Anticancer Non-narcotic Opium Alkaloid Papaverine Suppresses Human Glioblastoma Cell Growth

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Abstract. *Background: Glioblastoma (GBM) is the most aggressive type of primary malignant brain tumour. The interaction between high-mobility group box 1 (HMGB1) and receptor for advanced glycation end-products (RAGE) is important for tumour cell growth. Previously, we identified an anticancer candidate, papaverine, that inhibited the HMGB1–RAGE interaction. Materials and Methods: Our study assessed the anticancer effects of papaverine alone or in combination with temozolomide on U87MG and T98G human GBM cells using clonogenicity assays, as well as in a U87MG xenograft mouse model. The radiosensitizing efficacy of papaverine was measured based on the clonogenicity of T98G cells. Results: Papaverine significantly inhibited the clonogenicity of U87MG and T98G cells. Compared with single treatment, the combination of papaverine and temozolomide more highly suppressed the clonogenicity of T98G cells and delayed tumour growth in the U87MG xenograft mouse model. Furthermore, papaverine increased the radiosensitivity of T98G cells. Conclusion: Papaverine is a potential anticancer drug in GBM treatment.*

Glioblastoma multiforme (GBM) is one of the most aggressive primary malignant brain tumours, with a median survival time of ~14-15 months after diagnosis (1-5). Conventional treatments for patients with newly diagnosed

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GBM include surgery, radiotherapy, and chemotherapy with temozolomide. Temozolomide is the only standard chemotherapy available for GBM. It is the prodrug for an alkylating agent that transfers a methyl group to the O6, N7, and N3 positions of the purine bases of DNA. However, O6-methylguanine-DNA methyltransferase (MGMT) directly repairs the main cytotoxic lesion caused by temozolomide-mediated O6-guanine methylation. Therefore, MGMT activity may be the main mechanism underlying resistance to temozolomide (3, 4). In addition, mismatch repair and base excision repair contribute to resistance to temozolomide treatment (3, 4). Therefore, the discovery of next-generation anticancer drugs is important for improving the outcomes of GBM chemotherapy.

We previously evaluated the role of the interaction between high-mobility group box 1 (HMGB1) and receptor for advanced glycation end-products (RAGE) in facilitating tumour growth. HMGB1 is a non-histone DNA-binding nuclear protein that functions as an extracellular signalling molecule during inflammation, cell differentiation, cell migration, and tumour metastasis (6-9). HMGB1 has a high affinity for several receptors, including RAGE and toll-like receptors (TLR2, TLR4, and TLR9) (6-9). RAGE binds to multiple ligands including HMGB1, S100 family members, and amyloid-β (6-9). RAGE activation has been implicated in inflammation, tumour cell growth, migration, and invasion (6-9). Previously, we identified papaverine, a non-narcotic opium alkaloid and an inhibitor of the HMGB1–RAGE interaction, using *in silico* drug design and drug repositioning (10). Moreover, papaverine was found to suppress RAGE-dependent cell proliferation, migration, and invasion in HT1080 human fibrosarcoma cells (11).

Papaverine, a non-narcotic opium alkaloid (Figure 1), is isolated from *Papaver somniferum* (12). Medicinal papaverine, which is used as a smooth-muscle relaxant in the

treatment of vasospasm and erectile dysfunction, functions by inhibiting phosphodiesterase 10A (13-15). Papaverine reportedly shows selective anticancer effects on several types of tumour cells, including LNCaP (16, 17) and PC-3 prostate carcinoma (18); HT29 colorectal carcinoma (19); T47D (19), MCF-7, and MDA-MB-231 breast carcinoma (20); HT1080 fibrosarcoma (19); and HepG2 hepatocarcinoma cells (21). Benej et al. reported that papaverine radiosensitized A549 lung and EO771 breast tumour cells by targeting mitochondrial complex 1 (22). Recently, we demonstrated that papaverine suppressed the proliferation of human GBM temozolomide-sensitive U87MG and temozolomide-resistant T98G cells by inhibiting the interaction between HMGB1 and RAGE. In addition, papaverine reduced tumour volume in a human GBM U87MG xenograft mouse model (23).

