending); ^{1}H-NMR (400 MHz, DMSO-d_6): δ 10.81 (s, 1H, H9), 10.68 (s, 1H, H9″), 8.13 (s, 1H, H5′), 7.36 (d, *J* = 8.00 Hz, 1H, H5), 7.32 (d, *J* = 7.60 Hz, 1H, H5″), 7.26 (t, *J* = 7.60, 7.60 Hz, 2H, H8, H8″), 7.03-6.96 (m, 2H, H7, H7″), 6.43-6.90 (m, 2H, H6, H6″), 4.70 (br, 1H, OH), 4.60 (d, *J* = 6 Hz, 2H, H10), 4.02 (s, 2H, H1), 3.97 (d, *J* = 14 Hz, 1H, H6′a), 3.91 (d, *J* = 14 Hz, 1H, H6′b), 3.78 (d, *J* = 5.6 Hz, 2H, H1″), 3.72-3.51 (m, 2H, H10″), 3.50 (m, 1H, H3), 3.17-3.11 (m, 1H, H3″), 2.78 (dd, *J* = 15.6, 4.8 Hz, 1H, H4″b), 2.67 (dd, *J* = 15.2, 4.0 Hz, 2H, H4″a, H4b), 2.52-2.46 (m, 1H, H4a); ^{13}C-NMR (400 MHz, DMSO-d_6): δ 145.148, 136.454, 136.362, 132.282, 131.519, 127.561, 127.103, 125.135, 121.169, 120.811, 118.92, 118.660, 117.776, 117.707, 111.445, 111.316, 105.954, 105.573, 61.215, 59.125, 53.779, 53.253, 47.838, 46.045, 41.995, 24.889, 21.113; LRMS (ESI) m/z 468.50 [M+H]; HRMS (ESI) m/z calcd for [M^+] 467.5655, Found 468.2499 [M+H].

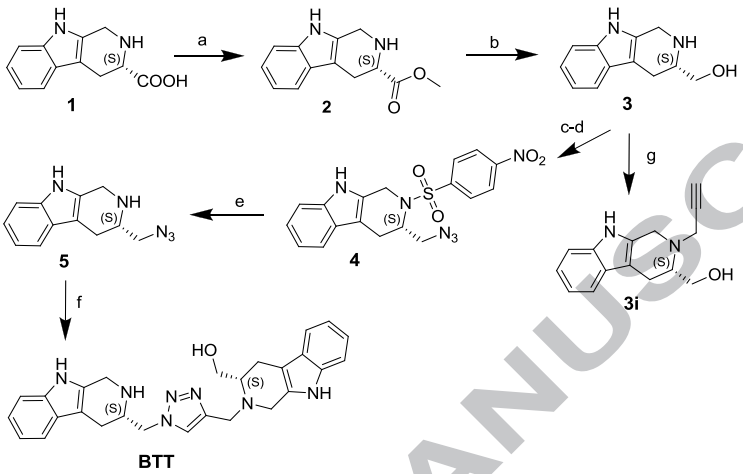

Figure 2. Synthesis of compound **BTT**. Reagents and conditions: (a) H_2SO_4, methanol, reflux, 18 h; (b) $NaBH_4$, ethanol, THF, reflux, 18 h; (c) 4-nitrobenzenesulfonyl chloride, TEA, CH_2Cl_2, rt, 4 h; (d) NaN_3, DMF, 70°C, 6 h; (e) K_2CO_3, thiophenol, DMF, 50°C, 2 h; (f) alkyne **3i**, $CuSO_4 \cdot 5H_2O$, sodium ascorbate, DMSO, rt, 24 h; (g) propargyl bromide, $NaHCO_3$, DMF, 70°C, 3 h.

