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PII: S0960-894X(19)30635-3

DOI: https://doi.org/10.1016/j.bmcl.2019.126685

Reference: BMCL 126685

To appear in:

Received date: 16 June 2019

Revised date: 10 September 2019

Accepted date: 12 September 2019

Please cite this article as: Z. Wang, C. Ma, Y. Wang, et al., Structural optimization and neurotrophic activity evaluation of neurotrophic gentiside derivatives, (2019), https://doi.org/10.1016/j.bmcl.2019.126685

Structural optimization and neurotrophic activity evaluation of neurotrophic gentiside derivatives

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ABSTRACT

C14 alkyl benzoate **ABG001**, derived from naturally occurring gentisides, was reported to exhibit neurotrophic activity which is similar to NGF (Nerve Growth Factor). In this research, **ABG001** was modified by the strategy of isosteric replacement and conformational restriction with the purpose of improving the bioactivity. The cellular neurotrophic activity of those **ABG001** derivatives were evaluated, among which 3-hydroxyquinolin-2-(1*H*)-one **A3** and 4-decylphenol ester **B7** displayed much better neurotrophic activity compared with **ABG001**, which highlights the potential of those novel scaffolds for future neurotrophic agent development.

Key Words: Gentisides, neurotrophic activity, isosteric replacement, conformational restriction, PC 12 cells

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Neurodegenerative diseases, which includes Alzheimer's disease, Parkinson's disease, Huntington's disease, and amyotrophic lateral sclerosis, are characterized by nervous system dysfunction resulting from progressive neuronal degeneration.[1] Additionally, spinal cord injury is a damage to the spinal cord resulting from trauma or disease.[2] About 50 million people in the world are suffering from neurodegenerative diseases or spinal cord injury, which costs over 600 billion dollars of annual healthcare budget.[3] The currently available therapies, which mainly relieve disease symptoms and control the damage, are insufficient to eradicate these diseases. It is well-known that promoting the survival and regeneration of neurons within the adult nervous system would be an ideal preventive and therapeutic strategy.[4] Nevertheless, such therapeutic approaches have not yet been successfully developed currently.

Neurotrophins are endogenous proteins that regulate neuron survival, development, and function.[5] Nerve growth factor (NGF), which acts as a prototypical neurotrophin, is essential for the development and survival of neuron cells, maintenance of synaptic connection and plasticity, and prevention of aging in the nervous system.[6] Nonetheless, the suboptimal pharmacokinetic properties of NGF restrict its clinical use such as poor *in vivo* stability and limited penetration of the blood-brain barrier owing to its large molecular size.[7] However, low-molecular weight compounds that can enhance or mimic the neurotrophic ability of NGF without those foregoing protein-based limitations have shown better clinical applications.[8] Over the past several decades, a lot of neurotrophic compounds were reported (**Figure 1**), for example, caffeic acid amide analogues synthesized by Omidreza Firuzi showed strong neurotrophic activity;[9] RC-33 could enhance the NGF-mediated neurite outgrowth by selectively activating σ1 receptors;[10] Verb-5 potentiated NGF-induced neurite outgrowth of PC-12D cell;[11] Sar-6 modified from Sarcodonin G exhibited excellent activity to promote NGF-induced neurite outgrowth;[12] Ribisin C was found to enhance the neurite outgrowth in the presence of NGF;[13] Piperodione isolated from the fruits of *P. retrofractum* promoted the neurite outgrowth of NGF.[14] It's worth mentioning that professor Jin-Ming Gao from Northwest A&F University is devoted to discovering neurotrophic natural products from native microbial and plant resources for decades, many natural compounds bearing fascinating chemical structures have showed good neurotrophic activity, such as Striatoids A−F, Cyafricanins G, Qinbunamides A-C and Neocyathins T. [15]

As listed above, most of these compounds exhibit neurotrophic activity in a NGF dependent manner,

and compounds with significant independent neuritogenic activity were rarely reported.