In the present study, we examined the anticancer effects of papaverine alone or in combination with temozolomide in human GBM U87MG and T98G cells as well as in the U87MG xenograft mouse model.

Materials and Methods

Reagents. Papaverine hydrochloride was obtained from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan). Papaverine was stored as a 30-mM stock solution in ultra-pure water at −20˚C. Temozolomide was obtained from LKT Laboratories, Inc. (St. Paul, MN, USA) and stored as a 150-mM stock solution in dimethyl sulphoxide (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) at −20˚C.

Cell culture. Human GBM U87MG and T98G cell lines were cultured as previously described (23). Briefly, U87MG and T98G cells were cultured in E-MEM and RPMI-1640, respectively, supplemented with 10% heat-inactivated foetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. Cells were grown in an incubator at 37˚C with 5% CO_2 at 100% relative humidity.

Colony-formation assay. Colony-formation assays were performed as previously described (24, 25). T98G Human GBM cells were dissociated using AccuMax (NACALAI TESQUE INC., Nijo Karasuma, Kyoto, Japan), suspended in medium, seeded into 6-well plates (200 cells per well) in triplicate, and incubated overnight. U87MG cells were dissociated using accutase (NACALAI TESQUE INC., Nijo Karasuma, Kyoto, Japan), suspended in medium, seeded onto poly-L-lysine coated 6-well plates (400 cells per well) in triplicate, and incubated overnight. T98G cells were then treated with papaverine (0.3, 1, 3, 10, and 30 μM) or temozolomide (10, 30, 100, 300, and 1000 μM). U87MG cells were then treated with papaverine (1, 3, 10, 30, and 100 μM) or temozolomide (10, 30, 100, 300, and 1000 μM). Dimethyl sulfoxide (DMSO) and ultra-pure water were used as controls. After incubation for 10 days, the cells were fixed with a 4% formaldehyde–phosphate-buffered saline (PBS) solution and stained with 0.1% (w/v) crystal violet. Colonies on the well were counted.

Gamma-ray irradiation. Gamma (γ)-ray irradiation was performed as previously described (25-27). T98G cells were γ-irradiated to different doses using a Cs137 Gammacells 40 Exactor (Best
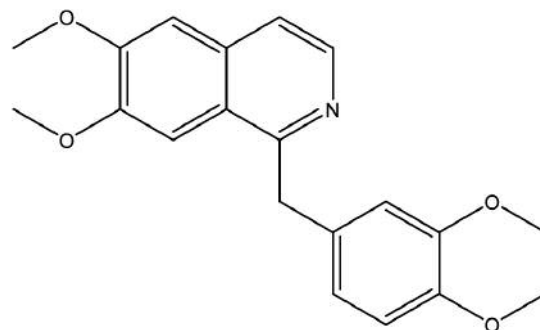
Theratronics, Ltd., Kanata, Ontario, Canada) at a γ-ray irradiation dose rate of 0.777 Gy/min.

U87MG human GBM xenograft mouse model. All animal studies were approved by the Animal Experimental Committee at the Tokyo University of Science (TUS) and performed in accordance with the TUS Guidelines for Animal Experiments (#Y16034 and #Y15052). These requirements are in accordance with the ethical guidelines for experimental animals in Japan. The animals were housed at 23˚C±2˚C under specific pathogen-free conditions with a 12/12 h light/dark cycle and provided a standard diet and water *ad libitum*. For heterotrophic/subcutaneous xenografts, 1×10⁶ U87MG cells re-suspended in 100 μl PBS were subcutaneously injected into the right leg of 5-week-old male BALB/c nude mice (weight, 20-22 g; n=5) (CLEA Japan, Inc., Tokyo, Japan). There were 5 mice in each treatment group. Before inoculation with the tumour cells, mice were anaesthetized with isoflurane (Escain® inhalation anaesthesia liquid 1 ml/ml, Pfizer Inc, NY, USA); papaverine was diluted in normal saline. After 7-10 days of inoculation, papaverine (40 mg/kg) or saline (vehicle control, solvent alone) was administered intraperitoneally twice a day for 4 days (n=5 per group). Temozolomide (5 mg/kg) or 1:3 DMSO/saline solution (vehicle control) was administered intraperitoneally once a day for 4 days (n=5 mice per treatment group). Tumour volume (V) was measured once every 3-4 days using the following formula: $V=ab^2/2$ (where a and b were the long and short diameters of the tumour, respectively). Mice were euthanized by isoflurane inhalation followed by cervical dislocation at the endpoint stage. In animal experiments, humane endpoint criteria were defined as follows: tumour burden >10% of body weight, tumour volume >2,000 mm³, or tumour largest dimension >20 mm.

