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의학박사 학위논문

Inhibition of STAT3 Enhances the Radiosensitizing Effect of Temozolomide in Glioblastoma Cells in vitro and in vivo

교모세포종에서 STAT3 억제를 통한 테모졸로마이드의 방사선감수성 증강에 대한 연구

2015년 8월

서울대학교 대학원
의학과 방사선종양학 전공
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Inhibition of STAT3 Enhances the Radiosensitizing Effect of Temozolomide in Glioblastoma Cells in vitro and in vivo

지도 교수 김 인 아

이 논문을 의학박사 학위논문으로 제출함

2015년 4월

서울대학교 대학원

의학과 방사선종양학 전공

한 태 진

한태진의 의학박사 학위논문을 인준함

2015년 7월

위 원 장 _____(인)

부위원장 _____(인)

위 원 _____(인)

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Abstract

Introduction: Despite aggressive treatment with radiation therapy plus concurrent and adjuvant temozolomide (TMZ), the prognosis for glioblastoma remain poor. We investigated the potential of targeting signal transducer and activator of transcription-3 (STAT3) to improve the therapeutic outcome of combined radiotherapy and TMZ and its associated mechanisms in glioblastoma.

Methods: We evaluated the preclinical potential of a STAT3 inhibitor, Cpd188 combined with temozolomide and radiation by using two established glioblastoma cell lines (U251, U87) and two patients-derived glioblastoma cell lines (GBL12, GBL28). Clonogenic assay was performed to determine the surviving fraction of cells after treatment, apoptosis was evaluated by caspase-3/7 assay and annexin V-FITC/PI double staining, modified Boyden chamber assay, wound healing assay, vasculogenic assay were performed to establish the association of epithlial-mesenchymal transition (EMT)-related mechanism. In vivo studies using 6- to 8-week old BALB/c nude mice bearing intracranial U251 xenografts, bioluminescence imaging was used to evaluate tumor growth. Markers associated with tumor microenvironmet were assessed by immunohistochemical analysis.

Results: Cpd188 potentiated the radiosensitizing effect of TMZ in U251 glioblastoma cell line which has high levels of p-STAT3 expression and in vitro. Increased radiosensitizing effects of TMZ were associated with the induction of apoptosis and the reversion of epithelial-mesenchymal transition (EMT). Cpd188 delayed in vivo tumor growth in combination with fractionated radiation and TMZ with improved survival rates compared to control (P=0.003) and radiation only group (P=0.007). Immunohistochemical staining of tumor sections showed that Cpd188 decreased the expression of CD31 (a marker of endothelial proliferation), vascular endothelial growth factor, and hypoxia-inducible factor-1α, suggesting that Cpd188 also has anti-angiogenic effects. We also confirmed the radiosensitizing effect of Cpd188 of GBL28 cell line which was originated from a patient who had a glioblastoma and also was confirmed high level of STAT3 expression and unmethylated MGMT. Cpd188 selectively sensitized glioblastoma cells to the cytotoxic effects of radiotherapy plus TMZ while not effecting the cell death of normal astrocytes.

Conclusions: These data indicate that Cpd188 has the potential to improve the therapeutic outcome of combined radiotherapy and TMZ in human glioblastoma, especially in patients whose tumor has a high level of STAT3 expression regardless of MGMT methylation status.

Keywords: glioblastoma, randiosentization, temozolomide, STAT3

Student Number: 2010-31150

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Introduction

Glioblastoma is the most common malignant primary brain tumor in adults and its extremely poor prognosis is known to be caused by a high degree of resistance to treatment (1). EORTC/NCIC phase III trial showed that the addition of temozomide (TMZ) to radiotherapy postoperatively for glioblastoma resulted in significant survival benefit. Since then maximal safe resection followed by concurrent radiotherapy and TMZ has been the standard of care for newly diagnosed glioblastoma. Nevertheless the median survival time was not exceeding approximately 15 months (2). Despite extensive translational research and conventional treatment to improve treatment outcome, there has been no significant improvement in overall survival for glioblastoma patients. Therefore, preclinical studies and clinical trials have been investigating whether it is possible to increase the therapeutic efficacy of TMZ by combining it with specific inhibitors for pro-survival signaling (3).

The signal transducers and activators of transcription (STATs) are a family of transcription factors that are activated by membrane-bound receptors and subsequently translocate to the nucleus to promote the expression of a variety of genes associated with cell survival, differentiation, and proliferation (4). In particular, STAT3 is a key candidate causing the malignant progression of glioma, and its aberrant activation has been a poor prognostic factor for survival in glioma

patients (5-9). Phosphorylated STAT3 (p-STAT3) leads to transcriptional activation of downstream genes involved in processes such as cell proliferation, differentiation, cell survival, angiogenesis, and cell cycle progression. Whereas normal STAT activation is highly regulated and transient, STAT3 is highly activated in diverse tumor types due to the dysregulation of cytokines, growth factors, or associated kinases (10). Interleukin-6, a major cancer-related inflammatory cytokine, regulates STAT3 activation and is upregulated in glioblastoma (11). The gene encoding epidermal growth factor receptor (EGFR) and its constitutively activated variant, EGFRvIII, are often overexpressed in malignant gliomas (12). On the cell surface, activated EGFR recruits and phosphorylates STAT3 at Y705. In turn, p-STAT3 enters the nucleus to activate the expression of several cancer-related genes (13). Thus, STAT3 is a convergence point of many signaling pathways and plays a major role in oncogenesis. Efforts are ongoing to develop anticancer drugs that target STAT3 (14-16). In malignant gliomas, a number of potential drugs inhibiting STAT3 activity have been investigated (17-23). Furthermore, STAT3 inhibition seems to increase TMZ efficacy by downregulating methyl-guanine methyl transferase gene (MGMT) expression in TMZ-resistant glioblastoma cell lines (24). Cpd188, a small molecule STAT3 inhibitor, inhibits STAT3 Y705 phosphorylation by targeting the STAT3 SH2 domain peptide-binding pocket (25). To the best of our knowledge, no study has reported the effects of Cpd188 on glioblastoma in combination with TMZ and radiation therapy.

In the present study, we investigated the in vitro effects of Cpd188 in

combination with radiotherapy with and without TMZ against a panel of glioblastoma cells. In addition to evaluating the effects of Cpd188 in vitro, we assessed the efficacy of Cpd188 in vivo as a single agent and in combination with TMZ. Taken together, this study shows that the inhibition of STAT3 could represent a strategy to increase the efficacy of TMZ combined with radiation against glioblastoma with activated STAT3.

Materials and Methods

Cell Culture

The human glioblastoma cell lines U251 and U87 were obtained from the American Type Culture Collection. The patient-derived glioblastoma cell lines GBL12 and GBL28 were kindly provided by Dr. Sun Ha Paek (Department of Neurosurgery, Seoul National University Hospital, Seoul, Korea). All procedures were performed according to a protocol approved by the institutional review board at the Clinical Research Institute, Seoul National University Hospital. Cells were maintained and cultured according to standard techniques at 37℃ in 5% (v/v) CO_2 using Dulbecco's modified Eagle's medium (DMEM). Glioblastoma cells were seeded into six-well plates in 10% fetal bovine serum. On the first day of treatment the media were replaced with vehicle control or Cpd188 (10 μM), with or without TMZ (25 μM), in DMEM without fetal bovine serum. The media containing drugs were replaced with DMEM media containing 10% fetal bovine serum after 24 hours.

