

Antitumor effects of ribavirin in combination with TMZ and IFN- β in malignant glioma cells

YUSHI OCHIAI^{1*}, KOICHIRO SUMI^{1*}, EMIKO SANO², SODAI YOSHIMURA¹, SHUN YAMAMURO¹, AKIYOSHI OGINO¹, TAKUYA UEDA², YUTAKA SUZUKI², TOMOHIRO NAKAYAMA³, HIROYUKI HARA⁴, YOICHI KATAYAMA^{1,5} and ATSUO YOSHINO¹

¹Department of Neurological Surgery, Nihon University School of Medicine, Tokyo 173-8610;

²Department of Computational Biology and Medical Science, Graduate School of Frontier Sciences,

The University of Tokyo, Chiba 277-8562; ³Division of Companion Diagnostics, Department of Pathology and Microbiology;

⁴Division of Functional Morphology, Department of Functional Morphology, Nihon University School of Medicine,

Tokyo 173-8610; ⁵Center for Brain and Health Sciences, Aomori University, Aomori 038-0003, Japan

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Abstract. The prognosis of glioblastoma, the standard chemotherapy agent for which is temozolomide (TMZ), remains poor despite recent advances in multimodal treatments. Therefore, it is necessary to identify and develop novel therapeutics for this malignant disease. Ribavirin, an anti-viral agent which is one of the standard agents for treatment of chronic hepatitis C in combination with interferon (IFN), was recently revealed to have an antitumor potential towards various tumor cells, including malignant glioma cells. The aim of the present study was to examine the antitumor effect of ribavirin in combination with TMZ and IFN- β on glioma cells and to evaluate the possibility that such combinations might represent a novel candidate for glioblastoma therapy. The combination of ribavirin with TMZ and IFN- β displayed a significant cell growth inhibitory effect with a ribavirin dose-dependency, including a relatively low concentration of ribavirin, on not only TMZ-sensitive but also TMZ-resistant malignant glioma cells. The antitumor efficacy of such a combination further indicated a synergistic interaction when assessed by the Chou-Talalay method. Furthermore, flow cytometry analysis suggested that apoptosis induction was one of the possible biological processes underlying the synergistic antitumor effect of these triple combination treatments. Therefore, such combinations may be potentially important in the clinical

setting for glioblastoma treatment, although further detailed studies, e.g. on the adverse effects, are required.

Introduction

Although advances in multimodal therapies including surgery, radiotherapy, and chemotherapy are being made, the prognosis for glioblastomas, the most common primary brain tumor in adults and classified as having WHO grade IV malignancy, has not improved adequately for more than 30 years. In 2009, the EORTC/NCIC reported final results indicating the benefits of concomitant and adjuvant temozolomide (TMZ: a relatively new alkylating agent) in addition to standard postoperative radiotherapy for glioblastomas (1). Subsequently, concomitant radiotherapy with TMZ followed by adjuvant TMZ chemotherapy has become the current and global standard treatment for malignant gliomas, especially glioblastomas. Even though such treatments show a survival benefit in glioblastoma patients, the prognosis deriving from these therapies remains unsatisfactory.

Ribavirin, first reported in 1972 by Sidwell *et al* (2) as an anti-viral agent, is a nucleic acid analog. To date, ribavirin has served as a therapeutic agent widely used against RNA and DNA viruses, and is in particular one of the standard agents for chronic hepatitis C combined with interferon (3). On the other hand, we and others have recently observed an anti-tumor effect of ribavirin on various tumor cells, including breast cancer, acute myeloid leukemia, and atypical teratoid/rhabdoid tumors, which is thought to be mediated via inosine-5'-monophosphate dehydrogenase (IMPDH), eukaryotic translation initiation factor 4E (eIF4E), histone methyltransferase enhancer of zeste homolog 2 (EZH2), extracellular regulated protein kinases (ERK), and/or mitogen-activated protein kinase interacting protein kinase 1 (MNK1) (3-11). To date, there have been only a few studies on the anti-tumor effect of ribavirin against glioma cells. Volpin *et al* (9) reported an anti-tumor effect of ribavirin including in combination with TMZ and irradiation on glioma cells and glioma stem-like cells *in vivo*

