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Neuritogenic activity of tetradecyl 2, 3-dihydroxybenzoate is mediated through the IGF-1 receptor/PI3K/MAPK signaling pathway

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Running title: Study on the action mechanism of ABG-001

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***Abbreviations*:** ABG-001, tetradecyl 2,3-dihydroxybenzoate; CREB, cAMP responsive element-binding protein; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethyl sulfoxide; ERK, extracellular signal-regulated kinase; *G. rigescens* French, *Gentiana rigescens*; IGF-1, insulin-like growth factor 1; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; MEK, mitogen-activated protein-extracellular signal-regulated kinase; MTT, 3-(4,5-dimethylthiazol-2-yl) -2,5-diphenyltetrazolium bromide; NGF, nerve growth factor; PI3K, phosphatidylinositol 3-kinase; PKA, protein kinase A; PKC,

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protein kinase C; PLC, phospholipase C; SAPK, stress-activated protein kinase; Trk A, tyrosine kinase A

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ABSTRACT

Tetradecyl 2,3-dihydroxybenzoate (ABG-001) is a lead compound derived from neuritogenic gentisides. In the present study, we investigated the mechanism by which ABG-00 1 induces neurite outgrowth in PC12 cells. Inhibitors of insulin-like growth factor 1 (IGF-1) receptor, phosphatidylinositol 3-kinase (PI3K) and extracellular signal-regulated kinase 1/2 (ERK1/2) significantly decreased ABG-001-induced neurite outgrowth. Western blot analysis revealed that ABG-001 significantly induced phosphorylation of IGF-1 receptor, AKT, ERK and cAMP responsive element-binding protein (CREB). These effects were markedly reduced by addition of the corresponding inhibitors. We also found that ABG-001-induced neurite outgrowth was reduced by protein kinase C (PKC) inhibitor as well as siRNA against the IGF-1 receptor. Furthermore, like ABG-001, IGF-1 also induced neurite outgrowth of PC12 cells, and low-dose NGF augmented the observed effects of ABG-001 on neurite outgrowth. These results suggest that ABG-001 targets the IGF-1 receptor and activates PI3K, mitogen-activated protein kinase (MAPK) and their downstream signaling cascades to induce neurite outgrowth.

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Introduction

Neurotrophic factors, such as nerve growth factors (NGFs), participate in neuronal development and in the survival and functional maintenance of neurons (McAllister 2001). Several studies have indicated that reduced neurotrophic support is a contributing factor in the pathogenesis of Alzheimer's disease, Parkinson's disease, and amyotrophic lateral sclerosis (Dawbarn et al., 2003). Therefore, neurotrophic factors are strong candidate therapeutic agents for chronic neurodegenerative diseases. Unfortunately, NGF cannot penetrate the blood–brain barrier because of its size, and this limitation hinders its applications in clinical therapy. As such, screening for NGF-mimicking chemical compounds with low molecular weights and the ability to pass through the blood–brain barrier is an active area of research.

Natural compounds, such as termitomycesphins A–D (Qi et al., 2000), linckosides A–E (Qi et al., 2002; Qi et al., 2004), panax ginseng (Yamazaki et al., 2001), and nardosinone (Li et al., 2003), as well as synthetic compounds such as 4,5-bis-epi-neovibsanin A (Chen et al., 2010), xaliproden (Marwan et al., 2009) and synthetic verbena chalcone derivatives (Clement et al., 2009), have recently been shown to have NGF-mimicking or NGF-enhancing activity related to neurite outgrowth of PC12 cells. Previous studies have identified diverse molecular signaling pathways participating in the neuron-protective activities of small natural compounds (Weng et al., 2011; Maceady et al., 2009). These pathways include selective actions of a number of protein and lipid kinase signaling cascades, the most notable of which involve the PI3K/AKT- and MAPK pathways that regulate pro-survival transcription factors

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and gene expression.

Gentiana rigescens French (*G. rigescens*) is a traditional Chinese medicine that is believed to remove heat and dampness from the human body. It is used to treat hypertension, cholecystitis, acute infective hepatitis, and cystitis. Pharmacodynamic studies have indicated that *G. rigescens* has anti-inflammation, anti-pathogen, antioxidant, antitumor and pro-immunity functions (Tang et al., 1977). In our previous study, we described the isolation of gentisides A−K, which are new neuritogenic compounds, from *G. rigescens* (Gao et al., 2010 a, b). These gentisides showed good neuritogenic activities in PC12 cells. To study their structure-activity relationships and to facilitate the discovery of lead compound in gentisides, we synthesized more than a hundred derivatives of gentisides and tested their neuritogenic activities in PC12 cells. We found that ABG-001 (Figure 1A) exhibited strong NGF-mimicking effects via the ERK signaling pathway (Luo et al., 2011). However, the molecular target of gentisides and the mechanism behind induction of neurite outgrowth in PC12 cells remain unknown. In the present study, we used inhibitors of the NGF signaling pathway and siRNAs in combination with Western blot assays to investigate the mechanism behind improvements in neurite outgrowth induced by ABG-001. We report that ABG-001 maybe target the IGF-1 receptor and activates PI3K, PKC/MAPK and the associated downstream signaling cascades to induce neuritogenic activity.

Materials and Methods

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Chemicals and Reagents

ABG-001 was synthesized and purified using HPLC in our laboratory (Luo et al., 2011). Dimethyl sulfoxide (DMSO), NGF, and the PI3K inhibitors LY294002 and wortmannin, the mitogen-activited protein-extracellular signal-regulated kinase (MEK)/ERK inhibitors U0126 and PD98059, the IGF-1 receptor kinase inhibitor T9576 (picropodophyllotoxin), PKC inhibitors (GF109203X, GO6983), a protein kinase A (PKA) inhibitor (H-89) were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Ras inhibitors (Sulindac sulfide and farnesylthiosalicylic acid) were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA) and Cayman Chemical (MI, USA). Phospholipase C (PLC) inhibitors (U73122 and U73343), a PKC inhibitors (RO318220), a JNK inhibitor (SP600125), a p38 MAPK inhibitor (SB203580), a tyrosine kinase A (TrkA) inhibitor (K252a), an insulin receptor inhibitor (HNMPA-(AM)3), an IGF-1 inhibitor (AG1024), and a Raf inhibitor (AZ628) were purchased from Santa Craz Biotechnology (CA, USA). IGF-1 was bought from Sino Biological Company (Beijing, China).

Cell Culture

A rat adrenal pheochromocytoma cell line, PC12, was purchased from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). PC12 cells were cultured in Dulbecco's modified Eagle medium (DMEM) (Thermo Scientific, Waltham, USA)

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containing 4 mM glutamine, 1.5 g/L sodium bicarbonate, 4.5 g/L glucose, 10 mM HEPES, and 1 mM sodium pyruvate and supplemented with 10% horse serum and 5% fetal bovine serum (Invitrogen, Grand Island, USA) in a 5% CO_2 incubator at 37 ^{o}C. Prior to treatment, cells were subcultured and allowed to attach overnight.

Neurite Outgrowth Assay

Briefly, 2×10^4 PC12 cells were seeded in the wells of a 24-well microplate and cultured under a humidified atmosphere of 5% CO_2 at 37 ^{o}C. The medium was replaced with 1 ml of serum-free DMEM medium containing the test sample or DMSO (0.5%) after 24 h. NGF was used as a positive control. The number of neurite-bearing cells was measured by counting cells in three arbitrary areas on the 24-well plate containing at least 100 single cells (not aggregated). A cell was identified as positive for neurite outgrowth if the outgrowths were at least twice the cell diameter (Jeon et al., 2010 a, b). Cells were visualized using phase contrast (200-fold magnification) with an Olympus microscope (Olympus, Model CK-2, China). About 100 cells were counted from a random site, and each experiment was repeated three times. If the neurite outgrowth of 100 cells observed in an experiment were twice the cell diameter, then the outgrowth is considered to be 100%. The results are expressed as mean ± SEM. For experiments involving inhibitors, we first performed dose-dependent investigation. The optimum concentration of inhibitors was then used to conduct subsequent experiments.