2.4 Biological Activity Assay

2.4.1 β-Secretase Inhibition Assay

The novel compound **BTT** was evaluated for BACE1 inhibitory action at a concentration of 25 µM. Briefly, the reaction wells containing 30 µL BACE1 enzyme stock solution 0.01 unit/µL, 20 µL of 5% DMSO of test compound and 50 µL of 50 µM β-secretase substrate IV from Calbiochem® were incubated at 37ºC for 30 min. The fluorescence was measured at E_x = 380 nm and E_m = 510 nm by using SpectraMax Gemini EM™.[14] Then, β-secretase inhibitor IV was used as positive control. The assays were run in triplicate. The IC_{50} values were calculated by using GraphPad Prism 4 in nonlinear regression curve fit.

2.4.2 Amyloid β Aggregation Inhibition Assay

The novel compound **BTT** was evaluated for the Aβ anti-aggregation at 100 µM. In each well of a transparent plate, 9 µL of 25 µM working solution of amyloid-β (1-42) peptide (Anaspec®) and 1 µL of the test compound were mixed gently and incubated in the dark at 37°C for 48 h. After incubation, 200 µL of 5 µM ThT (Sigma®) in 50 mM Tris buffer (pH 7.4) was added to each well. The fluorescence was measured at E_x = 446 nm and E_m = 490 nm by using SpectraMax Gemini EM™.[14]

2.4.3 Fe (II) Chelation Assay

The test compound was prepared in 50% DMSO at 100 µM. This solution (40 µL) was added to reaction wells containing 50 µL of 0.2 mM ferrous sulphate solution. Then, DI water was added to adjust the volume to 150 µL. The mixtures were incubated at room temperature for 10 min. After incubation, 50 µL of 1 mM ferrozine was added to the reaction mixture. The absorbance was measured at 562 nm by using infinite 200 pro™, Tecan.[14]

2.4.4 Free Radical Scavenging Assay

The compound **BTT** was prepared in 50% DMSO at 500 µM and evaluated for antioxidant activity at 100 µM. The reaction well containing 70 µL of DPPH solution (500 µM in methanol), 10 µL of methanol and 20 µL of test compound was incubated at room temperature in dark for 30 min. The absorbance was measured at 517 nm by using infinite 200 pro™, Tecan.[14]

2.4.5 Neuritogenic Activity Assay

P19 cells were grown in alpha minimal essential medium (α-MEM) supplemented with 7.5% newborn calf serum (NCS), 2.5% foetal bovine serum (FBS), and 1% antibiotics-antimycotic solution in a 5% CO_2 humidified atmosphere, at 37ºC. Cells in monolayer cultures were maintained in exponential growth by subculturing every 2 days.[19] Exponentially grown cultures were trypsinised and dissociated into single cells. P19 cells (2 x 106 cells/mL) were then suspended in 10 mL α-MEM supplemented with 5% FBS, 1% antibiotics-antimycotic solution and 0.5 µM all trans-retinoic acid (RA) and seeded onto a 100-mm bacteriological culture dish. The cells formed large aggregates in suspension. After 4 days of RA treatment, aggregates were dissociated by 5-mL glass measuring pipette, re-plated on poly-

L-lysine-pre-coated multi-well plates (multi-well plates were coated with 50 µg/mL poly-L-lysine dissolved in PBS for overnight and sterilised under UV light for 30 min) at 7 x 10^4 cells/mL (150 µL/well in 96-well plate and 1.5 mL/well in 6-well plate), in α-MEM supplemented with 10% FBS and 1% antibiotics-antimycotic solution, and incubated for 24 h. Cytosine-1-β-D-arabinoside or Ara-C (10 µM) was added at day one after plating and the medium was changed every 2-3 days. The differentiated neuronal cells, P19 derived-neurons, were used after day 14 of the differentiation process.[19-21]

