Inhibition of JNK Potentiates Temozolomide-induced Cytotoxicity in U87MG Glioblastoma Cells *via* Suppression of Akt Phosphorylation

VAN ANH VO[1*], JAE-WON LEE[1*], HEE JAE LEE[1], WANJOO CHUN[1], SO YOUNG LIM[2] and SUNG-SOO KIM[1]

[1]*Department of Pharmacology, School of Medicine, Kangwon National University, Chuncheon, Kangwon, Republic of Korea;*
[2]*Department of Anesthesiology and Pain Medicine, College of Medicine, Hallym University, Chuncheon, Republic of Korea*

Abstract. *Glioblastoma (formally glioblastoma multiforme, GBM) represents both the most common and most malignant variant among numerous of primary brain tumors. Temozolomide (TMZ) has been used for the treatment of glioblastoma. However, less than 1/3 of glioblastomas respond to TMZ-based therapies. Therefore, strategies to enhance the effect of TMZ are needed for more effective targeted therapeutics. Stress-activated protein kinases (SAPKs) JNK and p38 MAPK have been known to have apoptotic or anti-apoptotic effects depending on cell type and condition. On the other hand, Akt is a key regulator of cellular survival and has direct effects on the apoptosis machinery. In addition, it was discovered that Akt activation is primed by the activity of JNK. We, therefore, examined whether inhibition of JNK or p38 potentiates TMZ-induced apoptosis in U87MG cells via inhibition of Akt activation. TMZ significantly induced Akt activation as well as JNK or p38 activation. Inhibition of JNK suppressed Akt activation and potentiated TMZ-induced cytotoxicity. The phosphorylation of GSK-3β and Bad, the downstream mediators of Akt, was also suppressed by the inhibition of JNK. The present data strongly suggest that there may be a crosstalk between JNK pathway and Akt pathway in glioblastoma and that further investigation based on the present data may provide a valuable approach for enhancing TMZ-induced cytotoxicity in glioblastoma.*

Temozolomide (TMZ) is a DNA-alkylating agent and the most frequently used anti-glioma agent. However, the objective response rate in malignant glioma was 30% for newly-diagnosed patients and 11-25% for patients with progressive disease (1). In order to increase the response rate of malignant glioma to TMZ, it is necessary to understand the molecular mechanism of its resistance.

JNK has been established as a key kinase in death or survival of tumor cells. It has anti-apoptotic or pro-apoptotic effects, depending on the cell type and condition. When glioblastoma cells were treated with TMZ, JNK mediated the survival of glioblastoma cells through c-Jun-related responses (2, 3). Moreover, JNK can contribute to TMZ's resistance of glioblastoma cancer stem cells by controlling O-6-metnylguanine DNA methyltransferase (MGMT) expression (17). Similarly, the p38 pathway can also provide glioblastoma cells with TMZ's resistance by linking DNA mismatch repair to the G2 check point (12).

Akt, a family of serine/threonine-specific protein kinases, plays a key role in cell survival. Approximately 15 to 40% of glioblastoma multiforme shows mutation or loss of expression of the tumor suppressor gene *PTEN*, the negative regulator of Akt signaling (14). Thus, elevated activity of Akt may lead to failure of chemotherapy. Although Akt activity is mainly regulated by the phosphatidylinositol 3-kinase, JNK is also involved in the regulation of Akt activity, and therefore, phosphorylation of Akt at Thr450 by JNK primes Akt's activation (20) and phosphorylated Thr-Pro motifs of Akt on Thr450 are critical for the maintenance of Akt stability and activity (15). Another study revealed that the phosphorylation of Stat3 at Ser727 by JNK contributed to Akt activation in response to arsenic exposure and induced malignant transformation (16). Our previous study also showed that the

*These Authors contributed equally to this study.

Abbreviations: TMZ, Temozolomide; MAPK, mitogen-activated protein kinase; PARP, poly ADP ribose polymerase; PTEN, phosphatase and tensin homolog; JNK, c-Jun N-terminal kinase.

Correspondence to: Sung-Soo Kim, MD, Ph.D., Department of Pharmacology School of Medicine, Kangwon National University, 1 Kangwondaehak-gil, Chuncheon, Kangwon, 200-701, South Korea. Tel: +82 332508851, e-mail: ksslsy@kangwon.ac.kr

Key Words: Temozolomide, JNK, p38, Akt, glioblastoma, cytotoxicity.

levels of Akt activation are up-regulated following treatment with an anti-cancer drug, such as actinomycin D in primary astrocyte cells, which was attenuated by the JNK inhibition (data not shown). These reports suggest that, in glioblastoma, activation of Akt may be thought of as a feature against anti-cancer drugs with a possible crosstalk between the JNK and Akt pathways where the Akt activity may be regulated by manipulation of JNK. Therefore, the JNK pathway can be a target for the sensitization of glioblastoma to chemo-therapeutic methylating agents such as TMZ.