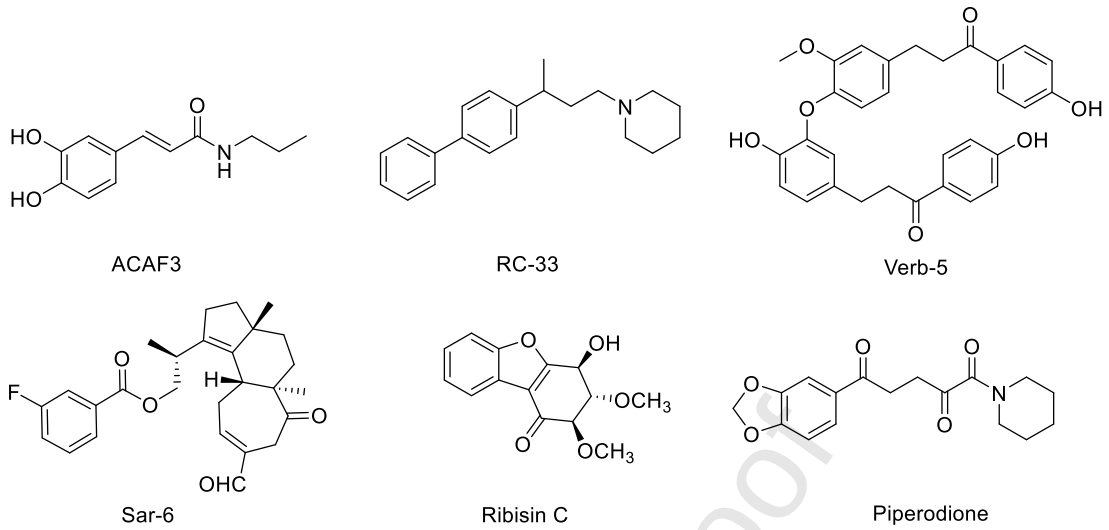


Figure 1. The structure of reported neurotrophic compounds

Gentisides isolated from Chinese medicine *Gentiana Rigescens* showed potent neurotrophic activity.[16] And further structural simplification led to compound **ABG001** with neurotrophic activity equivalent to NGF.[17] As shown in **Figure 2**, **ABG001** is a long-chain alkyl benzoate, and can be divided into three parts: 2,3-dihydroxy phenyl, ester group and a long alkyl chain. In this paper, **ABG001** was used as lead compound for structural optimization to explore novel NGF mimics as shown in **Figure 2**. The 2,3-dihydroxy phenyl group is unstable *in vivo* and easily oxidized to quinone which is detrimental for human body. The corresponding bioisosteres of 2,3-dihydroxy phenyl group was explored, such as pyrazolyl, 2-aminothiazole, 1H-benzo[*d*]imidazole, and quinoline-2,3-diol group, which could retain hydrogen bonds interactions and aromatic annulus (**Figure 2**, **Table 1**). Besides the long flexible alkyl chain may not be favorable for the binding with the target due to the entropy cost, therefore the proper restrictions to the long flexible chain could potentially improve the binding affinity. We introduced sterically equivalent 1,4-phenylene group and 1,1'-biphenyl group, which are effective templates for conformational restriction,[18] to replace four or eight methylene units of the long alkyl chain in **ABG001** and investigated the effects of distance (m) between newly incorporated group and ester group and length of the tail alkyl chain (n) on the neurotrophic activity (**Figure 2, Table 2** and **Table 3**).

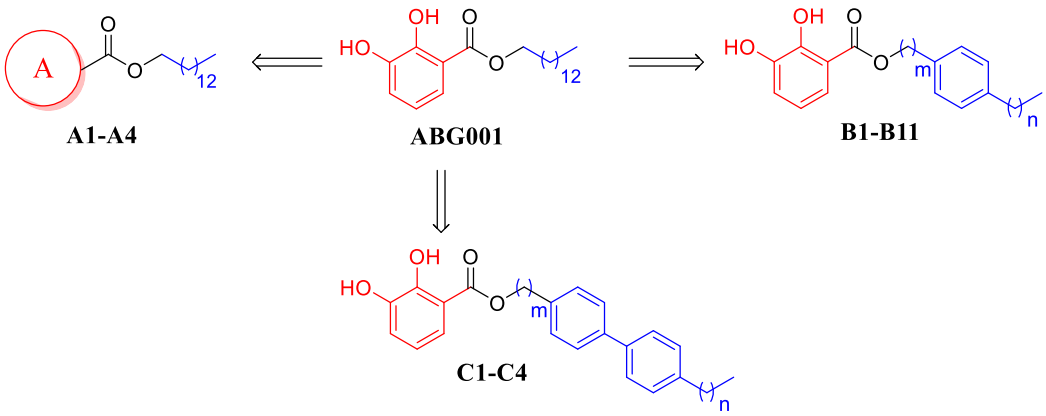
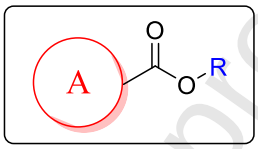


Figure 2. The design guideline of gentiside derivatives **A1-A4**, **B1-B11** and **C1-C4**.

Table 1 The structures of gentiside derivatives **A1-A4**



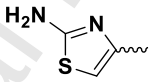
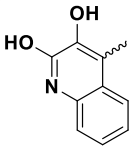
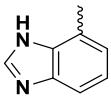
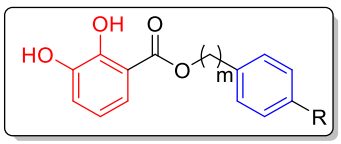
Compound	A	R
A1		$(CH_2)_{13}CH_3$
A2		$(CH_2)_{13}CH_3$
A3		$(CH_2)_{13}CH_3$
A4		$(CH_2)_{13}CH_3$

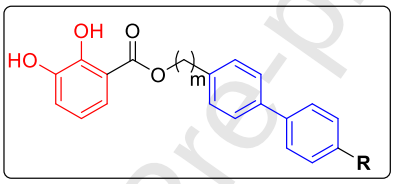
Table 2 The structures of gentiside derivatives **B1-B11**



Compound	m	R
B1	0	H
B2	1	H

B3	2	H
B4	3	H
B5	4	H
B6	10	H
B7	0	$(CH_2)_9CH_3$
B8	1	$(CH_2)_8CH_3$
B9	1	$(CH_2)_9CH_3$
B10	1	$CH=CH(CH_2)_6CH_3$
B11	1	$CH=CH(CH_2)_7CH_3$

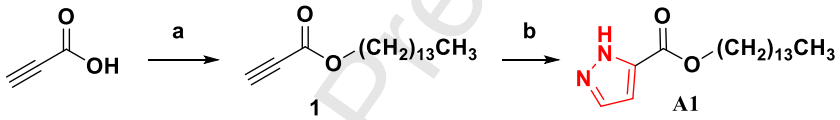
Table 3 The structures of gentiside derivatives **C1-C4**



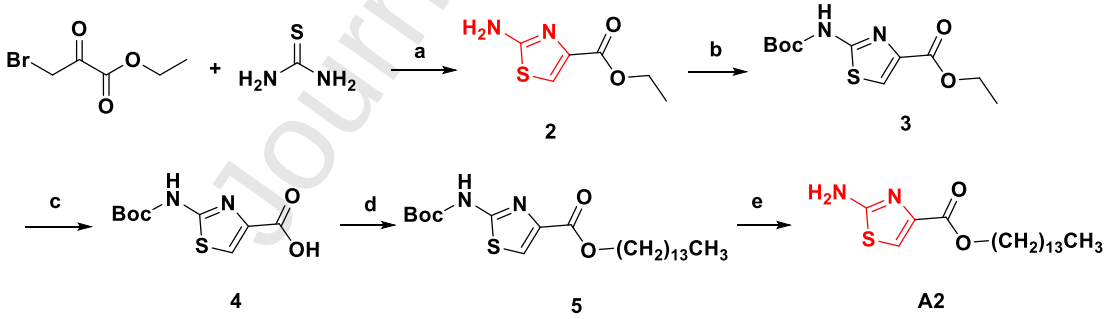
Compound	m	R
C1	0	H
C2	1	H
C3	0	$(CH_2)_5CH_3$
C4	1	$(CH_2)_4CH_3$