Correspondence to: Dr Atsuo Yoshino, Department of Neurological Surgery, Nihon University School of Medicine, 30-1 Oyaguchi-Kamimachi, Itabashi-ku, Tokyo 173-8610, Japan
E-mail: yoshino.atsuo@nihon-u.ac.jp

*Contributed equally

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and *in vitro*. More recently, we demonstrated an effect of ribavirin on apoptosis induction, cell cycle arrest, p53 pathway activation, and DNA damage (double-strand breaks: DSBs) in malignant glioma cell lines (10).

In the present study, we obtained further data by examining the effect of ribavirin in combination with TMZ and interferon-beta (IFN- β) on malignant glioma cells. The reasons for using TMZ and IFN- β in combination with ribavirin were that TMZ is a standard chemotherapeutic agent for glioblastomas as mentioned above, and IFN- β exhibits pleiotrophic biological activities against neoplasias (12-15) and acts as a drug sensitizer enhancing the anti-tumor effect when administered in combination with nitrosoureas (alkylating agents) against malignant gliomas (16). Furthermore, a synergistic anti-tumor effect between TMZ and IFN- β has been demonstrated in malignant glioma cells (17-19), and a significant anti-tumor effect of ribavirin in combination with IFN- α (grouped to type I IFNs, the same as IFN- β) has been observed in hepatoma cells and renal carcinoma cells (20,21). Based on the findings of the present study, the combination of these three agents could exert a synergistic anti-tumor effect in malignant glioma cells and provide an experimental basis for rational clinical treatments in glioblastoma patients.

Materials and methods

Cell lines and cell culture. Human malignant glioma cells of the A-172 (cell no. JCRB0228, lot no. 021999), AM-38 (cell no. IFO50492, lot no. 12082003), T98G (cell no. IFO50303, lot no. 1007), U-251MG (cell no. IFO50288, lot no. 12132002), and YH-13 (cell no. IFO50493, lot no. 1164) cell lines were obtained from Health Science Research Resources Bank (Osaka, Japan), and U-87MG (glioblastoma of unknown origin; catalog no.: HTB-14, lot no. 2497162) and U-138MG (catalog no. HTB-16, lot no. 1104428) were procured from the American Type Culture Collection. It has been confirmed by us that *O*⁶-methylguanine-DNA methyltransferase (MGMT: a key factor of alkylating agents) is expressed in T98G, U-138MG and YH-13 by RT-PCR and western blot analysis in a previous study (22). Consistent with an earlier report (23), it was also confirmed that T98G (237 Met→Ile) and U-251MG (273 Arg→His) have a point mutation of the *p53* gene. These cell lines were cultured in Dulbecco's modified Eagle's medium (Nissui Pharmaceutical) containing 10% fetal calf serum (Life Technologies) using plastic culture flasks (Corning®) in a standard humidified incubator at 37°C under a 5% CO₂, 95% air atmosphere.

Cell culture growth studies. Malignant glioma cell proliferation was evaluated using a Coulter Counter (Beckman Coulter) to determine the cell numbers in 24-well plates (Iwaki). Each well was seeded with 1×10⁴ cells and cultured for 24 h prior to treatment to allow adherence of the cells to the plate. The culture medium was replenished with fresh medium containing ribavirin (0.1-100 μ M; Sigma) alone, or in combination with TMZ (10 μ M; LKT Laboratories) and IFN- β (10 IU/ml; Toray), and the cells were cultured for 72 h. We selected the incubation conditions as 10 μ M TMZ and/or 10 IU/ml IFN- β , because these values represent clinically achievable concentrations of TMZ and IFN- β (19,24). Furthermore, in order to

assess whether the effect of the combination of these agents, TMZ, IFN- β , and/or ribavirin, was synergistic, the ribavirin was set at a clinically relevant concentration of 10 μ M (25). The proliferated cells were harvested with trypsin-EDTA solution (Invitrogen; Thermo Fisher Scientific, Inc.) and the numbers counted. The cell culture growth experiments were repeated a minimum of four times each.