In the present study, we revealed that TMZ induced Akt activation, while inhibition of JNK resulted in suppression of Akt activation and subsequent increased apoptotic cell death in U87MG glioblastoma cells.

Materials and Methods

Cell culture and drug treatment. U87MG glioblastoma cells were obtained from the Korean Cell Line Bank (KCLB), cultured in Dulbecco's modified Eagle's medium (DMEM; GIBCO BRL, Grand Island, NY, USA) plus 10% fetal bovine serum (FBS; Gibco BRL), 100 U/ml penicillin, 100 mg/ml of streptomycin (GIBCO BRL, Grand Island, NY, USA) at 37˚C in 5% CO_2. The JNK inhibitor (SP600125) and p38 inhibitor (SB203580) were purchased from Tocris Bioscience (Ellisville, MO, USA) and Calbiochem (Billerica, MA, USA), respectively. TMZ was purchased from Sigma Aldrich (St. Louis, MO, USA). In all experiments, cells were incubated in the presence of the indicated concentration of JNK or p38 inhibitors (10 μM) for 24 h in serum-free medium. With medium changes, TMZ (100 μM) and inhibitors were added to the medium and further incubated for 72 h.

Cell viability by MTT assay. U87MG glioblastoma cells were plated into 12-well culture plates and incubated at 37 ˚C under a 5% CO_2/95% humidified air incubator for 24 h. The cells were subsequently incubated with the JNK or p38 inhibitor for 24 h prior to the treatment with TMZ for another 72 h. Cell viability was determined using MTT 3-(4.5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay, which yields a blue formazan product in living cells, but not in dead cells or their lytic debris. Finally, cells were washed with PBS, dissolved in DMSO and absorbance was determined at 540 nm. The results were expressed as a percentage of surviving cells over control cells.

Western blot analysis. U87MG glioblastoma cells were pretreated with the JNK or p38 inhibitor for 24 h and challenged with TMZ. Cells were washed with PBS and lysed with lysis buffer [50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.02% sodium azide, 0.1% sodium dodecyl sulfate (SDS), 1% NP-40, 0.5% sodium deoxycholate and 1 mM phenyl methylsulfonyl fluoride]. Samples from these cell lysates were denaturated and resolved on 10% SDS-polyacrylamide gels and transferred to Hyond PVDF membrane (Amersham Biosciences, Piscataway, NJ, USA). Blots were blocked by incubation with 5% skim milk in TBST for 1 h at room temperature. Specific antibodies against p-Akt (Ser473), p-Akt (Tyr308), p-Akt (Thr450), Akt, p-GSK3β, GSK3β, p-Bad, Bad, cleaved PARP, cleaved caspase-3, p-JNK, JNK (Cell Signaling Technology, Inc.), p-p38, p38 (Santa Cruz Biotechnology Inc.), p-c-Jun, c-Jun, p-MAPKAP2, MAPKAP2 (Cell Signaling Technology, Inc.) and β-actin (Sigma-Aldrich, St. Louis, MO, USA) were diluted in 5% skim milk. After thoroughly washing with TBST, horse-radish peroxidase-conjugated secondary antibodies were applied. The blots were developed by enhanced chemi-luminesence detection (Amersham Biosciences).

Apoptosis analysis by flow cytometry. Cells were treated as indicated, harvested by trypsinization and stained with PE Annexin V and 7-Amino-Actinomycin (7-AAD) to measure cell apoptosis using the PE Annexin V Apoptosis Detection Kit I (BD Biosciences Pharmigen, address). Briefly, treated cells were washed twice with cold PBS, dissociated and resuspended in 1× Binding Buffer at a concentration of 1×10^6 cells/ml. One hundred μl of solution was transferred to a 5 ml culture tube and 5 μl of PE Annexin V and 7-AAD were added. The cells were gently vortexed and incubated for 15 min at room temperature (RT) in the dark. Each tube received 400 μl of 1× Binding Buffer. Stained cells were analyzed with FACS Calibur (supplier, address). At least 10,000 events were collected for each sample.

Statistical analysis. All data are given as mean±standard deviation (SD). Statistical significance was analyzed by two-tailed Student's *t*-test. Data with values of $p<0.05$ were considered as statistically significant. Single (*) and double (**) marks represent statistical significance in $p<0.05$ and $p<0.01$, respectively.