The target compounds were synthesized efficiently *via* the route outlined in **Scheme 1-4**. As shown in **Scheme 1**, The esterification reaction of propiolic acid with 1-tetradecyl alcohol in the presence of a catalytic amount of p-toluenesulfonic acid gave alkynes **1**, which reacted with trimethylsilyldiazomethane at room temperature to afford the pyrazolyl derivate **A1**. The **A2** were synthesized by a series of reactions as shown in the **Scheme 2**. Intermediate **2** was synthesized by cyclizing ethyl bromopyruvate and thiourea in ethanol at 78 °C, then was converted to 2-aminothiazole derivate **A2** by *t*-butoxycarbonyl protection, ester hydrolysis, followed by Yamaguchi esterification and deprotection of amino protecting group in order. As depicted in **Scheme 3**, Isatin was reacted with tetradecyl 2-diazoacetate in the presence of diethylamine, and the 3-hydroxyquinolin-2-(1*H*)-one derivative **A3** was obtained via subsequent Eistert ring expansion of the

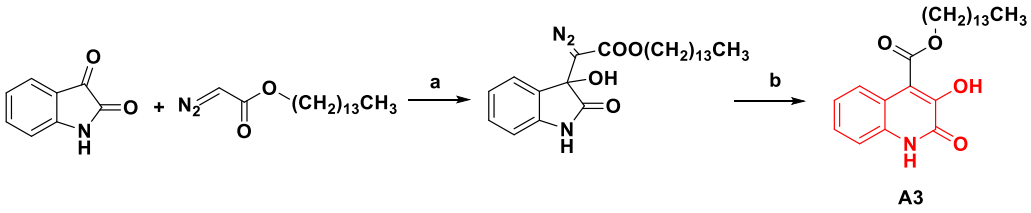
crude adducts after dilute aqueous acidic treatment. 1H-benzo[*d*]imidazole-7-carboxylic acid afforded desired compound **A4** using the N, N-dicyclohexylcarbodiimide as the dehydration agent (not shown in the scheme). The transformation of phenol followed by esterification, Fries rearrangement and ketone carbonyl reduction resulted in para alkyl-substitution phenol **7**. Commercially available methyl 4-formylbenzoate can react with octyltriphenylphosphonium bromide and nonyltriphenylphosphonium bromide respectively under alkaline conditions to get styrene **8a** and **8b**, followed by ester reduction with lithium aluminium hydride to further obtain **9a** and **9b**. The previous intermediates were carried on hydrogenolysis with 10% palladium on carbon to get the benzyl alcohol **10a** and **10b** in quantitative yield. The 1,1'-biphenyl derivates **11a** and **11b** are obtained by Suzuki cross-coupling reaction using corresponding phenyl bromide and phenylboronic acid. Finally, the target products **B1-B11** and **C1-C4** were obtained successively by esterification of 2,3-dihydroxybenzoic acid with the corresponding phenols or alcohols in dry acetonitrile using N, N-dicyclohexylcarbodiimide as condensation agent.



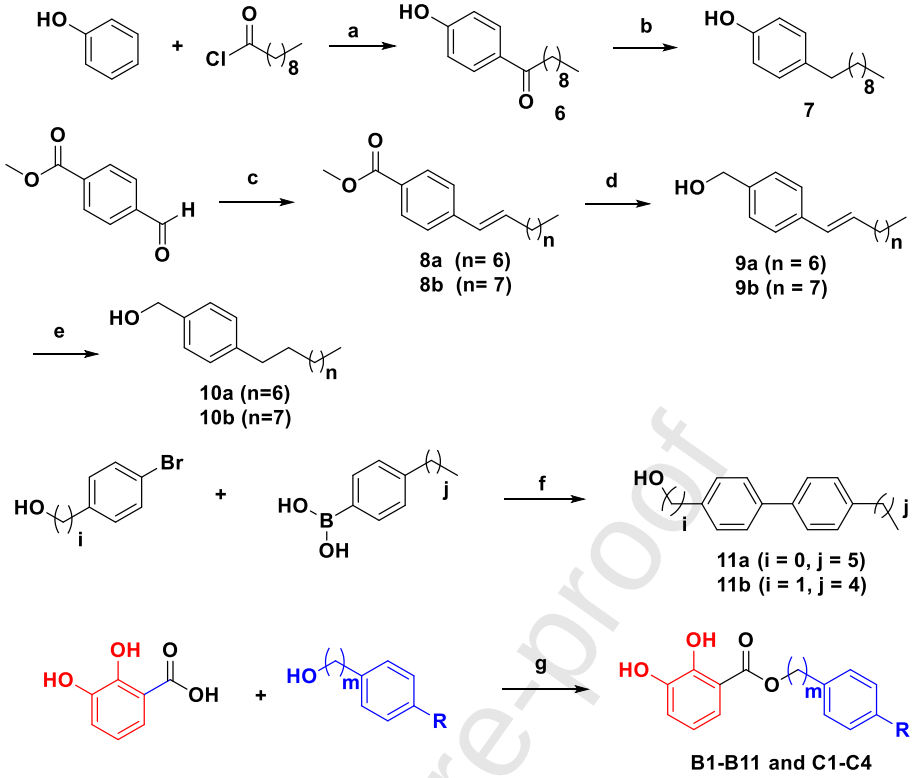
Scheme 1. Reagents and conditions: (**a**) 1-tetradecanol, p-toluenesulfonic acid, toluene, reflux, overnight, 84%; (**b**) trimethylsilyldiazomethane, *n*-hexane, rt, 2 h, 51%.



Scheme 2. Reagents and Conditions: (**a**) EtOH, 78°C, 4 h, 91%; (**b**) $(Boc)_2O$, pyridine, reflux, 6 h, 54%; (**c**) 1M LiOH, THF, rt, 4 h; (**d**) 2,4,6-trichlorobenzoyl chloride, DIPEA, 1-tetradecanol, DMAP, THF, 0°C, 1.5 h, rt, 1 h , 73%; (**e**) TFA/DCM = 4:1, rt, 4 h, 90%.



Scheme 3. Reagents and conditions: (**a**) Et_2NH, ethanol, rt, 72 h; (**b**) 1N HCl, rt, 2 h, 40%.