Synergism of drug combination treatment. Moreover, to confirm whether the anti-tumor effect of the combination with TMZ, IFN- β , and ribavirin was synergistic, a combination index (CI) was also calculated using the Chou-Talalay method (26). In the present study, we decided to calculate the CI at 50% inhibition of cell proliferation (IC₅₀). Two malignant glioma cell lines, A-172 and U-251MG, were therefore employed to calculate the CI, because their IC₅₀ values had been obtained on the basis of dose response curves. The IC₅₀ of TMZ, IFN- β , and ribavirin for A-172 was 52.4 μ M, 57.5 IU/ml, and 53.6 μ M, and for U-251 was 22.5 μ M, 26.4 IU/ml, and 257.7 μ M, respectively (7,15,22). Based on the Chou-Talalay method, CI values of less than 1 are indicative of synergism (the smaller the value, the greater the degree of synergy), those equal to 1 indicate additivity, and those of more than 1 are interpreted as antagonism. Values of CI < 0.4, between 0.4 and 0.8, and >0.8 indicate strong, medium and slight synergism, respectively (26,27). An additive effect is defined as a situation in which the final effect is equal to the sum of the effects of the drugs. Drug interactions are interpreted as antagonistic if they lead to a decrease in the effects of one or both drugs (26,27).

Assessment of apoptosis. From the results for the growth inhibitory effect and CI, U-251MG cells were subjected to further experiments. Apoptosis was analyzed by flow cytometry, using dual staining with an Annexin V-FITC/PI Apoptosis Detection Kit (BD Biosciences). Cells were seeded in 6-well plates (Iwaki) at 1×10⁶ cells and incubated for 24 h to adhere. The culture medium was then replenished with fresh medium containing ribavirin, TMZ, IFN- β , TMZ and IFN- β , or TMZ, IFN- β and ribavirin for 72 h (ribavirin, 10 μ M; TMZ, 10 μ M; IFN- β , 10 IU/ml). The cells were next washed in PBS and harvested using trypsin-EDTA solution. Following centrifugation and washing in PBS, the solution was agitated with 100 ml of binding buffer (Wako Pure Chemical Industries, Ltd.), into which 5 μ l of Annexin V Alexa Fluor 488 conjugate (Life Technologies; Thermo Fisher Scientific, Inc.) and 10 μ l of PI (Milenyi Biotech) were added, and incubated at room temperature for 10 min. An additional 400 μ l of binding buffer was added to give a total sample volume of 500 μ l. The fluorescence was measured with an FACSCalibur flow cytometer (Becton-Dickinson). The apoptotic cells were analyzed using Flowjo software (BioLegend). The experiments were repeated three times to confirm reproducibility.

Statistical analysis. Appropriate comparisons were made employing one-way analysis of variance followed by the Tukey-Kramer method among multiple comparisons using the software Stat View (Ver. 5.0; SAS Institute Inc.). Data were expressed as the means \pm standard error and were considered significant at P<0.05.