Results

TMZ increases phosphorylation of Akt, JNK and p38 in U87MG glioblasoma cells. Given the fact that Akt plays a key role in cell survival, the levels of Akt were examined in TMZ-treated U87MG glioblastoma cells. As shown in Figure 1A, TMZ increased phosphorylation of Akt in U87MG glioblastoma cells.

JNK has been reported to be involved in the regulation of death or survival of tumor cells. To investigate whether TMZ affects the activation of JNK, the levels of JNK phosphorylation were examined using western blotting. Phosphorylation of JNK was strongly induced with TMZ treatment in U87MG glioblastoma cells (Figure 1B). Similarly, TMZ also resulted in phosphorylation of p38 (Figure 1C).

Inhibition of JNK or p38 suppresses TMZ-induced Akt phosphorylation and its downstream activation in U87MG glioblastoma cells. Since the present data indicated that TMZ increased phosphorylation of JNK and p38 in U87MG glioblastoma cells, and JNK was reported to be involved in the regulation of Akt activity, a further study was carried-out to determine whether inhibition of JNK or p38 has any effect on Akt phosphorylation in TMZ-treated U87MG glioblastoma cells. As shown in Figure 2A, TMZ increased Akt phosphorylation at all three sites such as Serine 473, Tyrosine 308 and Threonine 450, which are needed for Akt's complete activity. However, inhibition of JNK or p38 significantly

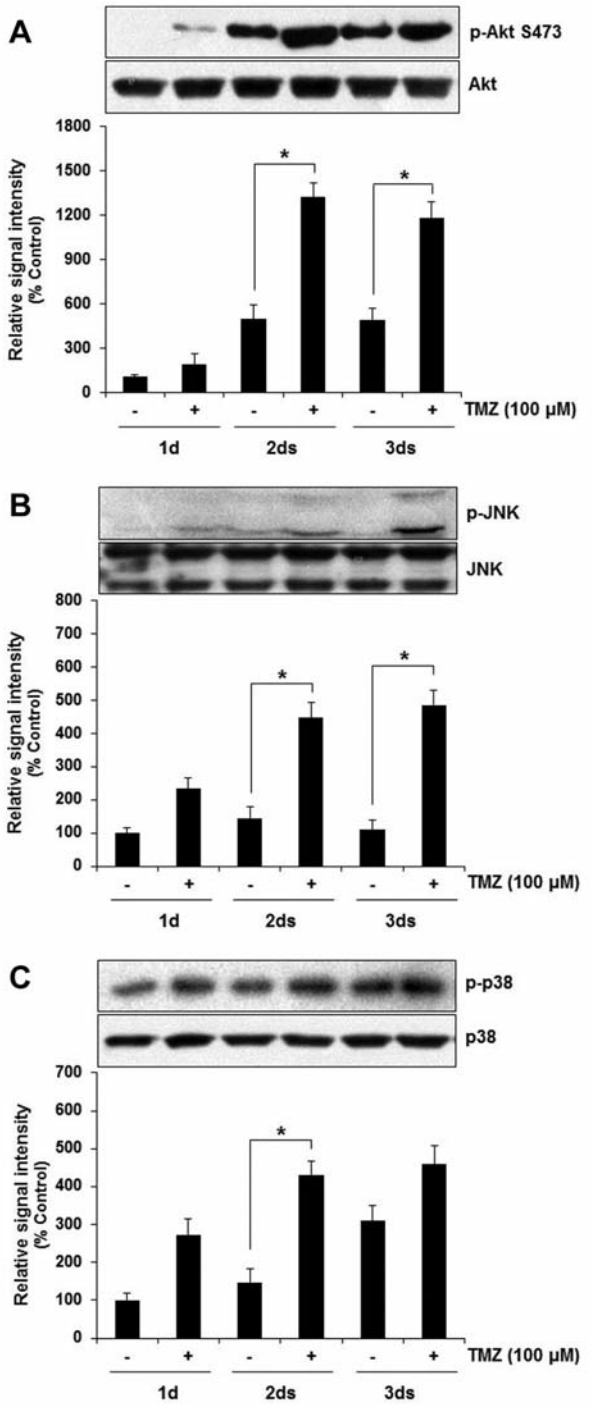

Figure 1. *TMZ increased phosphorylation of Akt, JNK and p38 in U87MG glioblastoma cells. Cells were treated with 100 μM of TMZ for 24, 48 and 72 h (A, B, C). Phosphorylations of Akt, JNK and p38 were analyzed by western blotting at each time point. Akt, JNK and p38 were used as internal standards for p-Akt, pJNK and p-p38, respectively. Quantitation of each protein was performed by densitometric analysis and phosphorylation level was normalized against the density of internal standard to obtain a relative signal density, which was expressed as % of control. Data are expressed as mean±S.D. *p<0.05 and **p<0.01 indicate statistically significant differences compared to control alone.*

attenuated TMZ-induced phosphorylation of Akt at all three sites in U87MG glioblastoma cells (Figure 2A).