Pharmacologic Inhibitors

Temozolomide (Schering-Plough, Kenilworth, NJ, USA) stock was stored as an 125-mM solution in dimethyl sulfoxide (DMSO; Sigma-Aldrich, St, Louis, MO, USA) at -20℃. The STAT3 inhibitor 4-((3-(carboxymethylsulfanyl)-4-hydroxy-1-naphthyl)sulfamoyl)benzoic acid (Cpd188) was purchased from Calbiochem (Darmstadt, Germany).

Cpd188 was stored as a concentrated stock solution in DMSO at -20°C and diluted in culture medium at the time of use. Control cells were treated with medium containing the same concentration of the drug carrier, DMSO.

Clonogenic assay

Clonogenic assays were performed as previously described (26). Identical numbers of cells were plated across the different treatment groups for each radiation dose. A specified number of cells were seeded into each wells of six-well culture plates and treated with temozolomide and/or Cpd188. After exposure of drugs, cells were irradiated with 6-MV X-ray from a linear accelerator (Clinac 21EX, Varian Medical Systems, Palo Alto, CA, USA) at a dose rate of 2.46 Gy/min and were incubated for colony formation for 14 to 21 days. Colonies were fixed with methanol and stained with 0.5% crystal violet; the number of colonies containing at least 50 cells was determined and surviving fraction was calculated. Radiation survival data were fitted to a linear-quadratic model using Kaleidagraph version 3.51 (Synergy Software, Reading, PA, USA). Each point on the survival curves represents the mean surviving fraction from at least three dishes. Sensitizer enhancement ratio (SER) was calculated as the ratio of the isoeffective dose at surviving fraction 0.5 and surviving fraction 0.05 in the absence of the drugs to that in the presence of the drugs.

Western Blot Analysis

Protein samples were electrophoresed and transferred to polyvinylidene difluoride membranes (Millipore Corp., Bedford, MA, USA). Membranes were blocked in phosphate-buffered saline (PBS) containing 0.1% Tween 20 and 5% powdered milk and probed with primary antibodies directed against p-Akt (Ser473), p-ERK (Tyr202/204), caspase-3, matrix metalloproteinase (MMP)-2, E-cadherin, and EphA2 (Cell Signaling Technology, Inc., Danvers, MA, USA) at 1:1000 dilutions. Antibodies against p-STAT3 (Tyr 705), vascular endothelial growth factor (VEGF), and β-actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA) were used at dilutions of 1:500 and 1:5000, respectively. Membranes were washed and incubated with peroxidase-labeled goat anti-rabbit or anti-mouse IgG secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) at a dilution of 1:5000.

Caspase-3/7 Assay

Cells (3×10^4 per well) were seeded into a 96-well plate in 200 μL culture medium. Cells were treated with TMZ with or without Cpd188 prior to irradiation. Caspase-3/7 activity was measured as per the manufacturer's instructions (Invitrogen, Life Technologies, Carlsbad, CA, USA).

Annexin V-fluorescein Isothiocyanate (FITC)/Propidium Iodide (PI) Double Staining

Apoptosis was evaluated using annexin V-FITC/PI double staining. Cells were seeded in 8-well chamber slides, treated with Cpd188 with or without TMZ prior to irradiation and stained with annexin V-FITC and propidium iodide according to the manufacturer's instructions (BD biosciences, San Jose, CA, USA). The cells were analyzed under a fluorescence microscope (Carl Zeiss, Goettingen, Germany).

Modified Boyden Chamber Assay

Cell invasion was measured using a transwell system (Corning, Rochester, NY, USA) that allows cells to migrate through 8-μm pores in polycarbonate membranes. Inserts containing cells were placed in 24-well plates (Corning) in starvation medium. Cells were trypsinized and resuspended, and an aliquot of 10^4 cells was added to the upper chamber. After 24 hours, inserts were fixed in methanol and stained with 1% crystal violet solution.

Wound Healing Assay

Cells were grown to confluence in 6-well plates (SonicSeal Slide; Nalge Nunc, Rochester, NY, USA) and starved as described above. Each well was divided into a 2x3 grid. Using a 1-mL pipette tip, a line was scratched in each hemisphere of the well to wound the cells. The medium was replaced with starvation medium. Images were taken of the intersections of the linear cell wound and each grid line. The migration rate was estimated from the distance that the cells moved, as

determined microscopically.

Vasculogenic Mimicry Formation Assay

The vasculogenic mimicry (VM) formation assay was performed using a commercialized Matrigel assay kit (BD Biosciences). ECM Matrigel (200 μL) was added to each well in 48-well tissue culture plates and incubated at 37℃ for 2 hours. Cells were treated with TMZ (25 μM) with or without Cpd188 (10 μM) and seeded onto the coated plate. After 24 hours of growth on the plate, VM formation was assessed using an inverted microscope.

In vivo Tumor Model

U251 cells had been modified to express a luciferase reporter (Promega, Fitchburg, WI, USA) in order to track tumor growth in vivo. Human glioblastoma xenografts were established by injecting 3×10^5 U251 cells intracranially into the right caudate-putamen of 6- to 8-week-old BALB/c athymic nude mice (Orient Bio Inc, Sungnam, Korea) with a 26G needle attached to a Hamilton syringe. All procedures were performed according to a protocol approved by the institutional animal care and use committee at the Clinical Research Institute, Seoul National University Bundang Hospital. Mice were randomized into groups of five animals per group on day 14 after injection, and the following treatments were started on day 15: control, Cpd188 only, TMZ only, TMZ combined with Cpd188, irradiation only,

Cpd188 combined with radiation, TMZ combined with radiation, and TMZ combined with Cpd188 and radiation. TMZ was administered orally in PBS at 25 mg/kg three times weekly. Cpd188 was dissolved in PBS and administered intraperitoneally at 20 mg/kg three times weekly for 3 weeks. When Cpd188 and TMZ were given in combination, TMZ was given 30 minutes after Cpd188. Starting on day 28 post-injection, mice were irradiated with a fractionated schedule (3 Gy) three times per week total 9 Gy using 6 MeV electron beam.

Bioluminescence Imaging

In vivo bioluminescence images were obtained using the IVIS Imaging System 100 series (Xenogen Corporation, Alameda, CA, USA). Beginning on day 14 post-injection, mice were injected with 150 mg/kg D-luciferin intraperitoneally. Fifteen minutes after injection, mice were anesthetized with 1−2% isoflurane. Images were acquired between 5 and 10 minutes, and peak luminescence signals were recorded. For statistical analysis comparing treatment groups, bioluminescence values from the last day where three mice in each group were alive were used.

Statistical Analysis

The results are expressed as the means ± standard deviation (SD) of three independent experiments. Data from these experiments were analyzed by Student's t test (SPSS v15.0 software). The Kaplan-Meier survival curves were constructed by GraphPad Prism 6.0 software

(GraphPad Software, San Diego, CA, USA), and a log-rank test was used to examine the differences between groups. Significant differences were established at $P<0.05$.