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Analysis of Cell Viability by MTT Assay

Cell viability was determined based on mitochondria-dependent reduction of MTT to purple formazan. Briefly, cells were incubated with ABG-001 at concentrations of 0, 0.3, 0.6, or 1 μM for 48 h. The medium was carefully removed by aspiration, and 0.5 ml of fresh medium containing MTT (0.2 mg/ml) was added to each well, and the plates were incubated at 37 ^{o}C for 2 h. The medium was then completely removed, and 0.2 ml of DMSO was added to each well to solubilize the formazan crystals. The resultant formazan was detected at 570 nm using a plate reader. All experiments were repeated at least three times.

Real-time PCR Analysis

Cells treated with siRNA against IGF-1 receptor and ABG-001 was collected, respectively. Total RNA was extracted using the Trizol Reagent (Beijing Cowin Biotech Company, Beijing, China) and RNA content was determined using a spectrophotometer. Transcription was performed using 2.5 μg of total RNA, Oligo(dT)$_{20}$ primers, and reverse transcriptase (Beijing Cowin Biotech Company, Beijing, China). Transcript levels were quantified by real-time PCR (AB SCIEX, Massachusetts, USA) and SYBR Premix EX Taq^{TM} (Takara, Otsu, Japan). PCR primers for rat IGF-1 receptor and *18S* RNA were as follows: for *IGF-1* receptor, sense: 5'-ATG GCT TCG TTA TCC ACG AC-3', and anti-sense: 5'-CGA ATC GAT GGT TTT CGT TT-3'; for *18S RNA*, sense: 5'-TAA CCC GTT GAA CCC CAT T-3', and anti-sense: 5'-CCA TCC AAT CGG TAG TAG CG-3'. cDNA was then amplified using Takara SYBR

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Premix Ex Taq™ under the following conditions: 95 °C for 2 min, followed by 40 cycles for 15 s at 95 °C, 15 s at 54.2 °C, and 20 s at 68 °C. All results were normalized to *18S* RNA levels, and relative mRNA transcript levels were calculated using the ΔΔCt formula. All samples were run in triplicate and the average values were calculated.

Western Blot Analysis

Approximately 1×10^6 PC12 cells were seeded in a 60 mm culture dish containing 5 ml DMEM and incubated for 24 h. To investigate the dose-dependent effect of ABG-001, various ABG-001 concentrations were added to the cultures at concentrations of 0, 0.3, 0.6, or 1.0 μM, after which they were incubated for 30 min or 16 h. To investigate the time-dependent effects of ABG-001, ABG-001 was added at a final concentration of 1 μM, after which the cultures were incubated for specific time periods. To investigate the effects of siRNA IFG-1 receptor on neurite outgrowth induced by ABG-001, ABG-001 was added after cell transfection of negative control, siRNA against IGF-1 receptor for 6 h. To prepare protein lysates, cells were collected in lysis buffer (1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 2 mM EDTA and 1% phosphatase inhibitor) and then sonicated using an ultrasonicator (Ningbo Scientz Biotechnology, Ningbo, China). The supernatant containing the proteins was collected by centrifugation at 12×10^3rpm for 15 min. The protein concentration was measured using the Bio-Rad Protein Assay Dye Reagent Concentrate (Bio-Rad Lab., CA, USA) at 595 nm. Protein lysates (15 μg) were separated by sodium dodecyl sulfate polyacrylamide gel

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electrophoresis and then transferred onto polyvinylidenedifluoride membranes. The membrane was incubated with primary antibodies followed by horseradish peroxidase-conjugated secondary antibodies. Antigens were visualized using chemiluminescent substrates (Amersham, GE Healthcare, NJ, USA). The primary antibodies used for immunoblotting are as follows: anti-44/42 MAPK antibody, anti-phospho-p44/42 MAPK (Thr202/Tyr204) polyclonal antibody, anti-IGF-1 receptor β antibody, anti-phospho-IGF-1 receptor β (Tyr1135/1136)/insulin receptor β (Tyr1150/1151), anti-phospho-CREB (Ser133), anti-CREB and anti-AKT antibodies (Signaling Technology, MA, USA), anti-phospho-AKT (Ser473) (Abcam, Hong Kong, China), and GAPDH antibody (Beijing Cowin Biotech Company, Beijing, China). The secondary antibodies used in this study are as follows: horseradish peroxidase-linked anti-rabbit and anti-mouse IgGs (Beijing Cowin Biotech Company, Beijing, China).

Cell Transfection

PC12 cells were transfected with FAM-siRNA to investigate transfection efficiency. A concentration of 120 nM which the transfection efficiency arrived 90% was used to perform the following experiment. The following primer sequences were used to generate siRNAs targeting the rat IGF-1 receptor and the negative control (Shanghai GenePharma Company, Shanghai, China): for IGF-1 receptor-530, sense: 5’-GCG GUG UCC AAU AAC UAC ATT-3’, anti-sense: 5’-UGU AGU UAU UGG ACA CCG CTT; for negative control, sense:

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5’-UUC GAA CGU GUC ACG UTT-3’, anti-sense: 5’-ACG UGA CAC GUU CGG AGA ATT-3’. Transfection of PC12 cells with siRNA was performed according to the manufacturer’s protocol (Invitrogen, Grand Island, USA). Briefly, the day before transfection, 2×10^4 cells were seeded in each well of 24-well plates and allowed to reach 70%–90% confluence in growth medium without antibiotics. Then, siRNA against IGF-1 receptor or negative control siRNA was used at a concentration of 120 nM with Lipofectamine 2000 (Invitrogen, Grand Island, USA) as the transfection agent. After 6 h of transfection, the medium in the plates was replaced with fresh medium containing 1 μM ABG-001 and incubated for additional 24 h. Cell morphologies were observed and recorded using a microscope fitted with a camera. Lengths of neurites were measured using Image J software (National Institute of Health, MD, USA).

Statistical Analysis

All experiments were independently performed three times, and each experiment was conducted with triplicate samples. Data are presented as mean ± SEM. The significant differences between groups were determined by ANOVA, followed by a two-tailed multiple t-tests with Student-Newman-Keuls through SPSS, biostatistics software. Values with $P < 0.05$ were considered significant.

Results

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ABG-001 Induces Neurite Outgrowth in PC12 Cells

Figure 1B shows the neuritogenic activity of ABG-001 in PC12 cells. ABG-001 induced neurite outgrowth in PC12 cells in a dose-dependent manner. The percentages of neurite-bearing cells treated with 0.3, 0.6, and 1.0 μM ABG-001 for 48 h were 57.7 ± 5.4%, 70.1 ± 3.6%, and 83.9 ± 5.8%, respectively, and were significantly higher than that of the control group ($P< 0.001$). This result suggests that ABG-001 has significant effects on neurite outgrowth in PC12 cells and is consistent with the findings of a previous study (Luo et al., 2011).

Effects of ABG-001 on PC12 Cells Viability

The effect of ABG-001 on PC12 cell viability was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. Figure 1C illustrates that PC12 cells treated with ABG-001 concentrations of 0.3, 0.6, and 1 μM for 48 h show viabilities of 82.6 ± 4.7%, 78.5 ± 11.3%, and 75.5 ± 7.1%, respectively. These results indicate that ABG-001 is weakly cytotoxic toward PC12 cells.

