

Synergistic effect of arsenic trioxide, vismodegib and temozolomide on glioblastoma

COSTANSIA BURETA^{1*}, YOSHINOBU SAITOH¹, HIROTO TOKUMOTO¹, HIROMI SASAKI¹,
SHINGO MAEDA², SATOSHI NAGANO¹, SETSURO KOMIYA¹,
NOBORU TANIGUCHI¹ and TAKAO SETOGUCHI^{2*}

Departments of ¹Orthopaedic Surgery and ²Medical Joint Materials, Graduate School of Medical and Dental Sciences,
Kagoshima University, Kagoshima 890-8520, Japan

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Abstract. The treatment of glioblastoma is a critical health issue, owing to its resistance to chemotherapy. The current standard of treatment is surgical resection, followed by adjuvant radiotherapy and temozolomide treatment. Long-term local treatment of glioblastoma is rarely achieved and the majority of the patients undergo relapse. Resistance to temozolomide emerges from numerous signalling pathways that are altered in glioblastoma, including the Hedgehog signalling pathway. Hence, further research is required to identify effective treatment modalities. We investigated the effect of vismodegib, arsenic trioxide and temozolomide on glioblastoma *in vitro* and *in vivo* to apply our findings to the clinical setting. WST-1 assay revealed that glioblastoma proliferation was inhibited following treatment with these drugs either in single or in combination; this synergistic effect was confirmed by CalcuSyn software. Western blot analysis revealed an increase in the expression of cleaved caspase-3 and γ H2AX. Furthermore, there was marked inhibition and decreased tumour growth in mice that received combination therapy, unlike those that received single agent or vehicle treatment. Our results revealed that the combination of arsenic trioxide/vismodegib and temozolomide may be an attractive therapeutic method for the treatment of glioblastoma.

Introduction

Glioblastomas (GBM, gliomas) are primary brain tumours of glial origin. They are the most common central nervous system

neoplasms in adults. Each year, 5-6 of 100,000 individuals are diagnosed with primary malignant brain tumours, of which ~80% are malignant gliomas and more than half of these are glioblastomas (1,2). There is a slight male predominance and individuals between 45-70 years of age are mainly affected (3). Despite the aggressiveness in approach which includes surgical resection, irradiation and chemotherapy, GBM is an aggressive neoplasm associated with high mortality resulting from infiltrative growth and recurrence with a uniformly fatal course.

Temozolomide (TMZ) is an alkylating agent used for GBM treatment (4). The approved dosage is 150-200 mg/square metre of body surface area, daily for 5 days of every 28-day cycle. A dosage of 75 mg/square metre for up to 49 days is safe (5); this extent of exposure to TMZ will damage the DNA repair enzyme encoded in the human as O⁶-methylguanine-DNA methyltransferase (MGMT) (4,6), but overexpression of MGMT in tumour cells confers resistance to TMZ and impairs therapeutic outcome.

The Hedgehog (Hh) signalling pathway was originally discovered in *Drosophila*, and regulates embryonic segment development (7). Hh signalling plays a crucial role in GBM tumour progression and pathogenesis. Its activation is mediated by sonic Hedgehog (Shh), which binds to its receptor patched (PTCH) to promote GLI1 activation. Activation of Hh/GLI1 thus promotes the resistance of glioma stem cells to TMZ (8,9).

Arsenic trioxide (As₂O₃, ATO), a Hh pathway inhibitor (10,11), is used as a therapeutic agent for acute promyelocytic leukaemia (APL) (12). It has also been reported to show a substantial effect in a wide range of other solid tumours including oesophageal (13), lung (14), liver (15), cervical cancer (16), prostate carcinoma (17) and osteosarcoma (18). Regardless of how sensitive different types of tumour cells are to this drug, there is a limitation in its clinical application in a wide range of haematological malignancies and solid tumours (19,20).

Vismodegib (VIS) is a small molecule inhibitor of smoothened (SMO). In the absence of PTCH1, VIS binds to SMO and inhibits the atypical activation of the Hh pathway (21).

In clinical practice, combination therapy is often used to enhance the cytotoxicity and reduce the adverse effects

Correspondence to: Dr Takao Setoguchi, Department of Medical Joint Materials, Graduate School of Medical and Dental Sciences, Kagoshima University, 8-35-1 Sakuragaoka, Kagoshima 890-8520, Japan

E-mail: setoro@m2.kufm.kagoshima-u.ac.jp

*Contributed equally

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of chemotherapeutic drugs (19,22). In the present study, we demonstrated that the combination of VIS, ATO and TMZ suppressed the growth of GBM.

Materials and methods

Cell line and reagents. Glioblastoma of unknown origin (GUO) [U-87MG (ATCC® HTB-14™) (RRID:CVCL_0022)] and U138MG human malignant GBM cell lines were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA), while U251MG was purchased from the Health Science Research Resource Bank (Osaka, Japan). All cell lines grown as monolayer cultures in minimum essential medium (MEM; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) were supplemented with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.) and 1% penicillin/streptomycin (Gibco; Thermo Fisher Scientific, Inc.) in a humidified atmosphere containing 5% CO₂ at 37°C.

