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Effect of brefelamide on proliferation of 1321N1 human astrocytoma cells induced by glial cell line-derived neurotrophic factor

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Malignant gliomas are highly resistant to chemotherapy and radiation and more effective options for treatment are urgently needed. We reported previously that the aromatic amide brefelamide, which is isolated from methanolic extracts of the cellular slime molds *Dictyostelium giganteum* and *D. brefeldianum*, hinders cellular proliferation in a glioma model utilizing 1321N1 human astrocytoma cells. Herein, we examined the mechanisms underlying the inhibition of 1321N1 cell proliferation by brefelamide. Glial cell line-derived neurotrophic factor (GDNF) was found to enhance the rate of proliferation of serum-free cultured 1321N1 cells, but did not affect proliferation in PC12 cells. Brefelamide pretreatment inhibited GDNF-induced cell proliferation and expression of rearranged during transfection (RET). GDNF enhanced the phosphorylation of extracellular signal-regulated kinase (ERK), AKT, and c-jun-N-terminal kinase (JNK); however, brefelamide pretreatment inhibited these effects. Brefelamide also reduced the expression of GDNF mRNA and GDNF secretion. Together, the findings from this study indicate that brefelamide inhibits the proliferation of 1321N1 cell via several mechanisms including reduced GDNF receptor expression and GDNF secretion, and reduced phosphorylation of ERK, AKT, and JNK.

1. Introduction

Glial cells provide support to neurons via neurotrophic factor secretion (Althaus and Richter-Landsberg 2000). Gliomas arising from glial cells are typical tumors of the central nervous system, and they express various growth factors and their corresponding receptors, which together contribute to malignancy (van der Valk et al. 1997). Gliomas are highly resistant to conventional treatments including chemotherapy and radiation, which makes them extremely challenging to treat.

The growth factor glial cell-derived neurotrophic factor (GDNF) promotes embryonic dopaminergic neuronal survival in the midbrain (Lin et al. 1993). GDNF has been shown to be a highly potent trophic factor for spinal motoneurons (Henderson et al. 1994) and central noradrenergic neurons (Arenas et al. 1995). The GDNF family comprises GDNF, artemin, neurturin and persephin, which specifically bind with GDNF family receptor (GFR) α1, GFRα2, GFRα3, and GFRα4, respectively (Lin et al. 1993). GDNF promotes glioma cell proliferation by enhancing expression of proliferating cell nuclear protein and Ki-67 (Qu et al. 2015). Furthermore, a high-level of expression of GDNF and GFRα1 has been demonstrated in human gliomas (Wiesenhofer et al. 2000).

Responses to GDNF are involved in a multicomponent receptor complex which consists of rearranged during transfection (RET) receptor tyrosine kinase and the glycosyl phosphatidylinositol-linked ligand-binding subunit GFRα (Sariola and Saarma 2003). RET has been shown to be expressed in various human cell types and tumors (Runeberg-Roos et al. 2007). Activation of RET receptors by ligand stimulation occurs via dimerization of the receptor and autophosphorylation of intracellular tyrosine residues, which in turn triggers intracellular signaling, mediated mainly through the MEK/extracellular signal-regulated kinase (ERK) and/or phosphatidylinositol-4,5-bisphosphate 3-kinase/AKT pathways (Salvatore et al. 2001; Knauf et al. 2003).

Brefelamide, an aromatic amide, is found in slime molds of the *Dictyostelium* family (Kikuchi et al. 2005). We previously demonstrated that brefelamide blocks 1321N1 human astrocytoma cell proliferation via G2/M arrest (Kikuchi et al. 2005). We also showed that ERK phosphorylation is inhibited by brefelamide via epidermal growth factor (EGF) receptor downregulation (Honma et al. 2009). In a recent study, we showed that brefelamide inhibits the proliferation of 1321N1 cells induced by hepatocyte growth factor (HGF) via a mechanism that involves inhibition of expression of HGF receptors and HGF secretion, and suppression of ERK and AKT phosphorylation (Honma et al. 2016). While the exact mechanisms underlying the inhibition of 1321N1 cell proliferation by brefelamide are not known, the effect may be mediated by growth factors and their activation of ERK and/or AKT (Honma et al. 2009, 2016).

The present study investigated the effect of brefelamide on the 1321N1 cell proliferation induced by GDNF. The findings indicate that brefelamide lowers the phosphorylation of ERK, AKT, and JNK via reduction of RET expression, GDNF mRNA expression, and GDNF secretion, resulting in inhibition of 1321N1 cell proliferation.