Effects of ABG-001 on the TrkA Signaling Pathway

We investigated the function of TrkA receptor in neurite outgrowth induced by ABG-001 using the TrkA receptor inhibitor K252a. Application of this inhibitor did not affect

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the neuritogenic activity of ABG-001 in PC12 cells (Figure 1D, Supplementary Figure 1A).

Effects of ABG-001 on the MAPK/ERK/CREB Signaling Pathway

We investigated the function of ERK1/2 activation in ABG-001-induced neurite outgrowth in PC12 cells. U0126 significantly attenuated the percentage of neurite-bearing cells from an initial value of 71.8 ± 5.5% to 32.4 ± 2% after cells were treated with 1 μM ABG-001 (Figure 2A, B, Supplemental Figure 2B). The average of neurite length of PC12 cells induced by NGF and ABG-001 were significantly reduced by U0126 (Figure 2C). Furthermore, we determined the effects of ABG-001 on ERK phosphorylation at the protein level. ERK1/2 phosphorylation was enhanced by ABG-001 at concentrations of 0, 0.6, 1.0 and 1.2 μM and reduced by U0126 (Figure 2D). ERK1/2 phosphorylation induced by ABG-001 began 8 h after treatment, and then gradually decreased after 48 h (Figure 2E). CREB phosphorylation induced by ABG-001 was initiated 8 h after treatment, and was maintained for 48 h (Figure 2F). Parallel blots were run and probed with antibodies to detect total ERK1/2, CREB, and GAPDH levels; results demonstrated that all proteins had been loaded at equivalent levels. These findings indicate that ERK/CREB signaling is involved in ABG-001-induced neurite outgrowth of PC12 cells.

Effects of ABG-001 on the PI3K/AKT Signaling Pathway

PI3K is critical for NGF-dependent survival of sympathetic neurons, and AKT, a major

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effecter, has the important role of integrating various survival signaling cascades (Crowder and Freeman, 1998; Pierchala et al., 2004). Here, specific inhibitors of PI3K, namely LY294002 and wortmannin, were used to examine the effects of ABG-001 on the PI3/AKT signaling pathway. ABG-001-induced neurite outgrowth and the average length of PC12 cell neuritis was markedly reduced by 20 μM LY294002 (Figure 3, A and B). LY294002 attenuated the percentage of neurite-bearing cells from an initial value of 70.9 ± 3.3% to 42.75 ± 1.2% after cells were treated with 1 μM ABG-001 (Figure 3C, Supplemental Figure 3A).

Next, we investigated AKT phosphorylation at the protein level at multiple time points (Figure 3D). AKT phosphorylation level increased with increase in treatment time in dose-dependent manner at ABG-001 concentrations of 0.0, 0.6, 1.0, and 1.2 μM. Furthermore, AKT phosphorylation was significantly decreased by LY294002 treatment (Figure 3E). ABG-001-induced neurite outgrowth was also reduced by wortmannin (Supplemental Figure 3B). The inhibitory effects of LY294002 were stronger than those of wortmannin. These results suggest that PI3K/AKT may exert an important effect on ABG-001-induced neuronal differentiation of PC12 cells.

Effects of ABG-001 on the Insulin/IGF-1 Signaling Pathway and PC12 Cells Differentiation

To determine the target of ABG-001, we focused on receptors located upstream of PI3K.

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As expected, HNMPA-(AM)3, the specific inhibitor of insulin receptor, slightly attenuated the percentage of neurite-bearing cells from an initial value of 69.4 ± 4.0% to 59.7 ± 4.3% after treatment with 1 μM ABG-001 (Figure 4A, Supplemental Figure 4A). However, the percentage of neurite-bearing cells was reduced from an initial value of 60.9 ± 5.5% to 41.9 ± 3.7% after treatment with the IGF-1 inhibitor AG1024 (Figure 4B, Supplemental Figure 4B). Changes in IGF-1 receptor phosphorylation were further observed after treatment with ABG-001. As shown in Figure 4D and 4E, ABG-001 induces dose-dependent enhancements in IGF-1 receptor phosphorylation, which peaks at 10 min; these enhancements are significantly reduced by AG1024. Furthermore, application of the tyrosine kinase IGF-1 receptor inhibitor T9576 led to significant decrease in the percentage of neurite outgrowth from PC12 cells from 83 ± 2.3% to 42.6 ± 2.3% (Figure 4C, Supplemental Figure 4C). To determine whether the IGF-1 signaling pathway is the primary mechanism by which ABG-001 affects PC12 cells, we examined the effects of IGF-1 on neurite outgrowth of these cells. As expected, IGF-1 significantly enhanced the neurite outgrowth of PC12 cells, just as ABG-001 and NGF did. This neurotrophic effect of IGF-1 on PC12 cells could also be inhibited by the IGF-R inhibitor T9576 (Figure 4F). These results suggest that ABG-001, like IGF-1, targets the IGF-1 receptor and regulates the expression of downstream genes to induce NGF-like effects in PC12 cells.

Effects of Knock-down IGF-1 Receptor by siRNA on Neurite Outgrowth after

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Treatment with ABG-001

To further verify that ABG-001 targets the IGF-1 receptor, we first used FAM-siRNA to determine the best siRNA transfection concentration. Photomicrographs of PC12 cells after transfection with FAM-siRNA are displayed in Figures 5A and 5B. Approximately 90% of the PC12 cells produced fluorescence at 120 nM. Therefore, we used this dose to transfect IGF-1 receptor siRNA into PC12 cells for 6 h and then treated with 1 μM ABG-001. The lengths of neuritis induced by ABG-001 were significantly reduced by IGF-1 receptor siRNA from 48.66 ± 4.17 to 28.22 ± 1.37 μm (Figures.5, C-E). The percentages of neurite outgrowth and gene expression level of IGF-1 receptor were also significantly decreased after treatment with IGF-1R siRNA (Figures.5, F-G). Furthermore, the total protein level of IGF-1 receptor and phosphorylation of IGF-1 receptor were both significantly reduced by treatment with siRNA against IGF-1 receptor (Figure.5, H). At the same time, phosphorylation of AKT and ERK were significantly lowered by siRNA against IFG-1 receptor (Figure 6F). These results are consistent with our earlier observations that ABG-001 acts through the IGF-1 receptor.

Effects of ABG-001 on the PKC, PLC, PKA, JNK, p38, Ras, Raf and NGF Signaling Pathways and PC12 Cell Differentiation

Since PKC is located downstream of PI3K, we investigated the function of PKC in ABG-001-induced neurite outgrowth using various PKC inhibitors (GO6983, GX109203X, and RO318220). RO318220 and GO6983 showed appreciable inhibitory effects (Figure 6, A

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and B, Supplemental Figure 1B). The percentage of neurite-bearing cells decreased from an initial value of 70.1 ± 2.8% to 36.0 ± 7.9% after treatment with RO318220 ($P< 0.01$). Likewise, the percentage of neurite-bearing cells decreased from an initial value of 55.1 ± 3.0% to 23.0 ± 7.9% after treatment with GO6983 ($P< 0.05$). Furthermore, low concentration of NGF could augment the effects of ABG-001 on neurite outgrowth of PC12 cells (Figure 6, C and D). These results show that PKC is involved in PC12 cell differentiation induced by ABG-001, but NGF signaling is not involved in the neurite outgrowth of PC12 cells induced by ABG-001.

In addition, inhibitors of PLC (U73122 and U73343), PKA (H-89), Jun N-terminal Kinase (JNK) (SP600125), p38 MAPK (SB203580), Raf (AZ628), and Ras (S3131, farnesylthiosalicylic acid) were used to investigate whether these signaling pathways are involved in neurite outgrowth mediated by ABG-001 in PC12 cells. Results showed that none of these inhibitors had significant effect on the neurite outgrowth induced by ABG-001. These results verify that the signaling pathways associated with these proteins are not involved in the NGF-mimicking effects of ABG-001.