Results

First, we evaluated p-STAT3 expression levels in a panel of glioblastoma cell lines. U251 and GBL28 cell lines, in particular, showed high levels of p-STAT3 expression among a panel of glioblastoma cell lines (Figure 1A). Next, we evaluated whether targeting STAT3 would radiosensitize U251 and U87 glioblastoma cell lines and further increase the radiosensitizing effect of TMZ. To determine whether the STAT3 inhibitor Cpd188 enhances the cytotoxic effect of radiotherapy plus TMZ, glioblastoma cell lines were pretreated with Cpd188 (10 μM), TMZ (25 μM), or both for 24 hours. Pretreatment with Cpd188 markedly caused a reduction in the levels of p-STAT3 but did not affect p-AKT or p-ERK expression in either cell line. U251 cells, which have high p-STAT3 expression, showed a high degree of radiosensitization by Cpd188 plus TMZ ($SER_{0.5}$, 1.43), whereas U87 cells with low p-STAT3 expression showed less radiosensitization ($SER_{0.5}$, 1.34; Figure 1B). Cpd188 preferentially potentiates the radiosensitizing effect of TMZ in glioblastoma cells with high levels of p-STAT3 expression. Radiation-induced cell death with 8 Gy was significantly enhanced in the combination treatment group compared to the single-drug groups for U251 cells (P=0.01 for TMZ and P=0.04 for Cpd188) based on unpaired t-test. For U87 cells, however, the combination treatment did not enhanced radiation-induced cell death with 8 Gy compared to the single-drug treatment (P=0.68 for

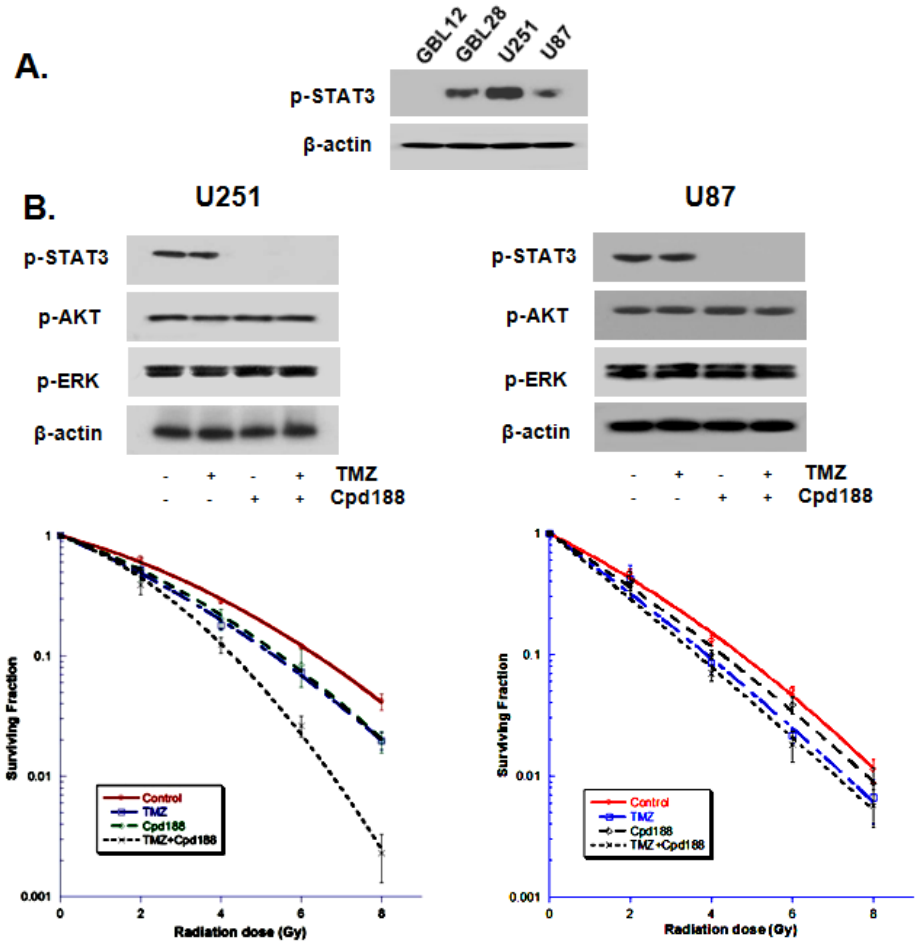

Figure 1. p-STAT3 protein expression and clonogenic survival. (A) High levels of p-STAT3 (Tyr705) protein expression in U251 and GBL28 cell lines and low levels of p-STAT3 expression in U87 and GBL12 cell lines. (B) Whereas U251 cells showed a high degree of radiosensitization, U87 cells showed less radiosensitization after combined treatment with Cpd188 and TMZ.

TMZ, P=0.27 for Cpd188).

Previous studies have shown that targeting STAT3 signaling induces cancer cell apoptosis (27). Annexin V-FITC/PI double staining and caspase-3/7 assay methods were employed to examine apoptotic cell death in U251 cells with high levels of p-STAT3 expression. As shown in Figure 2A, treatment with Cpd188 with or without radiation and/or TMZ produced more late-apoptotic cells. Additionally, co-treatment with Cpd188 with or without radiation and/or TMZ increased cleaved caspase-3 expression and caspase-3/7 activity within 24 hours after combination treatment in U251 cells (Figure 2B, $P<0.01$).

Radiation and/or TMZ treatment did not inhibit cell migration and invasion in U251 cells. However, treatment with Cpd188 combined with radiation with or without TMZ markedly inhibited the ability of U251 glioblastoma cells to migrate and invade (Figure 3A, B, $P<0.05$). To evaluate the inhibitory effect of each treatment on VM, we performed a VM formation assay using U251 glioblastoma cells. As shown in Figure 3C, neither radiation (6 Gy) nor TMZ treatment blocked VM formation. In contrast, treatment with Cpd188 combined with radiation with or without TMZ significantly impaired VM formation in U251 glioblastoma cells. Consistent with the reduction of invasion, migration, and VM formation, treatment with Cpd188 combined with radiation with or without TMZ decreased the expression of VEGF, MMP-2, and EphA2. In contrast, treatment with Cpd188 with or without TMZ resulted in up-regulation of epithelial marker E-cadherin (Figure 3C).

Next, we determined whether the enhancement of anti-tumor effects by

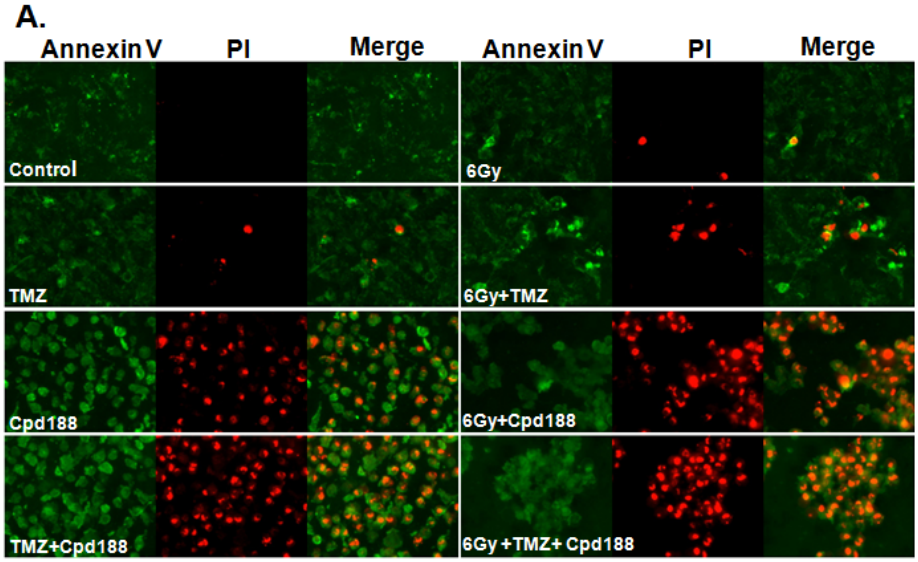

Figure 2. Cpd188 increased apoptosis in U251 glioblastoma cells. (A) Cpd188 treatment, with or without TMZ, and/or radiotherapy, increased green and red fluorescence in U251 cells.