Statistical analysis. All data are presented as mean and standard deviation. Significant differences among groups were assessed using the Student's *t*-test. Differences with $p<0.05$ were considered statistically significant.

Results

We previously investigated the anticancer activity of papaverine in several types of tumour cells, including GBM cells (11, 23). We evaluated the anticancer effects of
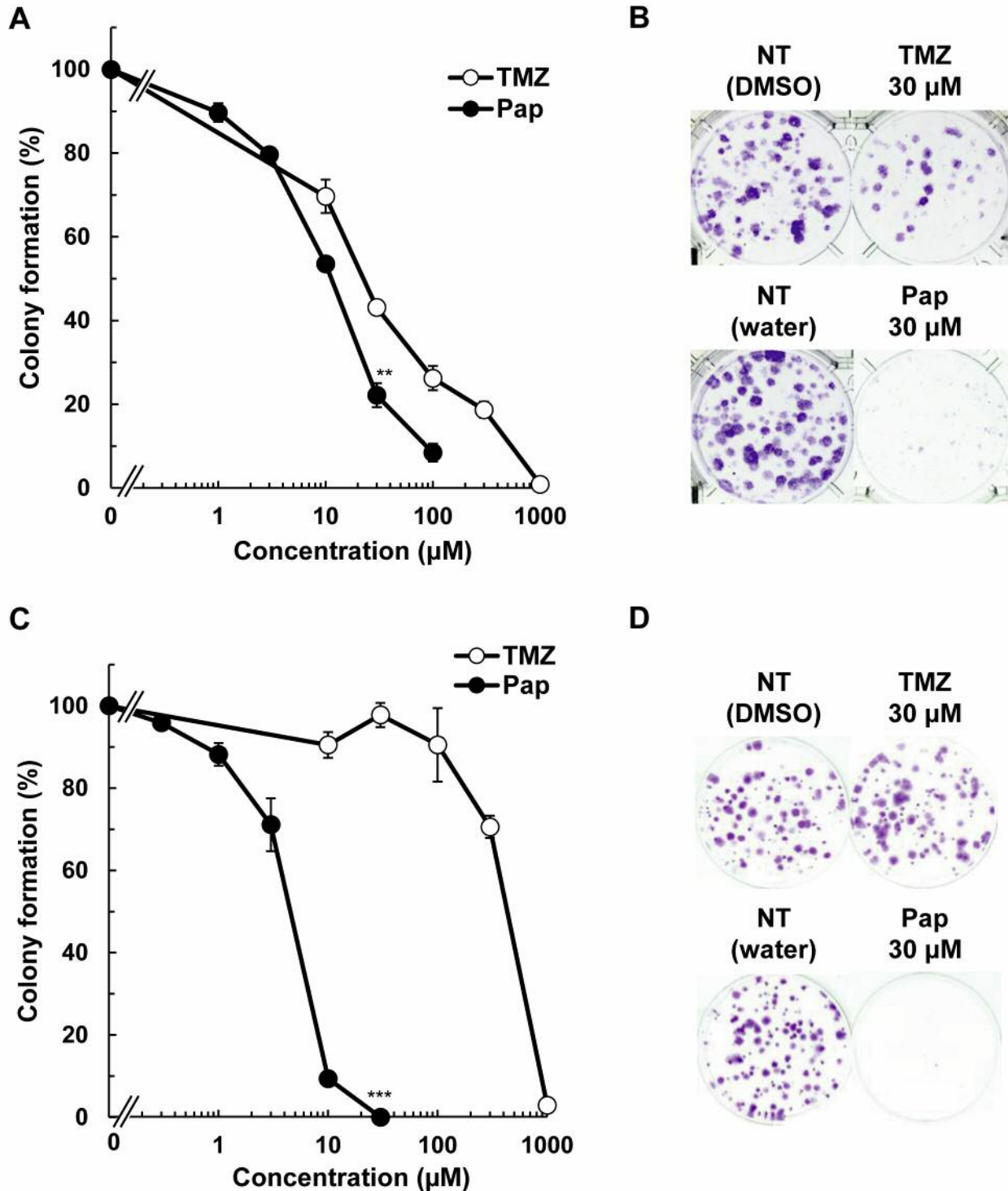