Glycogen synthase kinase-3 (GSK3)-β and Bad are downstream mediators of Akt. Bad is a Bcl-2 family member and the first component of the apoptotic machinery found to be phosphorylated by Akt. The Ser-136 phosphorylation sites play an important role in regulating Bad function (5). To confirm whether inhibition of JNK or p38 suppresses Akt activation, the levels of p-GSK3-β and p-Bad were examined. As shown in Figure 2B, inhibition of JNK or p38 significantly suppressed phosphorylation of GSK3-β and Bad.

JNK inhibitor (SP600125) and p-38 inhibitor (SB203580) specifically inhibited their own targets (Figure 2C). No noticeable cell death and phosphorylation of Akt were observed up to 10 μM of each inhibitor (data not shown).

Inhibition of JNK and p38 enhances TMZ-induced cytotoxicity in U87MG glioma cells. To evaluate the effects of inhibition of JNK or p38 in TMZ-induced U87MG glioblastoma cell death, cells were treated with JNK or p38 inhibitor for 24 h prior to TMZ treatment and further incubated for 72 h. TMZ treatment resulted in slightly attenuated cell viability in U87MG cells. However, inhibition of JNK significantly potentiated TMZ-induced cytotoxicity in U87MG cells (Figure 3A). Inhibition of p38 appeared to potentiate TMZ-induced cytotoxicity, although not statistically significant. Any noticeable cell death was not observed with 100 μM of TMZ, 10 μM of SP600125 and SB203580, respectively (data not shown).

Since cleaved PARP and cleaved caspase-3 have been reported to be apoptotic markers, the levels of these proteins were measured by western blot analysis. Treatment with TMZ did not cause any noticeable changes in the levels of cleaved PARP and cleaved caspase-3. However, addition of JNK or p38 inhibitors significantly increased the production of these proteins (Figure 3B). We further examined, using FACS analysis, whether inhibition of JNK or p38 increases apoptosis in TMZ-treated U87MG cells. FACS data clearly showed that apoptosis was noticeably increased when JNK inhibition preceded TMZ treatment (Figures 3C, D).

Discussion

Methylating agents, such as TMZ, are important compounds in the treatment of human gliomas, although only a limited percentage of gliomas respond to TMZ-based therapies. Even when a patient is treated with TMZ and radiation therapy, the median survival period is about 15 months (21). It is interesting to notice that most high-grade human gliomas have high levels of Akt activation, which is a consequence of *PTEN* deletion. It is believed that elevated Akt phosphorylation possibly functions as a mechanism of resistance to anticancer drugs. Particularly, Akt activity