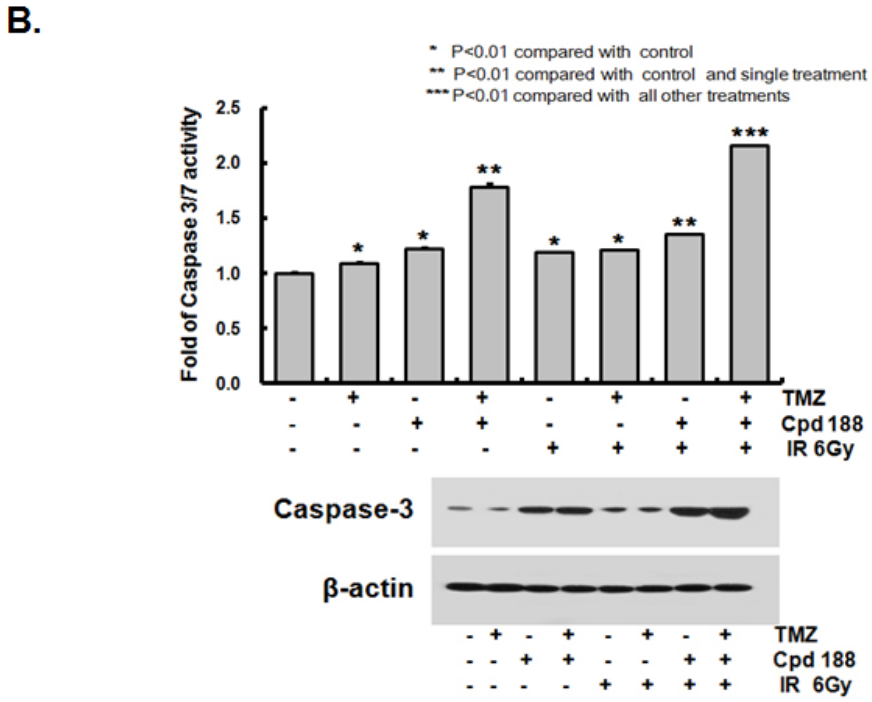

Figure 2. (B) Columns indicate means of caspase-3/7 activity compared to control. Error bars indicate SDs. The combination treatment of TMZ with Cpd188 increased caspase-3/7 activity and cleaved caspase-3 expression within 24 hours in U251 cells. Each experiment was repeated three times with similar results.

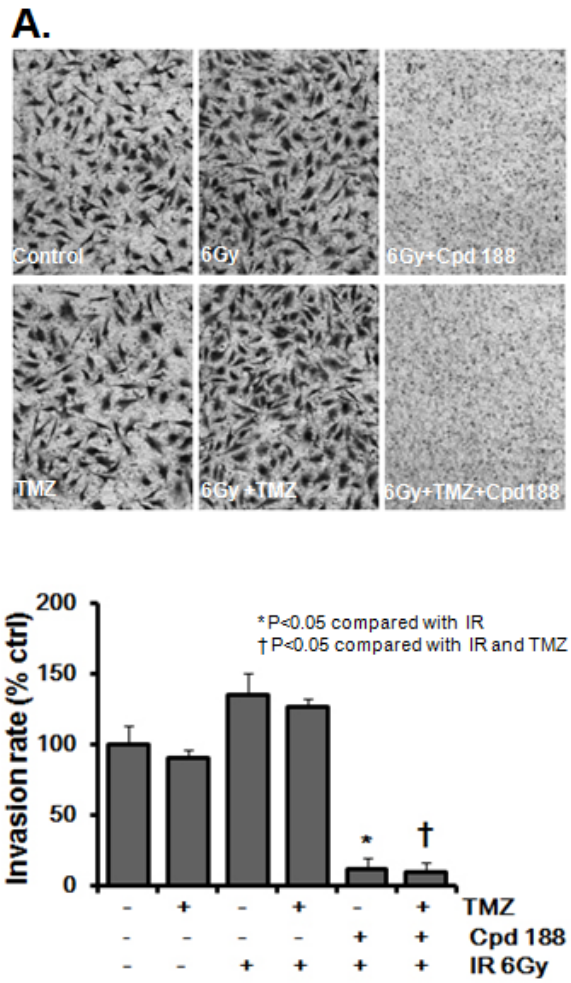

Figure 3. The effect on invasion, migration, and vasculogenic mimicry. (A) The effects on invasion was assessed in a modified Boyden chamber assay. Stained cells were analyzed in representative fields (100×).

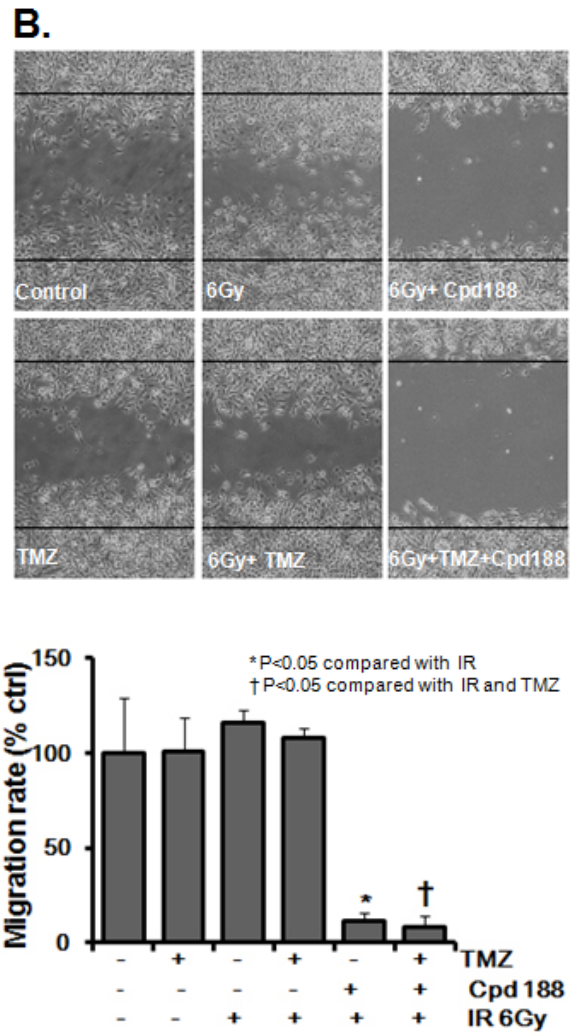

Figure 3. (B) The effects on migration were assessed in a wound healing assay. Stained cells were analyzed in representative fields (100×).