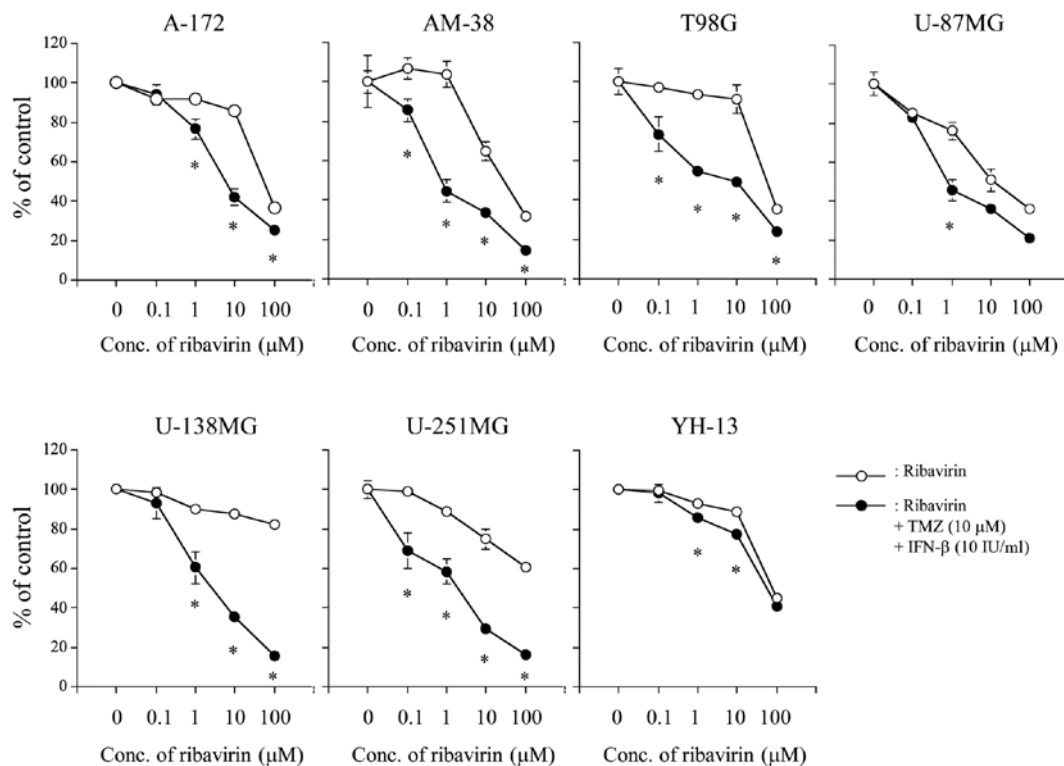


Figure 1. Cell growth inhibitory effect of a combination of ribavirin with or without TMZ and IFN- β in seven malignant glioma derived cell lines. Malignant glioma cells were exposed to medium containing various concentrations of ribavirin without IFN- β and TMZ (white circles) or with 10 IU/ml IFN- β and 10 μ M TMZ (black circle) for 72 h. The combination of ribavirin with TMZ and IFN- β exerted a more profound cell growth inhibitory effect compared with ribavirin alone, with a ribavirin dose-dependency in all malignant glioma cell lines. Data are presented as the mean \pm standard error. Significant differences between ribavirin alone and the triple combination were observed at each ribavirin concentration. * $P < 0.05$. IFN- β , interferon- β ; TMZ, temozolomide.

Results

Anti-tumor effects of a combination of ribavirin with TMZ and IFN- β . We have previously demonstrated an anti-tumor effect of ribavirin on malignant glioma cell lines (7). In that study, seven malignant glioma cell lines were exposed to 0.1-1,000 μ M of ribavirin and treated for 72 h, and it was found that ribavirin inhibited the growth of all seven cell lines in a dose-dependent manner (7).

To assess whether or not a combination of ribavirin with TMZ and IFN- β could produce a more profound anti-tumor effect as compared to ribavirin alone in malignant glioma cell lines, cells were incubated in culture medium containing various concentrations (0-100 IU/ml) of ribavirin alone or ribavirin with TMZ (10 μ M) and IFN- β (10 IU/ml) for 72 h. As shown in Fig. 1, the combination of ribavirin with TMZ (10 μ M) and IFN- β (10 IU/ml) revealed a significant cell growth inhibitory effect with a ribavirin dose-dependency in all seven malignant glioma cell lines. Such a cell growth inhibitory effect was also observed at a relative low concentration of ribavirin, 0.1 and 1 μ M. Furthermore, it was evident at 0.1 μ M of ribavirin in AM-38, T98G, U-87MG, and U-251MG cells.

In addition, we examined whether the combination of TMZ, IFN- β , and ribavirin displayed an enhanced cell growth inhibitory effect as compared to the control, TMZ alone, or TMZ and IFN- β . As demonstrated in Fig. 2, the cell growth inhibitory effect at 72 h was significantly higher in the group treated with TMZ (10 μ M), IFN- β (10 IU/ml), and ribavirin (10 μ M) as compared to the group treated with TMZ alone in

all seven malignant glioma cell lines. Furthermore, the combination of TMZ, IFN- β and ribavirin exerted a significantly enhanced growth inhibitory effect as compared to TMZ and IFN- β in the AM-38, U-87MG, U-138MG, and YH-13 cells.