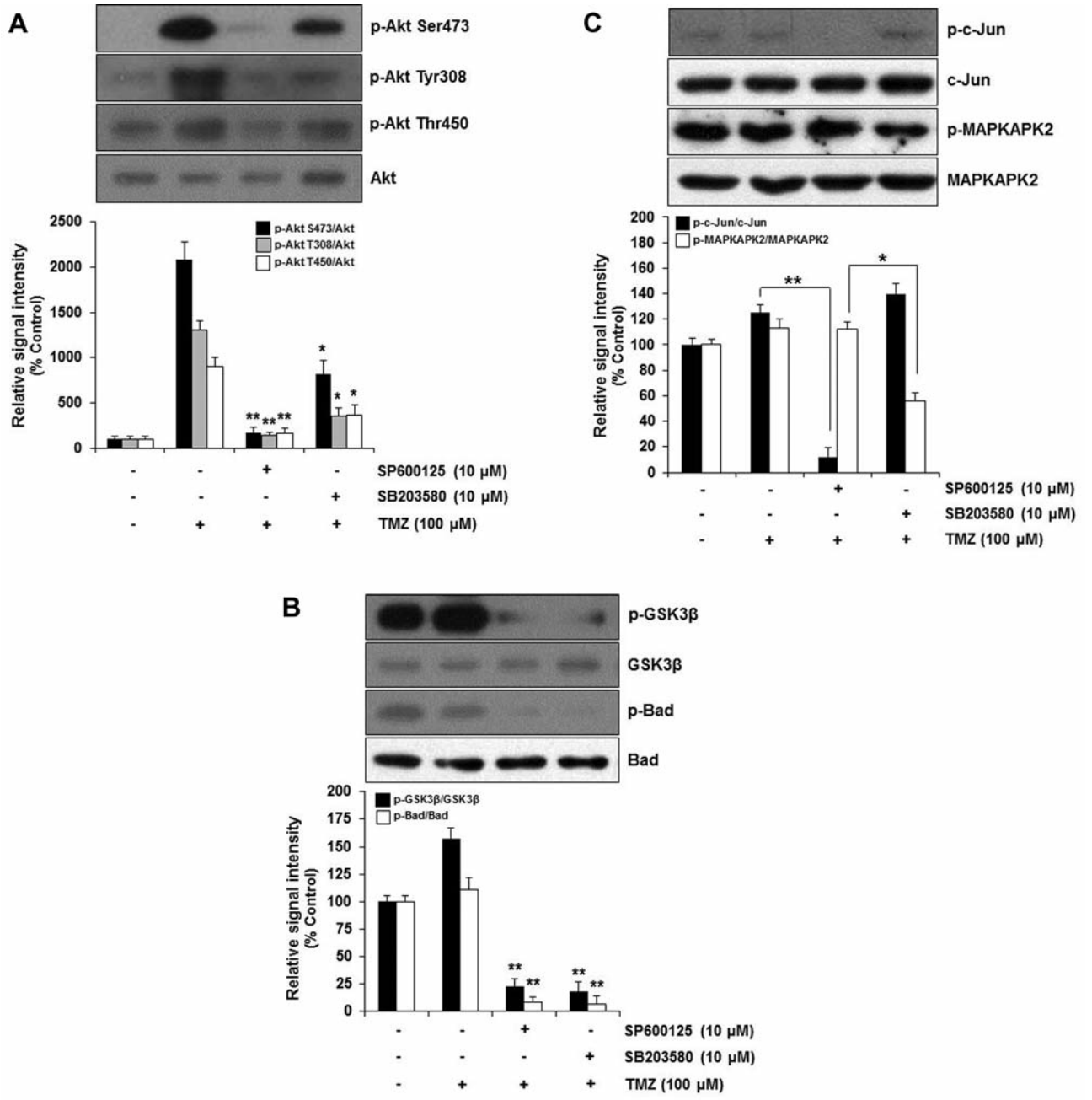

Figure 2. *Inhibition of JNK or p38 suppressed phosphorylation of GSK-3β and Bad, as well as activation of Akt. Cells were treated with 10 μM of SP600125 and SB203580 for 24 h prior to TMZ treatment (100 μM) and further incubated for 48 h. (A, B). The protein levels of p-AKT (Ser473, Thr308 and Thr450), p-Bad and p-GSK3β were determined by western blot analysis. Akt, GSK-3β and Bad were used as internal controls. Quantitation of each protein was performed by densitometric analysis and phosphorylation level was normalized against the density of internal standard to obtain a relative signal density, which was expressed as % of control. Data are expressed as mean±S.D. *p<0.05 and **p<0.01 indicate statistically significant differences from treatments with TMZ alone.*

enforced by cellular stress, like anticancer drug treatment, may attenuate cytotoxicity of anti-cancer drugs (2). In our previous study, Akt activation was increased with actinomycin D in primary astrocytes (data not shown). In the present study, Akt activation was also up-regulated in U87MG glioblastoma cells by TMZ treatment in a time-dependent manner (Figure 1A). Thus, TMZ treatment acted clearly as a kind of cellular stress (Figures 1B, 1C) and Akt