Figure 1. *Chemical structure of the non-narcotic opium alkaloid papaverine.*

Figure 2. *Anticancer effects of papaverine (Pap) in human glioblastoma (GBM) cells. A: Clonogenecity of U87MG human GBM cells after 10 days of treatment with papaverine and temozolomide (TMZ). Results are the average of three independent experiments performed in duplicate with error bars showing±SE. **p<0.05 (Pap at 30 μM vs. TMZ at 30 μM). B: Drug sensitivity of U87MG cells was obtained from the colony-formation assay. Cells were treated with 30 μM temozolomide or papaverine and incubated for 10 days. C: Clonogenecity of T98G cells after 10 days of treatment with papaverine and temozolomide. Results are the average of three independent experiments with error bars showing±SE (triplicates). ***p<0.001 (Pap at 30 μM vs. TMZ at 30 μM). D: Drug sensitivities of T98G cells were obtained from the colony-formation assay. Cells were treated with 30 μM TMZ or papaverine and incubated for 10 days. DMSO: Dimethyl sulfoxide; NT: Solvent alone (DMSO or water).*
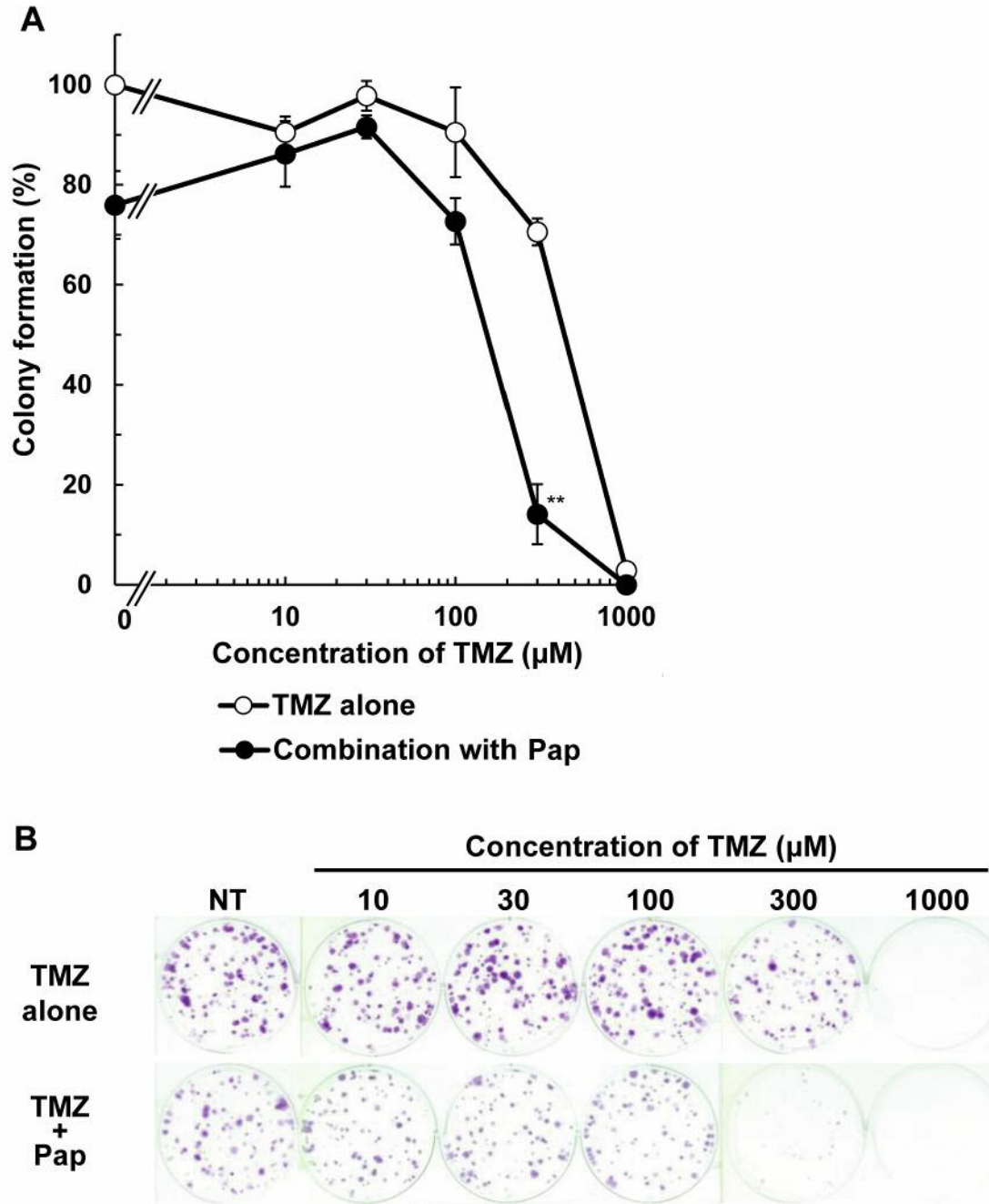
Figure 3. *Anticancer effects of papaverine (Pap) and temozolomide (TMZ) combination therapy in T98G human glioblastoma (GBM) cells. A: Clonogenicity of T98G human GBM cells after 10 days of combination treatment with papaverine and temozolomide or temozolomide alone. Results are the average of two independent experiments with error bars showing±SE in triplicates. **p<0.05 (TMZ alone vs. Pap+TMZ at 300 μM TMZ). B: Drug sensitivity of T98G cells was obtained from the colony-formation assay. Cells were treated with 30 μM temozolomide or papaverine and incubated for 10 days.*