2. Investigations and results

2.1. Effects of GDNF on the proliferation in 1321N1 cells

MTT assays were performed to determine the effect of GDNF on proliferation of 1321N1 human astrocytoma and PC12 rat pheochromocytoma cells after the cells had been treated with 10 or 100 ng/ml GDNF for 1 to 2 days. 1321N1 cells survival was enhanced by GDNF treatment concentration-dependently, while PC12 cell survival was unaffected (Fig. 1). Since GDNF has been shown to induce ERK and AKT phosphorylation in various types of cells (Salvatore et al. 2001; Knauf et al. 2003), we analyzed this phenomena in 1321N1 cells. GDNF was found to significantly upregulate ERK and AKT phosphorylation in 1321N1 cells at 5 min (Fig. 2). However, GDNF did not induce phosphorylation of ERK or AKT in PC12 cells (data not shown).

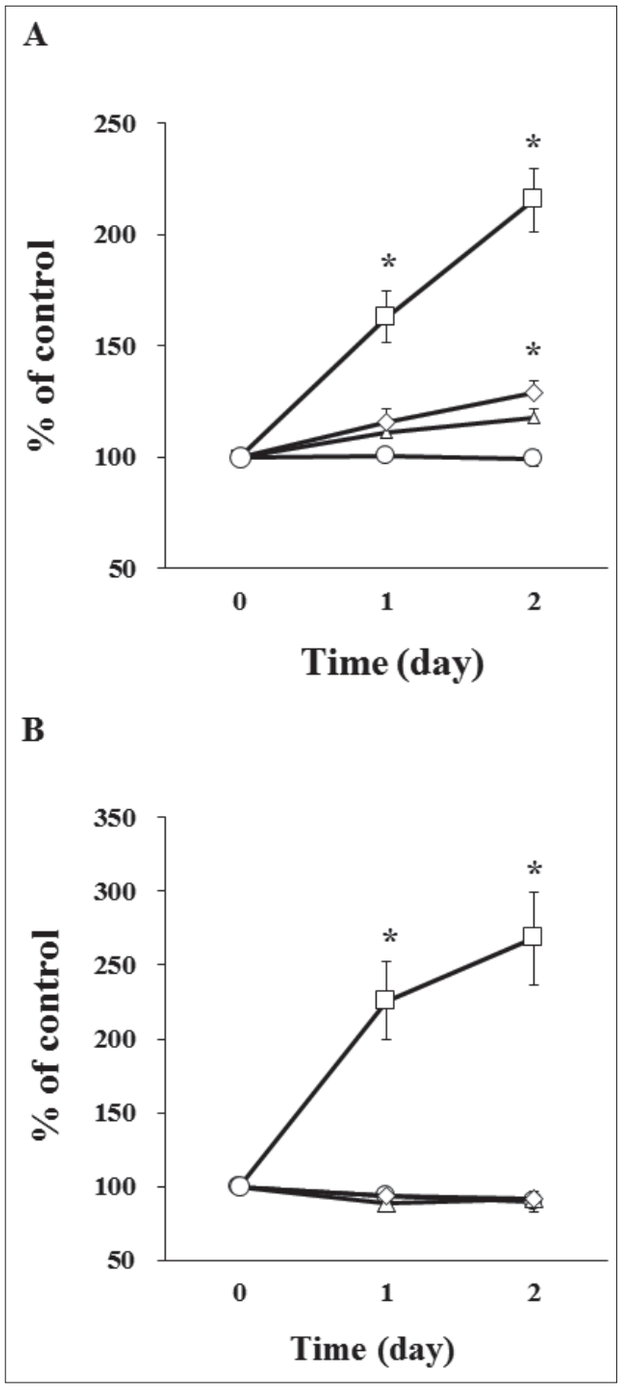

Fig. 1: Effect of GDNF on 1321N1 human astrocytoma cell and PC12 cell proliferation. 1321N1 human astrocytoma cells (A) and PC12 cells (B) were treated with vehicle (circles), 10 ng/ml GDNF (triangles), 100 ng/ml GDNF (rhombuses), or serum as a positive control (squares) for 1 or 2 days. Cell viability was determined by MTT assay. Data points indicate mean ± SEM from 6 separate replications. Asterisks represent a significant difference relative to the vehicle group ($P < 0.05$).