Viability assay: The assay was carried out on P19-derived neurons cultured in a 96-well plate. After 14 days of differentiation, the α-MEM supplemented with 10% FBS, 10 µM Ara-C and 1% antibiotics-antimycotic solution was removed and DMSO solutions of the sample, diluted with α-MEM supplemented with 10% FBS and 1% antibiotics-antimycotic solution in the presence of 10 µM Ara-C, were added to give the concentrations of 0.0001, 0.001, 0.01, 0.1, 1, and 10 µM. The concentration of DMSO was added to the cultures at 0.5% v/v. The α-MEM supplemented with 10% FBS, 10 µM Ara-C, and 1% antibiotics-antimycotic solution was added into control wells. The cells were incubated for 18 h at 37ºC. Then, 150 µL of the medium was removed, and 50 µL of XTT solution (1 mg/mL XTT in 60ºC α-MEM + 25 µM PMS) was added. After incubation at 37ºC for 4 hours, the 100 µL of PBS (phosphate buffer saline solution) pH 7.4 was added. The OD value was determined on a microplate reader at 450 nm. The data were expressed as the mean ± SEM (n = 3, each n was run in triplicate), with the medium as a control representing 100% cell viability. The concentration that enhanced survival of cultured neurons more than control will be further investigated for neuritogenicity.[22]

Neuritogenicity assay: The assay was carried out with P19-derived neurons cultured in a 6-well plate. The assay was performed in a replicate. After 14 days of differentiation, the α-MEM supplemented with 10% FBS, 10 µM Ara-C, and 1% antibiotics-antimycotic solution was removed and DMSO solution of the sample, diluted with the α-MEM supplemented with 10% FBS, 10 µM Ara-C, and 1% antibiotics-antimycotic solution, were added to give a final concentration of the samples (**6h**, **12c**, **12h** and **BTT**) at concentrations that enhanced the survival of cultured neurons more than the control. The concentration of DMSO was added to the cultures at 0.5% v/v. The α-MEM supplemented with 10% FBS, 10 µM Ara-C, and 1% antibiotics-antimycotic solution was added to control wells. The cells were incubated for 18 h at 37ºC. The morphology under a phase-contrast microscope was observed. The appearance of P19-derived neurons was compared to the control and measured for the length and number of neurites. The average length and number of neurites of 30 neurons from the assay were measured. The data were expressed as the mean ± SEM (n = 1).[22]

2.4.6 Cell Viability Assay

The SH-SY5Y cells were cultured in minimum essential medium (MEM): F-12 (1:1), 10% fetal bovine serum, MEM non-essential amino acids (0.5×), 0.5 mM sodium pyruvate and 100 units/ml of penicillin and 100 µg/mL of streptomycin. The cells were incubated under a humidified with 5% CO_2 at 37 °C.

SH-SY5Y cells were seeded in 96-well plates (2 × 10^4 cells per well) for 24 h. Then, cells were treated with 10 µM of test compounds for 2 h prior to exposure to 1 µM aggregated β-amyloid (1-42). The preparation of aggregated β-amyloid was done in a medium without serum, penicillin and streptomycin at 37 °C for 72 h. The aggregated β-amyloid treated cells were incubated for 24 h. After 24 h, 15 µL of MTT reagent (5 mg/mL MTT in serum free medium containing 10 µM HEPES) was added to each well and incubated at 37°C for 3 h. The medium was removed and 100 µL of 0.04 N HCl in isopropanol was added. The absorbance was measured at E_x = 570 nm and E_m = 630 nm using a Synergy™ HT multi-detection.[23]

Results and Discussion

Bis-Tryptoline Triazole (**BTT**) was designed to increase BACE1 inhibitory action by replacing the phenolic motif of compound **6h** with the tryptoline, a core structure of previously reported BACE1 inhibitors as displayed in Figure 3.[15] This modification altered the orientation of the structure and increased the hydrophobicity with intended to improve binding affinity. Moreover, the length between terminal aromatic rings was 13.2 Å, within the 13-14 Å, the optimal length for anti-Aβ aggregation activity.[14]

BTT was synthesised and its multifunctional mode of activity was determined in comparison with the previously reported compounds **6h**, **12c** and **12h**. The results in Figure 4 show that **BTT** possessed multifunctional activity with different degrees of potency between different actions i.e. high to moderate potency against BACE1 and amyloid aggregation, moderate to weak for metal chelation and antioxidant activity. As expected, the activity of **BTT** against BACE1 was higher than **6h** (IC_{50} 16.73 µM vs. > 50 µM) and the %inhibition of Aβ aggregation of **BTT** was greater than that of compound **12c.**