Temozolomide (TMZ) was purchased from LKT Laboratories Inc. (St. Paul, MN, USA), arsenic trioxide (ATO) was from Nihon Shinyaku Co., Ltd. (Kyoto, Japan) and vismodegib (VIS) was obtained from LC Laboratories (Woburn, MA, USA).

Cell viability assay. Cells were seeded at a density of 10³ cells/well in 96-well plates and treated with vehicle, 1 or 3 µM of ATO, 20 or 50 µM of VIS, and 300 or 1,000 µM of TMZ. Cell viability was assessed by adding to each well 10 µl of a tetrazolium salt (WST-1) (Roche Diagnostics, Basel, Switzerland) which was cleaved by mitochondrial dehydrogenase activity (18). Fluorescence intensity was measured after 2 h on a microplate reader.

Western blot assay. Briefly, cells were seeded at a density of 10⁵ cells/well in 6-well plates with vehicle, or 1 µM ATO, 30 µM VIS, or 300 and 600 µM TMZ in single or in combination of ATO and TMZ and a combination of VIS and TMZ for 48 h, washed with phosphate-buffered saline (PBS) and lysed using Mammalian Protein Extraction reagent (Thermo Fisher Scientific, Inc.), 3 mM p-APMSF (Wako Chemicals, Kanagawa, Japan) and 5 mg/ml aprotinin (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany). The lysates were centrifuged at 14,000 x g for 10 min at 4°C. Protein were determined by bicinchoninic acid (BCA) reagents A and B at 50:1. SDS-PAGE (4-15%) was conducted using 10 µg of each protein followed by immunoblotting on polyvinylidene difluoride (PVDF) membrane (Bio-Rad Laboratories, Hercules, CA, USA). Blocking was carried out with 5% skim milk for 1 h followed by incubation at 4°C with the following antibodies: γH2AX (cat. no. 2577), H2AX (cat. no. 2595), cleaved caspase-3 (cat. no. 9664) and caspase-3 (cat. no. 9665; all from Cell Signaling Technology Japan K.K., Tokyo, Japan) (23-26) at a dilution of 1:1,000 overnight and alpha-tubulin (cat. no. HRP-66031; ProteinTech Group, Inc., Rosemont, IL, USA) at a dilution of 1:5,000 for 1 h. Incubation at room temperature with horseradish peroxidase-conjugated anti-rabbit secondary antibodies (cat. no. 7074) or anti-mouse (cat. no. 7076) (Cell Signaling Technology Japan K.K., Tokyo, Japan) for 1 h at a dilution of 1:4,000. Signals were

analyzed using ECL Western Blotting reagent (Amersham; GE Healthcare Life Sciences, Little Chalfont, UK) and LAS 4000 Mini image analyzer (Fujifilm, Tokyo, Japan).

Drug combination studies. GUO, U251MG and U138MG cells were seeded in 96-well plates and treated with vehicle, single drug or a fixed drug ratio of the combined drugs. ATO and TMZ was used at 1:320, VIS and TMZ was used at 1:10. Cell viability was assessed by WST-1 assay. The CalcuSyn (version 2.11; Biosoft, Ferguson, MO, USA) median effect model was used to calculate the CI values and to analyse whether the drug combinations were synergistic, antagonistic, or additive. CI value of <1 indicates synergism, CI=1 indicates additivity, and CI >1 indicates antagonism (27).

Animal studies. Four-week-old male nude mice weighing 20 g (Japan SLC Inc., Hamamatsu, Japan) were used in the present study. Animal care and experimental procedures were specifically approved and carried out in accordance with the guidelines of the Institute of Laboratory Animal Sciences, Graduate School of Medical and Dental Sciences, Kagoshima University (Kagoshima, Japan) (no. MD 17101).

The animals were kept in a pathogen-free environment, with 12-h light/dark cycle at 24°C, 40-70% relative humidity and a free access to food and water *ad libitum*. They were allowed to habituate for 7 days prior to tumour inoculation. Briefly, 1x10⁷ GUO tumour cells in 50 µl MEM lacking FBS and antibiotics combined with 50 µl Matrigel (Corning Life Sciences, Tewksbury MA, USA) were inoculated subcutaneously into the flanks of nude mice. Tumours were allowed to grow for 7 days, and then mice were randomly divided into the control and the treatment groups (n=7 animals/group). They were administered intraperitoneally (i.p) with either TMZ (10 mg/kg/daily), ATO (2.5 µg/g/daily), or VIS (25 mg/kg/day) or in combination of ATO 2.5 µg/g/daily and TMZ 10 mg/kg/daily or VIS 25 mg/kg/daily and TMZ 10 mg/kg/daily, or with an equal volume of vehicle as the control. These drug concentrations were selected from published studies (8,14,15,22,28), and after conducting a pilot study, we used the minimum effective concentrations so as to be able to apply our results in clinical settings. Injections were given 4 days a week for 2 weeks. Tumour volumes were measured with callipers on alternative days with the longest diameter being the length and the perpendicular diameter being the width; volume was calculated using the formula (L x W²)/2. The maximum diameter exhibited by a single tumour was 17 mm. Twenty four days after tumour inoculation, animals were sacrificed by inhalation of CO₂ at a rate of 10-30%/min in an automatic euthanasia plastic chamber. The tumours were excised, weighed, formalin-fixed and paraffin-embedded. Paraffin sections (4 µm) were cut and stained with haematoxylin and eosin (H&E) for light microscopic evaluation.