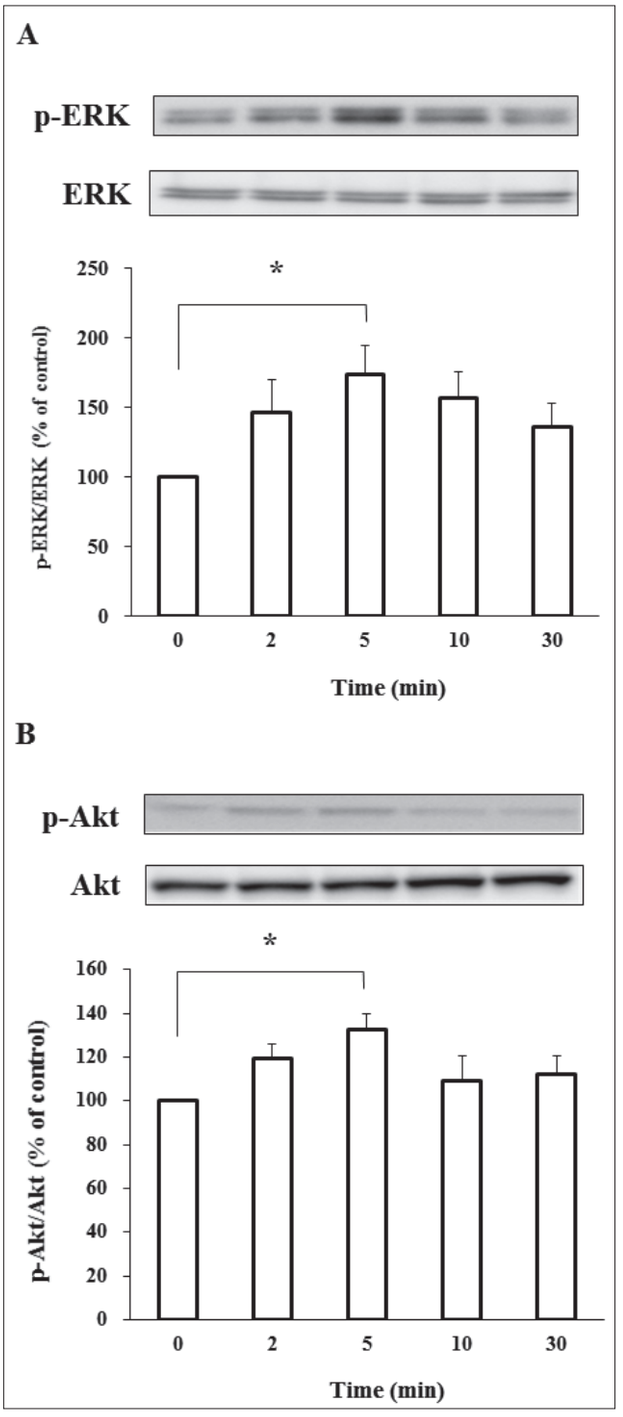

Fig. 2: Effect of GDNF on phosphorylation of ERK and AKT phosphorylation in 1321N1 human astrocytoma cells. Cells were combined with 100 ng/ml GDNF for the time periods shown. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was used to separate cellular proteins and immunoblotting was then carried out using anti-ERK or anti-phospho-ERK antibody (A), or anti-AKT or anti-phospho-AKT antibody (B). Columns indicate the mean ± SEM of 3 or 4 separate replications. Phosphorylation levels were normalized to the total levels of ERK or AKT and are represented as percentage of control. Asterisks represent a significant difference relative to the vehicle group ($P < 0.05$).

2.2. Effects of brefelamide on GDNF-induced proliferation in 1321N1 cells

MTT assays were conducted to investigate the effect of brefelamide (10 μM) on proliferation induced by GDNF following incubation of 1321N1 cells for 24 h before addition of GDNF at a concentration of 10 or 100 ng/ml. Brefelamide was found to inhibit the enhancement of 1321N1 cell viability by GDNF (Fig. 3A). Additionally, phosphorylation of ERK and AKT in 1321N1 cells was inhibited by brefelamide (Fig. 3B and 3C).

2.3. Effects of brefelamide on GFRα1 and RET expression in 1321N1 cells

We then investigated the effect of brefelamide on GFRα1 and RET expression in 1321N1 cells. Treatment of cells with brefelamide for 24 h led to significant inhibition of RET mRNA expression but did not affect expression of GFRα1 mRNA (Fig. 4).

