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Evaluation of Combination Treatment Effect With TRAIL-secreting Mesenchymal Stem Cells and Compound C Against Glioblastoma

HYE RIM HAN[1], SOON A. PARK[1], STEPHEN AHN[2], SIN-SOO JEUN[1,2*] and CHUNG HEON RYU[3*]

[1]*Department of Biomedicine and Health Sciences, College of Medicine, The Catholic University of Korea, Seoul, Republic of Korea;*
[2]*Department of Neurosurgery, Seoul St. Mary's Hospital, The Catholic University of Korea, Seoul, Republic of Korea;*
[3]*Department of Clinical Laboratory Science, Daejeon Health Institute of Technology, Daejeon, Republic of Korea*

Abstract. *Background/Aim: Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) triggers apoptosis of cancer cells and, when used in combination with other anticancer drugs, is regarded as an effective strategy for anticancer treatment. In this study, we investigated the efficacy of combination treatment with TRAIL-secreting human mesenchymal stem cells (MSC-TRAIL) and compound C, an AMP-activated protein kinase (AMPK inhibitor), on glioblastoma. Materials and Methods: The anticancer effect using MSC-TRAIL and compound C on glioma was evaluated in vitro and on in vivo models. Results: Combination treatment of MSC-TRAIL and compound C increased apoptosis by enhancing expression of B-cell lymphoma 2 (BCL2)-associated X protein (BAX) and reducing that of anti-apoptotic proteins cellular FLICE-inhibitory protein (FLIP), X-linked inhibitor of apoptosis (XIAP), and BCL2 in glioma. In addition, MSC-TRAIL and compound C combination increased caspase-3 cleavage and apoptotic cells in a mouse glioma model compared with the group treated with the agents alone. Conclusion: Our results suggest that MSC-TRAIL and compound C are a novel combination for treatment of glioma.*

Correspondence to: Sin-Soo Jeun, Department of Neurosurgery, Seoul St. Mary's Hospital, The Catholic University of Korea, 222 Banpo-daero, Seocho-gu, Seoul 137-701, Republic of Korea. Tel: +82 0222587535, Fax: +82 0222587791, e-mail: ssjeun@catholic.ac.kr and Chung Heon Ryu, Department of Clinical Laboratory Science, Daejeon Health Institute of Technology, 21 Chung jeong St., Dong-gu, Daejeon 34504, Republic of Korea. Tel: +82 0426709164, Fax: +82 0426709582, e-mail: ryuch@hit.ac.kr

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Glioblastoma multiforme (GBM) is one of the most aggressive and devastating types of cancer. GBM has the characteristics of infiltration into functional brain tissue and resistance to chemotherapy and radiotherapy (1). Conventional treatment such as surgery, radiotherapy, and chemotherapy lead to poor median survival rates (2). Therefore, a new strategy is needed to increase treatment efficacy.

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) selectively activates apoptosis in cancer cells, not normal cells (3, 4). However, the toxicity and short protein half-life of recombinant TRAIL present issues when being used to treat cancer, including glioma (5-7). Previously, use of TRAIL-secreting human bone marrow-derived mesenchymal stem cells (MSC-TRAIL) in combination with other agents showed increased anticancer efficiency against tumors including glioma (8-10).

AMP-activated protein kinase (AMPK) is a key energy sensor that modulates cell energy homeostasis and is related to the cell cycle and apoptosis (11). Recently, AMPK activation was found to promote many types of cancer growth and proliferation including in human malignant glioma (12-14). Compound C, also called a dorsomorphin, is commonly used as an inhibitor of AMPK, effectively blocking the action of AMPK. In addition, compound C exhibited therapeutic activity in various cancer types and enhanced efficiency of TRAIL in renal cancer (15-17). We hypothesized that compound C might be a potent sensitizer for increasing sensitivity to TRAIL-induced apoptosis in malignant glioma.

In this study, we examined the potential of combination treatment with MSC-TRAIL and compound C against glioma.

Materials and Methods

Reagents and cell culture. Human bone marrow-derived MSCs were obtained from the Catholic Institute of Cell Therapy (CIC, Seoul, Republic of Korea). MSCs were cultured in low-glucose Dulbecco's

modified Eagle's medium (DMEM; Thermo Fisher Scientific, Waltham, MA, USA) with 20% fetal bovine serum (Wisent Bioproducts, St-Bruno, QC, Canada) and Antibiotic-Antimycotic solutions (Gibco, Carlsbad, CA, USA). MSCs were used for the experiments after five to eight passages. Previously, we specifically described the adenovirus carrying the secretable trimeric form of the TRAIL gene (Ad-TRAIL) (18, 19). Human glioma cell lines (U87, U138) were obtained from the American Type Culture Collection. Glioma cell lines were cultured in high-glucose DMEM (Thermo Fisher Scientific). Firefly luciferase (Luc)-expressing U87 cells (U87-Luc) were stably transduced using a lentivirus-expressing Luc (20). All cells were incubated at 37˚C in a humidified atmosphere containing 5% CO_2.