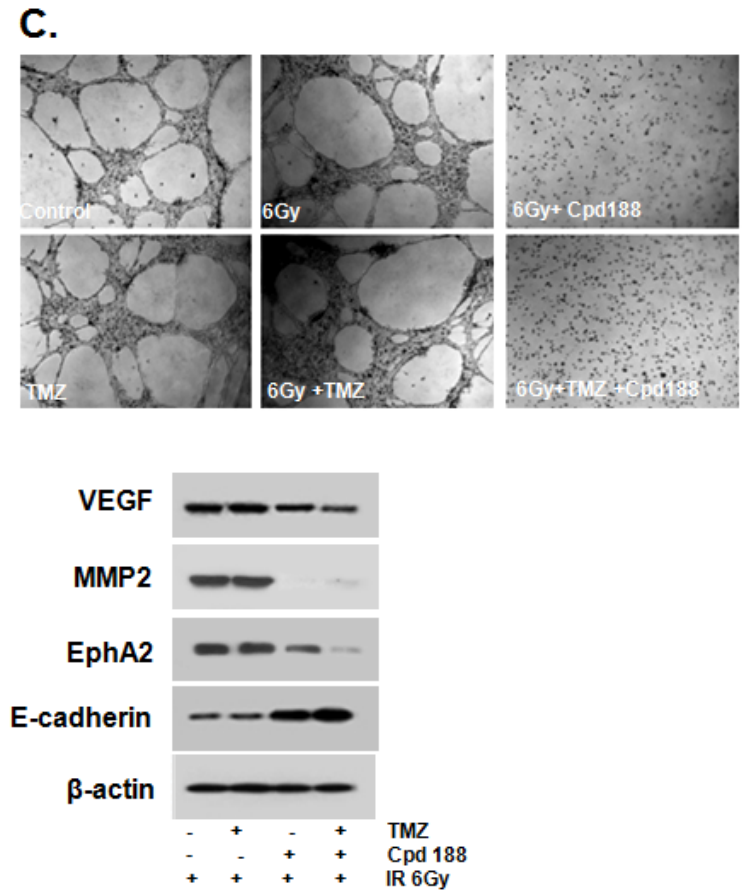

Figure 3. (C) The ability of U251 cells to undergo vasculogenic mimicry (VM) when plated on Matrigel was determined (200×). The treatment of Cpd188 combined with radiotherapy, with or without TMZ, resulted in down-regulation of VEGF, MMP-2, and EphA2 expression and up-regulation of E-cadherin expression.

Cpd188 and TMZ in vitro could be translated into in vivo tumor models. On day 35, the last day that three nude mice of each group were alive, median bioluminescence values showed a 6-fold decrease in volume for Cpd188 only (P=0.001), a 20-fold decrease for TMZ only (P=0.008), a 43-fold decrease for TMZ combined with Cpd188 (P=0.001), a 3-fold decrease for irradiation only (P=0.005), an 11-fold decrease for Cpd188 combined with radiation (P=0.001), a 28-fold decrease for TMZ combined with radiation (P=0.008), and an 83-fold decrease for TMZ combined with Cpd188 and radiation (P=0.001) relative to the control group (Figure 4A). In particular, levels of bioluminescence in the combination group (TMZ combined with Cpd188 and radiation) were the same as background, indicating very robust tumor regression. As shown in Figure 4B, Kaplan-Meier survival curves indicate that survival of mice increases after administration of TMZ, Cpd188, or TMZ and Cpd188 in nude mice bearing intracranial U251 xenografts. Survival in triple treatment group (radiotherapy plus TMZ and Cpd188) was significantly longer compared to control (P=0.003) and radiation only group (P=0.007). Mice treated with the combination Cpd188 plus TMZ exhibited longer survival relative to both Cpd188 monotherapy and TMZ monotherapy. While mice in the combination, TMZ with Cpd188, or TMZ with Cpd188 and radiation groups did have a trend for longer median survival than mice receiving TMZ alone or TMZ with radiation, In addition, there were no significant physical or behavioral differences in any of the treatment groups compared to the control group.

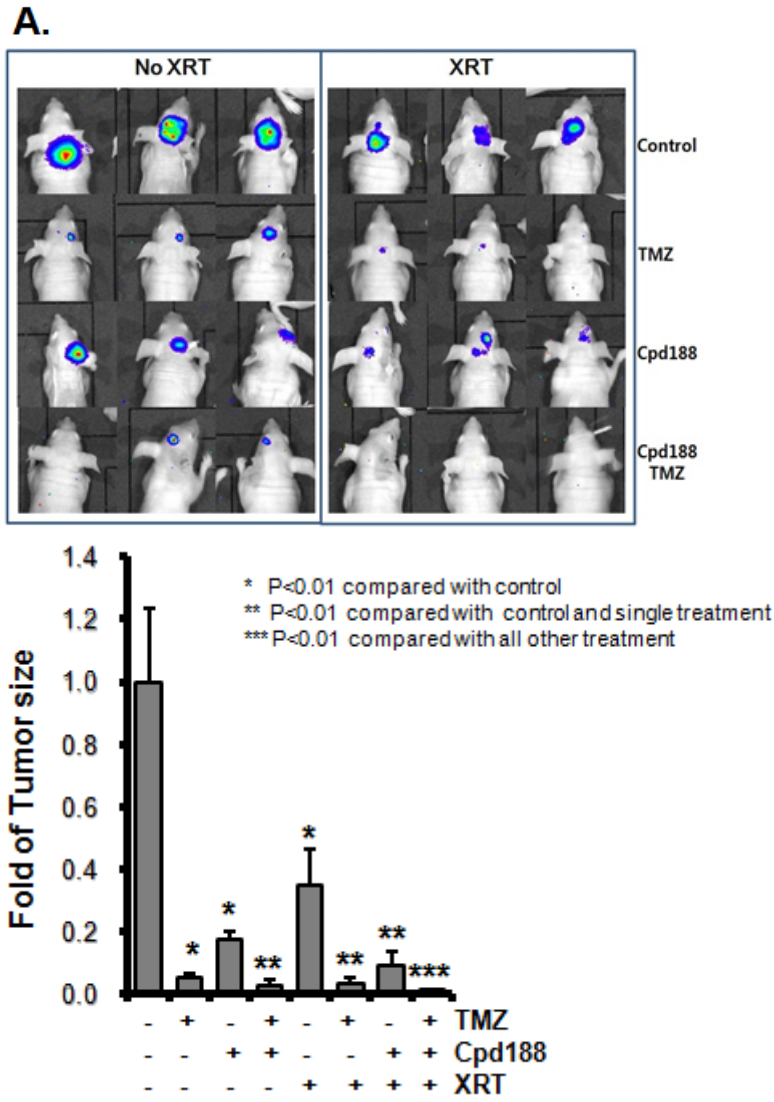

Figure 4. Synergistic antitumor activity of Cpd188 and TMZ in vivo. (A) Representative bioluminescence images in each treatment group on day 35. Tumor burden as measured by bioluminescence was decreased after TMZ and Cpd188 administration compared with TMZ or Cpd188 monotherapy. Bars indicate SDs.

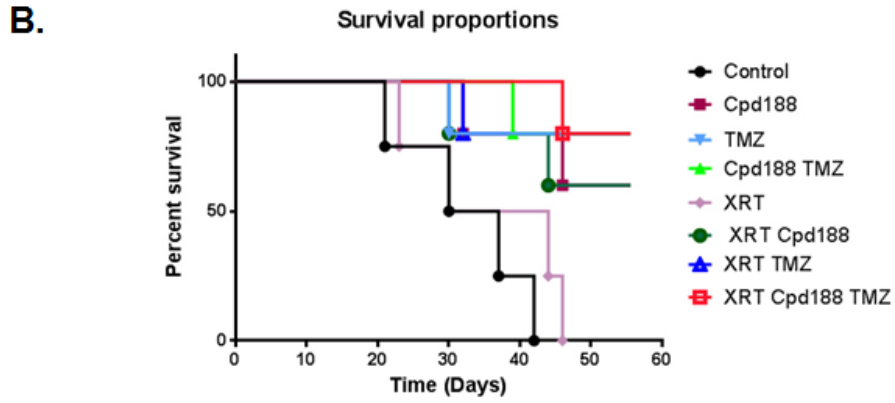

Figure 4. (B) Survival of mice increases after administration of TMZ, Cpd188, or TMZ+Cpd188 in nude mice bearing intracranial U251 xenografts. Mice treated with the combination Cpd188 plus TMZ exhibited longer survival relative to both Cpd188 monotherapy and TMZ monotherapy.