papaverine in temozolomide-sensitive (MGMT-negative) U87MG and temozolomide-resistant (MGMT-positive) T98G cells using a WST-8 assay. The 50% effective concentration (EC$_{50}$) values for papaverine were 29 and 40 μM in U87MG and T98G cells, respectively (23) Conversely, the EC$_{50}$ values for temozolomide were 42 and 390 μM in U87MG and T98G cells, respectively (23). Papaverine inhibited the clonogenicity of U87MG (EC$_{50}$=11 μM) and temozolomide-
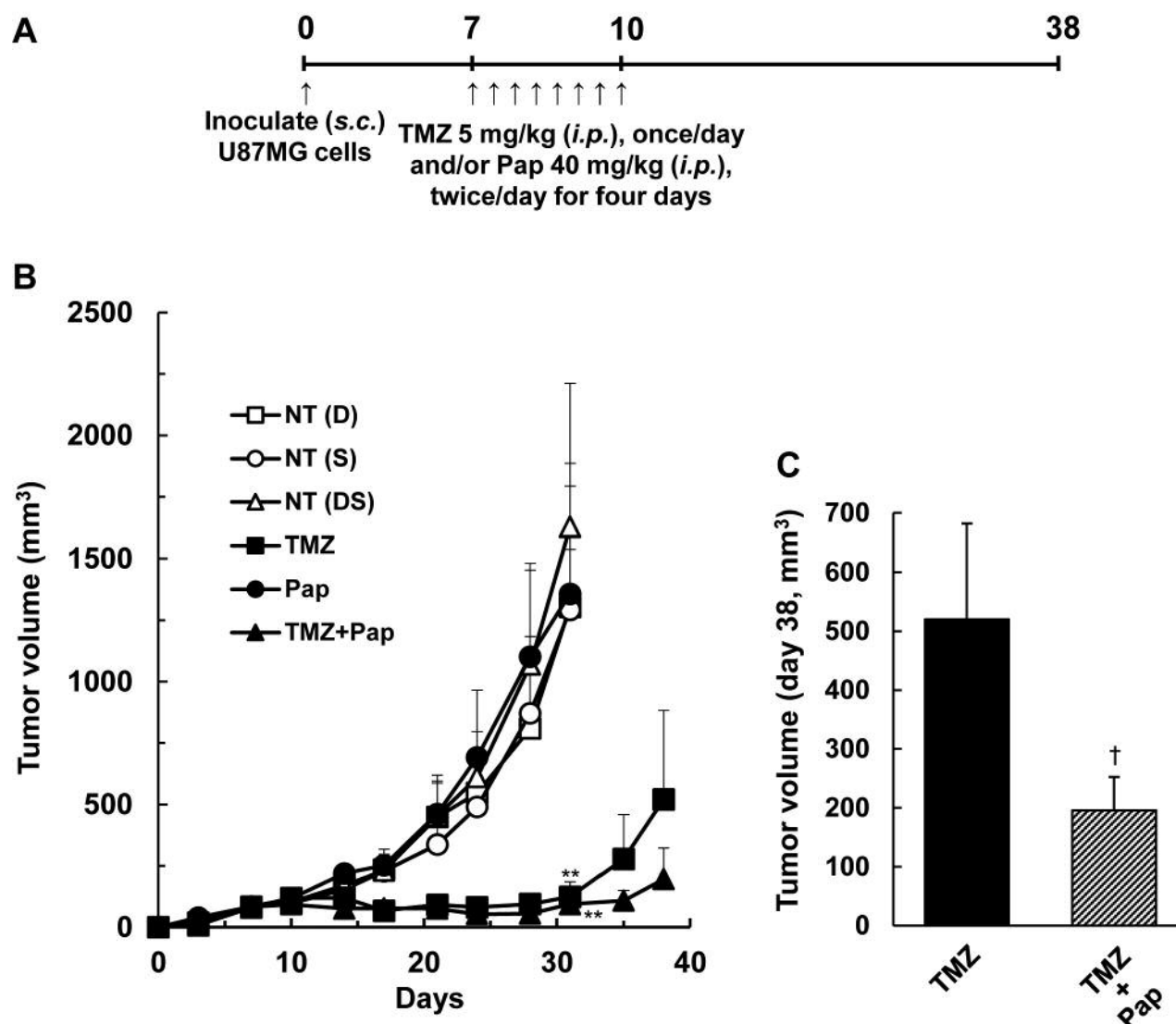
Figure 4. *Anticancer effects of papaverine (Pap) and temozolomide (TMZ) combination therapy in a xenograft mouse model of human glioblastoma (GBM). A: Experimental schedule. B: In order to assess the effects of papaverine monotherapy, and temozolomide and papaverine combination therapy in a xenograft model, 1×10^6 U87MG human GBM cells were subcutaneously injected into the right leg of 5-week-old male BALB/c nude mice. Seven days after inoculation, five mice per group were treated with papaverine (40 mg/kg; Pap) or saline [vehicle control, solvent alone; NT (S)] twice a day for 4 days or temozolomide (5 mg/kg; TMZ) or DMSO/saline [vehicle control, solvent alone; NT (D)] once a day for 4 days via i.p. administration. For combination therapy, mice were treated with papaverine (40 mg/kg, twice a day) and temozolomide (5 mg/kg, once a day) or solvent alone for 4 days via i.p. administration. Tumour volume was measured once every 3-4 days and was calculated as described in the Materials and Methods section. Results are the averages for groups of five mice each with error bars showing±SE. **p<0.05 [NT(D) vs. TMZ and NT(DS) vs. TMZ+Pap at day 31]. C: Tumour volumes following temozolomide monotherapy and combination therapy with papaverine and temozolomide in this xenograft model on day 38. Results are the averages for groups of five mice each with error bars showing±SE. †p>0.05 (TMZ vs. TMZ+Pap).*