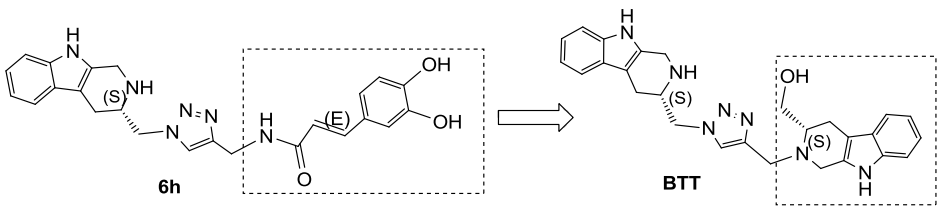

Figure 3. The structural design of **BTT**

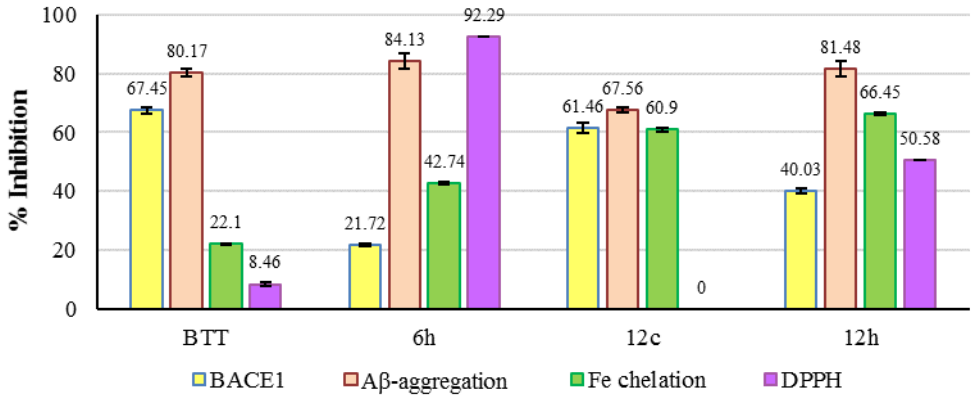

Figure 4. The multifunctional activity of **BTT** and related compounds; BACE1 inhibition was evaluated at 25 µM while other activities were tested at 100 µM

To gain further insight, the binding modes of all multifunctional compounds to the targets, BACE1 and Aβ were determined by molecular docking. All compounds are located in the substrate binding site of BACE1; **BTT** and **12c** occupy three pockets: S1, S2 and S4, while only two pockets are occupied by compounds **6h** (S1 and S2) and **12h** (S1 and S3) (Figure 5A). **BTT** interacted with Asp228, the catalytic amino acid, and Gly230 via hydrogen bonds. The increase in BACE1 inhibitory action of **BTT** compared to that of compounds **6h** and **12h** possibly results from the insertion of tryptoline moieties into S2 and S4 pockets (Tyr71, Thr72, Gln73, Gly230, Thr231, Thr232, Asn233 and Ser325), which provides the hydrophobic interactions and consequently leads to the increase in binding affinity (Figure 5B). The IC_{50} value of **BTT** (IC_{50} = 16.73 µM) is comparable with that of **12c** (IC_{50} = 20.75 µM) according to the similar binding mode.

