Lobarstin Enhances Chemosensitivity in Human Glioblastoma T98G Cells

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Abstract. *Background/Aim: Lobarstin is a metabolite occurring from the Antarctic lichen Stereocaulon alpnum. Human glioblastoma is highly resistant to chemotherapy with temozolomide. Lobarstin was examined for its effect on glioblastoma. Materials and Methods: Temozolomide-resistant T98G cells were subjected to toxicity test with temozolomide and/or lobarstin. DNA damage and recovery was assessed by the alkaline comet assay and expression of DNA repair genes was examined by RT-PCR and western blot analysis. Results: Lobarstin alone at 40 μM was toxic against T98G, but had no effect in primary human fibroblasts. Co-treatment of lobarstin with temozolomide yielded enhanced toxicity. Temozolomide-alone or with lobarstin co-treatment gave similar extent of DNA damage. However, the recovery was reduced in co-treated cells. Expression of DNA repair genes, O^6-methylguanine-DNA methyltransferase, poly(ADP-ribose) polymerase 1 and ligase 3 were reduced in lobarstin-treated cells. Conclusion: Enhanced sensitivity to temozolomide by lobarstin co-treatment may be attributed to reduced DNA repair.*

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Glioblastoma multiforme (GBM) is the most aggressive form of glioblastoma tumors and is accompanied by extremely poor prognosis, despite standard treatment with surgery, radiation therapy and chemotherapy (1). Better understanding of the disease at the molecular level has prompted the development of novel therapeutic strategies, aiming to enhance responsiveness to standard chemotherapy. Temozolomide (TMZ) is an alkylating agent most frequently used in GBM chemotherapy, that generates various methyl adducts on DNA, among which are at O^6-guanine, N^7-guanine and N^3-adenine (2). The cytotoxicity of TMZ is dependent on DNA repair systems, such as mismatch repair (MMR), O^6-methylguanine-DNA methyltransferase (MGMT) repair and base excision repair (BER). Many agents, including the MGMT inhibitor (3), poly(ADP-ribose) polymerase-1 (PARP1) inhibitor (4), ribonucleotide reductase inhibitors (5), anti-epileptic drugs (6, 7), resveratrol (8), rapamycin analogs (9) and cold atmospheric plasma (10), have been reported to enhance sensitivity of TMZ (11). However, further research remains to be performed until usage of these agents at the clinical level.

Several lichen extracts have been used for remedies in folk medicine, and recent research has identified various biological activities of lichen metabolites, including antibiotic, anti-mycobacterial, anti-viral, analgesic, and anti-pyretic properties (12, 13). We have recently reported isolation of several metabolites from the Antarctic lichen *Stereocaulon alpinum* with biological activities (12, 14, 15). In the present study, we report on the effects of lobarstin (15) in GBM T98G cells .

Materials and Methods

Cell culture. Human glioblastoma T98G cells (a generous gift from Dr. S. S. Kang of Gyeongsang National University; Jinju, Korea) and primary human fibroblasts (generous gift from Dr. J.H. Lee of Chungnam National University; Daejeon, Korea) were cultured in DMEM supplemented with 10% fetal bovine serum (JR Scientific; Woodland, CA, USA), 100 units/ml penicillin and 100 μg/ml streptomycin sulfate (Welgene; Daegu, Korea).

Reagents. TMZ was purchased from Sigma-Aldrich (T2577; St. Louis, MO, USA) and lobarstin was synthesized as described (15).

Cell viability assay. Primary human fibroblasts and T98G cells were seeded at 1,000 and 2,000 cells per well in 0.1 ml in 96-well flat-bottomed plates, respectively, and incubated overnight at 37˚C. After drug treatment for indicated times, water-soluble tetrazolium salt (WST) assay was performed with EZ-Cytox (DoGen; Seoul, Korea), as instructed by the manufacturer.

Reverse transcription-polymerase chain reaction (RT-PCR). RT-PCR was performed as described previously (16). Briefly, total RNA extracted with solution D was used to generate cDNA with M-MLV Reverse Transcriptase (Elpis Biotech; Daejon, Korea), followed by PCR with HiPi Plus Thermostable DNA Polymerase (Elpis Biotech). Primers used for PCR were the following: *MGMT*_F, GCAAT GAGAGGCAATCCTGT; *MGMT*_R, GTCG CTCAAACATCCATCCT; *GAPDH*_F, CTCAGACACCATGGGG AAGGTGA; GAPDH_R, ATGATCTTGAGGCTGTTGTCATA; *PARP1*_F, GCTCCTGAACAATGCAGACA; *PARP1*_R, CATT GTGTGTGGTTGCATGA; *LIG3*_F, GTGGATTTGGGCATGTA TCC; *LIG3*_R, GCCCATTCCCCCTATACTGT; *XRCC1*_F, GAGGATGAGGCCTCTCACAG;*XRCC1*_R, TCCTCTGTGTCCC CAGAATC; *MPG*_F, TGGCACAGGATGAA GCTGTA; *MPG*_R, GTGTCCTGCTCAGCCACTCT.