Cell viability assay. Cell viability was measured using a 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT; Sigma-Aldrich Co., St. Louis, MO, USA) allowing sensitive colorimetric detection using water-soluble tetrazolium salt that is reduced by dehydrogenases in living cells to give a colored product (formazan). Glioma cells were seeded in 96-well or 24-well plates to measure TRAIL-induced cytotoxicity and compound C-mediated cell death. Cells were treated with TRAIL (0-300 ng/ml) (R&D Systems, Minneapolis, MN, USA), compound C (0-20 μM; Selleckchem, Houston, TX, USA), or both and were analyzed 24 h later. Each well was measured for optical density (OD) at 570 nm using a Spectramax Plus 384 Microplate Reader (Molecular Devices, Sunnyvale, CA, USA).

Orthotopic xenografted mouse model and in vivo bioluminescent imaging analysis. The use of nude mice (6 to 8 weeks old, Male; Charles River Laboratories, Wilmington, MA, USA) was approved by the Institutional Animal Care and Use Committee of The Catholic University of Korea (approval number: 2017-0211-04). The intracranial xenograft mouse model of human glioma was established as specifically described in a previous study (20). To evaluate the therapeutic effects of the combined treatment, MSC-TRAIL (2×10^5 cells/5 μl of PBS) were transplanted intracranially at 10 days after cancer inoculation. Compound C was then injected in a mixture of saline at 10 mg/kg intraperitoneally for 5 days. To assess the inhibition of cancer growth, the animals were inoculated with U87-Luc. The substrate of luciferase, D-luciferin (150 mg of luciferin/kg of body weight; Xenogen, CA, USA), was injected intraperitoneally 10 min before visualization using the Optical *in vivo* Imaging System-IVIS Lumina XRMS (PerkinElmer Inc., Waltham, MA, USA).

Western blotting. Collection of mouse brain tissue protein and cell lysates and western blot analysis were described previously (20). Proteins were transferred to a nitrocellulose membrane (Invitrogen) using Trans-Blot® SD Semi-Dry Transfer Cell (BIO-RAD) and then incubated with primary antibodies against the cellular FLICE inhibitory protein (cFLIP; Alexis), X-linked inhibitor of apoptosis protein (XIAP), poly (ADP-ribose) polymerase (PARP), B cell lymphoma 2 (BCL2)-associated X protein (BAX), and BCL2 (Cell Signaling Technology, Beverly, MA, USA); β-actin (Sigma) was used as a loading control.

Immunohistochemistry and in vivo apoptosis assay. To evaluate cancer growth inhibition using histological analysis, the mice were sacrificed at day 20 after cancer inoculation during the imaging experiment. Mouse brains were perfused with 4% paraformaldehyde under deep anesthesia. Fixed tissues were cryosectioned (14-μm-thick sections) and stained with hematoxylin and eosin (H&E). Apoptotic activity was detected *via* staining using a terminal deoxyribonucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) assay kit (Roche, Mannheim, Germany) and developed with Cy3-conjugated streptavidin (Invitrogen). Nuclei were counterstained with 4,6-diamidino-2-phenylindole (Sigma).

Statistical analysis. All data are shown as mean±SD (standard deviation). Statistical differences between test conditions were determined using one-way ANOVA with Bonferroni multiple correction. Probability values less than $p<0.05$ were considered significant.

Results

The presence of TRAIL and of compound C increased glioma cell death. We examined the viability of glioma cells (U87, U138) to determine the effects of TRAIL and of compound C. TRAIL increased U87 cell death but did not increase U138 cell death, indicating a lack of sensitivity to TRAIL. Compound C increased cell death in U87 and U138 cells (Figure 1A). Next, we examined whether combined treatment with TRAIL and compound C increased glioma cell death compared to treatment with TRAIL or compound C alone. Combined treatment with TRAIL and compound C induced significant cell death in U87 and U138 cells (Figure 1B) compared to treatment with TRAIL or compound C alone. This indicates that compound C enhanced TRAIL-induced cell death.

Combined treatment with TRAIL and compound C modulated the expression of anti-apoptotic proteins and pro-apoptotic protein by glioma cells. We examined whether combined treatment with TRAIL and compound C affected glioma cells with regard to expression of apoptosis-related proteins. Expression of anti-apoptotic proteins such as cFLIP and XIAP greatly reduced with combined treatment compared to TRAIL or compound C alone treatment (Figure 2A). Increased apoptosis of glioma cells was verified by cleavage of PARP, a key apoptotic protein, in glioma cells. More cleavage of PARP was observed after combined treatment compared with TRAIL or compound C alone (Figure 2B), indicating that compound C enhanced TRAIL-induced apoptosis in glioma cells.