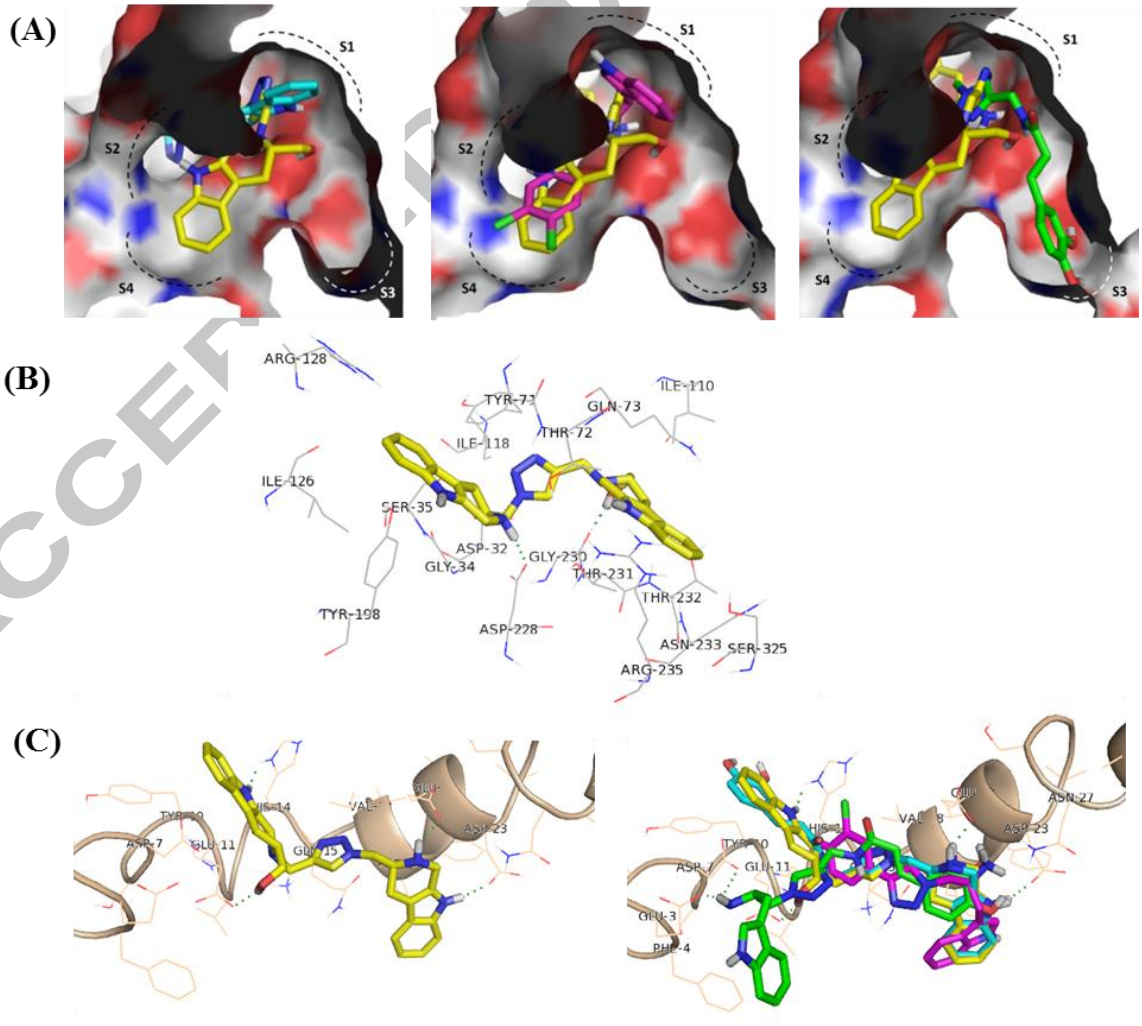

Figure 5. The binding modes of **BTT** (yellow) to two targets showing interacting amino acid and H-bond (dotted line): (A-B) BACE1 and (C) Aβ-amyloid superimposed with compounds **6h** (cyan), **12c** (magenta) and **12h** (green).

For Aβ aggregation, the modification did not improve the anti-amyloid aggregation of **BTT** because the binding mode of **BTT** with Aβ was similar to other multifunctional compounds. **BTT** formed four hydrogen bonds with Glu11, His14, Glu22, and Asp23 and interacted with Gln15, Val18, Phe19, Phe20, Glu22, and Asp23, which are the key amino acids in the self-aggregation region (Figure 5C). The docking results of all compounds against BACE1 and amyloid beta aggregation are shown in Table S1.