Discussion

ABG-001 (Figure 1A) is a leading compound derived from the neuritogenic compound gentisides. It possesses a long alkyl chain of 14 carbons, two close hydroxy groups on the benzene ring, and an ester linkage between the ring and alkyl chain. Results of experiments

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aimed at investigating its structure-activity relationships showed that the length of the alkyl chain, number and position of hydroxyl groups on the benzene ring, and the type of linking group between the benzene ring and the alkyl chain had distinct effects on its neuritogenic activities (Luo et al., 2011).

NGF is known to induce both neurite outgrowth and survival by binding and phosphorylating the TrkA receptor in cell membranes (Chao, 2003). Therefore, we used the TrkA receptor inhibitor K252a to examine whether the TrkA signaling pathway is involved in the neurogenic effects of ABG-001. Results indicated that K252a had no inhibitory effect on the neurogenic function of ABG-001 (Figure 1D and Supplemental Figure 1A). This result suggested that the TrkA receptor is not involved.

MEK and ERK1/2 have important functions in controlling gene transcription events leading to proliferation or differentiation of PC12 cells in response to NGF (Traverse et al., 1992; Rubinfeld and Seger 2005). NGF is known to promote PC12 cell survival via ERK1/2 signaling (Vaudry et al., 2002). In the present study, ABG-001 was confirmed to take part in neurite outgrowth of PC12 cells (Figure 2, A-C) and to induce ERK and CREB phosphorylation (Figure 2, D-G). Similar results were obtained when PD98089 was used (Supplementary Figure 2A). These results suggest that neurite outgrowth of PC12 cells after treatment with ABG-001 involves at least in part the MEK/ERK pathway. In addition, we found the early phase of CREB phosphorylation (Supplemental Figure 5). However, the phosphorlation of ERK at thw same time was not observed (data did not shown). At this point,

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maybe other signaling pathways take part in regulation of ABG-001 NGF mimic effects.

The Ras/MAPK (Ras/ERK1/2), PLC-γ, and PI3K/AKT signaling pathways (Kaplan and Miller 1997; Kusunoki et al., 2008) are important for the neurogenic effects of NGF. Specific inhibitors of Ras, PLC, PI3K/AKT, including S3131, farnesylthiosalicylic acid, U73122, U73343, LY294002, and wortmannin, were used in our study. Interestingly, only PI3K inhibitors were found to inhibit the neurogenic effects of ABG-001 (Figure 3, A-C) and phosphorylation of AKT by ABG-001 (Figure 3, C and D). These results indicate that PI3K/AKT-mediated signaling plays an important role in the neurogenic effects of ABG-001.

Insulin/IGF-1 exerts important growth-promoting effects by activating the PI3K/AKT signaling pathway (Cui and Almazan 2007). The biological actions of IGF-1 are mediated by IGF-1 receptor, a member of the receptor tyrosine kinase family that induces dimerization and activates several downstream pathways to transmit proliferative signals after extracellular stimulations (Bibollet-Bahena and Almazan 2009; Chitnis et al., 2008; Annenkov 2009). Our results show that ABG-001 induces IGF-1 receptor phosphorylation and that AG1024, T9576, and IGF-1 receptor siRNA inhibit PC12 cell differentiation induced by ABG-001 (Figures. 4–5). These results suggest that the NGF-mimicking effects of IGF-1 on PC12 cells may be mediated via ABG-001 binding to IGF-1 receptor and thereby activating PI3K and MAPK signaling cascades to induce neuritogenic activities. However, PC12 cell differentiation induced by ABG-001 was not completely inhibited by AG1024, T9576, and siRNA against the IGF-1 receptor. This suggests that other receptors may also contribute to the

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NGF-mimicking effects of ABG-001.

Some studies suggest that aside from the three signaling cascades described above, the stress-activated protein kinase (SAPK) /JNK pathway also affects differentiation (Gelderblam et al., 2004). Therefore, we investigated the possible involvement of the SAPK/JNK pathway in regulating the NGF-mimicking effects of ABG-001 using a specific inhibitor SP600125 and the P38 inhibitor SB203580. Differentiation induced by ABG-001 was only weakly inhibited when PC12 cells were treated with SP600125 and SB203580 (data not shown). These data suggest that the SAPK/JNK pathway does not participate in the neurogenic effects of ABG-001. Previous reports also indicated that cyclic AMP and PKC are involved in neuronal differentiation (Hansen et al., 2000; Kolkova et al., 2000). Therefore, the effects of cyclic AMP-dependent protein kinase A (PKA) and PKC were investigated to explore other possible signaling pathways regulating ABG-001-induced neurite outgrowth of PC12 cells. The inhibitory effects induced by PKC inhibitors were significant (Figure 6A, B), but that induced by H-89, a PKA inhibitor, was not (data not shown). Taking these findings together, we propose that the neuritogenic effect of ABG-001 is mediated by a signaling cascade that follows this order: IGF-1 receptor/PI3K/AKT-PKC/ERK/CREB (Figure 6, E, F).

Notably, the effective concentrations of some protein inhibitors (K252a, AG1024, T9576 and RO318220) used in our study were higher than those described in other reports (Tapley et. al., 1990; Wen et al., 2001; Linder et al., 2007; Alessi et al., 1997). In test experiments, we used the reported concentrations to investigate whether they have inhibitory effects on

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homologous proteins. These inhibitors did not produce inhibition of neurite outgrowth in PC12 cells (data not shown). We note that a few other studies also used higher concentrations of these inhibitors (Pandya et al., 2014; Kuo et al., 2014; Yang et al., 2009). It is possible that differences in the purity and sources of inhibitors account for the apparent discrepancy in the concentrations at which these inhibitors are effective.

In conclusion, we demonstrated that ABG-001 induced time- and dose-dependent phosphorylation of IGF-1 receptor, AKT, ERK, and CREB in PC12 cells. Moreover, inhibition of IGF-1 receptor, PI3K, PKC, and ERK activation by SC205907, LY294002, RO318220, and U0126 inhibitors, respectively, blocked the observed phosphorylation effects. In addition, siRNA against IGF-1 reduced the neurite outgrowth of PC12 cells induced by ABG-001 and IGF-1, like ABG-001, had NGF-mimicking effects. These results indicate that IGF-1 receptor-mediated MAPK activation is essential for ABG-001-induced neurite outgrowth in PC12 cells.

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Authorship contributions

Participated in research design: Qi, Osada

Conducted experiments: Tang, Gao, Cao

Contributed new reagents or analytic tools: Chen

Wrote or contributed to the writing of the manuscript: Xiang, Kawatani

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Footnotes

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There are no conflicts of interest.

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Figure legends

Figure 1 (A) Chemical structure of ABG-001. (B) Neurite outgrowth in PC12 cells of ABG-001 at different concentrations. (C) Percentage of viable PC12 cells after treatment with ABG-001 for 48 h. (D) Effects of TrkA inhibitor on the neurogenic effects of ABG-001 in PC12 cells. Percentage of neurite outgrowth as monitored using a phase-contrast microscope 48 h following ABG-001 treatment. Neurites were identified as cells bearing neurites that were at least twice the cell diameter. (Control: DMSO, 0.5%; Positive control: NGF, 40 ng/ml). *, *** Significantly different from the control group at the same time point at $P< 0.05$ or $P< 0.001$. ### Significantly different from the NGF group at the same time point at $P< 0.001$.