resistant T98G GBM cells (EC_{50}=4.5 μM) in a dose-dependent manner. Temozolomide yielded EC_{50} values of 23 and 430 μM against U87MG and T98G cells, respectively (Figure 2). Since the clonogenicity of GBM U87MG cells was very low, we evaluated the anticancer effect of papaverine in U87MG cells using the colony-formation assay

on poly-L-lysine coated plates. In addition, we assessed the effects the combination of papaverine and temozolomide on the clonogenicity of T98G cells. The addition of papaverine (2 μM=EC_{20}) significantly enhanced the anticancer effects of temozolomide in T98G cells. The EC_{50} for temozolomide was 430 μM when used alone and 150 μM when used in

combination with papaverine (Figure 3). These findings suggest that papaverine enhances the anticancer effects of temozolomide in T98G GBM cells.

The *in vivo* anticancer activity of papaverine in combination with temozolomide was measured in male BALB/c nude mice bearing U87MG GBM xenografts (Figure 4A). The tumour volume significantly decreased after treatment with temozolomide alone (5 mg/kg, *i.p.*, once/day for 4 days) as well as after combination treatment with temozolomide (5 mg/kg, *i.p.*, once/day for 4 days) and papaverine (40 mg/kg, *i.p.*, twice/day for 4 days) compared with that after the control treatment and treatment with papaverine alone (40 mg/kg, *i.p.* twice/day for 4 days). Combined treatment with temozolomide and papaverine was the most effective in inhibiting tumour growth (Figure 4B). At the end of the study period (31 days after treatment, *i.e.