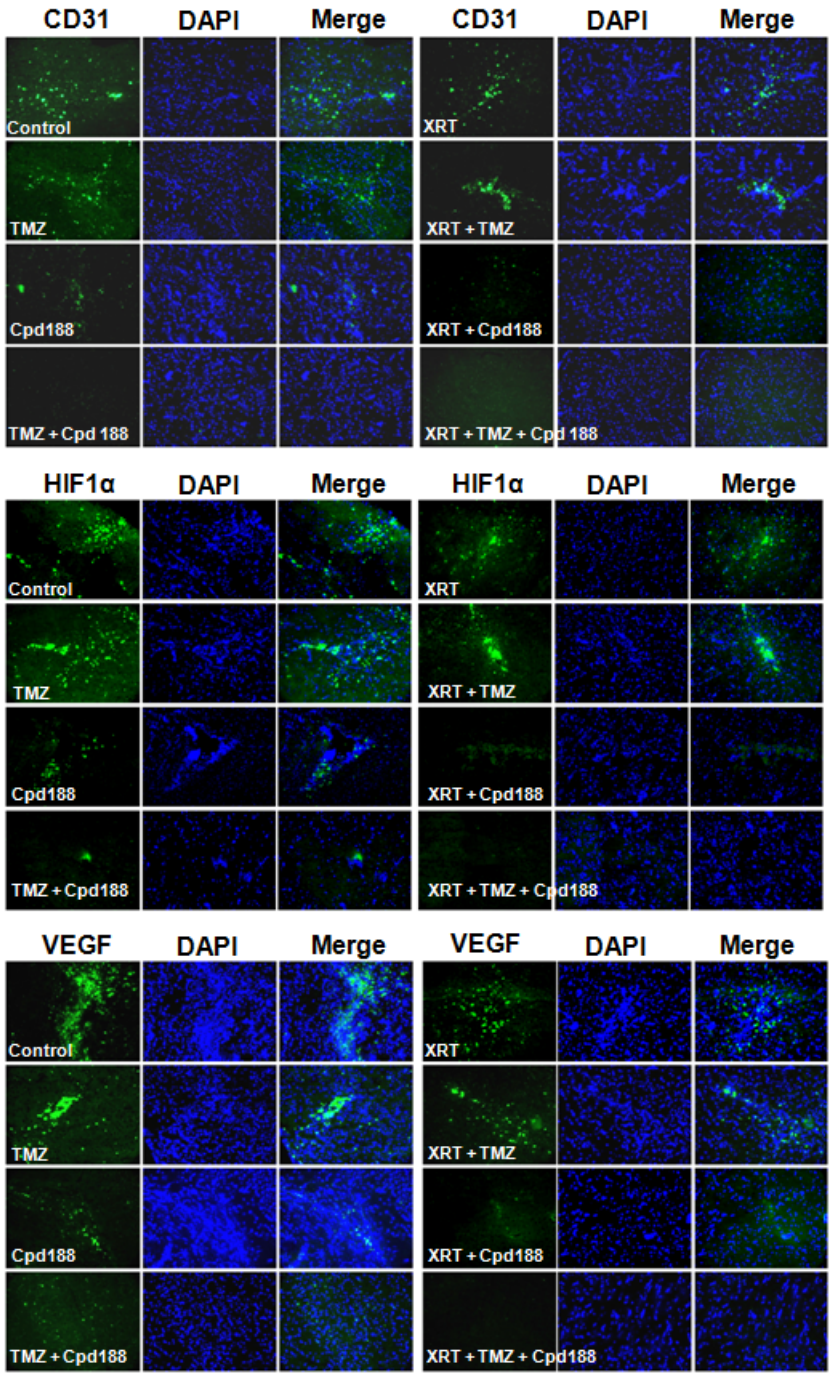
CD31
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Figure 5. Immunohistochemical analysis. Mice were treated with Cpd188, with or without TMZ, and with or without radiation, tumors were harvested, and representative tumor sections were stained for CD31, HIF-1α, or VEGF. Representative examples for staining of CD31 (top row, green), HIF-1α (middle row, green), and VEGF (bottom row, green) in tumor sections from various treatment groups (200×) are shown.

As shown in Figure. 5, a significant decrease in CD31, a marker of endothelial proliferation as well as HIF-1α and VEGF was observed after the treatment with Cpd188 with or without radiotherapy and/or TMZ. In contrast, TMZ with or without radiotherapy did not generate significant changes in the levels of CD31, VEGF, or HIF-1α staining. Consistent with this observation, the CD31, VEGF, and HIF-1α staining of cells treated only with Cpd188 and those that underwent combination treatment (Cpd188 plus TMZ) were comparable.

Finally, we confirmed that Cpd188 could radiosensitize patient-derived glioblastoma cell lines GBL12 and GBL28 and further increase the radiosensitizing effect of TMZ. The methylation status of MGMT gene promoter was determined retrospectively from the tumor tissue of cell line donors, and both of these cell lines had unmethylated MGMT gene promoter. While GBL12 cells, with low p-STAT3 expression, showed less radiosensitization by Cpd188 plus TMZ, GBL28 cells, with high p-STAT3 expression, showed a high degree of radiosensitization by Cpd188 plus TMZ (Figure 6A). Table 1 shows SER for each inhibitor alone and combined with TMZ in each glioblastoma cell line. Radiation-induced cell death with 8 Gy showed a trend for enhancement in the combination treatment group compared to the single-drug groups for GBL28 cells (P=0.09 for TMZ and P=0.08 for Cpd188) based on unpaired t-test. For GBL12 cells, the combination treatment did not enhanced radiation-induced cell death with 8 Gy compared to the single-drug treatment (P=0.67 for TMZ, P=0.21 for Cpd188). Figure 6B showed magnetic resonance images (MRI) of each

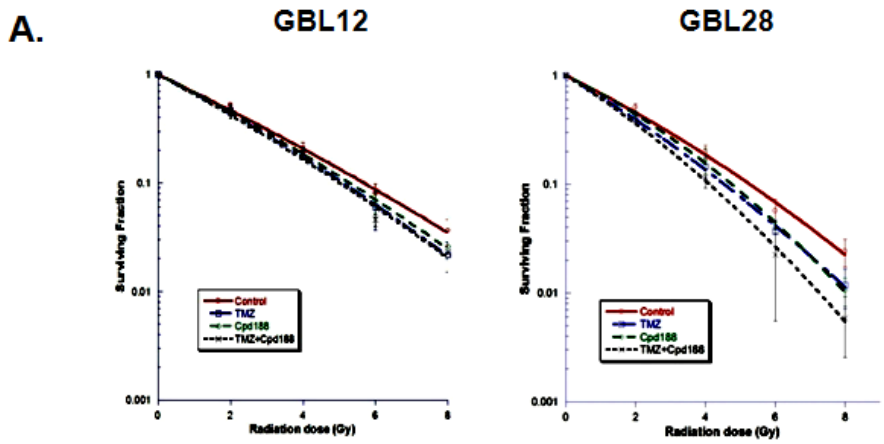

Figure 6. Clonogenic survival of patient-derived glioblastoma cell lines after each treatment and magnetic resonance images (MRI) of cell line donors before and after concurrent radiotherapy and TMZ. (A) Patient glioblastoma cell lines GBL12 and GBL28 were pretreated with the STAT3 inhibitor Cpd188 (10 μM) plus TMZ (25 μM) for 24 hours. While GBL28 cells showed a high degree of radiosensitization, GBL12 cells showed less radiosensitization after combined treatment with Cpd188 and TMZ. Points on the survival curves represent mean surviving fractions from experiments performed in triplicate.

B.