Combined treatment with MSC-TRAIL and compound C increased treatment efficiency in vitro. Previous results showed that MSC-based gene treatment had the advantages of offering continuous and concentrated local delivery of secretable TRAIL (8-10). Therefore, we used MSC-TRAIL in this study to increase efficiency over using TRAIL. We examined treatment using MSC-TRAIL combined with compound C and how it had an effect on cell death in glioma

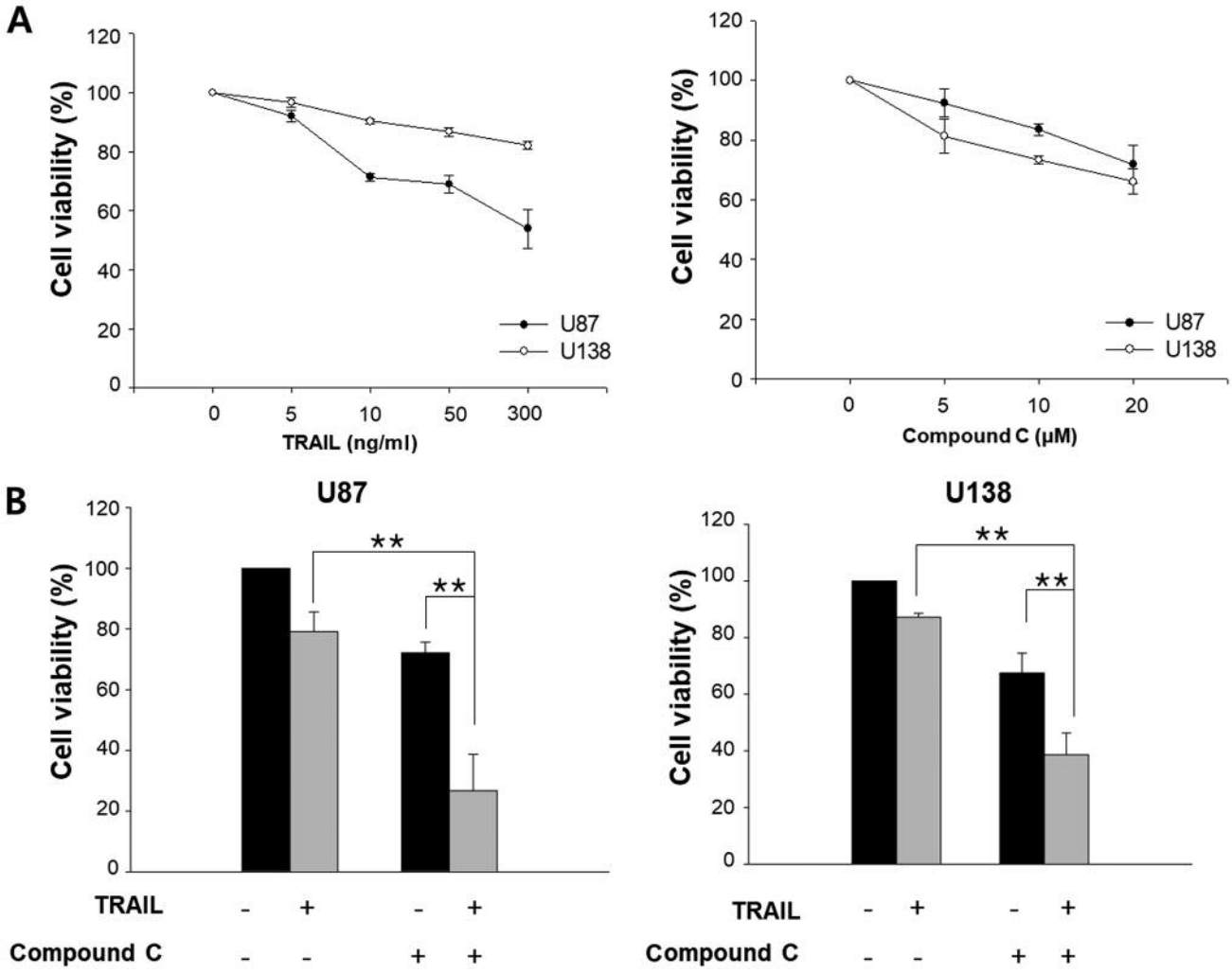

Figure 1. *Glioma cell death after combined treatment using Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) and compound C. A: Viability of U87 and U138 glioma cells treated with increasing doses of recombinant TRAIL (0-300 ng/ml) or compound C (0-20 μM) at 24 h using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. B: The cytotoxic effect on glioma cells treated with TRAIL (10 ng/ml) and compound C (10 μM) at 24 h with in comparison with the single treatment. Data are means±SD. **Significantly different at p<0.01, one-way ANOVA with Bonferroni multiple comparison test.*

cells. Co-culture experiments showed that MSC-TRAIL combined with compound C had therapeutic effects by significantly increasing glioma cells death than MSC-TRAIL or compound C alone (Figure 3A). In addition, we determined that MSC-TRAIL combined with compound C increased pro-apoptotic protein and reduce anti-apoptotic proteins in glioma cells. Expression of BAX, a pro-apoptotic protein, increased with combined treatment. The expression of the anti-apoptotic proteins BCL2, cFLIP and XIAP reduced using the combined treatment compared to treatment with TRAIL or compound C alone (Figure 3B). It was determined that MSC-TRAIL and compound C also increased treatment efficiency by increasing cell death through enhancing of apoptotic protein and reduction of anti-apoptotic proteins.