*, 38 days after tumour implantation), there was an approximately 62% reduction in mean tumour volume following combination treatment with temozolomide and papaverine (mean±SE=196±57 mm^3) compared with treatment with temozolomide alone (mean±SE=520±162 mm^3). These data indicate that while papaverine alone did not suppress tumour growth *in vivo*, combination therapy with temozolomide and papaverine was more effective in suppressing *in vivo* tumour growth than temozolomide alone in the U87MG xenograft model. This finding suggests that papaverine can be used in combination with temozolomide for GBM treatment.

We evaluated the radiosensitizing effect of papaverine in GBM cells by evaluating the sensitivity of T98G cells to γ-ray irradiation following treatment with papaverine (EC_{25}=3.1 μM) or temozolomide (EC_{25}=230 μM) (Figure 5). While the 37% sensitizer enhancement ratio (SER37) for γ-ray irradiation with papaverine was 1.28, it was 1.03 for that with temozolomide. Papaverine treatment significantly enhanced the γ-ray irradiation-induced loss of clonogenicity of T98G cells compared with temozolomide treatment. These data indicate that papaverine is a potential drug that may be useful in radiotherapy of GBM.

Discussion

Papaverine, a smooth muscle relaxant, is an opium alkaloid used for preventing intraoperative vasospasm during craniotomy (*e.g.* subarachnoid haemorrhage) (28-30). We identified papaverine as an inhibitor of the interaction between HMGB1 and RAGE using a unique *in silico* drug design system and a drug repositioning approach (10, 11, 23). Moreover, we demonstrated that papaverine inhibits the proliferation of U87MG and T98G GBM cells. In addition, papaverine dramatically suppressed tumour growth in a human GBM U87MG xenograft mouse model (23). In this study, we found that papaverine significantly suppressed the clonogenicity of temozolomide-sensitive U87MG and temozolomide-resistant T98G GBM cells (Figure 2). In
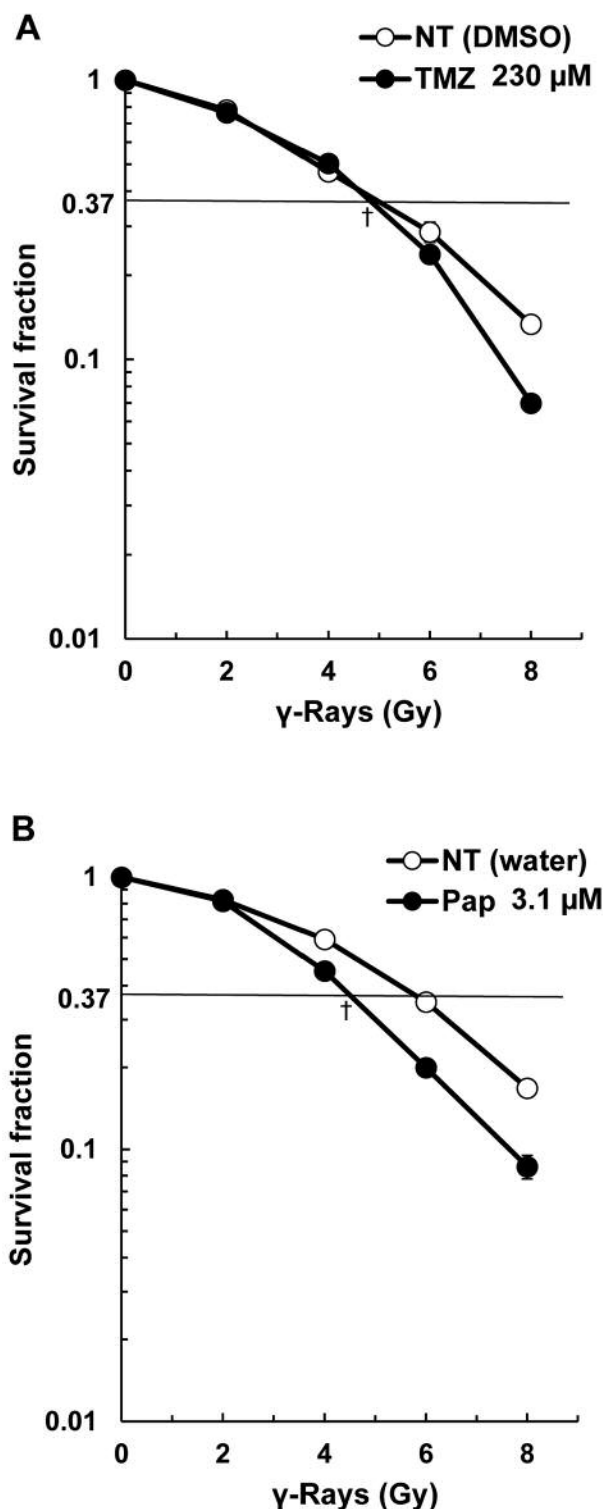