Western blot analysis. Western blot analysis was performed as described previously (17). Antibodies against MGMT (sc-33674) was from Santa Cruz Biotechnology (Santa Cruz, CA, USA), PARP1 (9532), XRCC1 (2735) and GAPDH (2118) were from Cell Signaling Technology (Danvers, MA, USA), LIG3 (GTX103197) and MPG (GTX101916) were from GeneTex (Irvine, CA, USA), and HRP-conjugated IgGs (111-035-003 and 115-035-003) were from Jackson ImmunoResearch Laboratories (West Grove, PA, USA).

Alkaline comet assay. Alkaline comet assay was performed with the CometAssay Kit (Trevigen; Gaithersburg, MD, USA) as instructed by the manufacturer. Images obtained by fluorescence microscopy (Olympus IX71 from Olympus; Tokyo, Japan) were subjected to analysis with Comet Assay IV v4.3 (Perceptive Instruments; Suffolk, United Kingdom). Statistical analysis of the results was performed as recommended by Bright *et al*. (18). Briefly, 50 measurements of tail intensity (TI, also known as %tail DNA) obtained per treatment were normalized and expressed as fold-change relative to the vehicle-treated group. Results from three independent experiments were analyzed using a one-way ANOVA, followed by post-hoc test using Scheffe (PASW Statistics for Windows, Version 18.0 from SPSS Inc.; Chicago, IL, USA) to examine for group differences.

Results

Reduced glioblastoma cell viability by lobarstin. Lobarstin (Figure 1A) was treated in various doses for three days on T98G glioblastoma cells. As seen in Figure 1B, toxic effect of lobarstin in T98G was seen at the concentration as low as 10 μM (n=5, p=0.002, Student's t-test). However, lobarstin had no effect on cell viability in human normal fibroblast at 40 μM (Figure 1B; n=3, p=0.108, Student's t-test). Because 40 μM was toxic to T98G cells (n=5, p=5.16E-05, Student's t-test) but was the highest tolerated dose in normal fibroblasts, we used the concentration of 40 μM as the treatment dose in further experiments.

Enhanced toxicity of TMZ by co-treatment with lobarstin. TMZ is used as a standard chemotherapeutic agent in glioblastoma, but it is less effective in patients who express *MGMT*, a gene responsible for repairing alkylation induced by TMZ at the O^6 position of guanine, than those who do not (19). We have chosen T98G cells to study the effect of lobarstin, because MGMT is known to be expressed in the specific cell line (Figure 2A), rendering cells more resistant to TMZ (20). Statistically significant toxicity of TMZ was seen in all conditions tested (Student's t-test, not shown), but the toxicity was more prominent at high doses of 500 and 750 μM (Figure 2B). Intriguingly, co-treatment of lobarstin with high doses of TMZ resulted in enhanced toxicity (Figure 2C). These results suggest that lobarstin treatment might have enhanced the toxicity of TMZ.

Reduced recovery from TMZ-induced DNA damage by lobarstin. Because TMZ is known to damage DNA by methylating guanine (at O^6 and N^7 positions) and/or adenine (at N^3 position) residues (2), we next quantified DNA damage by the alkaline comet assay (Figure 3). We first examined the effect of lobarstin on DNA damage. Lobarstin-alone at 40 μM for 26 h had a minimal effect on DNA damage, as the tail intensity (TI) was similar to that of vehicle-treated cells for 26 h (Figure 3, L *vs*. V). We next examined the effect of co-treatment on DNA damage, by treating T98G cells with 500 μM TMZ-alone, or with lobarstin for 2 h. Treatment time of 2 h was chosen because both conditions showed similar-extent DNA lesion [Figure 3, T(D) *vs*. LT(D)] and the time should be long enough to induce DNA damage but short enough not to overlap with the DNA repair system induced upon DNA damage [Figure 3A; Damage(D)]. Therefore, cells were washed after 2 h of drug treatment and incubated with fresh culture medium to measure recovery from DNA damage [Figure 3A; Recovery(R)]. When damaged cells were challenged with fresh medium for 24 h, the cells incubated with lobarstin-containing medium showed higher TI than those with vehicle [Figure 3, T(R) *vs*. LT(R)]. Using one-way ANOVA to examine the group differences, statistical