Figure 2 (A) Photomicrographs of PC12 cells after treatment with ABG-001 and U0126 for 48 h: (a) Control (0.5% DMSO), (b) NGF (40 ng/ml), (c) 1 μM ABG-001, (d) 40 ng/ml NGF + 10 μM U0126, and (e) 1 μM ABG-001 + 10 μM U0126. (B) Effect of U0126 on the neurite outgrowth in PC12 cells induced by ABG-001 at 1 μM. (C) The average of neurite length for control group was 0.175 ± 0.025 (μm); NGF at 40 ng/ml, 1.303 ± 0.117***; ABG-001 at 1 μM, 1.587 ± 0.158 ***; NGF + U0126, 0.717 ± 0.054$^{###}$; ABG-001 + U0126, 0.178 ± 0.031$^{\$\$\$}$. ABG-001 induced phosphorylation of ERK1/2 in a (D) dose- and (E) time-dependent manner (the cells treated with each agent for 16 h in dose-dependent experiment). (F) ABG-001 stimulated CREB phosphorylation. NGF was used as a positive

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control, and ERK and GAPDH antibodies were used as loading controls. ***Significantly different from control group at the same time point at $P< 0.001$. ###, $$$ Significantly different from NGF or ABG-001-treated group at $P< 0.001$.

Figure 3 (A) Effects of ABG-001 on the PI3K/AKT signaling pathway in PC12 cells. Morphological changes in PC12 cells after treatment with ABG-001 and LY294002 for 48 h: (a) Control (0.5% DMSO), (b) Positive control (NGF 40 ng/ml), (c) 1 μM ABG-001, (d) 40 ng/ml NGF + 20 μM LY294002, and (e) 1 μM ABG-001 + 20 μM LY294002. (B) The average of length neurite length for control group was 0.190 ± 0.042 (μm); NGF at 40 ng/ml, 1.576 ± 0.276 ***; ABG-001 at 1 μM, 1.486 ±0.231***; NGF + LY294002, 0.717 ±0.054$^{###}$; ABG-001 + LY294002, 0.178 ± 0.031$^{$$$}$. (C) Effects of LY294002 on ABG-001-induced neurite outgrowth. (D, E) ABG-001 stimulated phosphorylation of AKT in a time- and dose-dependent manner (the cells treated with each agent for 30 min in dose-dependent experiment). NGF was used as a positive control, and AKT and GAPDH antibodies were used as loading controls. ***represents significant difference compared with control group at $P< 0.001$. ###, $$$represent significant difference compared with NGF or ABG-001-treated group at $P< 0.001$.

Figure 4 (A, B and C) Effects of insulin/IGF-1 inhibitors on the neurogenic effects of ABG-001 in PC12 cells. Cells were pretreated with 10 μM HNMPA, 0.1 μM AG1024, 0.1μM

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T9576, specific inhibitors of insulin, and IGF-1 inhibitors for 30 min, followed by addition of 1 μM ABG-001. (D) Time- and (E) dose-dependent changes in phosphorylation of the IGF-1 receptor (the samples were got after treated ABG-001 for 5 min). NGF was used as a positive control, and IGF-1 receptor and GAPDH antibodies were used as loading controls. (F) Effects of IGF-1 and T9576 on the neurite outgrowth of PC12 cells. *** Significantly different from the NGF-treated group at the same time point at $P< 0.001$. ###Significantly different from the ABG or IGF-1-treated groups at the same time point at $P< 0.05$ or $P< 0.01$

Figure 5 Effects of IGF-1 receptor siRNA on the neurogenic effects of ABG-001 in PC12 cells. The microphotograph of PC12 cells after transfection FAM-siRNA (A, B) and IGF-1 receptor siRNA (C, D). (E) The change of neurite length in PC12 cells after treated IGF-1 receptor siRNA. (F) The gene expression of IGF-1 receptor after treated IGF-1 receptor siRNA and ABG-001. (G) The neurite outgrowth percentage of PC12 cells after treated IGF-1 receptor siRNA and ABG-001. (H) The western blot analysis for IGF-1 receptor after treating siRNA of IGF-1 receptor. Cells were transfected with Lipofectamine 2000 and 120 nM IGF-1 receptor siRNA for 6 h and then treated with 1 μM ABG-001. The ABG-001 treatment group was used as a positive control. **, *** Significantly different from the ABG-001-treated group at the same time point at $P< 0.01$, $P< 0.001$. #Significantly different from negative siRNA-treated group at the same time at $P< 0.05$.

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Figure 6 (A, B) Effects of PKC inhibitors on the neurogenic effects of ABG-001 in PC12 cells. (C, D) Effect of NGF on the neurogentic effects of ABG-001 in PC12 cells treated for 24 h. (E) Proposed mechanism of ABG-001 in induction of neurite outgrowth in PC12 cells. ABG-001 induces neurite outgrowth through activation of the IGF-1 receptor, PI3K/AKT-PKC, and MAPK/ERK-dependent pathways in PC12 cells (Figures. 2–6). (F) The change in AKT and ERK phosphorylation after treatment with siRNA against IGF-1 receptor. Cells were pretreated with 0.1 μM RO318220 and 5 μM GO6983 specific inhibitors of PKC for 30 min and then administered 0.3 or 1 μM ABG-001. NGF was used as a positive control. ** Significantly different from the control group at the same time point at $P< 0.01$. $^{\#, \#\#,}$ $^{\$\$}$Significantly different from the NGF or ABG-001-treated group at the same time point at $P< 0.05$ or $P< 0.01$.