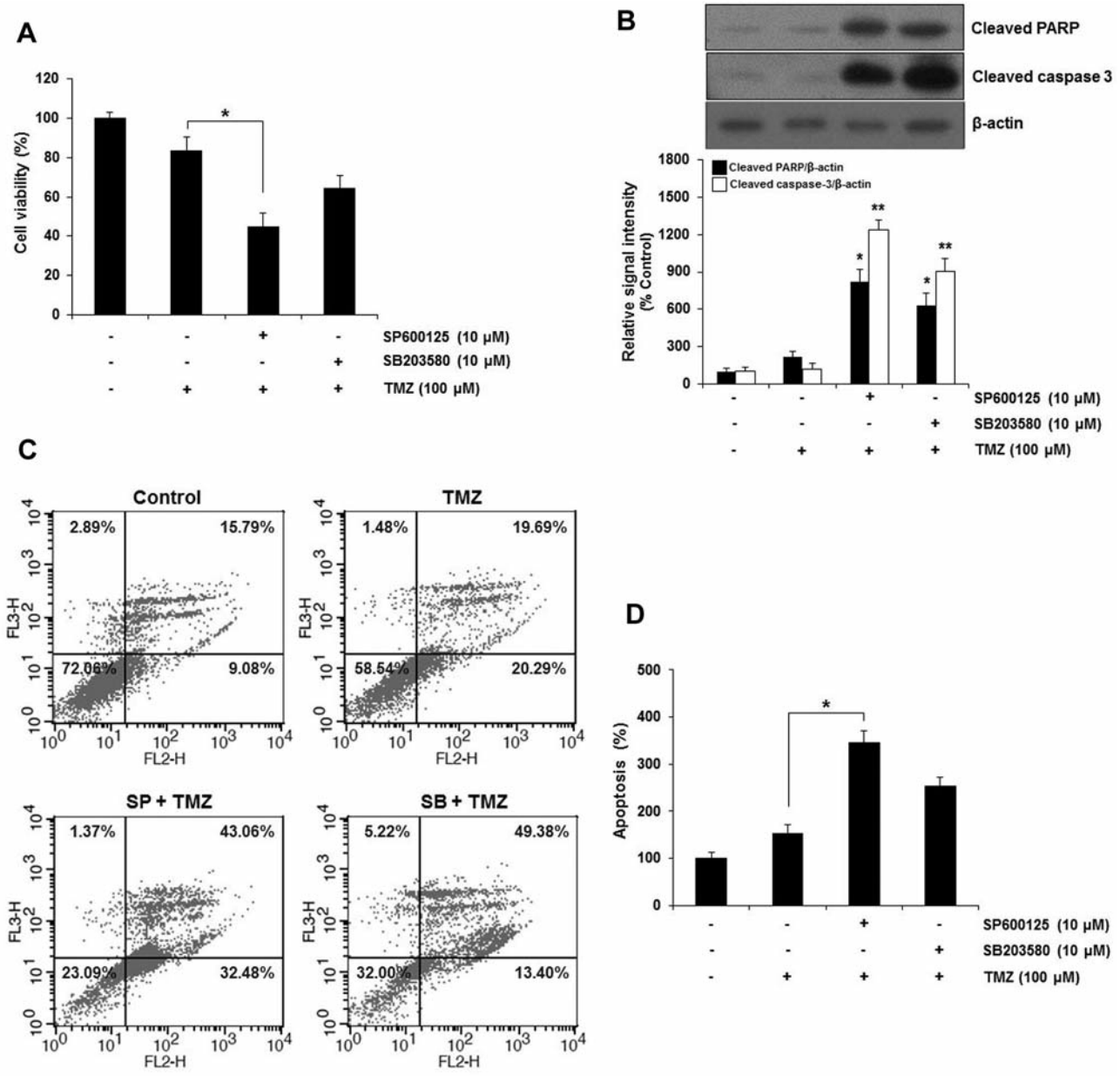

Figure 3. *TMZ-induced apoptosis was enhanced by inhibition of JNK or p38 in U87MG glioblastoma cells. Cells were treated with inhibitors of JNK or p38 prior to TMZ treatment and further incubated for 72 h. (A) Cells were treated with 10 μM of JNK or p38 inhibitor for 24 h prior to TMZ treatment (100 μM) and further incubated for 72 h. Cell viability was measured using the MTT assay. (B) The protein levels of cleaved PARP and cleaved caspase-3 were determined by western blot analysis. β-actin was used as an internal control. (C, D) FACS assay was carried-out to determine the apoptotic cell death. Significant apoptotic cell death was observed with inhibition of JNK or p38 in TMZ-induced U87MG glioblastoma cells. Data are expressed as mean±S.D. *p<0.05 and **p<0.01 indicate statistically significant differences from treatments with TMZ alone.*

activation may be used as a defense mechanism of glioblstoma cells against anti-cancer drugs.

Akt is a family of serine/threonine-specific protein kinases, coded by 3 genes *Akt1*, *Akt2* and *Akt3*, activated through phosphorylation by phosphoinositide 3-OH kinase (PI3-kinase) at four sites (Ser473, Thr308, Thr450 and Ser124) (5). Ser473 and Thr308, required for Akt activity, are inducibly phosphorylated after treatment of cells with extracellular stimuli. In contrast, Thr450 and Ser124 appear to be basally phosphorylated independently of cell stimulation or PI3K activation and most likely render Akt competent to undergo activation at both Thr308 and Ser473, which requires PI3K