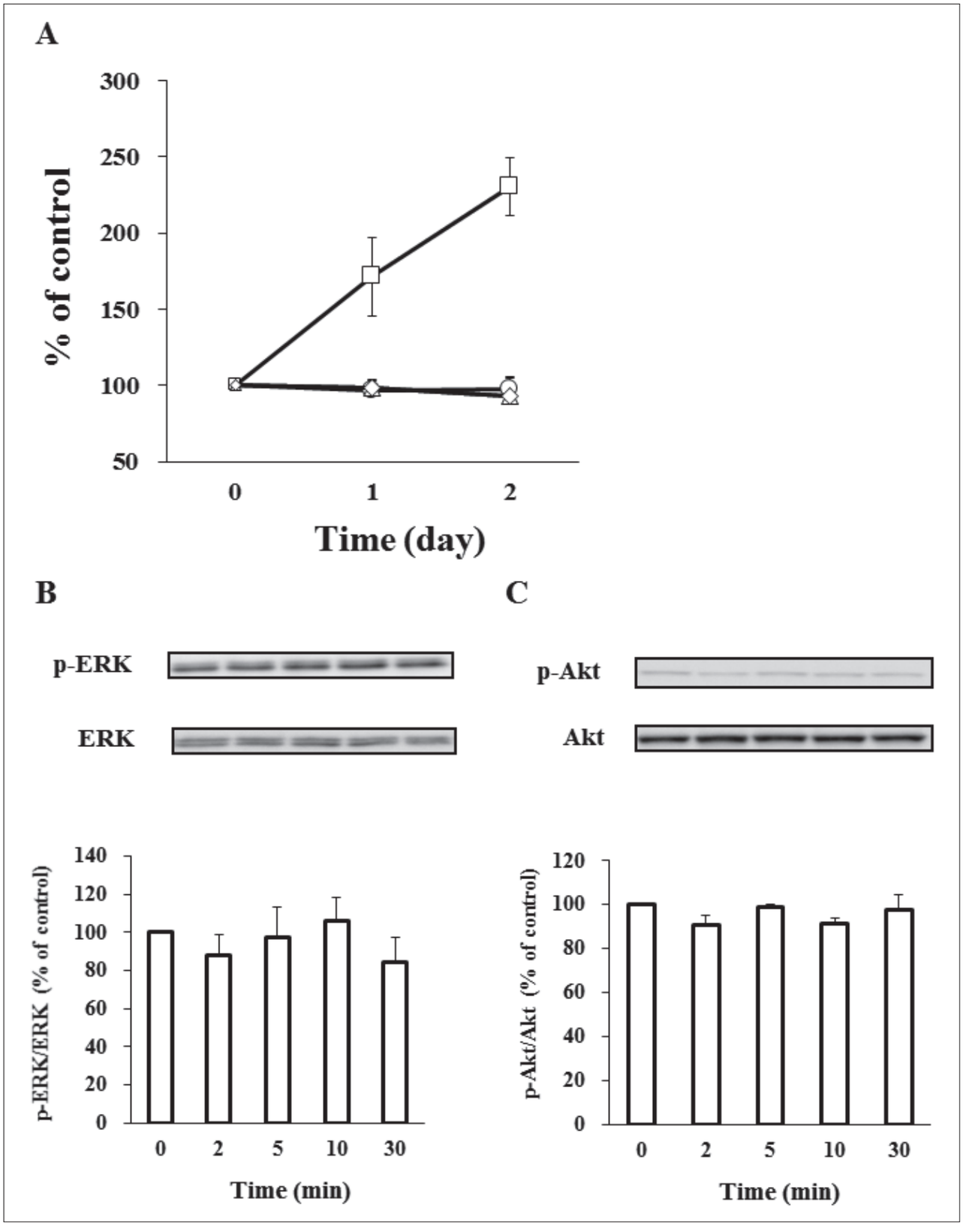

Fig. 3: Effect of brefelamide on cell proliferation, and phosphorylation of ERK and AKT induced by GDNF in 1321N1 human astrocytoma cells. (A) Cells were treated with 10 µM brefelamide and incubated for 24 h prior to being stimulated with GDNF. 1321N1 cells were treated with vehicle (circles), 10 ng/ml GDNF (triangles), 100 ng/ml GDNF (rhombuses), or serum as a positive control (squares) for 1 or 2 days. Cell viability was determined by MTT assay. Data points indicate mean ± SEM from 6 separate replications. Asterisks represent significant differences relative to the vehicle group ($P < 0.05$). Cells were treated with 10 µM brefelamide and cultured for 24 h; they were then stimulated with 100 ng/ml GDNF for the time periods indicated. SDS-PAGE was used to separate cellular proteins and immunoblotting was then carried out using anti-ERK or anti-phospho-ERK antibody (B), or anti-AKT or anti-phospho-AKT antibody (C). Columns indicate mean ± SEM. of 3 separate replications. Phosphorylation levels were normalized to the total levels of ERK or AKT and are represented as percentage of control.