Combined treatment with MSC-TRAIL and compound C increased treatment efficiency in glioma-bearing mice. We evaluated the therapeutic efficacy of this treatment in intracranial xenograft mouse models. To monitor cancer growth every 7 days after tumor inoculation using *in vivo* bioluminescent imaging analysis, we established glioma-bearing mice with U87-Luc cells. A reduce in bioluminescence expression was observed in mice treated with the combination of MSC-TRAIL and compound C compared with mice treated with MSC-TRAIL or compound C alone (Figure 4A). In addition, H&E staining revealed that cancer growth was inhibited in the mice treated with the combination of MSC-TRAIL and compound C compared to PBS or individual treatment (Figure 4B). These results indicate that treatment

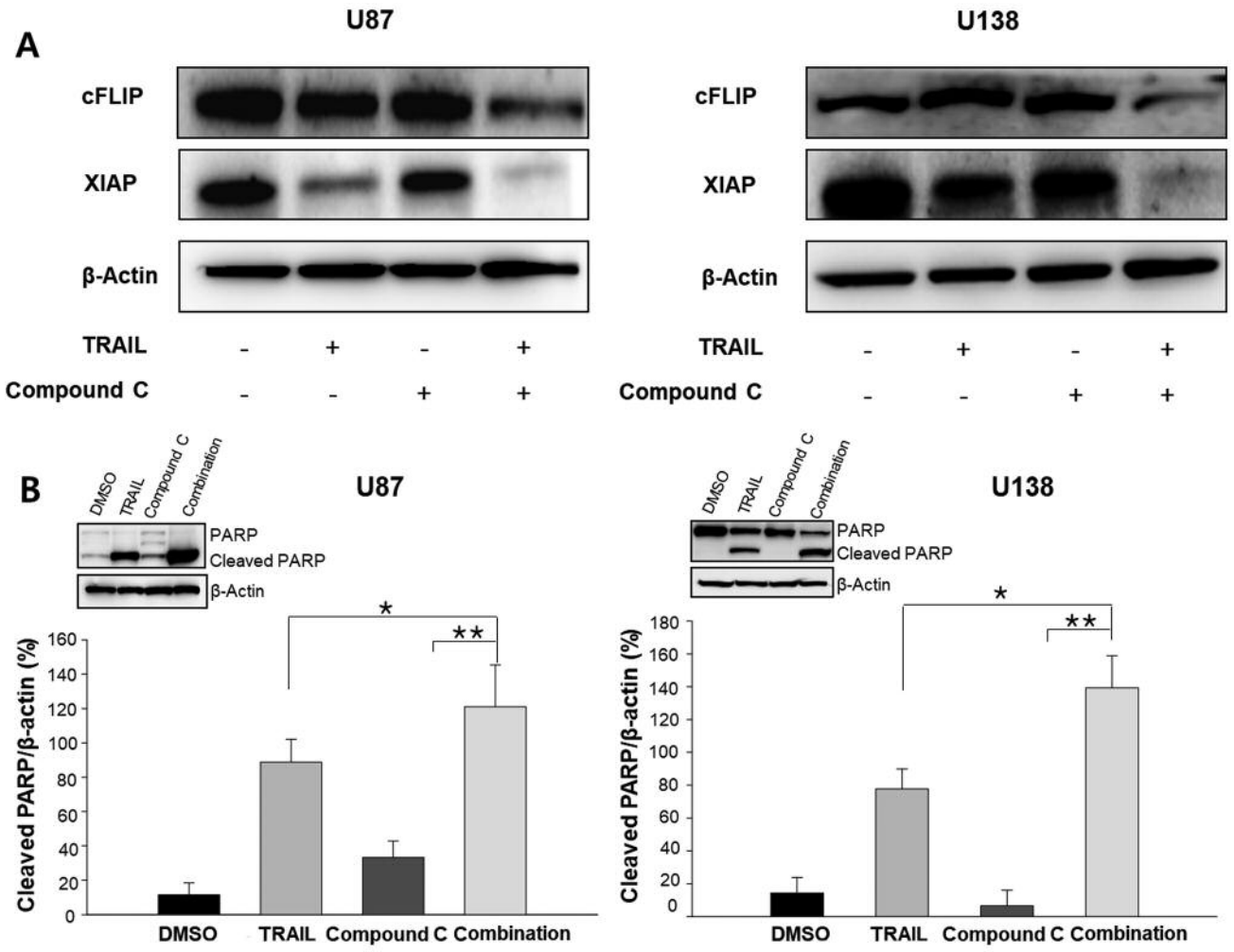

Figure 2. *Down-regulation of anti-apoptotic proteins cellular FLICE inhibitory protein (cFLIP) and X-linked inhibitor of apoptosis protein (XIAP) and up-regulation of pro-apoptotic protein cleaved poly (ADP-ribose) polymerase (PARP) with combined treatment using tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) and compound C. A: Western blot analysis of cFLIP and XIAP in glioma cells treated with recombinant TRAIL (10 ng/ml) with or without compound C (10 μM) after 24-h treatment. B: Western blot analysis of PARP and cleaved PARP in glioma cells in response to 24-h treatment with TRAIL (10 ng/ml) with and without compound C (10 μM). Data are means±SD. Significantly different at *p<0.05 and **p<0.01, one-way ANOVA with Bonferroni multiple comparison test.*

with the combination of MSC-TRAIL and compound C reduced cancer growth in glioma-bearing mice.

Combined treatment with MSC-TRAIL and compound C increased apoptosis in intracranial glioma. To determine whether the apoptotic effects were involved in the anticancer activity of MSC-TRAIL and compound C, brain tissue sections from glioma-bearing mice were analyzed by immunohistochemistry 20 days after treatment. TUNEL and cleavage of caspase 3 staining demonstrated a significant increase in number of apoptotic cells in the group treated with MSC-TRAIL and compound C together compared to those treated with each treatment alone (Figure 5). These results indicate that the anticancer activity of the combination treatment of MSC-TRAIL and compound C is involved in apoptosis and enhances apoptotic cell death in intracranial glioma.

Discussion

In this study, we evaluated the possibility of combined treatment with TRAIL and compound C, an AMPK inhibitor, to reduce glioma cell viability and induce apoptosis. In addition, we investigated how MSC-TRAIL and compound C might increase treatment efficiency to glioma *in vitro* and *in vivo* through enhancement of anticancer effects with the increase of apoptosis.