upon exposure of cells to extracellular stimuli (5). Although phosphorylation of Akt at Thr450 by JNK primes Akt's activation in heart cells, there are reports showing that JNK was excluded as a kinase for Thr450-Pro phosphorylation (13) and that Thr308 and Ser473 phosphorylation was not dependent on Thr450 or Ser124 (10). Therefore, the observation that JNK mediates Akt phosphorylation on Thr450 might be cell type-specific. The present study showed that the inhibition of JNK attenuated Akt phosphorylation at all three sites; Ser473 and Tyr308, which are essential for its activity, and Thr450, which plays a role in the priming Akt activation at other sites (Figure 2A). As Thr450 phosphorylation is essential for the other two sites of phosphorylation and full activity of Akt in cardiomyocytes (20), it is reasonable to think that, in U87MG glioblastoma cells, JNK inhibition also suppresses Thr450 phosphorylation in Akt, resulting in reduced phosphorylation of both Ser473 and Thr308. Inhibition of p38 with a 10 μM concentration of SB203580 could suppress Akt activation by inhibiting phosphorylation in Thr450, which is a site for the phosphorylation of JNK. Considering that isoforms of JNK2 of a molecular mass of 55,000 are the principal active JNK isoforms present in primary glial tumors (22) and that JNK2/3 can be inhibited by SB203580 (3, 4), then a 10 μM concentration of SB203580 seems to be enough for inhibition of Thr450 phosphorylation of Akt, but not enough for inhibition of c-Jun phosphorylation (Figure 2C).

Reduced activity of Akt by JNK inhibition was further confirmed by monitoring the phosphorylation of GSK-3β and Bad. Akt was found to directly phosphorylate and inactivate GSK3, which regulates a number of substrates involved in glycogen synthesis, as well as proliferation (5). Akt also phosphorylates Bad, the pro-apoptotic Bcl-2-related protein, one of the first direct downstream targets of Akt-mediated cell survival (6-8). As expected, inhibition of JNK down-regulated phosphorylation of GSK3β and Bad at their specific sites for phosphorylation by Akt, which are Ser9 and Ser136, respectively (Figure 2B).

Because the activation of JNK could suppress cell death in a glioma cell line by c-Jun related response and Akt can protect cells lacking Bad expression (5, 9, 18), it is considered that Bad is not the only target for the survival effects of JNK activity in this study. It is however, clear that reduced cell viability (Figure 3A), decreased Bad phosphorylation (Figure 2B), increased apoptotic markers such as cleaved-PARP and cleaved-caspase3 (Figure 3B) and increased apoptosis (Figures 3C, 3D) imply strong activation of an intrinsic apoptotic pathway, which was triggered by the interference with the Akt signaling pathway via JNK inhibition.

As it is evident from the literature, concerning the investigation of finding target pathways for increasing the chemotherapeutic effect in glioblastoma treatment, inhibition of the Akt signaling pathway can result in potentiation of anticancer drug activity (11, 19). This study revealed that JNK inhibition can be a tool for the regulation of Akt pathway's activity, which can be used to increase apoptotic cell death in glioblastoma cells. Taken together, the elucidation of interaction between the Akt pathway and other signaling pathways, including JNK pathway, might be necessary for enhancing the anticancer effect of TMZ for the treatment of glioblastoma.

Acknowledgements

This work was supported by the 2013 research grant from Kangwon National University (120131314).

References

1 Hand book of Brain tumor chemotherapy. 31.
2 Clark AS, West K, Streicher S and Dennis PA: Constitutive and inducible Akt activity promotes resistance to chemotherapy, trastuzumab, or tamoxifen in breast cancer cells. Mol Cancer Ther *1*: 707-717, 2002.
3 Clerk A and Sugden PH: The p38-MAPK inhibitor, SB203580, inhibits cardiac stress-activated protein kinases/c-Jun N-terminal kinases (SAPKs/JNKs). FEBS Lett *426*: 93-96, 1998.
4 Coffey ET, Smiciene G, Hongisto V, Cao J, Brecht S, Herdegen T and Courtney MJ: c-Jun N-terminal protein kinase (JNK) 2/3 is specifically activated by stress, mediating c-Jun activation, in the presence of constitutive JNK1 activity in cerebellar neurons. J Neurosci *22*: 4335-4345, 2002.
5 Datta SR, Brunet A and Greenberg ME: Cellular survival: a play in three Akts. Genes Dev *13*: 2905-2927, 1999.
6 Datta SR, Dudek H, Tao X, Masters S, Fu H, Gotoh Y and Greenberg ME: Akt phosphorylation of BAD couples survival signals to the cell-intrinsic death machinery. Cell *91*: 231-241, 1997.
7 Datta SR, Katsov A, Hu L, Petros A, Fesik SW, Yaffe MB and Greenberg ME: 14-3-3 proteins and survival kinases cooperate to inactivate BAD by BH3 domain phosphorylation. Mol Cell *6*: 41-51, 2000.
8 Datta SR, Ranger AM, Lin MZ, Sturgill JF, Ma YC, Cowan CW, Dikkes P, Korsmeyer SJ and Greenberg ME: Survival factor-mediated BAD phosphorylation raises the mitochondrial threshold for apoptosis. Dev Cell *3*: 631-643, 2002.
9 Green BD, Jabbour AM, Sandow JJ, Riffkin CD, Masouras D, Daunt CP, Salmanidis M, Brumatti G, Hemmings BA, Guthridge MA, Pearson RB and Ekert PG: Akt1 is the principal Akt isoform regulating apoptosis in limiting cytokine concentrations. Cell Death Differ *20*: 1341-1349, 2013.
10 Guo H, Gao M, Lu Y, Liang J, Lorenzi PL, Bai S, Hawke DH, Li J, Dogruluk T, Scott KL, Jonasch E, Mills GB and Ding Z: Coordinate phosphorylation of multiple residues on single AKT1 and AKT2 molecules. Oncogene 2013.
11 Hirose Y, Katayama M, Mirzoeva OK, Berger MS and Pieper RO: Akt activation suppresses Chk2-mediated, methylating agent-induced G2 arrest and protects from temozolomide-induced mitotic catastrophe and cellular senescence. Cancer Res *65*: 4861-4869, 2005.