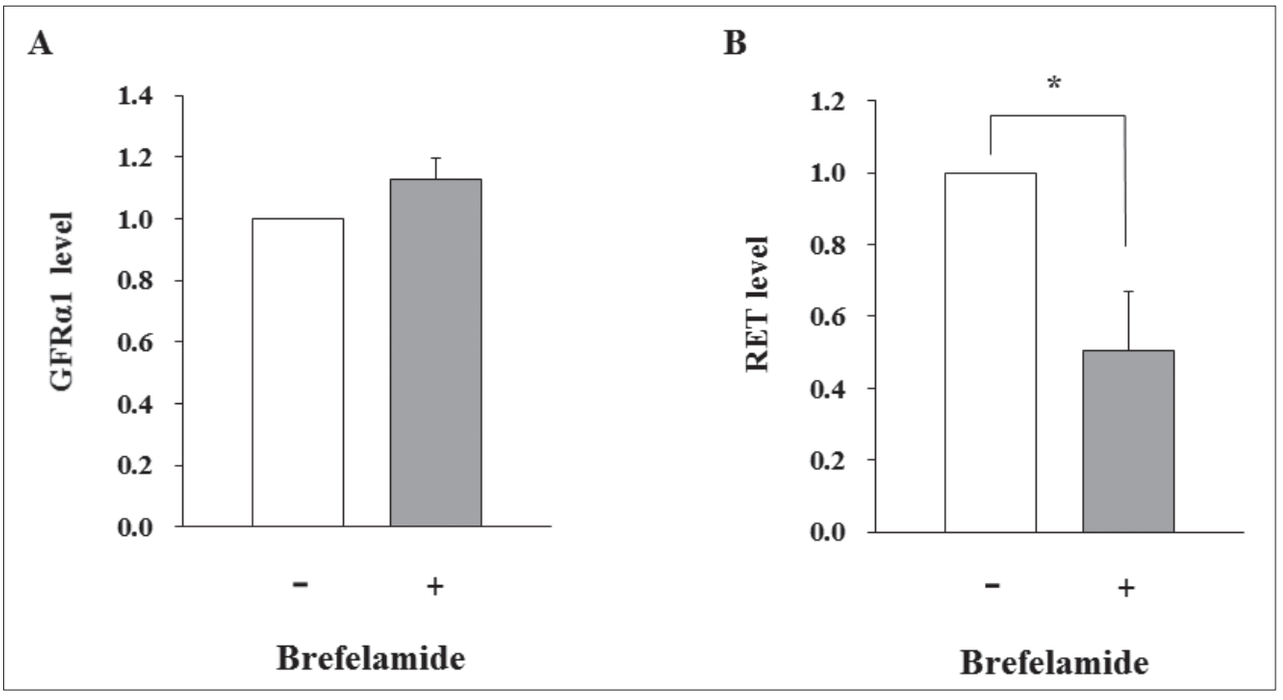

Fig. 4: Effect of brefelamide on expression of GFRα1 and RET mRNA in 1321N1 human astrocytoma cells. Real-time PCR analysis of the expression of GFRα1 (A) and RET (B) mRNAs. Columns indicate mean ± SEM of 3 separate replications. The asterisk represents a significant difference relative to the group not treated with brefelamide ($P < 0.05$).

2.4. Effects of brefelamide on GDNF-induced JNK phosphorylation in 1321N1 cells

Given that inhibition of RET activity has been shown to block JNK phosphorylation (Zhou et al. 2015), we examined the induction of JNK phosphorylation by GDNF. We found that GDNF led to a significant increase in JNK phosphorylation in 1321N1 cells at 5 min (Fig. 5A). Additionally, JNK phosphorylation induced by GDNF in 1321N1 cells was inhibited by brefelamide (Fig. 5B).

2.5. Effects of brefelamide on expression of GDNF mRNA and secretion of GDNF protein in 1321N1 cells

Real-time PCR and ELISA were conducted to assess GDNF mRNA levels and secretion of GDNF protein in 1321N1 cells incubated with brefelamide. Brefelamide treatment led to significant suppression of both the levels of GDNF mRNA and secretion of GDNF protein (Fig. 6).

3. Discussion

The present study showed suppression of GDNF-induced proliferation by brefelamide in a model of glioma utilizing 1321N1 human astrocytoma cells. It is assumed that the effect of brefelamide is mediated via several mechanisms including reduced expression of RET and secretion of GDNF secretion, together with reduced phosphorylation of ERK, AKT, and JNK in 1321N1 cells.

In a previous study, we found that brefelamide blocks 1321N1 cell proliferation (Honma et al. 2009). We therefore investigated the influence of brefelamide on proliferation of 1321N1 cells induced by GDNF, which is known to promote the differentiation, survival, and organogenesis of a number of cell types. We found that GDNF led to upregulation of 1321N1 cell proliferation, but did not affect PC12 cell proliferation. Blockade of GDNF downstream signaling has been shown to impair neurite outgrowth in PC12 cells (Zhou et al. 2013). It can therefore be surmised that treatment with GDNF stimulates neurite outgrowth, indicative of differentiation as opposed to proliferation of PC12 cells.

GDNF has been shown to stimulate ERK and AKT phosphorylation in various types of cells (Salvatore et al. 2001; Knauf et al. 2003). ERK has been shown to control cell cycle progression, while AKT is known to play a role in cell survival and proliferation (Brazil and Hemmings 2001). Futami and Sakai (2009) reported that proliferation of NB-39-nu neuroblastoma cells is enhanced by GDNF via ERK and AKT activation. In our study, GDNF treatment was found to enhance both the survival of cells as well as ERK and AKT phosphorylation at 5 min in 1321N1 cells. In addition, brefelamide was found to inhibit cell proliferation and phosphorylation of ERK and AKT induced by GDNF. Dihydroartemisinin, which is closely related chemically to artemisinin, an anti-malarial drug, has been shown to inhibit the proliferation of glioma cells, an effect which occurs in parallel with ERK and AKT inactivation (Du et al. 2015). Therefore, ERK and AKT inhibition would likely be a highly effective strategy for the treatment of gliomas.