Drug synergy analysis of a combination of ribavirin with TMZ and IFN- β . Our results indicated that the combination of TMZ, IFN- β and ribavirin displayed significant growth inhibition in malignant glioma cells. When TMZ, IFN- β , and ribavirin were applied in combination, a synergistic effect was detected as analyzed by the Chou-Talalay method in malignant glioma cells. The CI value was 0.68 (medium synergism) in A-172 and 0.98 (slight synergism) in U-251MG at the IC_{50} level, respectively.

Apoptosis induced by a combination of TMZ, IFN- β , and ribavirin in U-251MG cells. The induction of apoptosis by TMZ, IFN- β , ribavirin, TMZ and IFN- β , or TMZ, IFN- β and ribavirin in U-251MG cells was examined by Annexin V/PI double staining and evaluated using flow cytometry. The distribution of apoptotic cells (Annexin V-positive, early-stage apoptosis; Annexin V/PI-positive, late-stage apoptosis) after 72 h treatment is illustrated in Fig. 3. In particular, the proportion of Annexin V/PI positive cells (late-stage apoptosis) following treatment with three agents (TMZ, IFN- β and ribavirin; mean=10.56%) was higher than that for each single agent (means: TMZ, 5.57%; IFN- β , 5.74%; and ribavirin, 5.57%, respectively). The results indicated that the apoptotic cells after treatment with three agents tend to be increased, showing