12 Hirose Y, Katayama M, Stokoe D, Haas-Kogan DA, Berger MS and Pieper RO: The p38 mitogen-activated protein kinase pathway links the DNA mismatch repair system to the G2 checkpoint and to resistance to chemotherapeutic DNA-methylating agents. Mol Cell Biol *23*: 8306-8315, 2003.
13 Ikenoue T, Inoki K, Yang Q, Zhou X and Guan KL: Essential function of TORC2 in PKC and Akt turn motif phosphorylation, maturation and signalling. EMBO J *27*: 1919-1931, 2008.
14 Knobbe CB, Merlo A and Reifenberger G: Pten signaling in gliomas. Neuro Oncol *4*: 196-211, 2002.
15 Liao Y and Hung MC: Physiological regulation of Akt activity and stability. Am J Transl Res *2*: 19-42, 2010.
16 Liu J, Chen B, Lu Y, Guan Y and Chen F: JNK-dependent Stat3 phosphorylation contributes to Akt activation in response to arsenic exposure. Toxicol Sci *129*: 363-371, 2012.
17 Okada M, Sato A, Shibuya K, Watanabe E, Seino S, Suzuki S, Seino M, Narita Y, Shibui S, Kayama T and Kitanaka C: JNK contributes to temozolomide resistance of stem-like glioblastoma cells via regulation of MGMT expression. Int J Oncol *44*: 591-599, 2014.
18 Peltzer N, Vanli G, Yang JY and Widmann C: Role of mTOR, Bad, and Survivin in RasGAP Fragment N-Mediated Cell Protection. PLoS One *27*: e68123, 2013.
19 Pozsgai E, Bellyei S, Cseh A, Boronkai A, Racz B, Szabo A, Sumegi B and Hocsak E: Quercetin increases the efficacy of glioblastoma treatment compared to standard chemoradiotherapy by the suppression of PI-3-kinase-Akt pathway. Nutr Cancer *65*: 1059-1066, 2013.
20 Shao Z, Bhattacharya K, Hsich E, Park L, Walters B, Germann U, Wang YM, Kyriakis J, Mohanlal R, Kuida K, Namchuk M, Salituro F, Yao YM, Hou WM, Chen X, Aronovitz M, Tsichlis PN, Bhattacharya S, Force T and Kilter H: c-Jun N-terminal kinases mediate reactivation of Akt and cardiomyocyte survival after hypoxic injury *in vitro* and *in vivo*. Circ Res *98*: 111-118, 2006.
21 Stupp R, Mason WP, van den Bent MJ, Weller M, Fisher B, Taphoorn MJ, Belanger K, Brandes AA, Marosi C, Bogdahn U, Curschmann J, Janzer RC, Ludwin SK, Gorlia T, Allgeier A, Lacombe D, Cairncross JG, Eisenhauer E and Mirimanoff RO: Radiotherapy plus concomitant and adjuvant temozolomide for glioblastoma. N Engl J Med *352*: 987-996, 2005.
22 Tsuiki H, Tnani M, Okamoto I, Kenyon LC, Emlet DR, Holgado-Madruga M, Lanham IS, Joynes CJ, Vo KT and Wong AJ: Constitutively active forms of c-Jun NH2-terminal kinase are expressed in primary glial tumors. Cancer Res *63*: 250-255, 2003.

Received June 11, 2014
Revised July 9, 2014
Accepted July 10, 2014