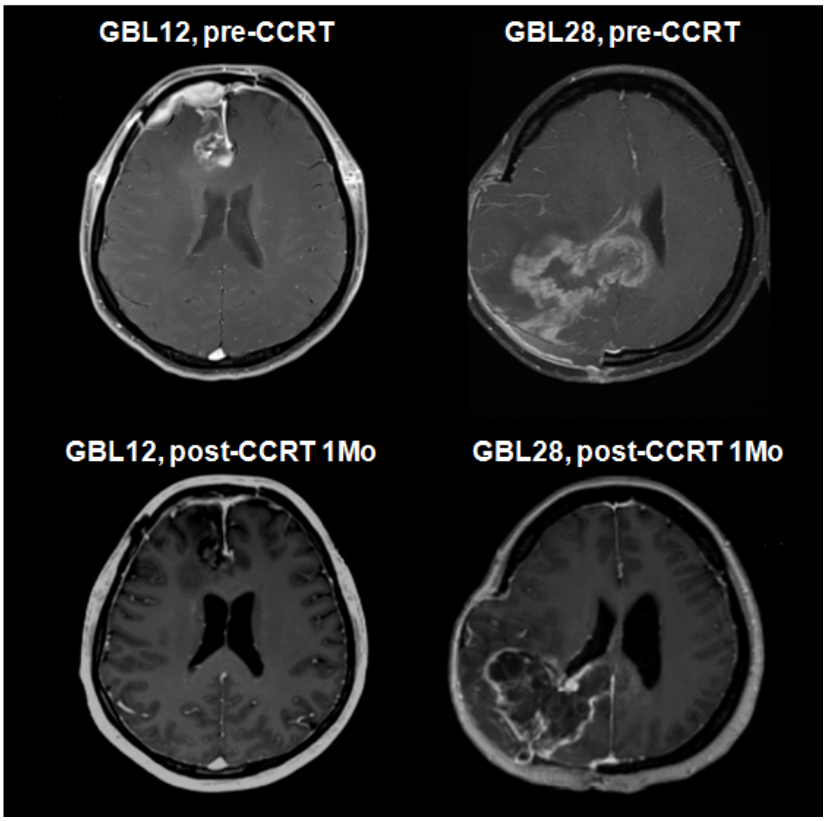

Figure 6. (B) MRI images of each cell line donors were taken before the start of concurrent chemoradiotherapy (CCRT) with TMZ, and after 1 month completion of CCRT.

Cell line	Inhibitor	$SER_{0.5}$	$SER_{0.05}$
U251	Cpd188 TMZ TMZ + Cpd188	1.25 1.32 1.43	1.17 1.17 1.51
U87	Cpd188 TMZ TMZ + Cpd188	1.07 1.33 1.34	1.09 1.18 1.24
GBL12	Cpd188 TMZ TMZ + Cpd188	1.08 1.08 1.08	1.09 1.14 1.14
GBL28	Cpd188 TMZ TMZ + Cpd188	1.17 1.27 1.40	1.06 1.06 1.39

Table 1. SER (Sensitizer enhancement ratio) for each inhibitor alone and combined with TMZ in each glioblastoma cell line.

patient whose cells were derived, before and after, a concurrent radiotherapy and TMZ. Although these are only illustrative examples, patient who had tumor with low p-STAT3 expression is still alive after 26 months of radiotherapy and TMZ, while patient with high p-STAT3 expression died after 8 months of radiotherapy and TMZ.

Discussion

Recently, several studies have focused on aberrant signal transduction in glioblastoma and resistance mechanisms of glioblastoma to TMZ and radiotherapy (3). One frequently dysregulated pathway is STAT3 signaling, which is activated by various mechanisms in glioblastoma. STAT3 is an oncogene and is a member of the STAT protein family, which are signaling intermediates that mediate the actions of many cytokines and growth factors (5). Previous studies showed that aberrant activation of STAT3 was identified not only in glioblastoma but also in many other cancers (10). Further, it has recently been demonstrated that the expression of p-STAT3 correlates with epithelial mesenchymal transition via EGR/EGFR pathway, reducing E-cadherin expression and increasing mesenchymal proteins including TWIST (28-33). STAT3 is also essential for the aggressiveness of gliomas (6-9). Local invasive growth is a key feature of glioblastoma, and this highly invasive/migratory characteristic is considered to be a major therapeutic obstacle for glioblastoma treatment (34). The addition of Cpd188 to radiotherapy with or without TMZ markedly suppressed invasion/migration of U251 glioblastoma cells compared to TMZ monotherapy. Additionally, VM is as the process whereby non-endothelial tumor cell-lined microvascular channels are formed (35), and traditional anti-vascular therapies aiming at endothelial cells have no significant effects on malignant tumors with VM (36). In this study,

Cpd188 was also able to inhibit VM formation effectively in U251 glioblastoma cells.

STAT3 also does not seem to be essential for the survival of untransformed cells but is indispensable for many different tumor cells (37), providing another reason to target STAT3. In our results, Cpd188 selectively sensitized glioblastoma cells to the cytotoxic effects of radiotherapy plus TMZ while not effecting the cell death of normal astrocytes (Figure 7).

Further, glioblastoma is highly proliferative and resistant to apoptosis, and STAT3 acts as a suppressor of apoptosis in a number of cancer cells (38). Several human tumor xenograft studies in mice have repeatedly demonstrated that inhibiting STAT3 results in decreased tumor growth and improved animal survival by inducing apoptosis in tumor cells (27) and enhancing anti-tumor immune-mediated cytotoxicity (39). Potential drugs targeting STAT3 or downstream pathway have been investigated to identify their inhibitory effect on glioblastoma growth in vitro (9,18,21-23). Some small molecules were reported to suppress phosphorylation of STAT3 directly and inhibit STAT3 DNA binding (18). Other drugs decreased activation of STAT3 via JAK2 inhibition (21,22). The effect of STAT3 inhibitors on glioblastoma cell growth were also investigated in mice tumor model with constitutively activated STAT3, and survival gain with STAT3 inhibitors in monotherapy or combined therapy with radiotherapy have been reported (17,19,20). Kohsaka et al. suggested that STAT3 inhibition could overcome TMZ resistance in glioblastoma and STAT3 inhibitor might

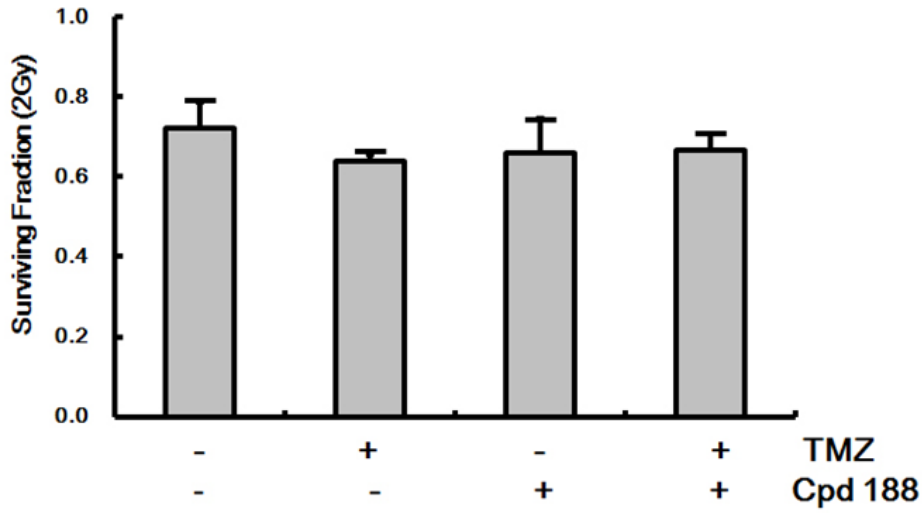

Figure 7. Cpd188 did not increased toxicity of normal cells

be one of the candidate drugs for combination therapy with TMZ for patients with TMZ-resistant glioblastoma (24). In our study, Cpd188 preferentially potentiated the radiosensitizing effect of TMZ in glioblastoma cell lines that expressed high levels of p-STAT3 by augmenting caspase-3/7 activity and cleaved caspase-3 expression in vitro. Moreover, our in vivo data showed that the treatment of Cpd188 effectively increased anti-tumor activity of radiotherapy with or without TMZ, resulting in tumor growth delay and improvement in survival rate.