Statistical analysis. Statistical analyses were carried out using Steel-Dwass test, as a post hoc test for pairwise comparisons following a significant Kruskal-Wallis test with Excel Statistics 2013 (Microsoft Excel; Microsoft Corp., Redmond, WA, USA) and KyPlot 5.0 (KyensLab Inc., Tokyo, Japan). P-values of <0.05 were considered to indicate a statistically significant result.

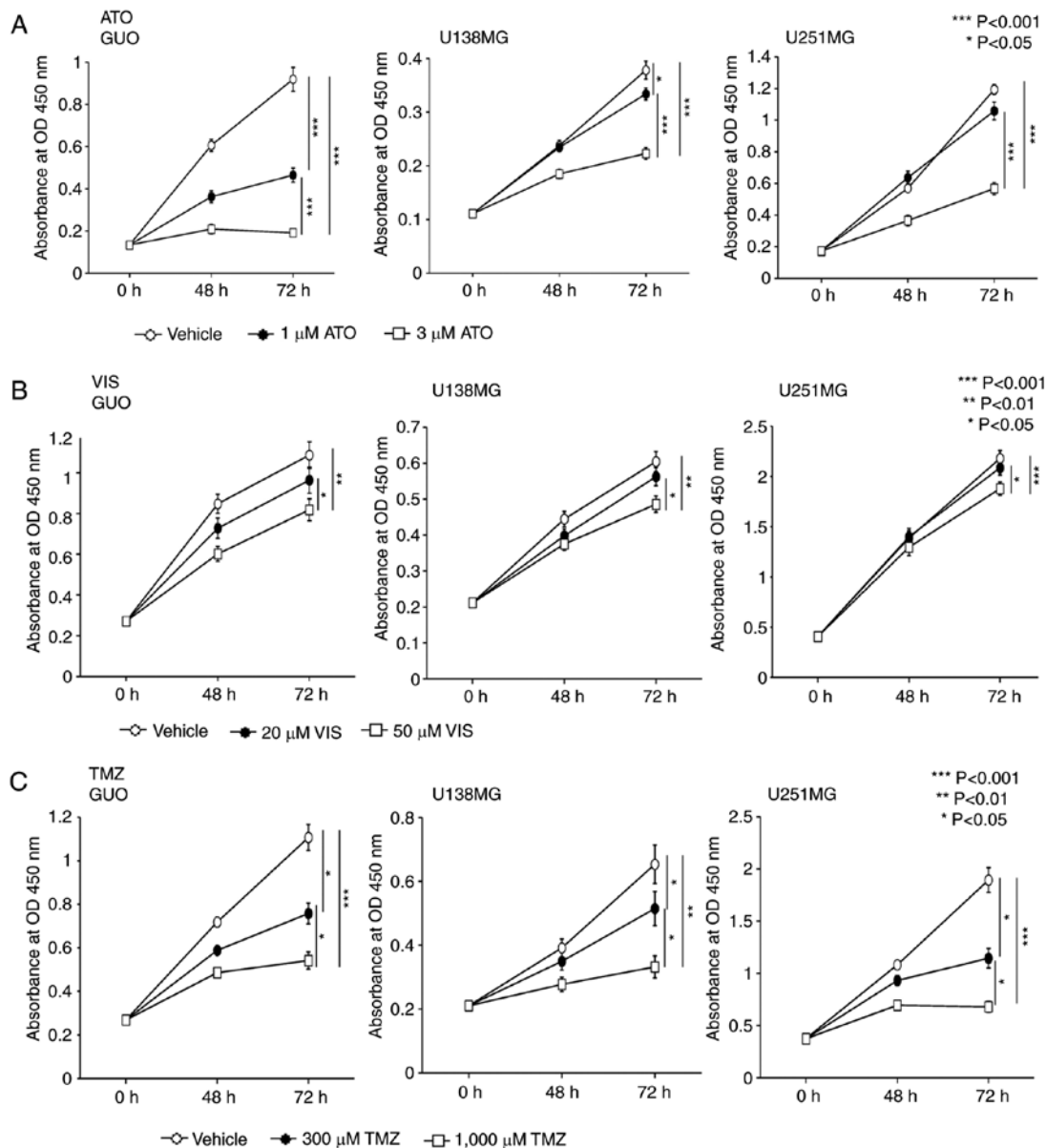


Figure 1. ATO, VIS and TMZ hinder the growth of human GBM cell lines, GUO, U138MG and U251MG. WST-1 assay demonstrated that (A) 1 μ M ATO significantly inhibited the growth of GUO and U138MG cells but did not significantly inhibit the proliferation of U251MG. Yet, 3 μ M ATO significantly inhibited the proliferation of all the cell lines used. WST-1 assay demonstrated that (B) a concentration of 50 μ M VIS was significantly more effective in inhibiting the proliferation of the GUO and U138MG and U251MG cells compared to 20 μ M of VIS which showed only a slight inhibitory effect when compared with the vehicle control. (C) WST-1 assay showed that treatment with 300 and 1,000 μ M TMZ significantly inhibited the growth of human GBM cells lines, GUO, U138MG and U251MG cell lines in a dose-dependent manner. The experiment was carried out in triplicate producing similar results. $P < 0.05$ was considered significant (Kruskal-Wallis test). Error bars represent the mean \pm SD. ATO, arsenic trioxide; VIS, vismodegib; TMZ, temozolomide; GBM, glioblastoma; GUO, glioblastoma of unknown origin; OD optical density.

Results

Single-agent efficacy of ATO, VIS and TMZ on the growth of GBM cells. To examine the efficacy of ATO, VIS and TMZ on the growth of GBM cells *in vitro*, GUO, U138MG and U251MG human GBM cell lines, were used. WST-1 results showed that, there was a dose-dependent inhibition in cell proliferation when all the cell lines were treated with 