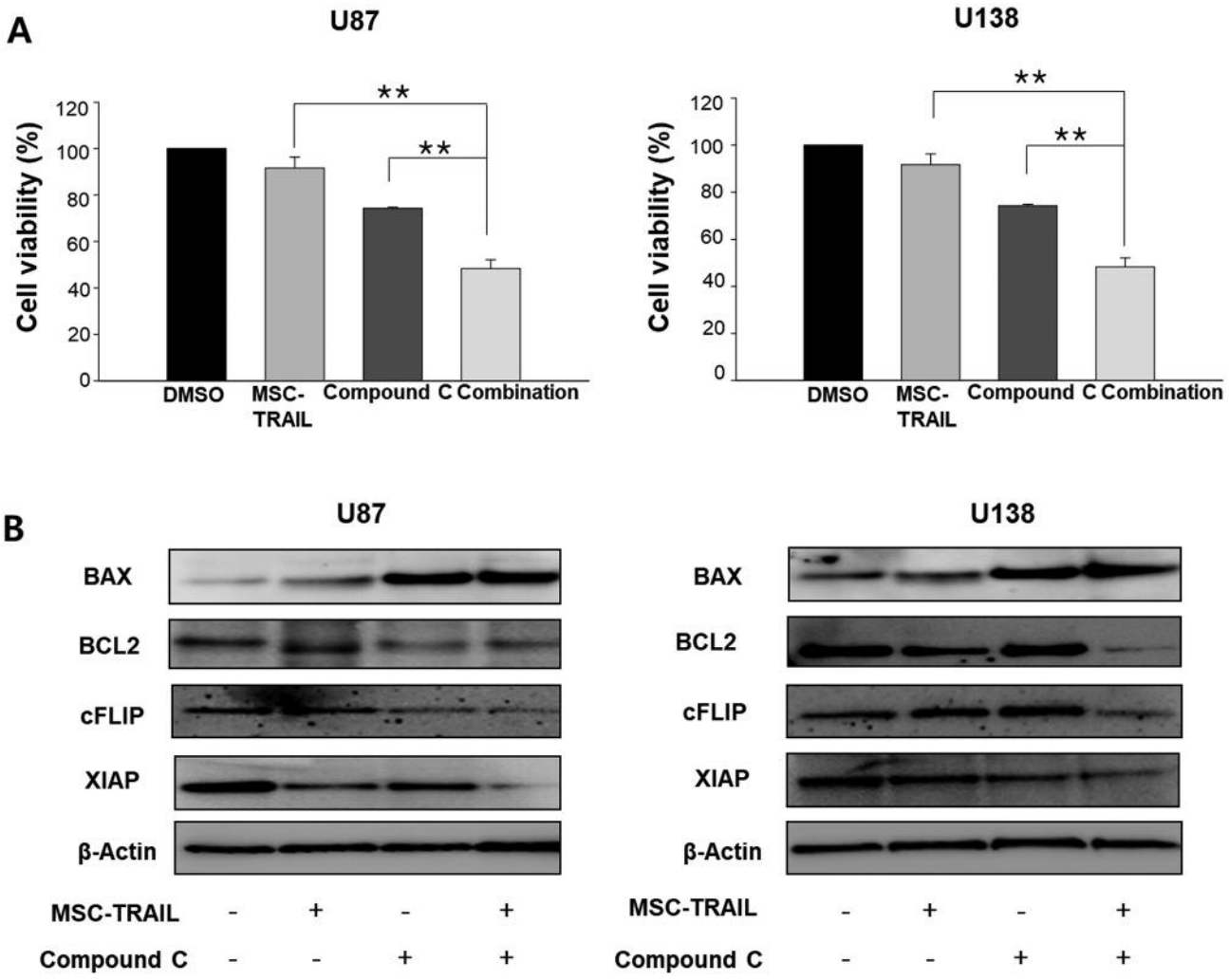

Figure 3. *Effects of tumor necrosis factor-related apoptosis-inducing ligand-secreting human bone marrow-derived mesenchymal stem cells (MSC-TRAIL) combined with compound C on U87 and U138 glioma cells. A: Glioma cells (5×10^4) were seeded in lower wells of 24-well plates, and MSC-TRAIL (1×10^4) were seeded in Trans well inserts (0.4 μm pores). The effects of MSC-TRAIL combined with compound C (10 μM) on glioma cells were analyzed by co-culture using Transwell plates, and the viability was determined at 24 h by MTT assay. Data are means±SD. **Significantly different at $p<0.01$, one-way ANOVA with Bonferroni multiple comparison test. B: The effects of MSC-TRAIL combined with compound C (10 μM) on glioma cells were determined at 24 h by western blot analysis using antibodies against pro-apoptotic protein B-cell lymphoma 2 (BCL2)-associated X protein (BAX) and anti-apoptotic proteins BCL2, cellular FLICE inhibitory protein (cFLIP) and X-linked inhibitor of apoptosis (XIAP).*

In previous reports, TRAIL treatment with chemotherapy, radiotherapy, or other means of therapy were considered as a strategy to increase TRAIL treatment efficiency (8-10). Recently, compound C, an AMPK inhibitor, was used to arrest cancer growth and inhibit cell cycle in cancer, including glioma (21-23). Here, we studied the anticancer effects of combined treatment with TRAIL and compound C on U87 and U138 glioma cells. Although TRAIL enhanced U87 cell death, TRAIL did not reduce U138 cell death. U138 did not respond to TRAIL alone treatment, which was in accordance with previous results (24). This indicated that TRAIL alone is not sufficient to induce death of all glioma cells. Therefore, we combined treatment of TRAIL and compound C for glioma cells and observed significant death of U87 and U138 glioma cells. Our results indicate that compound C increases sensitivity to TRAIL in glioma cells. In addition, we applied MSC-TRAIL with other anticancer agents against glioma (8-10) since MSC-TRAIL overcomes the limitations of conventional TRAIL treatment (28) such as short half-life and low transport towards cancer sites (29).

It is important to enhance the effects of TRAIL in many cancer types to activate pro-apoptotic proteins and reduce