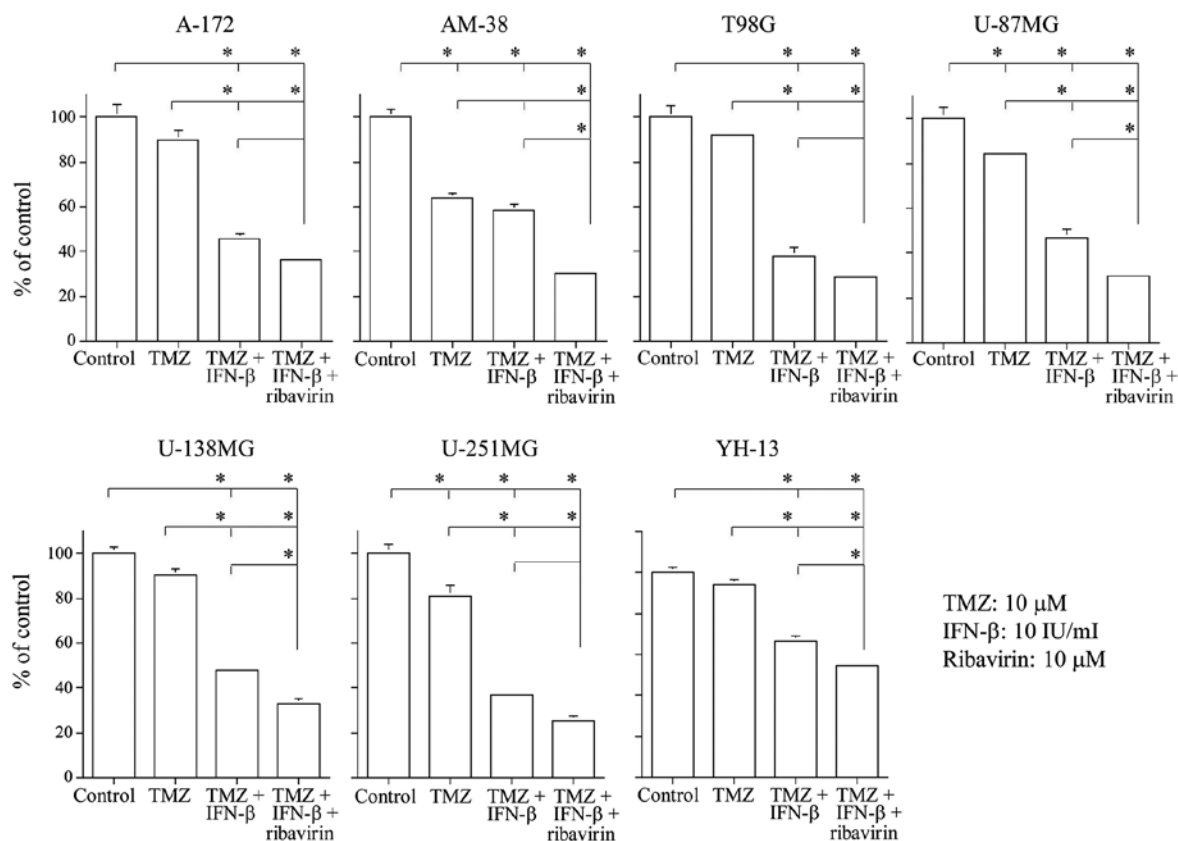


Figure 2. Cell growth inhibitory effect of a combination of TMZ alone, TMZ and IFN-β, or TMZ, IFN-β and ribavirin in seven malignant glioma cell lines. A significant synergistic cell growth inhibitory effect was observed with the combination of TMZ, IFN-β and ribavirin in all seven glioblastoma cell lines. The results are presented as the mean \pm SE. The Tukey-Kramer test was employed to identify which group differences accounted for the significant P-values *P<0.05. IFN-β, interferon-β; TMZ, temozolomide.

an elevation of more than 2% between each treatment, in U-251MG cells. However, the fact that statistically significant differences were not found among the treatments might reflect the small numbers in each group.

Discussion

TMZ has become the current first-line chemotherapeutic agent, but it does not provide satisfactory benefits for glioblastoma patients. Further studies are needed to improve the clinical therapeutic efficacy and to establish a therapeutic strategy for glioblastomas. On the other hand, we demonstrated a dose-dependent anti-tumor efficacy of ribavirin for malignant glioma cell lines (7). Recently, Volpin *et al* (9) showed that ribavirin (30 μM) inhibited cell proliferation and migration, and increased cell arrest and cell death in glioma cells, potentially through modulation of the eIF4E, EZH2, and ERK pathways. Furthermore, they indicated that ribavirin combined with TMZ (100 μM) and irradiation (5 Gy) could potentially enhance the anti-tumor response in glioma cells and glioma stem-like cells, and that animals treated with a combination of ribavirin, irradiation, and TMZ had a significantly increased survival time (9). More recently, we found that ribavirin (10 μM) exerts an anti-tumor effect on malignant glioma cells via the biological processes of induction of DSBs, cell cycle arrest in G₀/G₁, and both exogenous and endogenous apoptosis (10). Moreover, such effects might not be dependent on MGMT expression, which is closely correlated with resistance to TMZ treatment (10).

In the present study, the combination of ribavirin with TMZ and IFN-β (clinically achievable concentrations) revealed a more profound cell growth inhibitory effect as compared to ribavirin alone with a ribavirin dose-dependency, which was observed from a relatively low concentration of ribavirin, in all seven malignant glioma cell lines (Fig. 1), suggesting that ribavirin has some anti-tumor effect as a medical drug. Although TMZ (10 μM) did not show a cell growth inhibitory effect in A-172, T98G, U-138MG, and YH-13, the combination of ribavirin with TMZ and IFN-β did exhibit a significant cell growth inhibitory effect in these cell lines. Out of the four cells, U-138MG and YH-13 displayed a significant cell growth inhibitory effect when treated with ribavirin, TMZ, and IFN-β as compared to the effect without ribavirin (Fig. 2). These findings suggested that the combination of ribavirin with TMZ and IFN-β revealed a more significant cell growth inhibitory effect in not only TMZ sensitive cells but also in TMZ resistant cells. Furthermore, in this study, the anti-tumor efficacy of a combination of these three agents on A-172 and U-251MG cells indicated a synergistic interaction when assessed by the Chou-Talalay method [A-172 and U-251MG were used in the analysis because the IC₅₀ for each drug had been obtained (7,22)]. Such a combination of ribavirin with TMZ and IFN-β might therefore be considered as an effective treatment in certain glioblastoma cells, although, to the best of our knowledge, no report has yet described the detailed effect of such a combination on glioma cell lines and other cell lines.