1 or 3 μ M of ATO although 1 μ M ATO did not show significant inhibition in the U251MG cell line (Fig. 1A). A concentration of 50 μ M VIS was significantly more effective in inhibiting the proliferation of the GUO and U138MG and U251MG cells compared to 20 μ M of VIS which did not show a significant inhibitory

effect when compared with the vehicle control (Fig. 1B). TMZ when used at a concentration of 300 or 1,000 μ M significantly inhibited the proliferation of GUO, U138MG and U251MG cells (Fig. 1C).

Combination of a Hedgehog (Hh) inhibitor and a standard chemotherapeutic drug hinders the proliferation of GBM cells *in vitro*. We then examined the effect of treating these cell lines with Hh inhibitors VIS or ATO in combination with TMZ in a dose-dependent manner. Five different concentrations were used at a ratio of 1:320 for ATO:TMZ with the highest concentration being 2:640, and 1:10 for VIS:TMZ with the highest concentration being 64:640 (Table I). When combination

Table I. Combination index (CI) for a standard anticancer drug when combined with a Hedgehog inhibitor.

Cell line	Drugs	CI ED ₅₀	CI ED ₇₅	CI ED ₉₀	Dm	m	r
U87MG	ATO+TMZ (CR 1:320)	0.91	0.70	0.56	1.53	0.82	0.99
U138MG	ATO+TMZ (CR 1:320)	0.63	0.67	0.78	4.4	0.53	0.98
U251MG	ATO+TMZ (CR 1:320)	0.49	0.59	0.93	1.1	0.69	0.98
U87MG	VIS+TMZ (CR 1:10)	0.5	0.4	0.35	57.49	0.82	0.96
U138MG	VIS+TMZ (CR 1:10)	0.53	0.54	0.57	130.66	0.79	0.96
U251MG	VIS+TMZ (CR 1:10)	0.28	0.19	0.15	87.84	0.98	0.94

The CI, Dm and r values were calculated using CalcuSyn software. 'Dm' being the median-effect dose, 'm' the exponent defining the shape of the dose effect curve and 'r' indicates the goodness of fit for the data (where $r=1$ is a perfect fit). The r value of the median effect plot for the cell culture system should be $r>0.95$. $CI < 1$ indicates synergism, 1 indicates additivity and >1 indicates antagonism.