The present study showed that RET mRNA expression is inhibited by brefelamide. Recent studies have identified several promising therapeutic molecules that act as specific inhibitors of GDNF receptors in gliomas. Cabozantinib, which is a potent RET inhibitor, suppresses medullary thyroid cancer tumor cell growth in both in vivo and in vitro models (Bentzien et al. 2013). Vandetanib, which inhibits vascular endothelial growth factor receptor, RET receptor tyrosine kinase, and EGF receptor, has been shown to inhibit proliferation of glioblastomas in isolated cells (Yiin et al. 2010) animal models (Navis et al. 2011), and human clinical trials (Broniscer et al. 2010). Herein, we discovered that treatment with brefelamide significantly reduced the expression of GDNF mRNA and secretion of GDNF. Activation of RET by GDNF has been shown to induce expression of GDNF (Cerchia et al. 2006), which suggests that RET inhibition by brefelamide could be an effective strategy for the treatment of brain tumors. A previous study demonstrated that JNK and Src activation is required for the migration of olfactory ensheathing cells induced by GDNF/Ret (Cao et al. 2006). We therefore investigated whether phosphorylation of JNK is induced by GDNF in 1321N1 cells. GDNF led to a significant increase in the phosphorylation of JNK, while

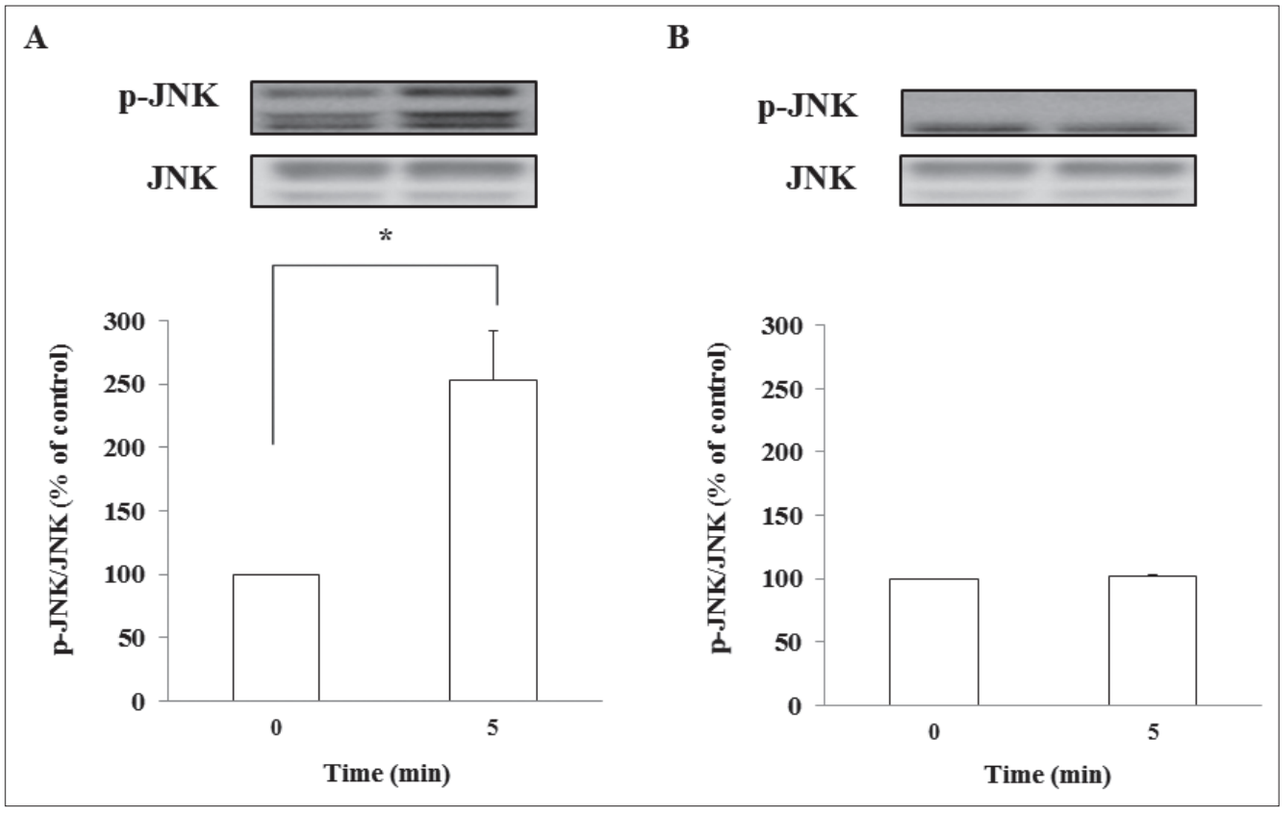

Fig. 5: Effect of brefelamide on phosphorylation of JNK induced by GDNF in 1321N1 human astrocytoma cells. Cells were treated without (A) or with (B) 10 μM brefelamide, cultured for 24 h, and stimulated with 100 ng/ml GDNF for 5 min. SDS-PAGE was used to separate cellular proteins and immunoblotting was then carried out using anti-JNK or anti-phospho-JNK antibodies. Columns indicate mean ± SEM of 4 separate replications. Phosphorylation levels were normalized to the total levels of JNK and are presented as percentage of control. The asterisk represents a significant difference relative to the vehicle group ($P < 0.05$).