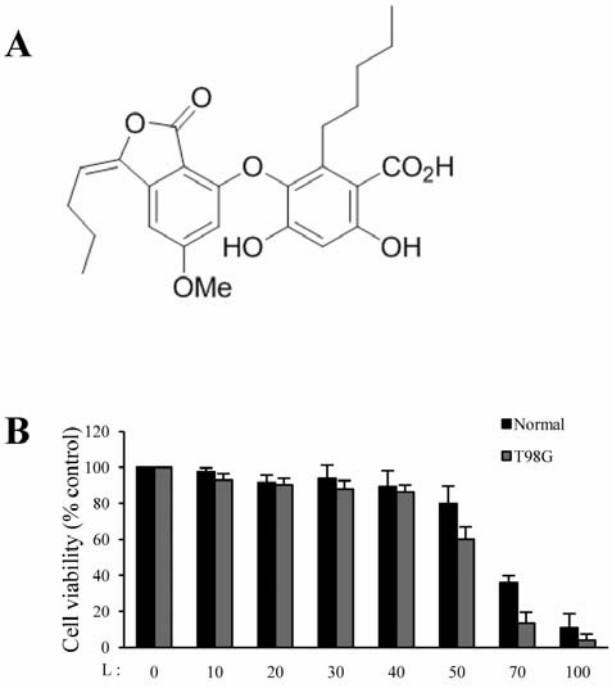

Figure 1. *Chemical structure and cytotoxicity of lobarstin. (A) Chemical structure of lobarstin. (B) Primary human fibroblasts (Normal) and T98G (T98G) cells were tested for cell viability with lobarstin, as described in Materials and methods. Cells were treated with indicated doses of lobarstin for 72 h. L, lobarstin concentration (μM). Results are shown as average of three (Normal) and five (T98G) independent experiments with standard deviation as error bars.*