Scheme 4. Reagents and conditions: (**a**) $AlCl_3$, 130 °C, 1 h; (**b**) NH_2-NH_2, DEG, KOH, 250 °C, 2 h, 190 °C, 4 h, 82%; (**c**) ylide agents, *n*-BuLi, THF, -20 °C, 1 h, rt, 2 h, 53% for **8a**, 59% for **8b** ; (**d**) $LiAlH_4$, THF, 0 °C, 2 h, 94% for **9a** and **9b**; (**e**) Pd/C, H_2, MeOH, rt, overnight; (**f**) $Pd(PPh_3)_4$, K_2CO_3, DMF, EtOH, H_2O, 90 °C, overnight, 86% for **11a** and 67% for **11b**; (**g**) DCC, DMAP, CH_3CN, 50 °C, 1 h, 65 °C, 3 h, 37%-69%.

Undifferentiated rat pheochromocytoma cell (PC-12 cell), which can be induced to differentiate into sympathetic-like neurons after exposure to NGF, is a useful model system to evaluate the neurotrophic activity of compounds in vitro.[19] The effects of the target compounds on neurite outgrowth of undifferentiated PC-12 cells were evaluated by morphological observations and quantitative analysis of percentage of neurite-bearing cells.

The effect of different bioisosteres (**A1**-**A4**) on the neurotrophic activity was first investigated. As depicted in **Figure 3**, the 2,3-dihydroxy phenyl group replaced by pyrazolyl heterocycle (**A1**), 2-aminothiazole heterocycle (**A2**) or 1H-benzo[*d*]imidazole heterocycle (**A4**) are detrimental to the neurotrophic activity, while 3-hydroxyquinolin-2-(1*H*)-one scaffold (**A3**) could improve the activity.

Next, we evaluated the effect of the distance (m) between newly incorporated phenyl group and ester group (**B1**-**B6**) on the neurotrophic activity. As **Figure 3** shows, the phenolic ester **B1** (m=0) and benzyl ester **B2** (m=1) displayed much better neurotrophic activity, and longer distances led to impaired activity

(**B3**-**B6)**, suggesting that the distance (m) is of critical importance to the neurotrophic activity. Using **B1** and **B2** as templates, to determine the the impact of the length of alkyl chain (n) on the activity, we introduced 1,4-phenylene group to replace the four methylene units in the alkyl chain of **ABG001** to obtain compounds **B7** and **B8.** To our delight, the two compounds exhibited remarkably increased activity compared with **ABG001**. While further increasing the alkyl chain by one carbon (**B9**) resulted in slight reduction in potency compared with **B8**. When double bonds are introduced into the alkyl chain of **B8** and **B9** , the activity decreases considerably compared with saturated carbon chains (**B10** vs **B8**, **B11** vs **B9**). After that we replace the eight methylene units in the long fat chain with a biphenyl group to further enhance the rigidity of the compound and also evaluated the effect of the distance (m) between newly incorporated group and ester group on the neurotrophic activity (**C1**-**C4**). Unfortunately, the neurotrophic activity of all compounds in this series was significantly decreased compared with the corresponding phenyl substituted compounds (**C1** vs **B1**, **C2** vs **B2**, **C3** vs **B7** and **C4** vs **B8**).

Particularly, 3-hydroxyquinolin-2-(1*H*)-one derivative **A3**, phenol ester **B7** and benzyl ester **B8** displayed even better activity than lead compound **ABG001** (**Figure 3**). To the best of our knowledge, compounds bearing quinoline motif was first reported to have neurotrophic activity. **Figure 4** shows the morphological changes of PC12 cells after treatment with our artificial compounds bearing good bioactivity (**A3**, **B7** and **B8**) in comparison with the solvent control (0.5% DMSO), and the positive control (**NGF** and **ABG001**). Control cells (without any test compound) showed very few short neurite outgrowth. When treated with **A3** (1 μM), **B7** (1 μM) and **B8** (1 μM), the cells extended long multipolar neurite outgrowths 48 h after treatment, which were similar to those produced following treatment with **NGF** (40 ng/mL) and **ABG001** (1 μM).

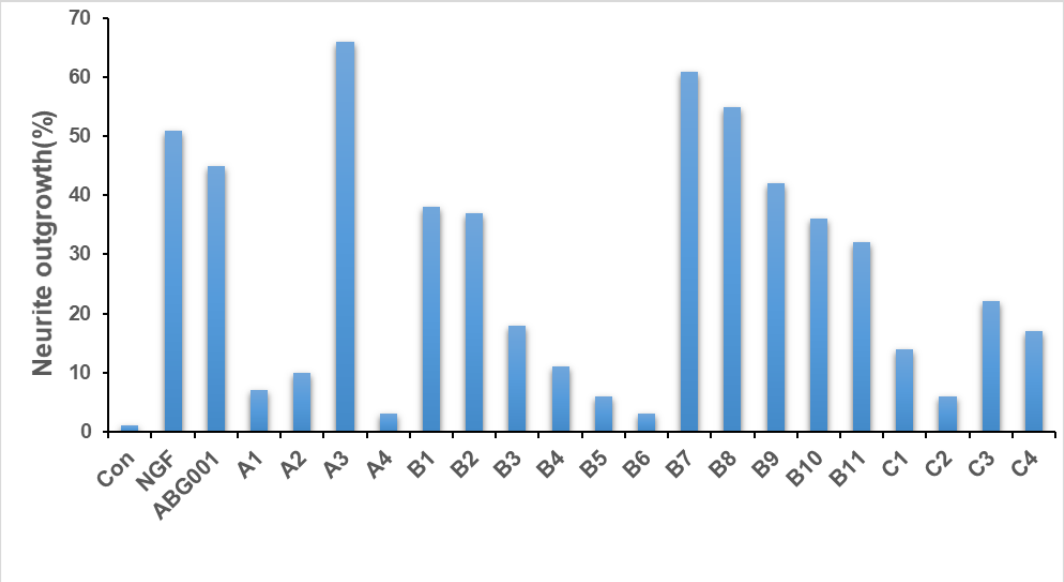
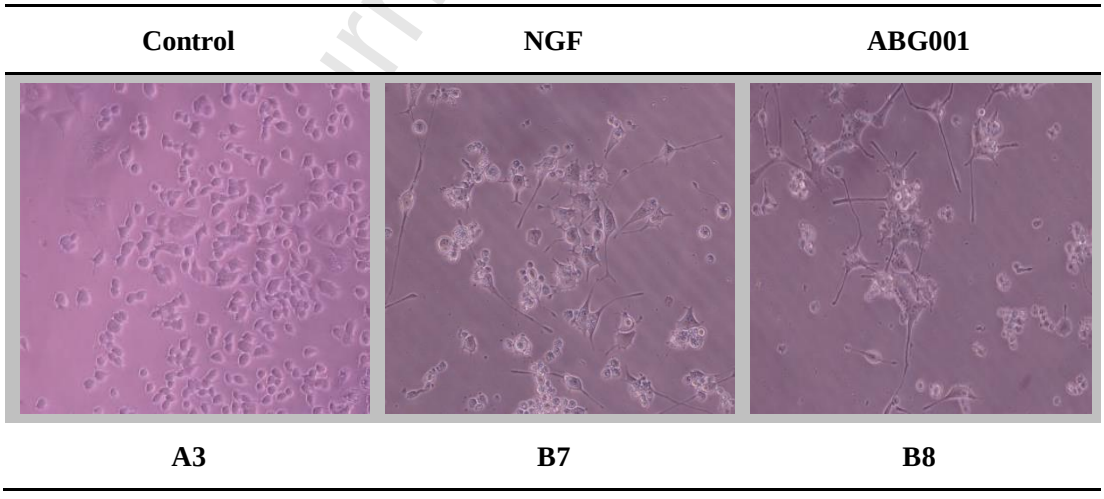