Figure 5. *Radiosensitization of T98G human glioblastoma (GBM) cells by papaverine (Pap). Clonogenicity of T98G GBM cells was assessed following γ-ray irradiation and treatment with temozolomide (TMZ) (A) or papaverine (B) for 10 days. Results are the average of three independent experiments with error bars showing±SE in triplicates. †p>0.05 (NT vs. TMZ and NT vs. Pap at SER37).*

addition, combination treatment with temozolomide and papaverine suppressed the clonogenicity of T98G GBM cells (Figure 3) and tumour growth in the U87MG xenograft mouse model. This combination treatment was more effective than temozolomide or papaverine monotherapy (Figure 4B and C). Meanwhile, papaverine monotherapy did not delay tumour growth in this xenograft model (Figure 4B). Recently, we reported that papaverine suppressed tumour growth in U87MG xenograft mice using a preventative effect study model (23). We determined that the anticancer efficacy of papaverine is related to the tumour volume at the stage of treatment. These findings suggest that papaverine can be used in combination with temozolomide to prevent the recurrence of GBM after resection. Indeed, standard treatment for GBM involves surgery/surgical resection of tumour along with radiation and adjuvant chemotherapy with temozolomide (4, 31). However, several limitations and risk factors are associated with radiation therapy, including the invasive nature of GBM, radiation-induced necrosis and neuronal damage, and radioresistance of some tumours (4, 32). Papaverine may be effective in reducing radioresistance and preventing adverse side-effects associated with radiation therapy for GBM.

Interestingly, we found that papaverine dramatically enhanced the radiosensitivity of temozolomide-resistant T98G GBM cells (Figure 5B). Benej et al. previously demonstrated that papaverine radiosensitizes A549 lung and EO771 breast cancer cells by inhibiting mitochondrial complex 1 (22). These results indicate that papaverine is a potential radiosensitizing agent that can be useful in treating various cancer types, including GBM. Importantly, papaverine was shown to enhance the reversible opening of the blood–brain barrier (33) and to mediate transient blood–brain barrier permeability (34). In conclusion, our findings suggest that papaverine can serve as an anticancer drug in GBM treatment.

Conflicts of Interest

None.

Authors' Contributions

Conceived and designed the experiments: AS. Performed the experiments: MI, AS, and MS. Analyzed the data: MI, AS, MS, YY, YA, KI, and ST. Wrote the article: AS.

Acknowledgements

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