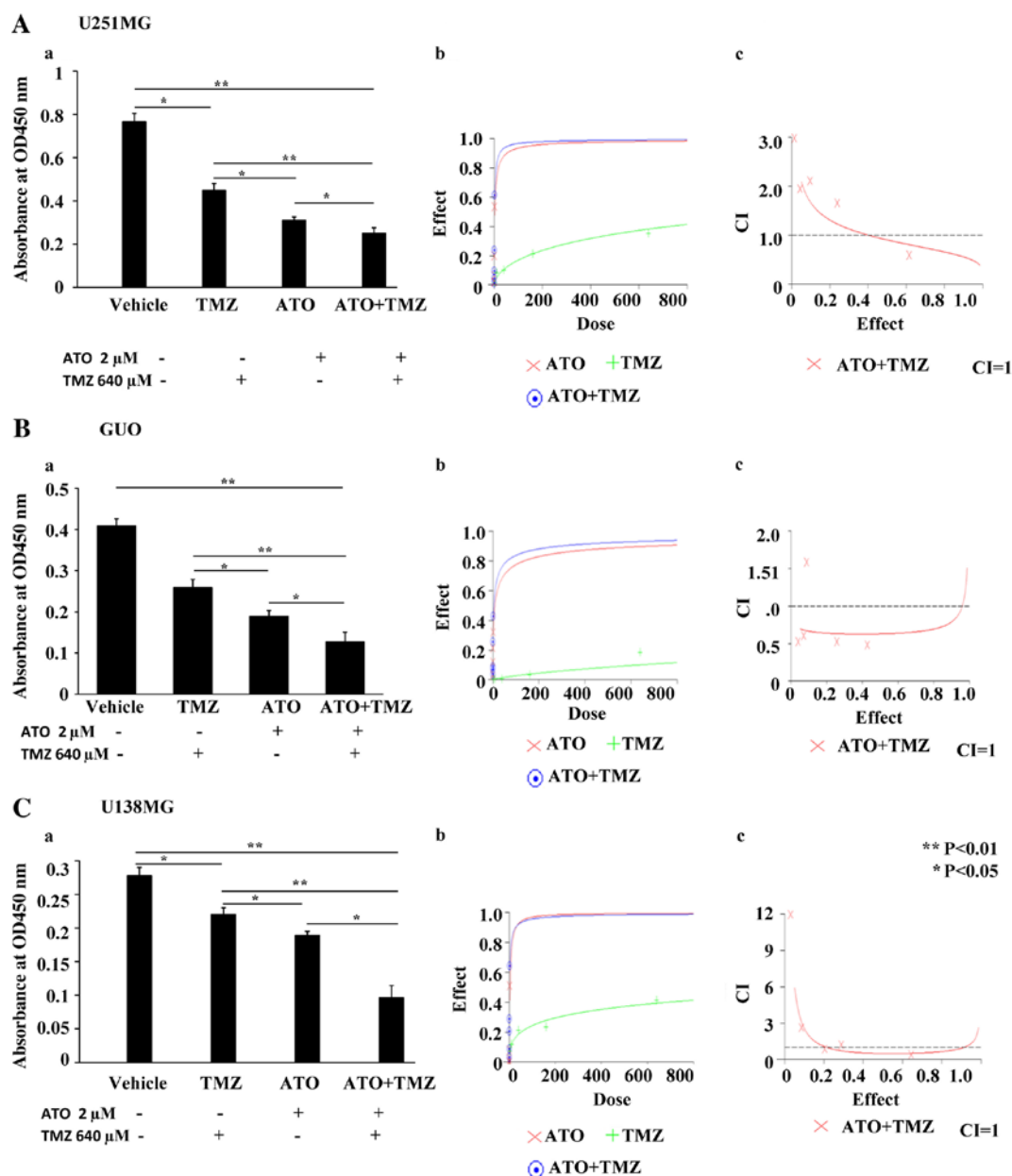


Figure 2. The proliferation of human GBM cells: (A) U251MG, (B) GUO and (C) U138MG cells was inhibited following treatment with a combination of ATO and TMZ. (Aa-Ca) This proliferation effect was analyzed by WST-1 assays. (Ab-Cb) The dose-effect curves of ATO and TMZ in single and in combination are shown. (Ac-Cc) The combination-index and fractions affected were graphed in combination of ATO and TMZ. All the experiments were repeated three times generating similar results. $P < 0.05$, was considered significant (Kruskal-Wallis test). Error bars represent the mean \pm SD. ATO, arsenic trioxide; VIS, vismodegib; TMZ, temozolomide; GBM, glioblastoma; GUO, glioblastoma of unknown origin. CI value of <1 indicates synergism, $CI=1$ indicates additivity, and $CI > 1$ indicates antagonism.