Figure 3. Effects of compounds A1-A4, B1-B11 and C1-C4 on neurite outgrowth in PC12 cells. Undifferentiated PC12 cells were treated with Con (0.5% DMSO), NGF (40 ng/mL), ABG001(1 μM), A1-A4 (1 μM), B1-B6 (30 μM), B7-B11 (1 μM) and C1-C4 (10 uM) for 48 h to evaluate their neurotrophic effects by using phase-contrast microscope. Five images were selected randomly under a microscope for each well. At least 100 cells in each of five randomly separated fields were scored and the proportion of cells with neuritis greater than or equal to the length of one cell body were positive for neurite outgrowth, and expressed as a percentage of the total cell number in five fields (Y-axis). Experiments were repeated at least three times. Significant differences between each groups were tested by ANOVA, followed by two-tailed multiple t-tests using SPSS biostatistics software (IBM; Armonk, NY, U.S.A.), values represent the mean ± SE (n =3). ** $p < 0.01$ compared with control.



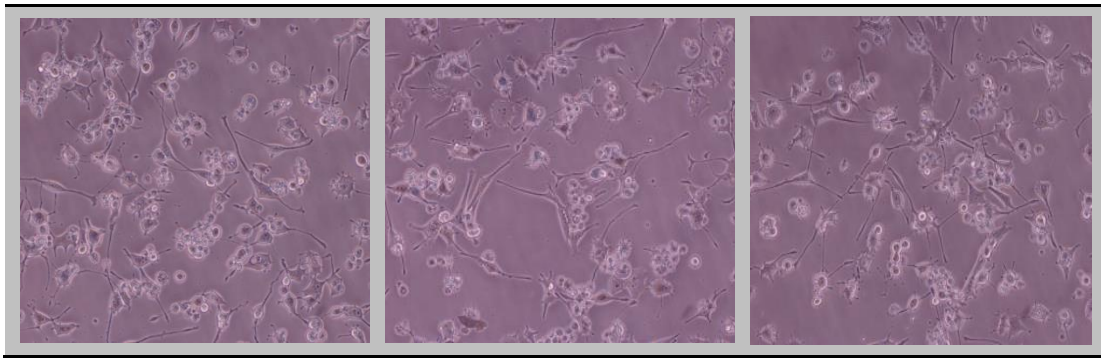


Figure 4. Photomicrographs of **PC12** cells under a phase-contrast microscope (the magnification is ×400) 48 h after treatment with 0.5% DMSO (Control), NGF (40 ng/mL), ABG001 (1μM), A3 (1μM), B7 (1μM), or B8 (1μM).

Sequentially, the dose-response test and the cell viability test of the three promissing compounds (**A3**, **B7** and **B8**) in PC12 cells were executed. As can be seen from the cell viability curve (**Figure 5**), considerable toxicity to PC-12 cells was detected when concentrations of these compounds were above 5 μM, also obviously **A3** and **ABG001** showed the higher toxicity when tested in high concentrations (10 μM) compared with **B7** and **B8**. It determined that the optimal concentration should not exceed the 1 μM. Besides the three compounds showed dose-dependent activity when evaluated in the concentration range of 0.1 to 1 μM (**Figure 6**).

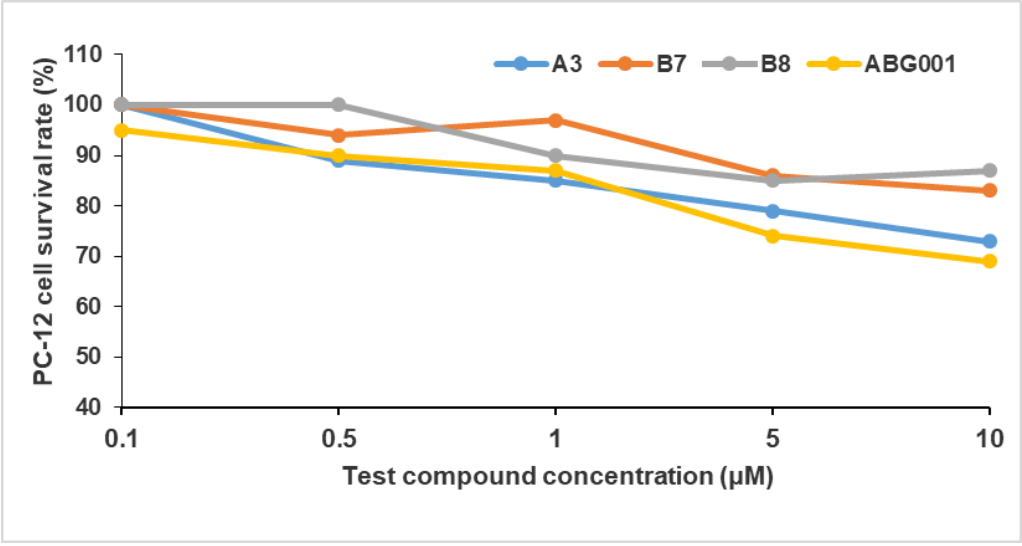


Figure 5. PC-12 cells survival rate of A3, B7, B8 and ABG001 48 h after treatment. ** $p < 0.01$ compared with control (0.5% DMSO).