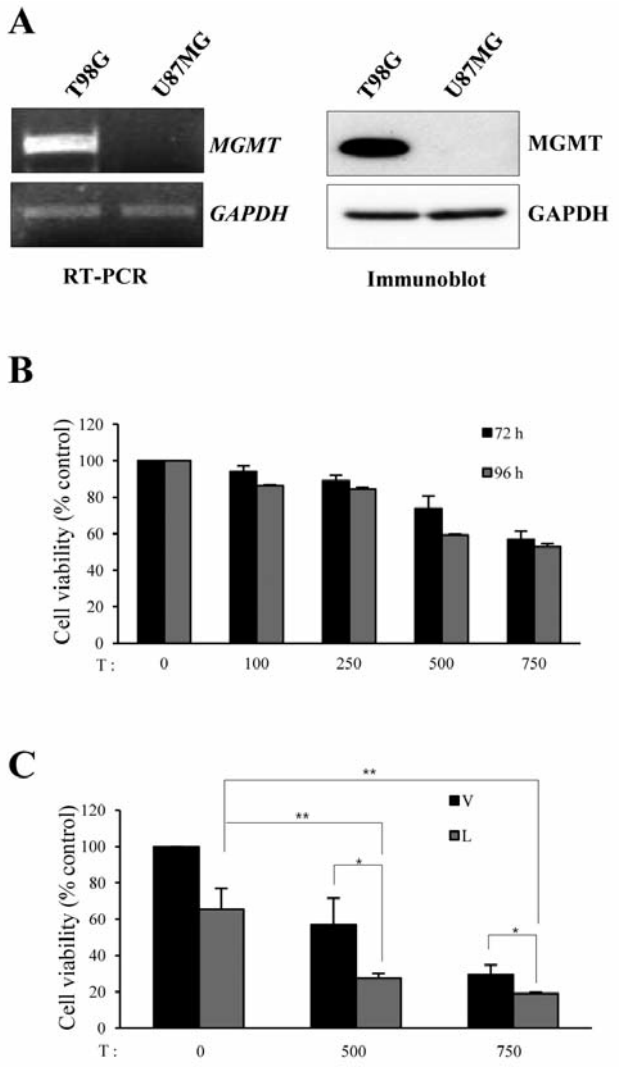

Figure 2. *Lobarstin potentiates the sensitivity of TMZ in MGMT-positive T98G cells. (A) Expression of MGMT in T98G cells. Expression at the mRNA (left panels, RT-PCR) and protein (right panels, Immunoblot) levels were shown. MGMT-negative U87MG cells were used as negative control for MGMT expression. GAPDH was used as loading control. (B) Cytotoxicity of TMZ on T98G cells. T98G cells were treated with the indicated doses of TMZ for 72 h or 96 h. (C) The effect of concomitant treatment of lobarstin and TMZ on T98G cell viability. Cells were treated with indicated combination of drugs for 72 h. T, TMZ concentration (μM); V, vehicle; L, 40 μM lobarstin. Student's t-test, *p<0.05, **p<0.01. Results are shown as representative (A) or average of three independent experiments with standard deviation as error bars (B and C).*

significance was seen between the groups [$F(1,5)=4058.828$, $p<0.001$]. Results obtained by utilizing the post-hoc test using Scheffe were as follows: (1) Significant difference observed between V and T(D), and between T(D) and T(R), $p<0.001$; (2) significant difference between L and LT(D), and between LT(D) and LT(R), $p<0.001$; (3) not significant difference between V and L, $p=1.000$; (4) not significant between T(D) and LT(D), $p=0.998$; and (5) significant difference between T(R) and LT(R), $p<0.001$. Taken together, these results suggest that lobarstin-alone may not induce DNA damage, but the DNA damage induced by TMZ may be sustained in the presence of lobarstin.

Reduced expression of DNA repair genes induced by lobarstin. Because lobarstin-treated cells showed greater DNA damage in the alkaline comet assays, we hypothesized that the DNA repair system may be affected by lobarstin. Treatment of T98G cells with lobarstin-alone for up to 48 h resulted in reduced expression of MGMT and PARP1 proteins, enzymes implicated in DNA repair, in a time-dependent manner (Figure 4A, left panels). Reduced expression was also seen at the transcription level (Figure 4A, right panels). Moreover, co-treatment of lobarstin with TMZ resulted in lesser expression of MGMT, PARP1, LIG3 and XRCC1 (Figure 4B). These results suggest reduced expression of DNA repair genes as a possible mechanism for enhanced sensitivity seen with lobarstin co-treatment.