Poor prognostic impact of p-STAT3 expression has been well documented in various types of malignancy (40-46). Abou-Ghazal et al. reported that p-STAT3 expression was associated with the poor prognosis in patients with anaplastic astrocytoma (6). Tu et al. also described association with the poor prognosis and p-STAT3 expression in glioma patients (47). In present study, Cpd188 enhanced radiation effect on patient-derived glioblastoma cell lines with TMZ, especially in cell lines with high levels of p-STAT3 expression. These findings suggest that these strategies are potentially beneficial in the patients who had p-STAT3 expression. In our study, donor of GBL28 cell line died earlier than GBL12 donor. However, if effective ways against p-STAT3 other than TMZ had existed, the clinical outcomes could have been altered.

Five-year follow-up analysis of EORTC/NCIC trial revealed that the methylation status of MGMT promoter was the strongest predictor for benefit from the addition of TMZ (48). Glioblastoma patients with a

high level of MGMT protein expression are resistant to TMZ, in other words, therapeutic benefit of TMZ for those with unmethylated MGMT promoter are remarkedly limited (49). U251 and U87 cell lines are known to be MGMT-negative due to methylated MGMT promotor (50), whereas patient-derived cell lines; GBL12 and GBL28 had unmethylated MGMT promoter. Nevertheless, GBL28 cells with high p-STAT3 expression showed a high degree of radiosensitization by Cpd188 plus TMZ. These finding correspond closely with results by another investigator (24), which is a strong suggestion that combination of Cpd188 with TMZ has the potential to be successful regimen for patients with unmethylated MGMT as well as those with methylated MGMT.

Genotoxic radiotherapy activates various radiation response genes and cytokines and alters peritumoral microenvironment, in turn increases invasion and metastasis of surviving tumor cells. These enhance resistance to treatment and rapid tumor repopulation (51). In present study, radiation and/or TMZ showed rather detrimental effects on invasion and migration (Figure 3A, 3B). These findings are comparable with the results by Li et al. These authors showed that radiation significantly promoted the invasion of A549 cells in a dose-dependent manner compared with the untreated controls. In addition, they showed that radiation induces STAT3 phosphorylation, promotes its nuclear localizationanner, and inhibition of STAT3 phosphorylation suppressed the radiation-induced expression of MMP-2 and invasion of A549 cells (52). Gao et al. detected nucleus translocation of p-STAT3 in A549

cells by immunofluorescence (53).

Involvement of STAT3 with DNA damage repair process was suggested one of the possible mechanism of radiation-induced resistance, however, a role for STAT3 or its contribution in response to DNA damage has not been identified clearly. A few studies for non-brain tumor reported the potential correaltionship between STAT3 and DNA repair. Courapied et al. proposed that cdk5-STAT3 pathway reduced DNA damage in response to topoisomerase I inhibition via activation of Eme1 in human colorectal cell line (54). Barry et al. showed that STAT3 was necessary for efficient repair of damaged DNA via ATM-Chk1/2 pathways in mouse embryonic fibroblast (55).

Taken together, these results showed that the addition of Cpd188 increased the anti-tumor effects of radiotherapy with or without TMZ both in vitro and in vivo. The beneficial effects of Cpd188 in combination with other agents are associated with the induction of apoptosis and the inhibition of migration/invasion and angiogenesis. Therefore, our data suggest that targeting STAT3 signaling using Cpd188 may provide a potential therapeutic intervention for glioblastoma to improve the therapeutic outcome of TMZ combined with radiotherapy, especially in patients with high levels of p-STAT3 expression regardless of MGMT methylation status.

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초 록

서론: 교모세포종은 수술 후 방사선치료-테모졸로마이드 동시 병용요법과 테모졸로마이드 보조요법과 같은 적극적인 치료에도 불구하고 예후가 매우 불량하다. 본 연구는 Signal Transducer and activator of transcription-3 (STAT3)에 대한 표적 억제가 방사선치료와 테모졸로마이드에 대한 치료 감수성을 증강시킬 수 있는 방법인지 확인하고 관련 기전을 규명하기 위하여 시행하였다.

방법: STAT3 억제제인 Cpd188을 테모졸로마이드와 방사선조사 병용에 추가적으로 처리하였을 때의 효과를 평가하기 위해, 교모세포종 세포주 U251, U87과 환자로부터 얻은 세포주 GBL12, GBL28을 배양하여 대상 세포생존을 결정하기 위한 세포집락 검사 (clonigenic assay), 세포사의 방식이 세포고사임을 확인하기 위한 caspase-3/7 assay 및 annexin V-FITC/PI 이중 염색 검사, epithelial-mesenchymal transition (EMT) 관련 기전이 관여하는지 확인하기 위한 modified Boyden chamber assay, wound healing assay, vasculogenic mimicry assay 등을 시행하였다. 한편, 6-8주 연령의 BALB/c 무흉선 누드 마우스에 U251 세포를 두개내 이식한 종양 모델에서 이종이식 종양의 크기를 생체발광영상(bioluminescence; BLI)을 이용하여 평가하였고, 마우스에서 얻은 조직 절편을 가지고 종양미세환경과 관련되어 있는 표지자를 면역조직화학 염색으로 분석하였다.

결과: Cpd188은 p-STAT3 발현이 증가한 U251 세포주에서 테모졸로마이드 및 방사선조사와 병용 시 세포살상 효과를 증강시켰다. 그 증강 기전에 세포고사 유도 및 EMT의 복귀가 관련되었다. Cpd188은 방사선조사 및 테모졸로마이드와 병용투여 시 마우스 종양 성장을 지연시켰고 생존율도 증가시키는 경향을 보였다 (대조군과 비교 시 P=0.003, 방사선조사 단독치료군과 비교 시 P=0.007). 종양 절편을 면역조직화학염색한 결과 Cpd188은 CD31, VEGD (vascular endothelial growth factor), HIF-1α (hipoxia-inducible factor-1α)의 발현을 감소시켰는데, 이는 Cpd188이 신생혈관 생성을 억제하는 효과가 있음을 시사한다. 또한, p-STAT3 발현 정도가 높고 methyl-guanine methyl transferase (MGMT) 메틸화가 되어있지 않은 GBL28 세포주에서도 테모졸로마이드와 방사선조사의 병용시 세포살상 효과가 증강되었다. 이러한 Cpd188의 방사선치료와 테모졸로마이드의 세포살상 효과 증강은 정상 교세포에서는 관찰되지 않았다.

결론: STAT3 억제제인 Cpd188가 배양 및 마우스 이식 교모세포종 세포주에서 방사선조사와 테모졸로마이드 병용 시의 세포치사 효과를 향상시켰다. 그러한 효과는 특히 p-STAT3 발현이 높은 종양에서 뚜렷하였으나 MGMT 메틸화 상태에 따른 영향은 없었다.

주요어: 교모세포종, 방사선 감수성, 테모졸로마이드, STAT3

학 번: 2010-31150