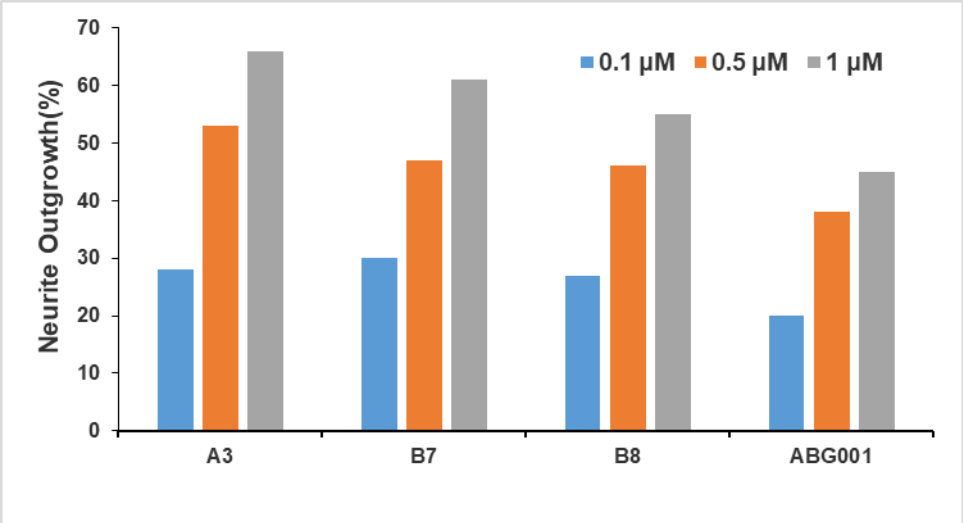


Figure 6. Dose-dependent curve of the NGF-mimicking activity of A3, B7, B8 and ABG001 48 h after treatment. ** $p < 0.01$ compared with control (0.5% DMSO).

In summary, one series of neurotrophic gentiside derivatives with isosteric replacement or the long alkyl chain optimization of the lead compound **ABG001** were reported. 3-hydroxyquinolin-2-(1*H*)-one scaffold with excellent neurotrophic activity is first reported. The most potent compounds **A3** and **B7** exhibited better neurotrophic activity profiles than **ABG001**, which could be served as new leads for further exploration in the future neuritogenic agent development. Our research also highlight the importance of employing isosteric replacement and conformation restriction strategy to the lead optimization in drug discovery.

Acknowledgements

We gratefully acknowledge the financial support from National Natural Science Foundation of China (Grant No. 81773576).

Supplementary data

Supplementary data is available on the publishers' web site along with the published article.

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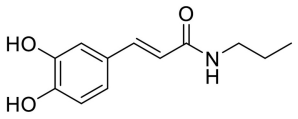

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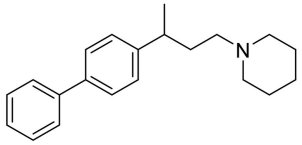

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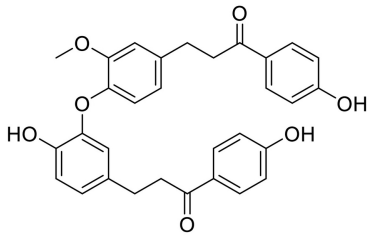

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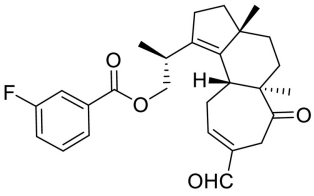

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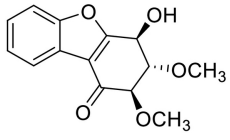
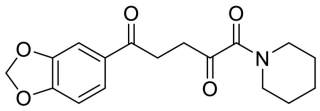