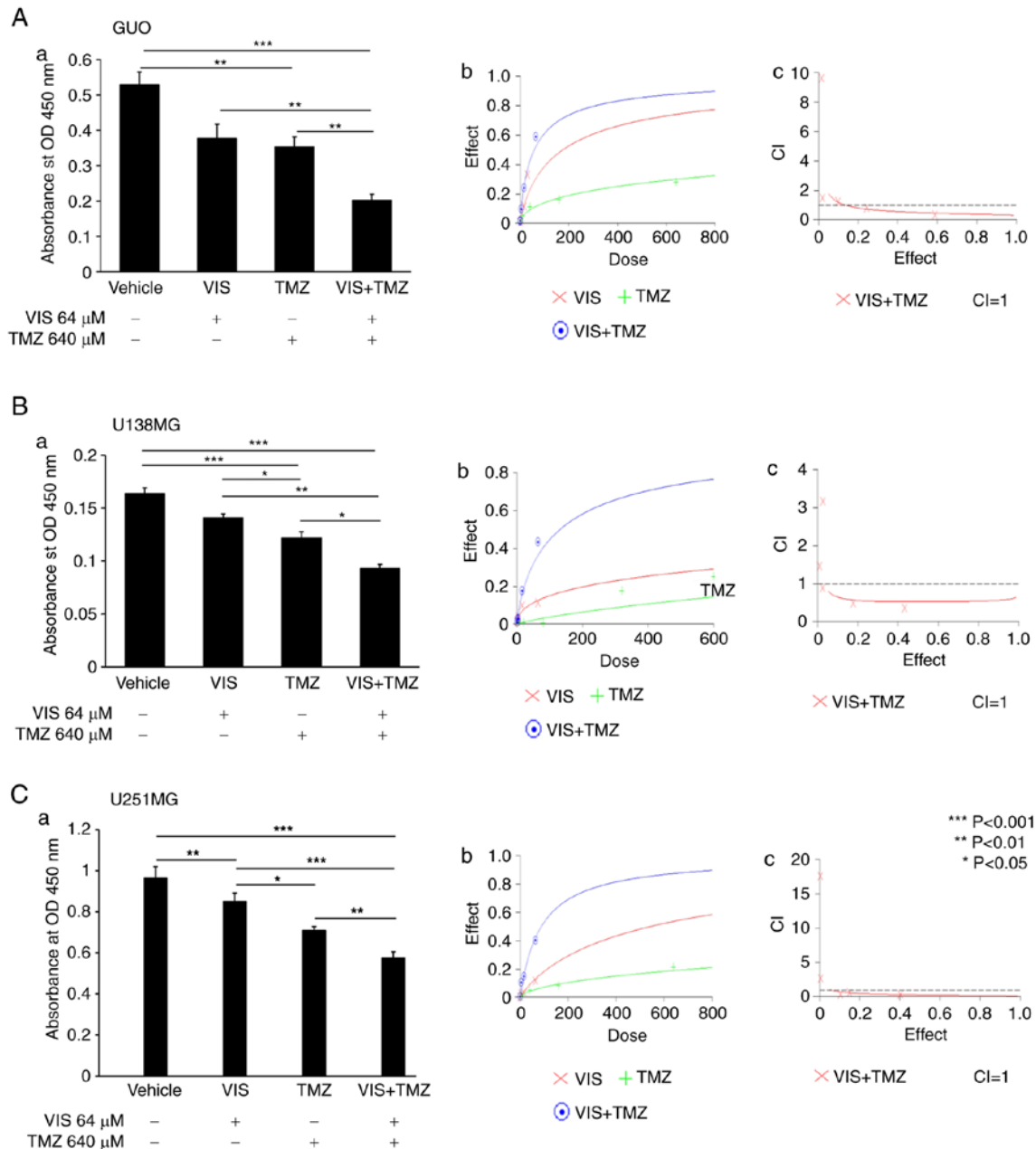


Figure 3. The proliferation of human GBM cells: (A) GUO, (B) U138MG and (C) U251MG cells was inhibited following treatment with a combination of VIS and TMZ. (Aa-Ca) This proliferation effect was analysed by WST-1 assays. (Ab-Cb) The dose-effect curves of VIS and TMZ in single and in combination are shown. (Ab-Cc) The combination-index and fractions affected were graphed in combination of VIS and TMZ. All the experiments were repeated three times generating similar results. P<0.05 was considered significant (Kruskal-Wallis test). Error bars represent the mean \pm (SD). ATO, arsenic trioxide; VIS, vismodegib; TMZ, temozolomide; GBM, glioblastoma; GUO, glioblastoma of unknown origin. CI value of <1 indicates synergism, CI=1 indicates additivity, and CI >1 indicates antagonism.

treatment was used, there was marked inhibition in the proliferation of GBM cell lines, unlike with the use of the single agents. This was shown by assessing the synergistic effect of these drugs by CalcuSyn software version 2.11 (Figs. 2 and 3).

Combination of a Hedgehog (Hh) inhibitor and standard anticancer drug triggered apoptosis of GBM cells in vitro. We next examined the ability of 300 μ M TMZ when combined with 1 μ M ATO/30 μ M VIS to cause DNA damage and apoptosis in GBM cells following treatment for 48 h. Western blot analyses using γ H2AX and cleaved caspase-3 revealed that there was higher expression of γ H2AX and cleaved caspase-3

when the drugs were combined, unlike when they were used as single agents (Fig. 4).

Combination of ATO/VIS and TMZ prevents GBM proliferation in vivo. Mouse xenograft models showed that the combination of ATO and TMZ, VIS and TMZ, significantly inhibited GBM proliferation *in vivo* compared with the vehicle or single drug administration (Fig. 5A and B). We measured the body weight of the mice during the treatments so as to assess the toxicity of these combination treatments. We found that there was no significant difference between the body weights of the control and the treatment groups (Fig. 5C).