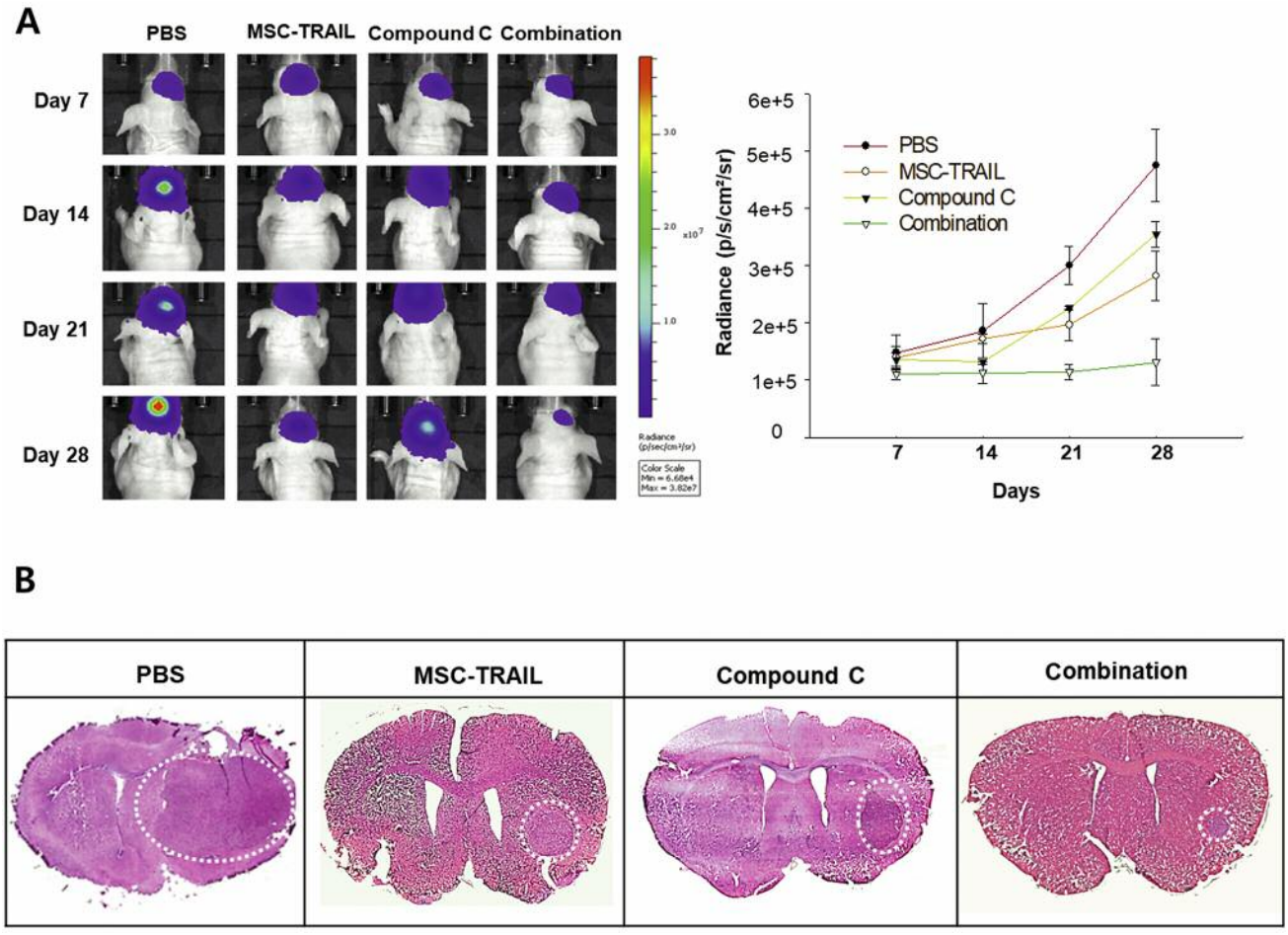

Figure 4. *In vivo effects of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-secreting human bone marrow-derived mesenchymal stem cells (MSC-TRAIL) and compound C on glioma-bearing mice. A: Glioma-bearing mice were treated with PBS, MSC-TRAIL (2×10^5), compound C (10 mg/kg), or MSC-TRAIL and compound C (n=5/group). The effects were shown by inoculation with U87-Luc cells after treatment using the Optical in vivo imaging System-IVIS Lumina XRMS (PerkinElmer). B: Images of hematoxylin and eosin staining of brain tissue from each treatment group showing tumor size at day 20 after cancer inoculation. Images were captured using Pannoramic MIDI. Magnification, 1×. Dotted line: Tumor edge.*

anti-apoptotic protein expression to promote apoptosis. cFLIP inhibits caspase-8 and XIAP, inhibitors of the apoptosis protein family, by blocking effector caspase-3, -7, and -8 which blocks the extrinsic TRAIL-induced apoptosis pathway (30). Combination treatment of MSC-TRAIL and compound C significantly enhanced apoptosis compared to alone treatment through reduction cFLIP and XIAPWe also observed an influence on BAX, which induces the intrinsic TRAIL-induced apoptosis pathway, and BCL2, which blocks BAX expression (31). MSC-TRAIL and compound C increased BAX and reduced BCL2 expression in glioma cells. Our results indicate that combined treatment with MSC-TRAIL and compound C enhanced pro-apoptosis factors and diminished anti-apoptosis factors in glioma cells. Based on our *in vitro* results, we observed the tumoral area through *in vivo* bioluminescent imaging analysis verified by H&E stain. Both *in vivo* bioluminescent imaging analysis and H&E staining showed that MSC-TRAIL with compound C reduced the tumoral area in glioma mouse models compared to treatment with MSC-TRAIL or compound C alone. In addition, we observed that MSC-TRAIL and compound C increased apoptosis *in vivo* using TUNEL and staining for cleavage of caspase 3 stain. Our results indicate that combination treatment with MSC-TRAIL and compound C increased glioma treatment efficiency by reducing the tumor extent and increasing apoptosis.

We examined, for the first time, combined treatment of glioma with MSC-TRAIL and compound C. Our results show that MSC-TRAIL combined with compound C is a useful strategy to increase anticancer effects against glioma through