Ribisin C



Piperodione

Figure 1

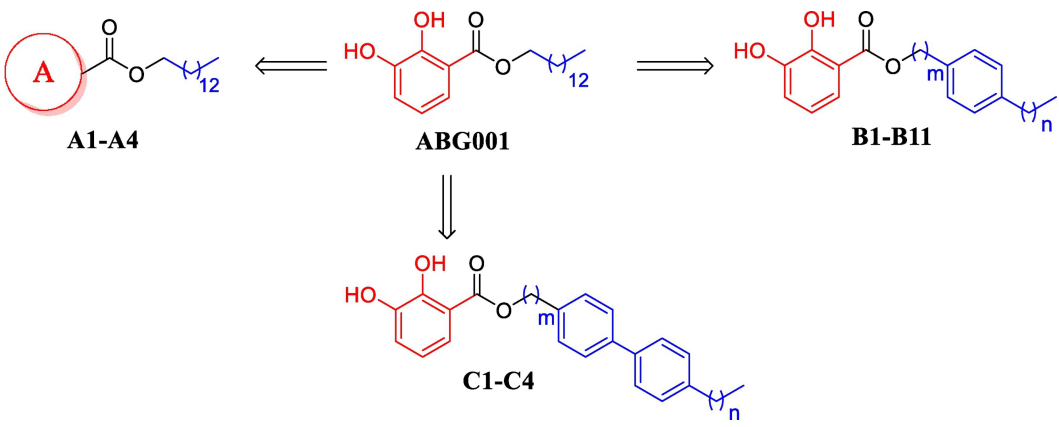


Figure 2

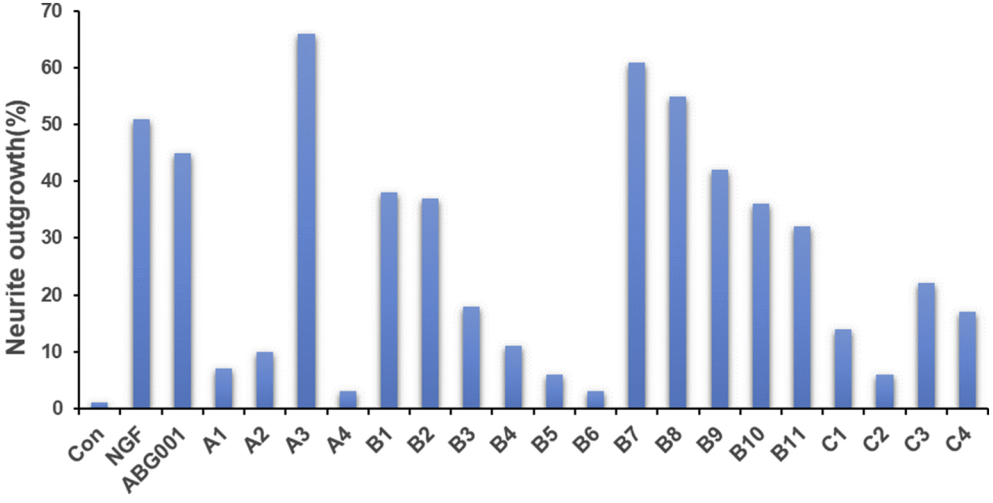

Neurite outgrowth(%)
70
60
50
40
30
20
10
0
Con
NGF
ABG001
A1
A2
A3
A4
B1
B2
B3
B4
B5
B6
B7
B8
B9
B10
B11
C1
C2
C3
C4