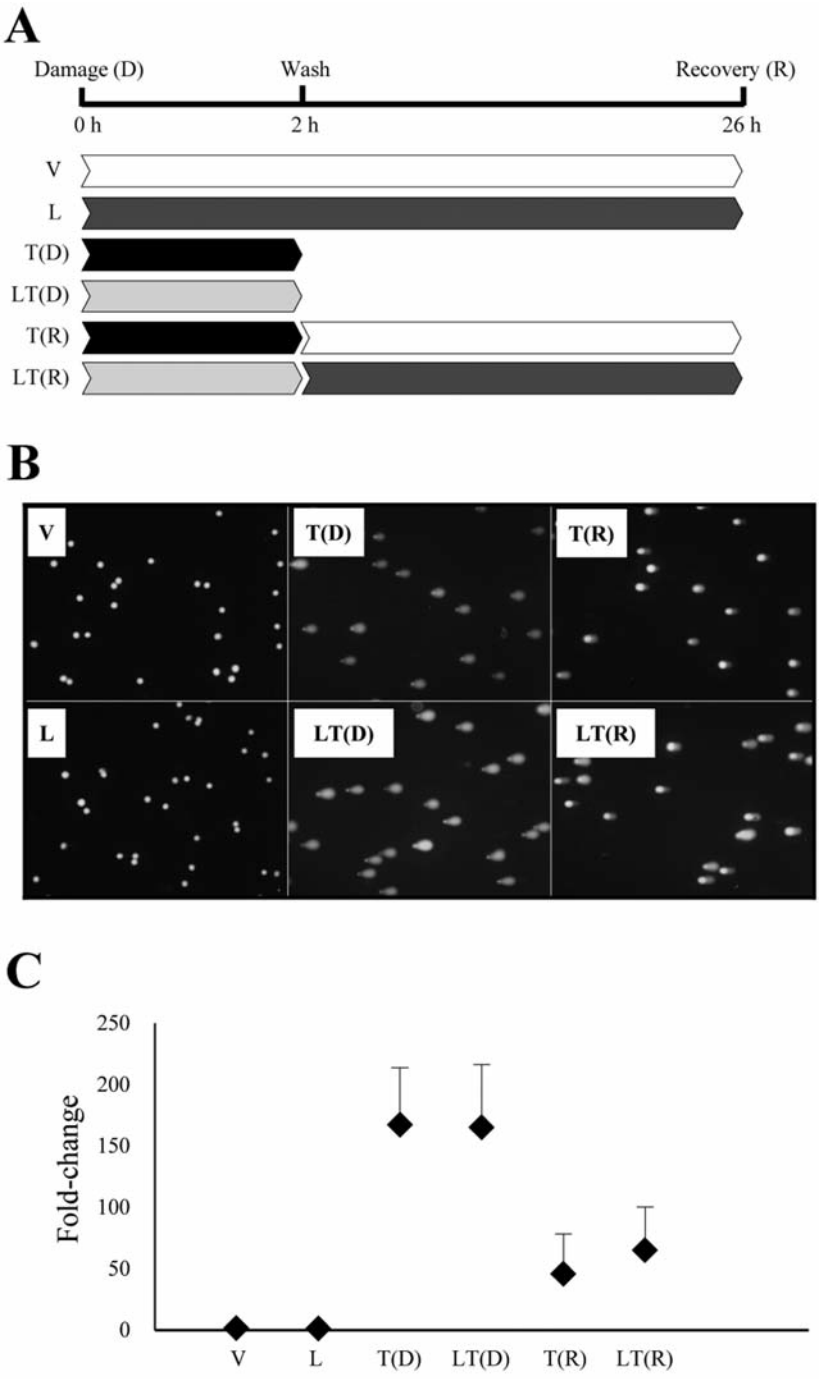

Figure 3. *Effect of lobarstin on DNA damage and recovery. (A) Experimental paradigm of lobarstin and/or TMZ treatment for alkaline comet assay. Damage (D) and recovery (R) are defined in the solid line shown on the top (not drawn to scale). Six different experimental conditions are shown underneath the solid line as arrows [V, Vehicle; L, lobarstin; T(D), TMZ (damage); LT(D), lobarstin and TMZ (damage); T(R), TMZ (recovery); and LT(R), lobarstin and TMZ (recovery)]. The alkaline comet assay was performed at the end of each arrow. (B) Fluorescent image of cells subjected to alkaline comet assay. (C) Summary of alkaline comet assay. Results are shown as average of fold-change relative to the vehicle-treated group from 50 measurements obtained per treatment and three independent experiments (total of 150 measurements) and standard deviation as error bars.*

Discussion

In the present study, we reported on the effect of lobarstin, a lichen metabolite, in enhancing the sensitivity of TMZ in chemo-resistant GBM T98G cells. We observed reduced recovery of DNA damage in cells co-treated with lobarstin and TMZ, and we suggested the reduction in DNA repair genes expression as a responsible molecular mechanism (Figure 5).

Reduction of MGMT following TMZ treatment has been reported previously (21). However, induction of MGMT expression by TMZ has been reported as well (22). Such discrepancies may be attributed to differences in the