These multifunctional compounds (**BTT**, **6h**, **12c** and **12h**) were evaluated for the effect on neurite outgrowth of P19-derived neurons using quercetin and geldanamycin as positive controls. The P19 cell line was selected in this experiment because P19 cells can differentiate to cholinergic neurons under 4-5 days of retinoic acid treatment.[20] Thus, this P19 cell line was suitable as an activity testing model for AD. Quercetin was selected as a reference compound because its effects are related to our designed compounds namely antioxidant, amyloid aggregation inhibitor and neurite outgrowth inducer.[24-26] Quercetin mediated its neurite outgrowth inducing effect through the elevation of intracellular cAMP and GP-43 expression levels.[26] Geldanamycin (GA) was also included as a positive control as it possessed neuroprotection against neuronal plaque formation in animal models[27,28] as well as augmented neurite outgrowth *in vitro* and accelerate axonal regeneration *in vivo*.[29] The exact mechanism of GA is still not fully revealed, the plausible mechanisms of GA is binding to heat shock protein 90 (Hsp90)[27] and upregulation of heat shock protein 72.[28,29] Before the evaluation of BTT for neuritogenic activity, the viability assay was performed at six serial concentrations (0.0001, 0.001, 0.01, 0.1, 1, and 10 μM). The results are shown in supplementary data. All compounds were non-toxic at 1 nM and significantly increased the neuronal viability at *p*-value < 0.05 (Figure 6).

The neuritogenic activity was determined at a lowest non-cytotoxic concentration (1 nM); the morphology, length and numbers of neurites were observed under a phase-contrast microscope after treatment with the test compounds (Figure S3). All multifunctional compounds showed significant increase in neurite number, 113-136% increase when compared with control. Moreover, the number of neurites was significantly greater in cells treated with multifunctional compounds than that of quercetin and geldanamycin treated control. The neurons incubated with test compounds increased the neurite length up to 57-93% compared with control (Table 1).

Only compounds **BTT** and **12c**, which showed good inhibition against BACE1, significantly increased neurite length more than quercetin and geldanamycin, the well-reconized neuroprotectors and neurite outgrowth inducers. Taken together, compound **BTT** showed the best neuritogenic activity by increasing both neurite length and number. Between multifunctional modes of activity, it appeared that BACE1 inhibitory activity played a significant role in neurite outgrowth, as **BTT** with anti-amyloid aggregation (IC_{50} = 66.36 μM) moderate showed greater neuritogenic activity than quercetin, a potent amyloid aggregation inhibitor (IC_{50} = 7.94 μM).[30]

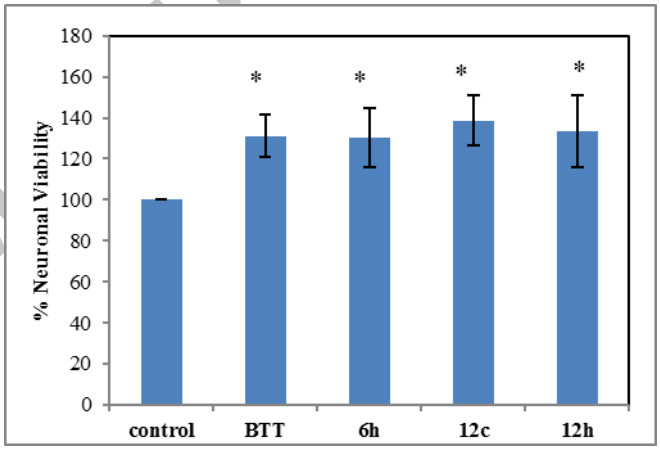

Figure 6. The effect of compounds (1 nM) on the neuronal viability of P-19 derived neurons. The data were expressed as the mean ± SEM (n = 3, each n was run in triplicate), with the medium as a control representing 100% cell viability using *t*-test analysis with *p < 0.05.