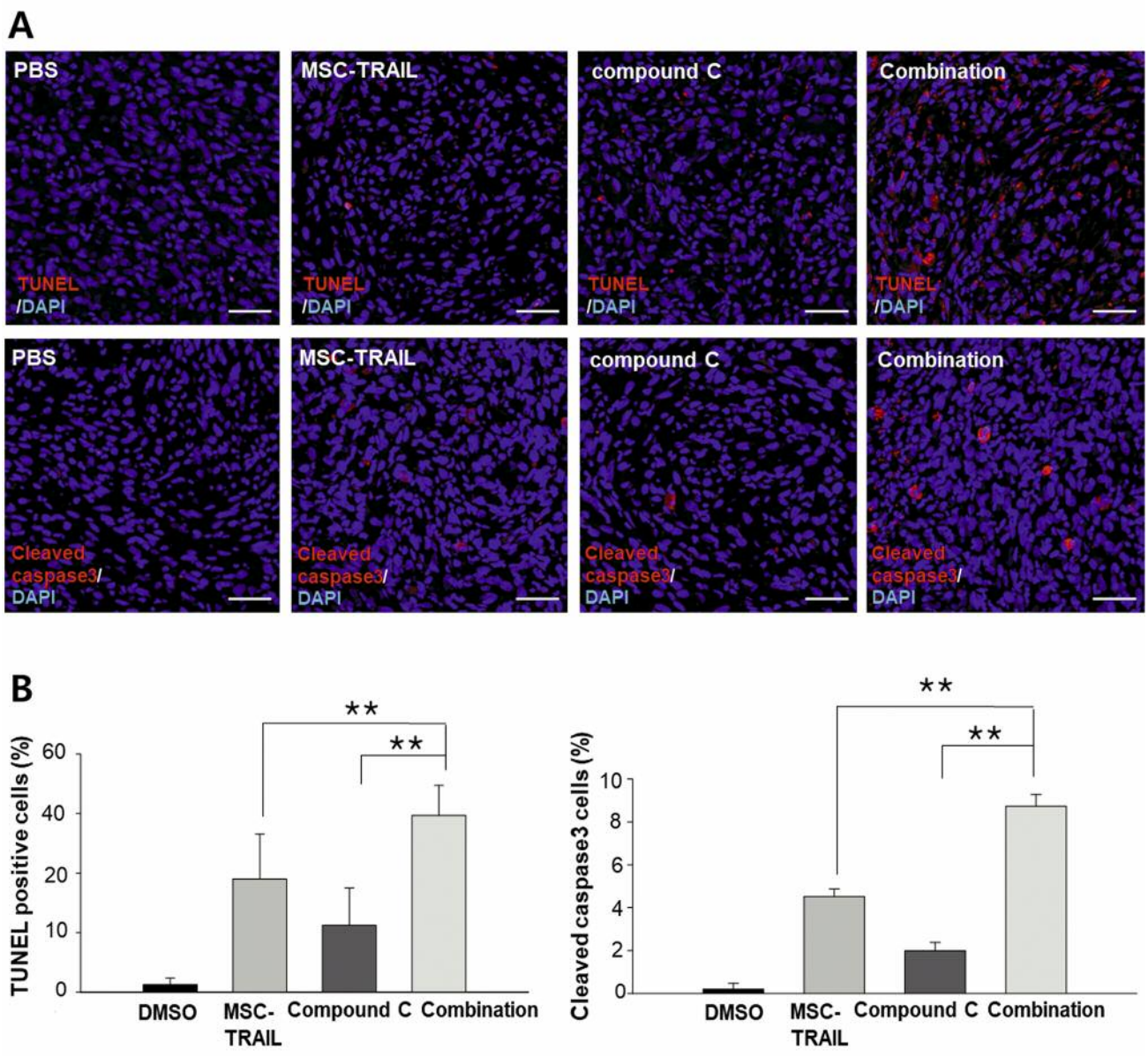

Figure 5. *Histological analysis of apoptotic activity in the xenograft mouse model. Glioma-bearing mice were treated with phosphate-buffered saline (PBS), tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-secreting human bone marrow-derived mesenchymal stem cells (MSC-TRAIL) (2×10^5), compound C (10 mg/kg), or MSC-TRAIL and compound C (n=5/group). A: Cryosections of brain tissue from each group at day 20 were stained by terminal deoxyribonucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) (red; upper panel) and for cleaved caspase 3 (red; bottom panel). TUNEL and caspase 3 cleavage staining showed apoptotic cell death in brain tumor. Nuclei were counterstained with 4,6-diamidino-2-phenylindole (DAPI, blue). B: TUNEL-positive cells and cells with caspase-3 cleavage in brain tissue were quantified by computerized image analysis. Scale bar=50 μm, Magnification: 200×; Data are means±SD. **Significantly different at p<0.01 one-way ANOVA with Bonferroni multiple comparison test.*

reducing expression of anti-apoptotic proteins and enhancing that of pro-apoptotic proteins. Therefore, we suggest that combined treatment with MSC-TRAIL and compound C has potential to enhance anticancer effects against glioma.

Conflicts of Interest

The Authors declare that they have no conflicts of interest in regard to this study.

Authors' Contributions

HHR wrote the draft article, performed most of the experiments and collected data. SAP editing of the article and analyzed statistical data analysis. AS prepared the MSC and tested the cell *in vitro*, and SSJ and CHR contributed to the conception and design of the study, interpretation of data, and editing of the article. All Authors read and approved the final version of the article.

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