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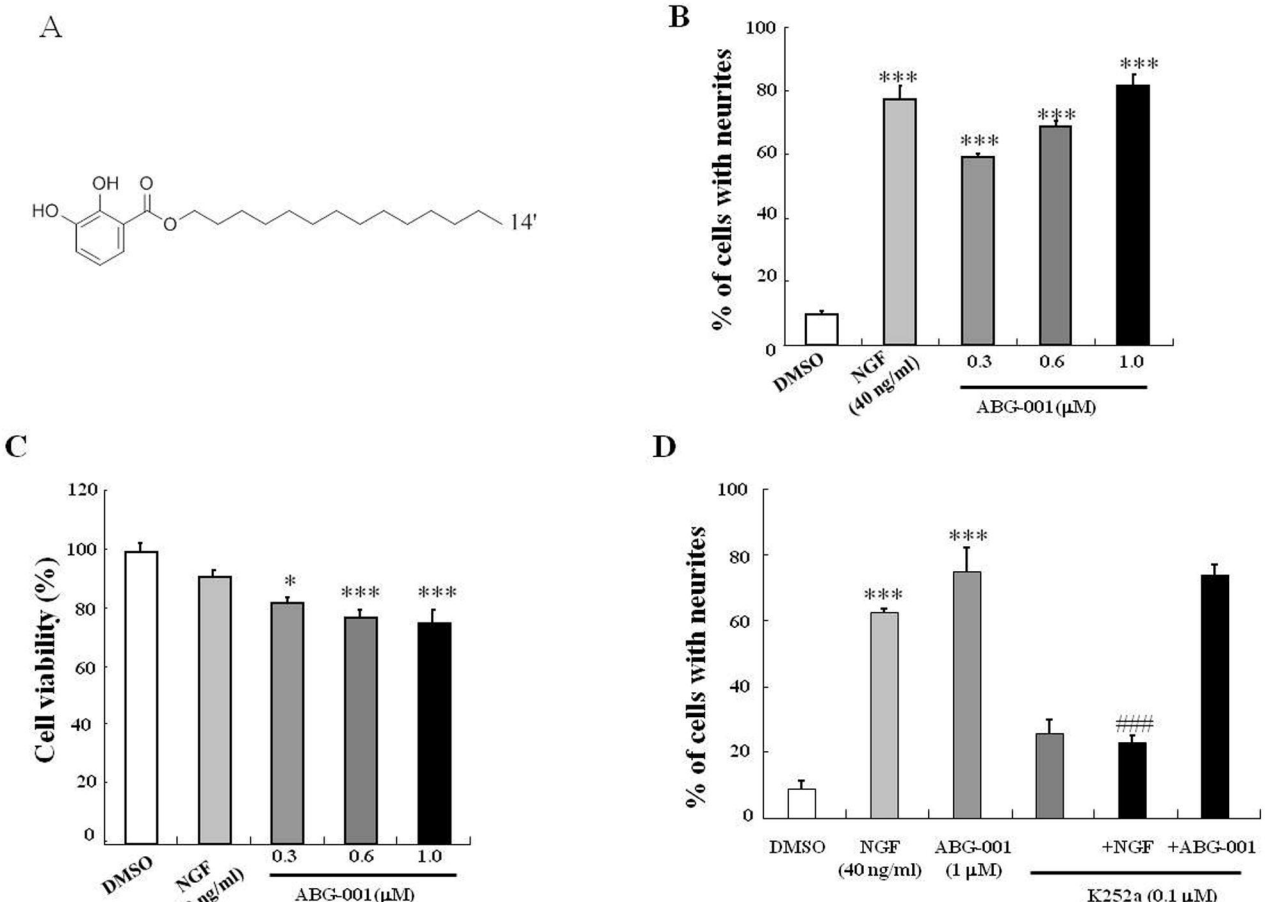
A
OH
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14'
B
% of cells with neurites
100
80
60
40
20
0
***
***
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***
DMSO
NGF (40 ng/ml)
0.3
0.6
1.0
ABG-001(μM)
C
Cell viability (%)
120
100
80
60
40
20
0
*
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DMSO
NGF (40 ng/ml)
0.3
0.6
1.0
ABG-001(μM)
D
% of cells with neurites
100
80
60
40
20
0
***
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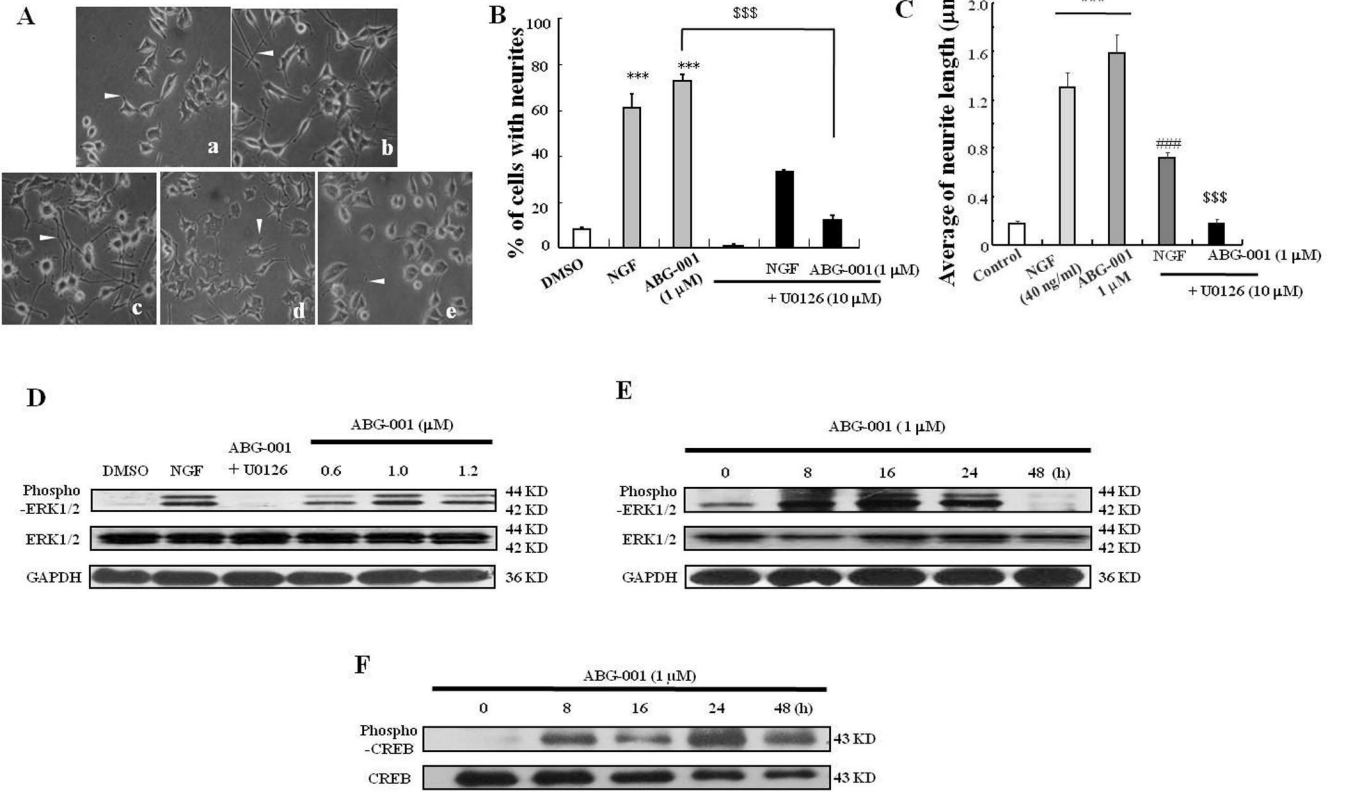
###
DMSO
NGF (40 ng/ml)
ABG-001 (1 μM)
+NGF
+ABG-001
K252a (0.1 μM)

Figure 1


A
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b
c
d
e
B
% of cells with neurites
100
80
60
40
20
0
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***
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DMSO
NGF
ABG-001 (1 μM)
NGF
ABG-001(1 μM)
+ U0126 (10 μM)
C
Average of neurite length (μm)
2.0
1.6
1.2
0.8
0.4
0
***
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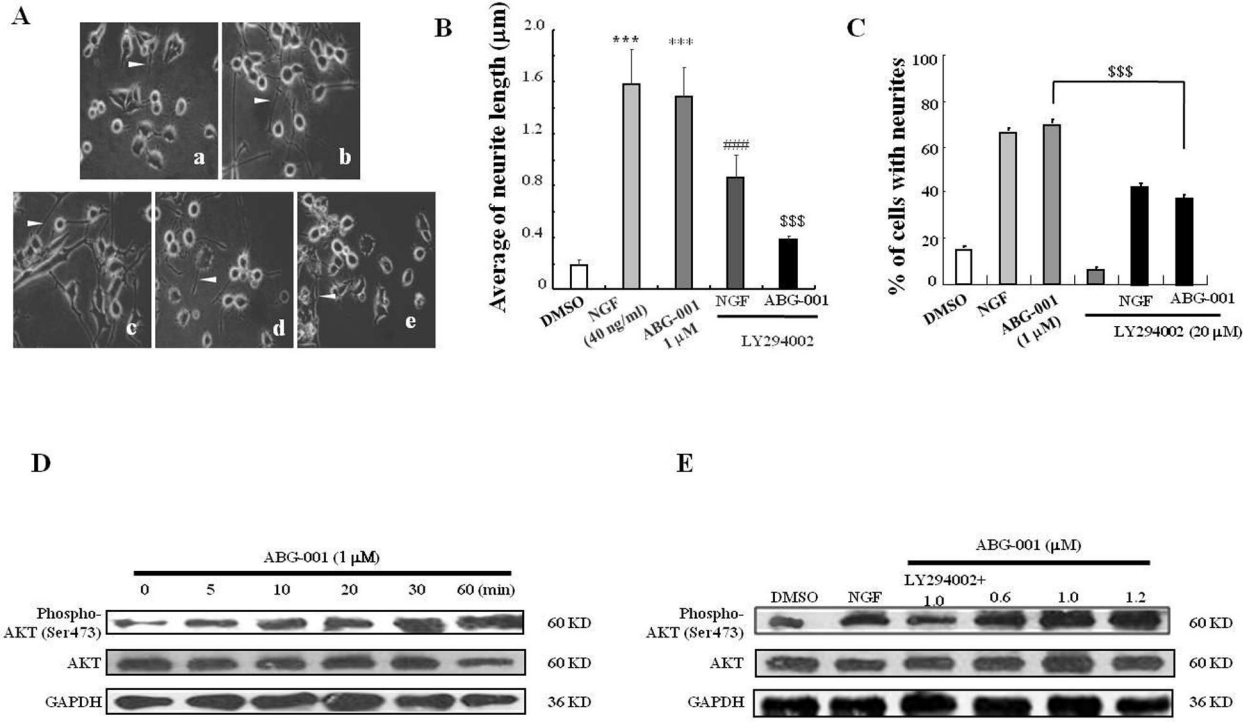
Control
NGF (40 ng/ml)
ABG-001 1 μM
NGF
ABG-001 (1 μM)
+ U0126 (10 μM)
D
ABG-001 (μM)
DMSO
NGF
ABG-001 + U0126
0.6
1.0
1.2
Phospho -ERK1/2
ERK1/2
GAPDH
44 KD
42 KD
44 KD
42 KD
36 KD
E
ABG-001 (1 μM)
0
8
16
24
48 (h)
Phospho -ERK1/2
ERK1/2
GAPDH
44 KD
42 KD
44 KD
42 KD
36 KD
F
ABG-001 (1 μM)
0
8
16
24
48 (h)
Phospho -CREB
CREB
43 KD
43 KD