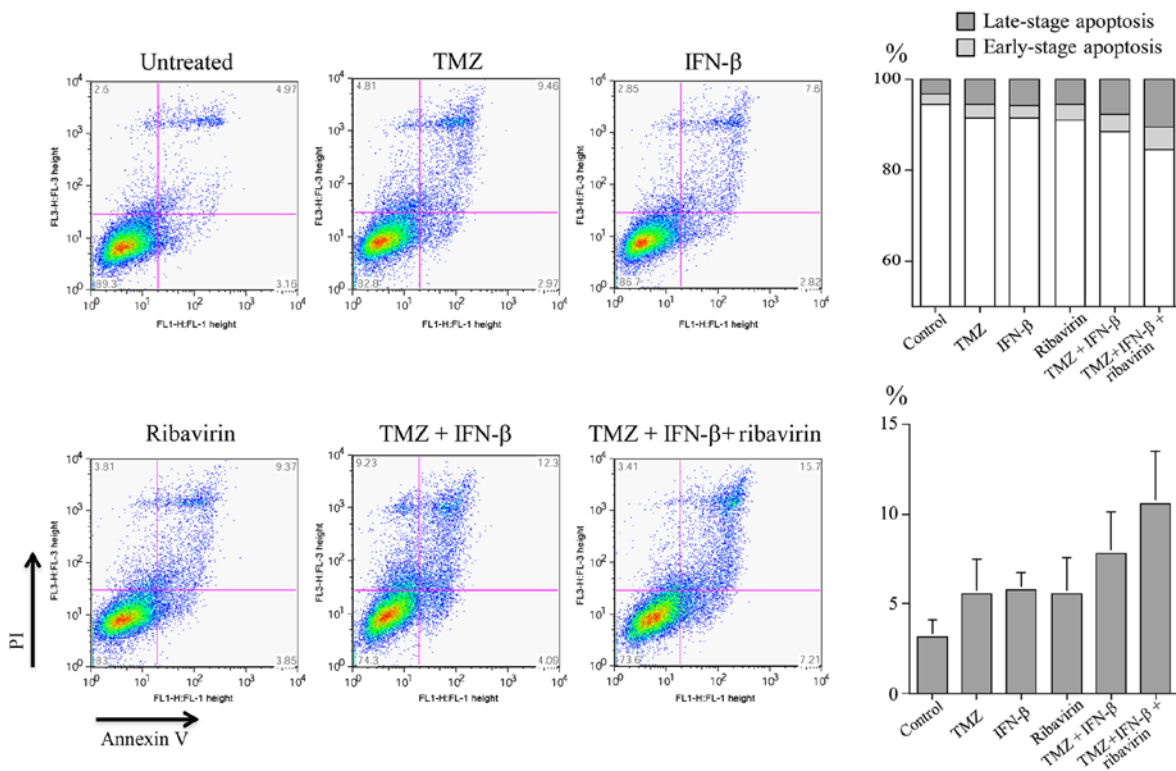


Figure 3. Induction of apoptosis by TMZ, IFN- β , ribavirin, TMZ and IFN- β , or TMZ, IFN- β and ribavirin in U251MG cells. The ratio of detection of Annexin V-positive and Annexin V/PI-positive cells, indicating early-stage apoptosis, and late-phase apoptosis or necrosis, respectively, was measured by fluorescence-activated cell sorting after 72 h of treatment. The percentage of Annexin V-positive cells and the percentage of Annexin V/PI-positive cells was increased as shown at the bottom right and top right, respectively. The mean distributions of apoptotic cells with each drug and the combination are illustrated on the right. IFN- β , interferon- β ; TMZ, temozolomide.