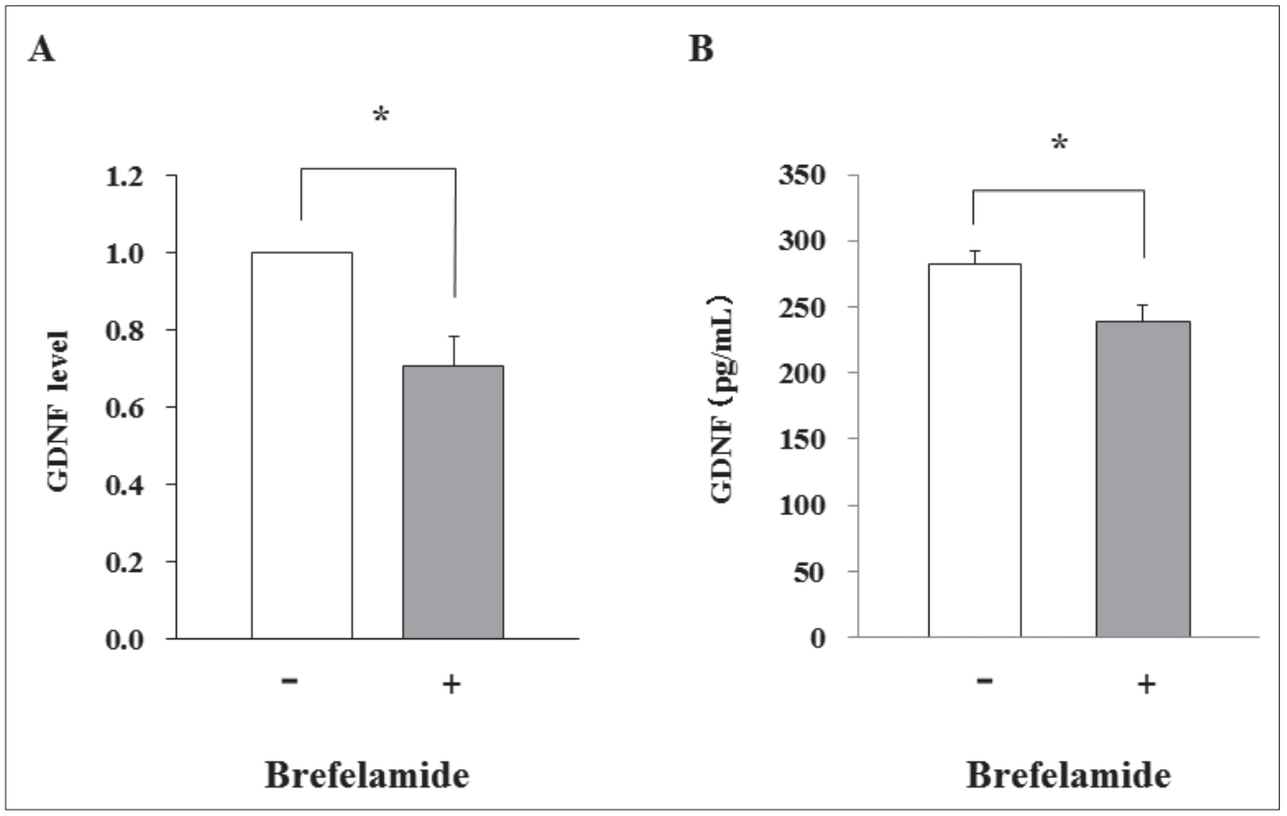

Fig. 6: Effect of brefelamide on secretion of GDNF in 1321N1 human astrocytoma cells. (A) Real-time PCR analysis of GDNF mRNA expression. Columns indicate mean ± SEM of 6 separate observations. The asterisk represents a significant difference relative to the group not treated with brefelamide ($P < 0.05$). (B) Expression of GDNF protein analyzed by ELISA. Columns indicate mean ± SEM of 5 separate observations. Asterisks represent a significant difference relative to the group not treated with brefelamide ($P < 0.05$).

the phosphorylation of JNK induced by GDNF was inhibited by brefelamide. Vandetanib is known to downregulate the Rho GTPase-JNK pathway via suppression of RET activity (Zhou et al. 2015). Additionally, in breast cancer cells JNK inhibition has been shown to reduce G2/M transition, proliferation, and apoptosis (Mingo-Sion et al. 2004). We demonstrated previously that treatment with brefelamide hinders 1321N1 human astrocytoma cell proliferation as a result of G2/M arrest (Kikuchi et al. 2005). Consequently reduced phosphorylation of JNK in 1321N1 cells may be an important mechanism underlying the suppression of glioma progression by brefelamide.