Figure 3

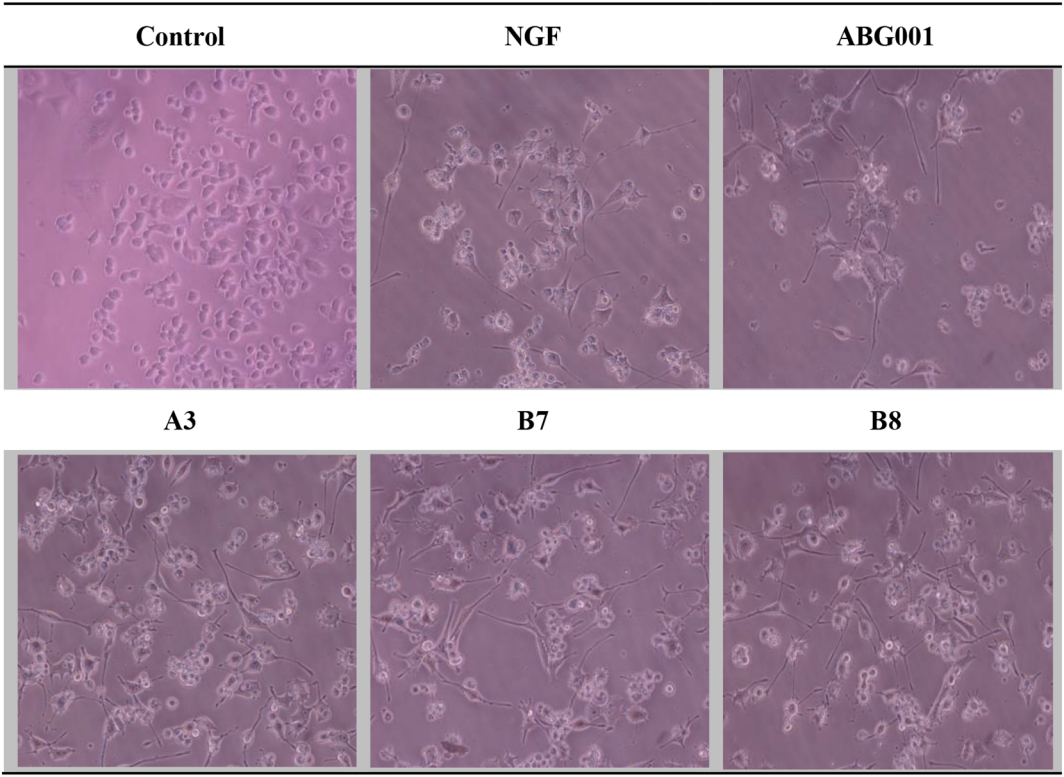


Figure 4

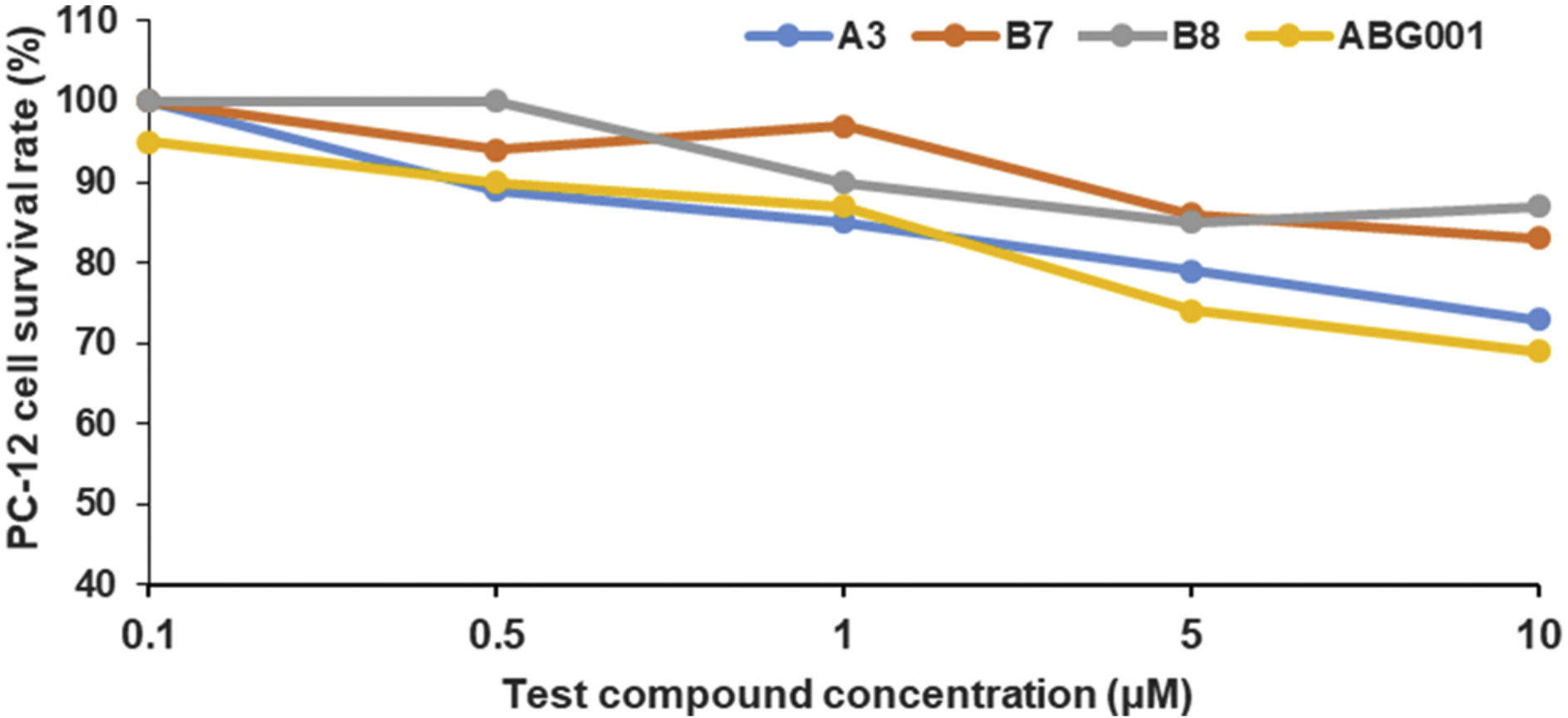

A3
B7
B8
ABG001
PC-12 cell survival rate (%)
110
100
90
80
70
60
50
40
0.1
0.5
1
5
10
Test compound concentration (μM)

Figure 5

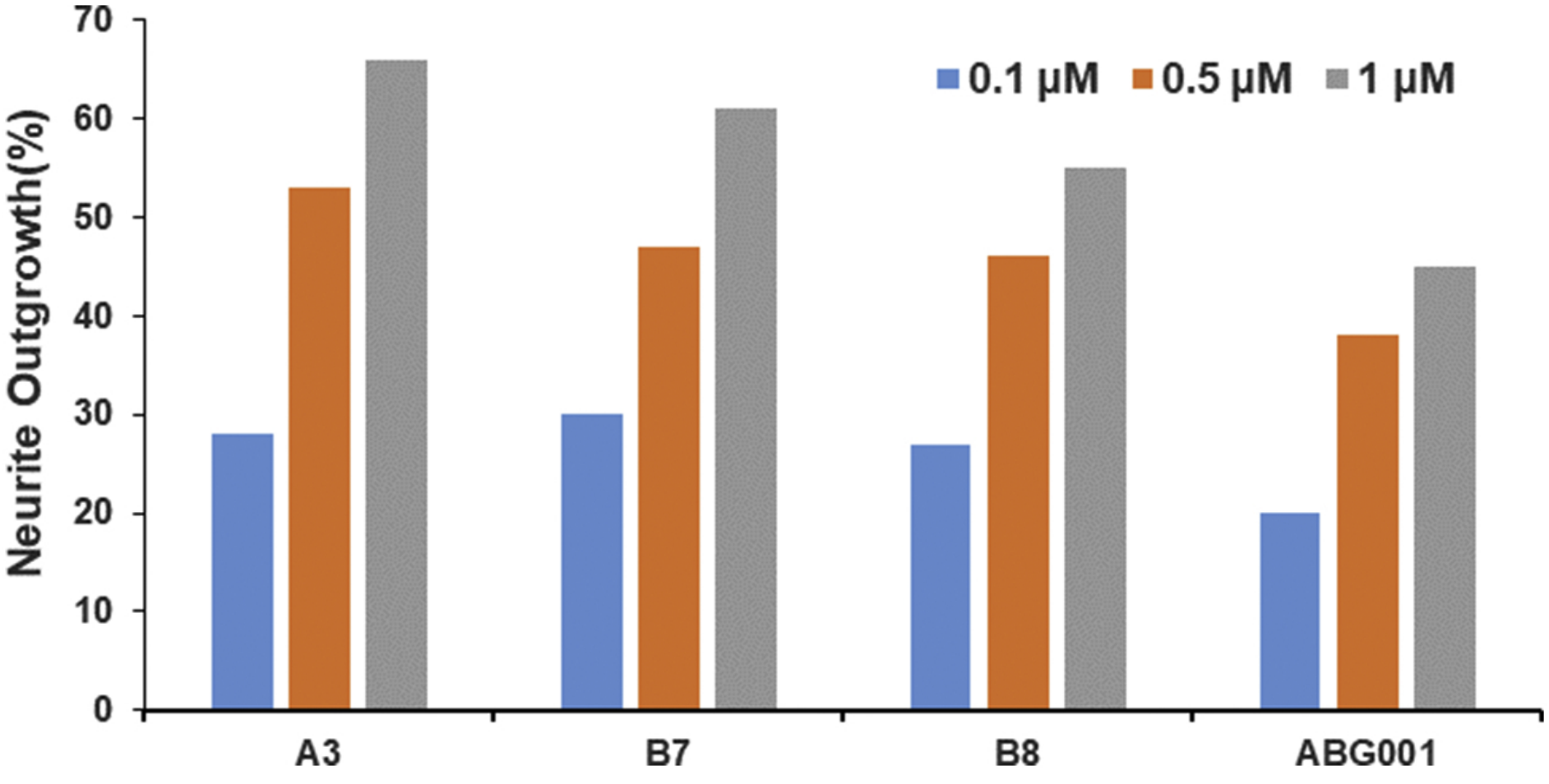

Neurite Outgrowth(%)
70
60
50
40
30
20
10
0
0.1 μM
0.5 μM
1 μM
A3
B7
B8
ABG001

Figure 6