Figure 2


A
a
b
c
d
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B
Average of neurite length (μm)
2.0
1.6
1.2
0.8
0.4
0
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DMSO
NGF (40 ng/ml)
ABG-001 1 μM
NGF
ABG-001
LY294002
C
% of cells with neurites
100
80
60
40
20
0
$$$
DMSO
NGF
ABG-001 (1 μM)
NGF
ABG-001
LY294002 (20 μM)
D
ABG-001 (1 μM)
0
5
10
20
30
60 (min)
Phospho-AKT (Ser473)
60 KD
AKT
60 KD
GAPDH
36 KD
E
ABG-001 (μM)
LY294002+
DMSO
NGF
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0.6
1.0
1.2
Phospho-AKT (Ser473)
60 KD
AKT
60 KD
GAPDH
36 KD

Figure 3

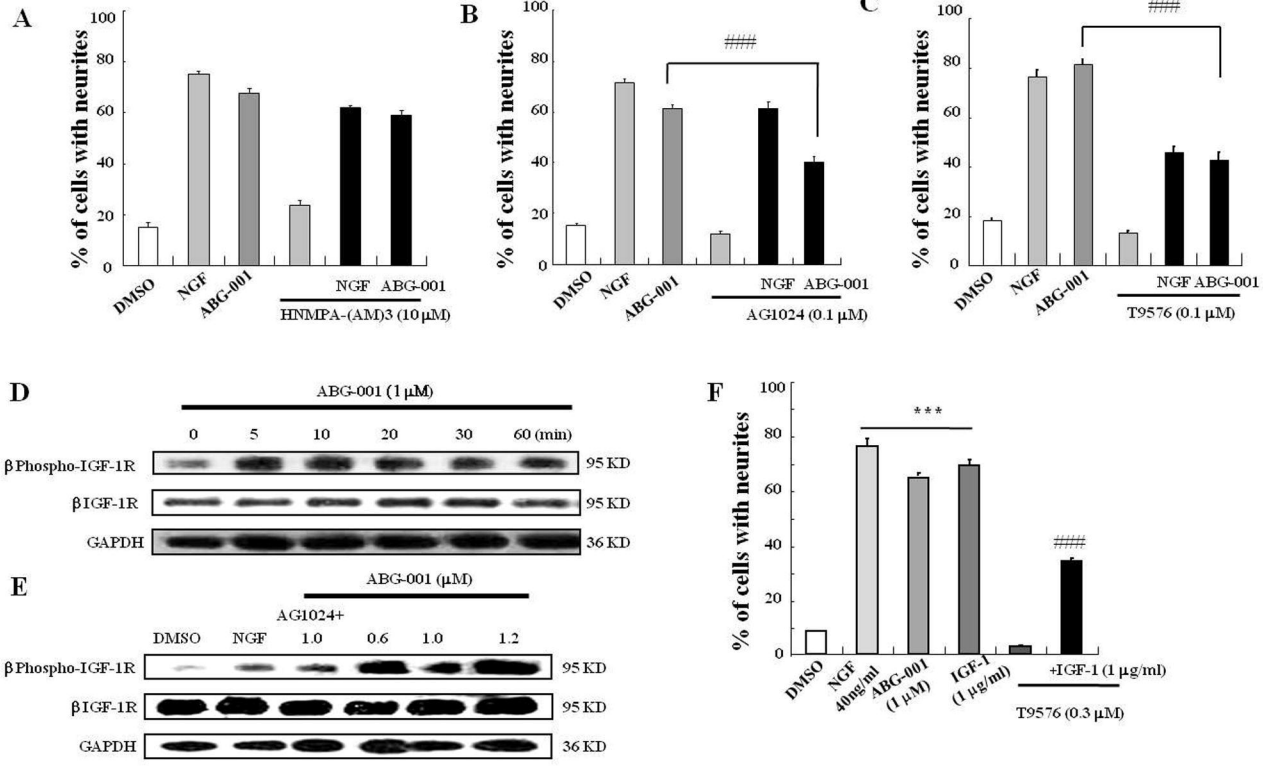
A
% of cells with neurites
DMSO
NGF
ABG-001
NGF ABG-001
HNMPA-(AM)3 (10 μM)
B
###
AG1024 (0.1 μM)
C
T9576 (0.1 μM)
D
ABG-001 (1 μM)
0 5 10 20 30 60 (min)
βPhospho-IGF-1R
βIGF-1R
GAPDH
95 KD
36 KD
E
ABG-001 (μM)
AG1024+
DMSO NGF 1.0 0.6 1.0 1.2
F
***
NGF 40ng/ml
ABG-001 (1 μM)
IGF-1 (1 μg/ml)
+IGF-1 (1 μg/ml)
T9576 (0.3 μM)