The present study demonstrated that treatment with brefelamide leads to inhibition of 1321N1 human astrocytoma cell proliferation induced by GDNF and lowers the expression of RET, and GDNF mRNA, secretion of GDNF, and phosphorylation of ERK, AKT, and JNK. Additional studies are necessary to clarify the exact mechanism by which brefelamide inhibits the proliferation of 1321N1 cells. Nevertheless, brefelamide may represent an effective treatment option for the therapy of gliomas.

4. Experimental

4.1. Brefelamide synthesis

Synthesis of brefelamide [*N*-(3-(2-amino-3-(4-hydroxyphenoxy)phenyl)-3-oxopropyl)-4-hydroxybenzamide] was carried out according to methods described previously (Kikuchi et al. 2005). The compound was dissolved in a stock solution of 50 mM dimethyl sulfoxide, which was then diluted with complete growth medium to the required concentration before use (final DMSO concentration: <0.1%).

4.2. Cell culture

1321N1 human astrocytoma cells were grown in DMEM with 5% FBS, 50 U/ml penicillin, and 50 mg/ml streptomycin in a humidified incubator with 5% CO_2 (Honma et al. 1999). PC12 rat pheochromocytoma cells were cultured in DMEM with 10% FBS, 5% horse serum, 50 U/ml penicillin, and 50 mg/mL streptomycin.

4.3. MTT assay

MTT assays were carried out as reported previously (Honma et al. 2016). 1321N1 and PC12 cells were seeded in 96-well plates (density = 2.5×10^4 cells/mL). After seeding for 2 days, the medium was substituted with serum-free DMEM, and the cells were re-incubated for 24 h in the presence or absence of 10 µM brefelamide, followed by GDNF treatment (Wako Pure Chemicals). MTT was then added to the wells and the cells were incubated for an additional 4 h at 37 °C. A plate reader (Sunrise, Tecan, Switzerland) was then used to measure absorbance at 590 nm.

4.4. Western blotting

Western blotting was carried out according to previously reported methods (Honma et al. 2006). Protein-transferred polyvinylidene difluoride membranes were incubated with 0.5% dry skim milk in Tris-buffered saline with Tween 20 for 2 h and subsequently incubated with anti-ERK, anti-phospho-ERK, anti-AKT, anti-phospho-AKT, anti-JNK, or anti-phospho-JNK antibodies. Membranes were then incubated with anti-rabbit horseradish peroxidase-linked immunoglobulin G (Cell Signaling Technology Inc.) at a 1:3000 dilution for 2 h at 25 °C . The density of bands was measured with Image Gauge software (Fujifilm). Data represent the ratio of phosphorylated to total ERK, AKT, or JNK.

4.5. Real-time RT-PCR

Real-time RT-PCR was carried out as reported previously (Honma et al. 2016). The following primer pair sequences were used: human *GFRα1*, 5′- TCGGGCAATACACACCTCTGT -3′-(sense) and 5′- CTTGGAGGAGCAGCCATTGA -3′ (antisense) (Bottner et al. 2013); human *RET*, 5′- AAGGAGATGGCAAAGGGATCAC -3′ (sense) and 5′- TTGATGTCTTGGGTCTCCACAA -3′ (antisense) (Bottner et al. 2013); human *GDNF*, 5′- TTTAGGTACTGCAGCGGCTCTT -3′ (sense) and 5′- TCACTCACCAGCCTTCTATTTCTG -3′ (antisense) (Narantuya et al. 2010); human glyceraldehyde-3-phosphate dehydrogenase (*G3PDH*), 5′- ACCACAGTCCATGCCATCAC-3′ (sense) and 5′- TCCACCACCCTGTTGCTGTA -3′ (antisense). The expression of target genes was normalized to the expression of *G3PDH* gene.

4.6. Enzyme-linked immunosorbent assay

GDNF secretion from cultured 1321N1 cells was quantified using a Human GDNF ELISA Kit (Sigma-Aldrich). Analyses were carried out according to the manufacturer's instructions. All samples were tested in duplicate.

4.7. Statistical analysis

Results are expressed as mean ± standard error of the mean (SEM). Significant differences between values were analyzed using Student's *t*-test for two-sample comparisons, and multiple comparisons were performed using one-way ANOVA with Dunnett's test. Statistical significance was defined as a *P*-value less than 0.05.

Conflicts of interest: None declared.

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