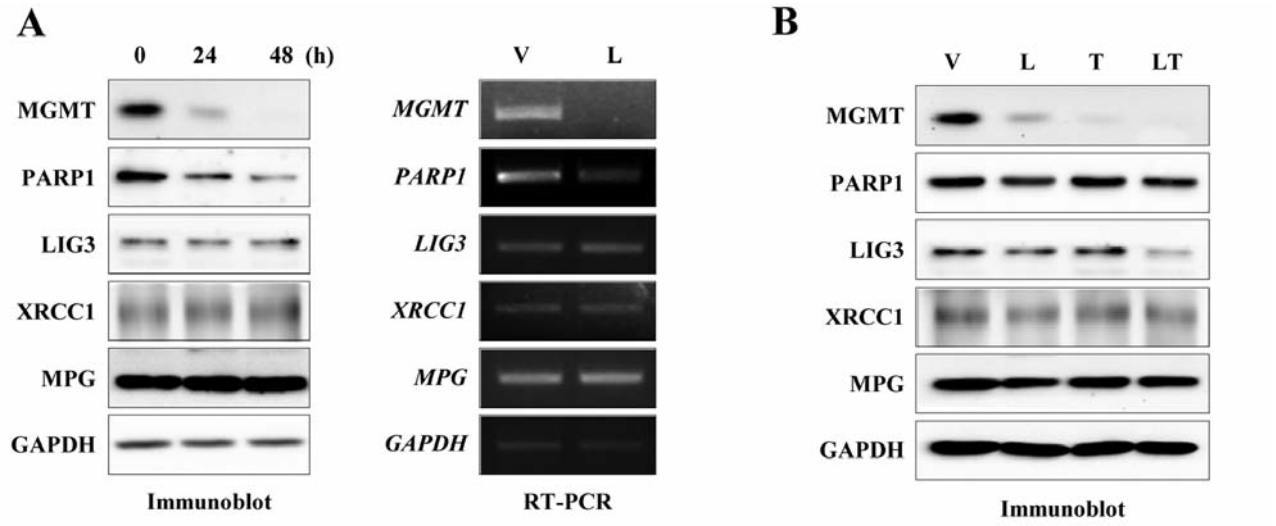

Figure 4. *Effect of lobarstin on the expression of DNA repair genes. (A) The effect of lobarstin on DNA repair genes. Cells were treated with 40 μM lobarstin for indicated times (left panels) or for 24 h (right panels) and subjected to immunoblot and RT-PCR, respectively. (B) The effect of 40 μM lobarstin and/or 500 μM TMZ on DNA repair genes. Cells were treated with indicated drugs for 24 h and subjected to immunoblot. Shown are representative results of three independent experiments. V, Vehicle; L, lobarstin-only; T, TMZ only; LT, lobarstin and TMZ.*

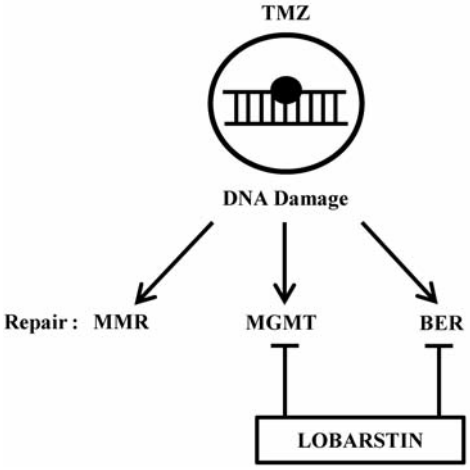

Figure 5. *Role of lobarstin in GBM. In MGMT-positive, TMZ-resistant T98G cells, the sensitivity to TMZ was enhanced by lobarstin co-treatment. Recovery from TMZ-induced DNA damage was attenuated by concomitant lobarstin treatment, accompanied by reduced expression of genes in the MGMT and BER pathways.*

condition of TMZ treatment, as prolonged exposure to low-dose TMZ is suggested as a mechanism for acquired chemoresistance (23, 24). Although we used a high dose of TMZ at 500 μM, long-term effect of lobarstin treatment on the expression of MGMT and other DNA repair genes remains to be examined. Furthermore, it would be intriguing to investigate the effect of lobarstin with other clinically-utilized therapies, such as radiotherapy (25), carmustine (26) and bevacizumab (27).

Despite standard therapy, GBM is known to present an extremely high incidence of recurrence (~90%) (28). It has been suggested that a sub-population of therapy-resistant cells with stem cell-like characteristics are responsible for tumor re-growth (29). Although these cells may be highly heterogeneous, thus extremely difficult to characterize, eradication of these tumor-initiating cells would be pivotal in the complete cure of this fatal disease (29, 30). It would be interesting to examine the effect of lobarstin in a population of cells called side-population, which behaves like stem cells and is resistant to chemotherapeutic treatments (31).

The molecular mechanism of lobarstin action also remains to be investigated. Because gene expression of multiple genes were affected by lobarstin, lobarstin may modulate the expression and/or activity of transcription factor(s). GATA4 may be an interesting candidate to examine further, because the expression of the BER enzyme alkylpurine DNA N-glycosylase was reduced by GATA4 (32). Recently, we have reported on lobaric acid, a related metabolite isolated from *Stereocaulon alpinum*, as a potent inhibitor of protein tyrosine phosphatase N1 (PTPN1) (12). Although the exact biological activity of lobarstin remains to be elucidated, testing the possibility as a phosphatase inhibitor would be intriguing, as PTPN1 is actively pursued as a drug target for various diseases, including cancer (33, 34).

In conclusion, in the present study, we showed the effect of lobarstin in enhancing the sensitivity of GBM cells to TMZ. We also suggested a reduced DNA repair gene

expression as a possible mechanism for this phenomenon. These results open the possibility of lobarstin as a potential factor for GBM combination therapy.

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