Figure 4

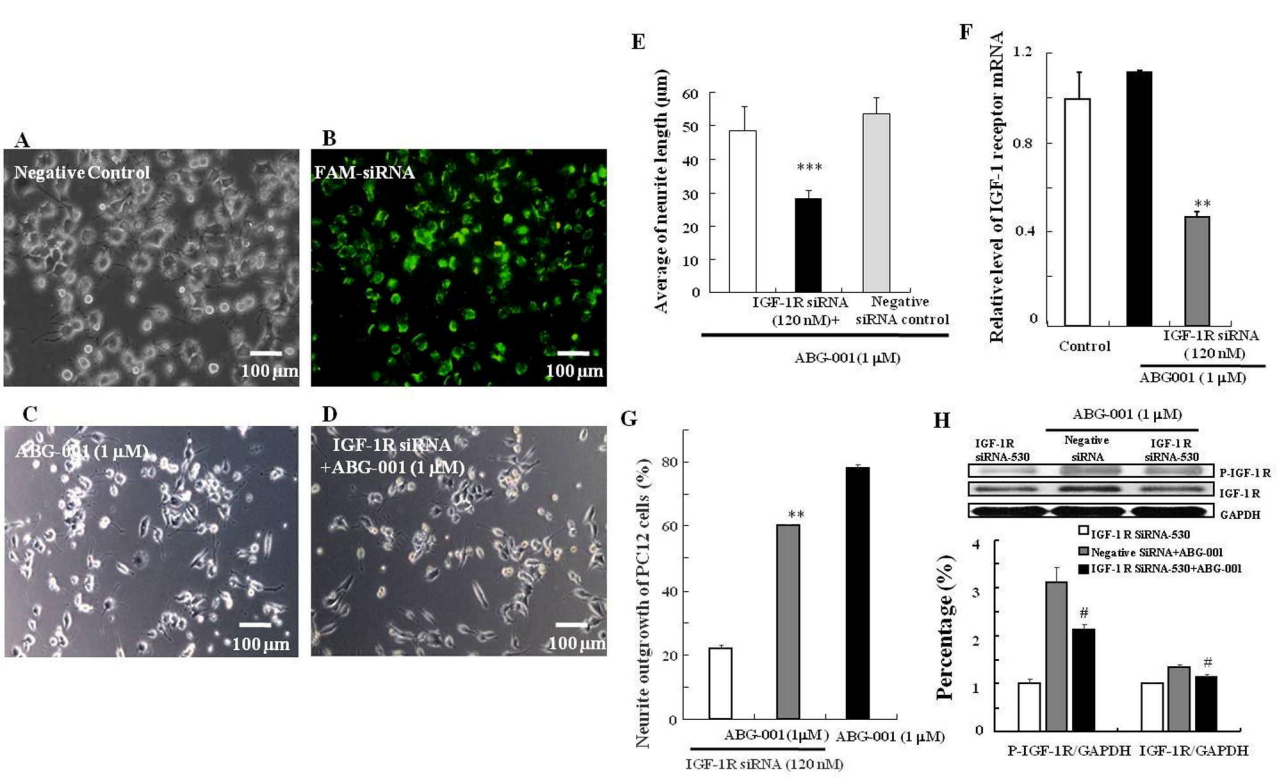
A
Negative Control
100 μm
B
FAM-siRNA
100 μm
C
ABG-001 (1 μM)
100 μm
D
IGF-1R siRNA
+ABG-001 (1 μM)
100 μm
E
Average of neurite length (μm)
60
50
40
30
20
10
0
***
IGF-1R siRNA
(120 nM)+
Negative
siRNA control
ABG-001(1 μM)
F
Relative level of IGF-1 receptor mRNA
1.2
0.8
0.4
0
**
Control
IGF-1R siRNA
(120 nM)
ABG001 (1 μM)
G
Neurite outgrowth of PC12 cells (%)
80
60
40
20
0
**
ABG-001(1μM)
ABG-001 (1 μM)
IGF-1R siRNA (120 nM)
H
ABG-001 (1 μM)
IGF-1R
siRNA-530
Negative
siRNA
IGF-1 R
siRNA-530
P-IGF-1 R
IGF-1 R
GAPDH
IGF-1 R SiRNA-530
Negative SiRNA+ABG-001
IGF-1 R SiRNA-530+ABG-001
Percentage (%)
4
3
2
1
0
#
#
P-IGF-1R/GAPDH
IGF-1R/GAPDH

Figure 5

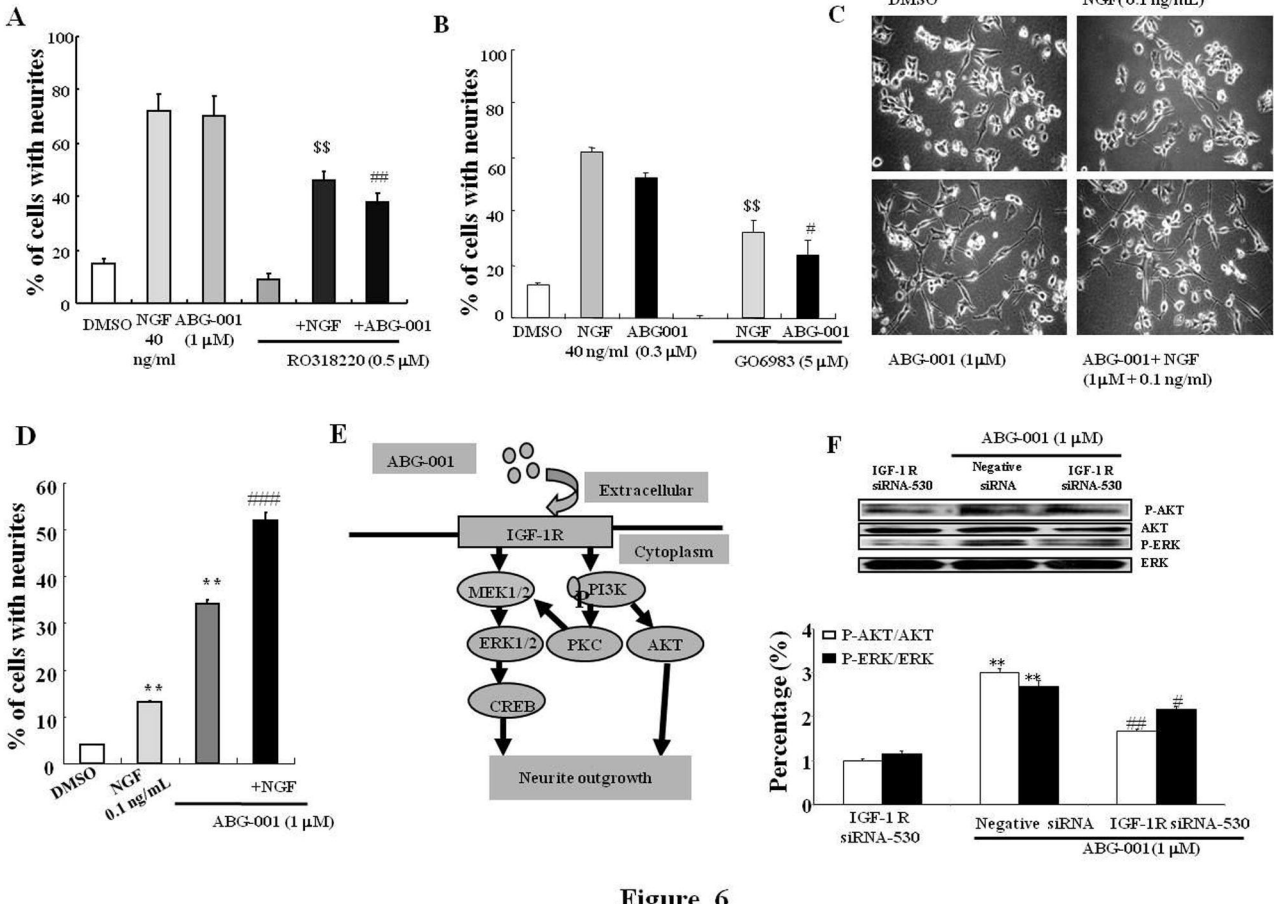
A
% of cells with neurites
DMSO
NGF 40 ng/ml
ABG-001 (1 μM)
+NGF
+ABG-001
RO318220 (0.5 μM)
$$
##
B
% of cells with neurites
DMSO
NGF 40 ng/ml
ABG001 (0.3 μM)
NGF
ABG-001
GO6983 (5 μM)
$$
#
C
DMSO
NGF(0.1 ng/mL)
ABG-001 (1μM)
ABG-001+ NGF (1μM+0.1 ng/ml)
D
% of cells with neurites
DMSO
NGF 0.1 ng/mL
+NGF
ABG-001 (1 μM)
**
**
####
E
ABG-001
Extracellular
IGF-1R
Cytoplasm
MEK1/2
PI3K
ERK1/2
PKC
AKT
CREB
Neurite outgrowth
F
ABG-001 (1 μM)
IGF-1 R siRNA-530
Negative siRNA
IGF-1 R siRNA-530
P-AKT
AKT
P-ERK
ERK
P-AKT/AKT
P-ERK/ERK
Percentage (%)
IGF-1 R siRNA-530
Negative siRNA
IGF-1R siRNA-530
ABG-001(1 μM)

Figure 6