Table 1. The neuritogenic activity at concentration of 1 nM in P19-derived neurons

Compounds	Neurite length		Neurite number	
	Average ± SE (μm)	% Increase	Average of ± SE	% Increase
Control	68.70 ± 6.37	0.00	2.30 ± 0.21	0.00
0.5% DMSO	70.00 ± 5.50	1.89	2.43 ± 0.23	5.65
BTT	132.76 ± 13.12*,**	93.25	5.43 ± 0.26*,**	136.09
6h	108.23 ± 9.44*,**	57.54	5.13 ± 0.38*,**	123.04
12c	126.50 ± 10.08*,**	84.13	5.03 ± 0.25*,**	118.70
12h	111.44 ± 8.86*,**	62.21	5.33 ± 0.39*,**	131.74
Geldanamycin	109.33 ± 9.06*,**	59.14	3.57 ± 0.34*,**	55.22
Quercetin	108.80 ± 19.86 *,**	58.37	3.23 ± 0.28*,**	40.43

*p < 0.05 compared with control, **p < 0.05 compared with 0.5% DMSO
t-Test was calculated by OriginPro 8.5.1

In addition, the increasing length of neuron cells is related to BACE1 inhibitory activity: **BTT** > **12c** > **12h** > **6h**. This result might involve an increase of soluble β-amyloid precursor protein alpha (sAPPα) by the inhibition of BACE1, as reported by the research group of Mattsson and Porteius.[31] This sAPPα can stimulate neurite outgrowth by binding with $p75^{NTR}$.[32] Moreover, contactin-2 has been recently reported to be the substrate of BACE1. The role of contactin-2 is regulating axon guidance, neurite outgrowth and cell adhesion.[33]

Apart from BACE1, the neuritogenic activity might contribute to anti-amyloid aggregation action, as compounds **6h** and **12h** with moderate inhibition effects on BACE1 were able to enhance the neurite outgrowth. Amyloid aggregation inhibitory activity might partially contribute to the neuritogenic activity. This inhibition of amyloid formation might reduce the activation of Rho GTPase activity yielding decreasing of Rho Kinase (ROCK II), the collapsin response mediator protein-2 (CRMP-2) phosphorylate enzyme. The phosphorylation of CRMP-2 reduced tubulin assembly in neurites causing microtubule disassembly.[34, 35] It is plausible that the inhibitions of BACE1 and amyloid aggregation are the key factors in the promotion of neuron outgrowth. In addition, **BTT** was determined for the protective effect against amyloid-induced toxicity in SH-SY5Y neuronal cells using curcumin, a potent antioxidant and anti-amyloid aggregation (IC_{50} = 0.63 μM)[36] as control. The result showed that **BTT** was able to protect neurons against amyloid-induced toxicity and increased cell viability over control significantly (Figure 7).

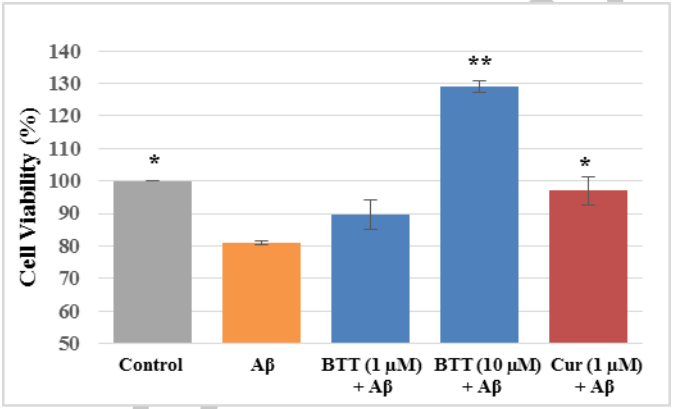

Figure 7. The protective effect of BTT against Aβ induced neuronal toxicity by MTT viability assay using *t*-test analysis with *$p < 0.05$, **$p < 0.01$ vs Aβ treated cells. *t*-Test was calculated by SPSS 17.0.

Beside amyloid generation and aggregation partway, neuritogenicity of **BTT** might be form other mechanism because **BTT** can increse cell viability in amyloid-induced toxicity over the control significantly. Thus, other proposed neurite outgrowth mechanism of **BTT** such as inducing of cAMP level and Gap-43 expression and decreasing of protein level (raf1, ERK, Akt1 and CDK4) will be invatigated in further study. [26, 37]

3. Conclusions

We successfully designed multifunctional compounds **BTT**, **6h**, **12c** and **12h** to exert the neuritogenic activity at the nanomolar level. The results reported herein support the validity of the multifunctionality approach in drug development for AD.

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Supplementary data

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