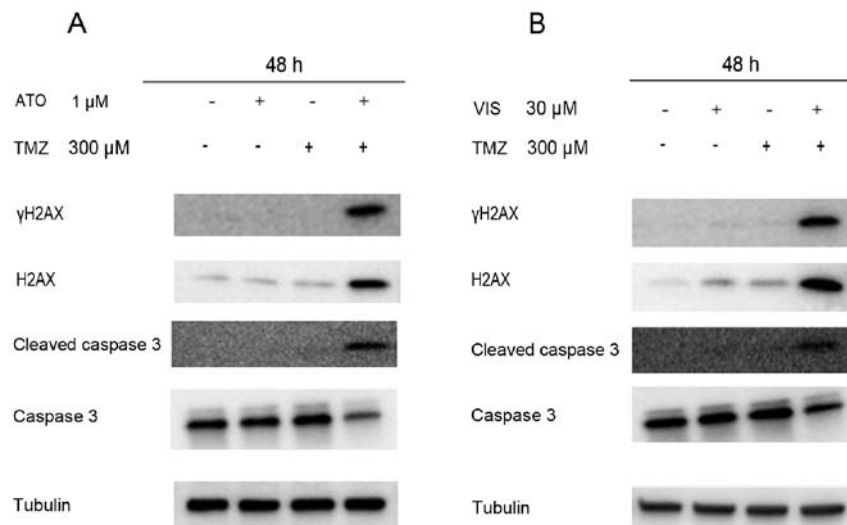


Figure 4. Combination of ATO/VIS and TMZ increased DNA damage and apoptosis, as shown by the increased density of γ H2AX and cleaved caspase-3. ATO, arsenic trioxide; TMZ, temozolomide.

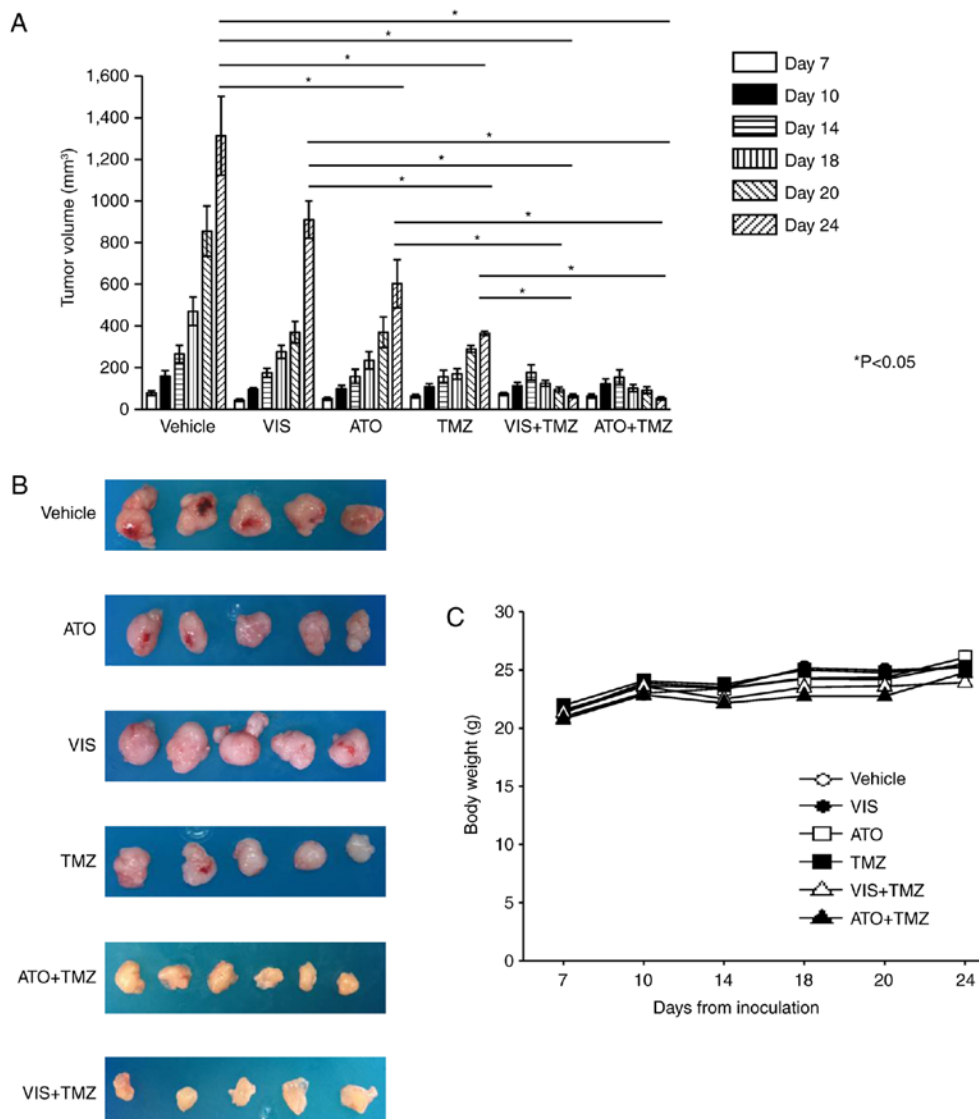


Figure 5. Synergistic chemotherapeutic activity of ATO/VIS and TMZ combination on mouse GUO-derived xenografts. After the establishment of flank tumours, mice were administered i.p. with 2.5 μ g/g ATO, 25 mg/kg/day VIS and 10 mg/kg/day TMZ, or a combination of both regimens for 4 days/week. (A) Tumour volumes (n=7). (B) Representative images of the excised tumours from the mice. P < 0.05 was considered significant (Steel-Dwass test). (C) Body weight of mice. ATO, arsenic trioxide; VIS, vismodegib; TMZ, temozolomide; GUO, glioblastoma of unknown origin.