Although the mechanism underlying the anti-viral and anti-tumor effects of ribavirin has not yet been fully elucidated, several participating/possible processes have been mentioned above. Kast *et al* (28) pointed out the following major mechanisms of action, particularly related to the anti-viral action: i) actual intermingling within the viral RNA; ribavirin enters the cell via a nucleoside transport mechanism, subsequently inhibiting/altering viral RNA synthesis, ii) structural analogy to GTP; incorporation into the cell passively, then binding/inhibiting RNA polymerase/RNA synthesis, iii) immune clearance; immune-stimulation by up-regulation of cytokines to shift the Th1/2 cell balance to Th1 dominance, iv) inhibition of eIF4E; thereby inhibiting mRNA capping and translation initiation, v) modulation of IFN-related gene expression, vi) inhibition of IMPDH; with consequent depletion of intracellular GTP, and vii) RNA mutagen; following triphosphorylation, ribavirin triphosphate is incorporated into replicating RNA viral RNA polymerases with consequent induction of viral mutagenesis. These processes are attractive as factors in the repurposing of the anti-viral ribavirin as an anti-tumor agent, and also in the mechanism underlying the synergistic effect of ribavirin in combination with other agents. In particular, the modulation of IFN-related gene expression is thought to be a potential factor in the synergistic effect of ribavirin with IFN (13,19), since IFN exerts a priming effect on the ribavirin-induced IFN-related gene (29). Further studies investigating the molecular mechanisms are clearly needed to reaffirm the efficacy of these combinations.

In the present study, the flow cytometry analysis might indicate that apoptosis induction represented one possible biological process associated with the synergistic anti-tumor effect of triple combination treatment, because the apoptotic cells after such triple treatment tended to be increased in U-251MG, although no statistically significant differences were observed. Schlosser *et al* (20) demonstrated that ribavirin and IFN enhanced apoptosis and caspase activation in hepatoma cells. Further, Teng *et al* (21) showed that ribavirin in combination with IFN could significantly inhibit the cell proliferation and migration, induce apoptosis, arrest the cell cycle, and decrease IL-10 production in renal carcinoma cells. On the basis of these findings, we propose that ribavirin in combination with TMZ and IFN- β could increase apoptosis in glioblastoma cells.

Finally, and very importantly, we need briefly to mention other effects of the drug combination used in this study. Chemotherapy-induced toxicity is a major problem in anti-tumor therapy. A cell growth inhibitory effect was also observed at a relatively low concentration of ribavirin (0.1 and 1 μ M) in some glioma cell lines, AM-38, T98G, U-87MG, U-138MG, and U2-51MG, combined with 10 μ M TMZ and 10 IU/ml IFN- β : both concentrations are clinically achievable (19,24). Ribavirin at 10 μ M is considered a clinically relevant concentration, because when administered at a dose of 800 mg/day as a therapeutic agent for chronic hepatitis C, the blood concentration of ribavirin reached 13 μ M and the cerebral spinal migration of ribavirin was 70% (10,25). In addition, Casaos *et al* (11) employed 50 μ M ribavirin as a

clinically appropriate concentration in their *in vitro* experiments. A very low concentration of ribavirin should help to reduce the adverse events of ribavirin, such as anemia, and be easier for clinical use. In addition, ribavirin is relatively inexpensive, and therefore beneficial in relation to the rising medical costs particularly of tumor treatment. Since the clinical use of triple combination therapy could lead to combined toxicity in patients, further studies are needed to investigate the extent of efficacy at various doses and times of use of these combinations.

In conclusion, we have provided evidence that ribavirin in combination with TMZ and IFN- β can induce synergistic anti-tumor effects involving of cell growth inhibition in glioma cells, and could be of potential importance in the clinical setting.

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Availability of data and materials

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

Authors' contributions

AY contributed to the experimental concept and design. YO and ES developed the experimental design, performed most of the experiments and some of the data analysis, and wrote part of the draft manuscript. KS and AY also conducted part of the data analysis and contributed to the writing of the manuscript. SYo, SYa and AO were involved in the conception and design of the study, undertook part of the experiments, analyzed the data, and contributed to the writing of the draft manuscript. TU, YS, TN, HH and YK supervised the study (including the experimental design), performed some of the experiments, analyzed data, helped prepare the draft manuscript, and proof-read the manuscript. YO and KS contributed equally to this work. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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