Discussion

The emergence of chemotherapeutic drug resistance is a major limitation of therapy for glioblastoma (GBM) patients. In spite of the fact that temozolomide (TMZ) is the standard regimen for GBM, these tumours are highly resistant to chemotherapy (3). On account of the mechanism responsible for such resistance, several factors have been stipulated, and DNA repair-related genes such as *MGMT*, *MSH2* and *MSH6* have been recognised as critical factors involved in the survival of the tumour after treatment with alkylating agents (29-31). *MGMT* expression is also associated with GLI1 activity due to an apparent GLI1-binding site in the *MGMT* gene promoter (32). Ulasov *et al* further consolidated the possible link between Hedgehog (Hh) activity and therapeutic resistance to TMZ by their experiments with CD133⁺ glioma stem cells (33).

Arsenic trioxide (ATO) has been approved as an anticancer agent for acute promyelocytic leukaemia (APL) by the Food and Drug Administration (FDA) and Pharmaceutical and Medical Devices Agency (PMDA) in Japan (34). Inhibition of the Hh pathway by ATO could be a useful additional therapy to the standard chemotherapy for GBM. Several mechanisms have been stipulated to account for the inhibition of the Hh pathway by ATO. In a recent study, ATO was shown to inhibit the transcription of GLI target genes and promote apoptotic cell death in osteosarcoma cells due to increased DNA damage (18). Other authors have also reported inhibition of the expression of *GLI2* and downregulation of the expression of *SMO* and *PTCH* by ATO (11,22,35). Our findings delineate that ATO hindered the proliferation of GBM cells both *in vivo* and *in vitro*.

Vismodegib (VIS) is the first Hh inhibitor to be approved by the FDA for the treatment of basal cell carcinoma (BCC) (36,37). Smoothened (SMO) inhibitors such as VIS have been evaluated in recent clinical trials (37). Targeting the Hh pathway with VIS blocks aberrant signalling caused by mutational inactivation of the negative regulator *PTCH1* or mutational activation of *SMO* (37,38).

Despite the impressive tumour regression achieved by targeting the Hh pathway with ATO and VIS, resistance has also been reported (39,40) thus, conferring the need for a combination therapy (19).

The combination of ATO (Hh/GLI inhibitor) and alkylating agents has been reported to synergistically inhibit the proliferation of cells with inherited or acquired drug resistance (41). Silencing of *GLI1* in GBM has also been reported to promote sensitivity to TMZ by broadly reducing efflux behaviour attributed to multidrug transporters (42). Our findings revealed that combined treatment with either ATO and TMZ or VIS and TMZ was better at inhibiting GBM growth *in vitro* and *in vivo* than single-drug therapy. Among the two combination treatments, a combination of ATO and TMZ has the most promising potential, due to the effectiveness of ATO at a low concentration, compared to VIS. We believe this is the first study to show the synergistic effect of ATO/VIS with TMZ on GBM as determined by the CI-isobologram method of Chou (43) and Chou and Talalay (44).

Other authors including Nagao-Kitamoto *et al* (22) and Saitoh *et al* (27) also reported that combined administration

of VIS and ATO inhibited Hh pathway activation and tumour growth compared with single-agent therapy. These combinations could reduce the effective concentration of each drug and hence decrease toxicity. In the present study, there was marked inhibition of GBM growth when TMZ was combined with either ATO or VIS.

GBM is a very heterogeneous and genomically unstable tumour (45,46), hence posing the need to identify GBM patients with activated Hh pathway before commencement of treatment with Hh inhibitors. A recent study showed the usefulness of a five-gene Hh signature that can strongly identify activated Hh in medulloblastoma (47), and can thus be used for screening patients who have high chances of benefiting from Hh inhibitor therapies such as GBM patients. There is a high likelihood that the pleiotropic effect of ATO and off-target effects of SMO have a high possibility of affecting the growth inhibition of GBM. However, combination of TMZ with either ATO or VIS showed a promising therapeutic effect for GBM.

In conclusion, these findings denote that a combination of Hh pathway inhibitors and TMZ may be an important and safe therapeutic approach for the treatment of GBM.

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Availability of data and materials

All data generated or analysed during this study are included in this published article.

Authors' contributions

CB and TS developed the experimental design, conducted the experiments and drafted the manuscript. YS, HT, HS, SM, SN, SK and NT collected, analysed and interpreted the data. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

Animal care and experimental procedures were specifically approved and carried out in accordance with the guidelines of the Institute of Laboratory Animal Sciences, Graduate School of Medical and Dental Sciences, Kagoshima University (Kagoshima, Japan) (no. MD 17101).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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