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ORIGINAL ARTICLE

Use of an anti-viral drug, Ribavirin, as an anti-glioblastoma therapeutic

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The median survival for glioblastoma patients is ~15 months despite aggressive surgery and radio-chemotherapy approaches. Thus, developing new therapeutics is necessary to improve the treatment of these invasive brain tumors, which are known to show high levels of the eukaryotic initiation factor, eIF4E, a potent oncogene. Ribavirin, the only clinically approved drug known to target eIF4E, is an anti-viral molecule currently used in hepatitis C treatment. Here, we report the effect of ribavirin on proliferation, cell cycle, cell death and migration of several human and murine glioma cell lines, as well as human glioblastoma stem-like cells, *in vitro.* In addition, we tested ribavirin efficacy *in vivo,* alone and in combination with temozolomide and radiation. Our work showed that ribavirin inhibits glioma cell growth and migration, and increases cell cycle arrest and cell death, potentially through modulation of the eIF4E, EZH2 and ERK pathways. We also demonstrate that ribavirin treatment in combination with temozolomide or irradiation increases cell death in glioma cells. Finally and most importantly, ribavirin treatment *in vivo* significantly enhances chemo-radiotherapy efficacy and improves survival of rats and mice orthotopically implanted with gliosarcoma tumors or glioma stem-like cells, respectively. On the basis of these results, we propose that ribavirin represents a new therapeutic option for glioblastoma patients as an enhancer of the cytotoxic effects of temozolomide and radiotherapy.

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INTRODUCTION

Ribavirin is one of the standard treatments for patients with hepatitis C.[1–3] This broad-spectrum anti-viral drug, part of the synthetic purine nucleoside analog family, was developed more than 40 years ago as a therapeutic agent against RNA and DNA viruses.[1,4] Recent evidences reveal that ribavirin shows unexpected anti-tumor activity in various tumor cells, such as leukemic and breast cancer cells.[5–8] However, the mechanisms responsible for its efficacy against tumor cells are not clear. At the molecular level, ribavirin offers multiple potential mechanisms of action and interesting targets, which could explain its anti-cancer activity.[5,9,10] For instance, ribavirin has been described as a regulator of extracellular regulated protein kinases (ERK) in the mitogen-activated protein kinase (MAPK) pathway in cancer cells.[11] Another study demonstrated that ribavirin decreases the expression of the histone methyltransferase enhancer of zeste homolog 2 (EZH2), which has a role in transcriptional repression through H3K27 tri-methylation and is considered an attractive epigenetic target for cancer therapy.[6] Finally, it has been shown that ribavirin directly binds to the eukaryotic translation initiation factor and onco-protein, eIF4E.[12,13] Ribavirin disrupts eIF4E binding to the 5′cap of mRNA, subsequently disrupting translation and proliferation.[12,13] Interestingly, eIF4E has been shown to promote tumorigenesis, is overexpressed in many human malignancies, and is usually a marker of poor prognosis.[14–16]

Indeed, eIF4E over-expression has been identified in 30% of human cancers including high-grade astrocytic neoplasms, such as glioblastoma (GBM).[15] In accordance with the WHO classification, GBMs are grade IV astrocytic brain tumors and are one of the most common, aggressive and deadly human cancers.[17] Despite the standard treatments, which combine surgery, radiotherapy and chemotherapy, including temozolomide (TMZ) and/or carmustine wafers, the median survival does not exceed 21 months.[18–20] Therefore, identifying and developing new therapeutics is critical. The use of ribavirin for breast cancer and leukemia patients has been clinically beneficial and the combination of ribavirin with current chemotherapeutic agents has shown synergistic effects in patients with acute myeloid leukemia (AML) suggesting that ribavirin could become a potential adjuvant therapy that might enhance the effects of radiation and chemotherapy.[7,8,21,22] Only one previous study reported efficacy of ribavirin against malignant gliomas *in vitro*. In this study, the authors demonstrated that ribavirin had anti-glioma properties and that its potential effects could be related to the modulation of the platelet-derived growth factor receptor alpha (PDGFR-A).[23]

On the basis of these data, the aim of our work was to determine more thoroughly the anti-tumor activity of ribavirin on several glioma cell lines (U87, U251, LN18, SF767, T98G, 9L, F98, C6) and GBM stem-like cells (GB1A, 1113) *in vitro* and *in vivo,* and to evaluate whether ribavirin could successfully enhance the cytotoxic effects of TMZ or radiotherapy. First, we demonstrate that ribavirin inhibits glioma cell proliferation, increasing cell cycle arrest and cell death processes. In addition, we observed that ribavirin impairs GBM cell migration and adhesion capacities.

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Mechanistically, we provide evidence that ribavirin treatment modulates several known oncogenic pathways, such as eIF4E, ERK and EZH2, in glioma cell lines and GBM stem-like cells. Translating our *in vitro* findings *in vivo*, we demonstrate in rat and mouse models of GBM that ribavirin treatment significantly improves the survival of the treated animals compared with the vehicle-treated groups. Finally, we show *in vitro* and *in vivo* that ribavirin, in combination with TMZ and irradiation (IR), potentiates the cytotoxic effects and anti-tumoral response in glioma cells. As standard GBM treatments commonly fail to cure gliomas, ribavirin represents a potential important step forward for these patients.

RESULTS

Ribavirin decreases glioma cell proliferation through cell cycle arrest and cell death

It has previously been shown that ribavirin affects viability and cell growth of several cancer cell lines.[6,8,11,12,22–24] In glioma cell lines, it has been established that the IC50 of ribavirin is 10–100 μM.[23] In order to confirm the effect of ribavirin on glioma cell growth, we specifically selected a panel of five different human glioma cell lines (U87, U251, LN18, SF767, T98G), three rat glioma cell lines (9L, F98, C6), and two human GBM stem-like cells (GB1A, 1113). We treated these glioma cells with 30 or 50 μM of ribavirin and subsequently assessed viable cell number over 6 days (Figure 1a, Supplementary Figure S1A and data not shown). As shown in Figure 1a, ribavirin treatment (30 μM) leads to a significant decrease in all glioma cell growth. Ribavirin's effect on GBM cell growth was particularly significant at day 4 (96 h) after treatment. Interestingly, GB1A and 1113 GBM stem-like cells present an increased sensitivity to ribavirin with an anti-proliferative effect starting at day 2 (48 h). To assess the direct effect of ribavirin on glioma cell proliferation and exclude toxicity from indirect effects, such as glucose exhaustion or acidification of the extracellular media, we repeated this time-course experiment and supplied GBM cells with fresh media every day for the time of the assay. In these conditions, the ribavirin effect on cell growth was exactly the same and we did not notice any significant difference suggesting that ribavirin exerts a targeted effect on glioma cells (data not shown). Together, these results suggest that ribavirin impairs glioma cell growth and particularly GBM stem-like cell growth.

To understand the ribavirin-mediated decrease in glioma cell growth, we first looked at the Ki67 proliferative marker over 72 h by flow cytometry. Ki67 protein is a reliable marker for determining the active and resting fraction within a cell population as Ki67 is expressed during all active phases of the cell cycle but is absent from arrested cells (G_0).[25–27] Performing this time-course experiment on U87, U251, LN18, 9L, GB1A and 1113 cells, we observed that ribavirin significantly increases the number of Ki67-negative cells over time, starting at 24 h (Figure 2a). These results indicate that inhibition of glioma cell growth due to ribavirin treatment might be due to a cell cycle arrest in G_0.

Given that cell death and apoptosis often are consequences of cell cycle arrest,[25] Annexin-V/propidium iodide staining was

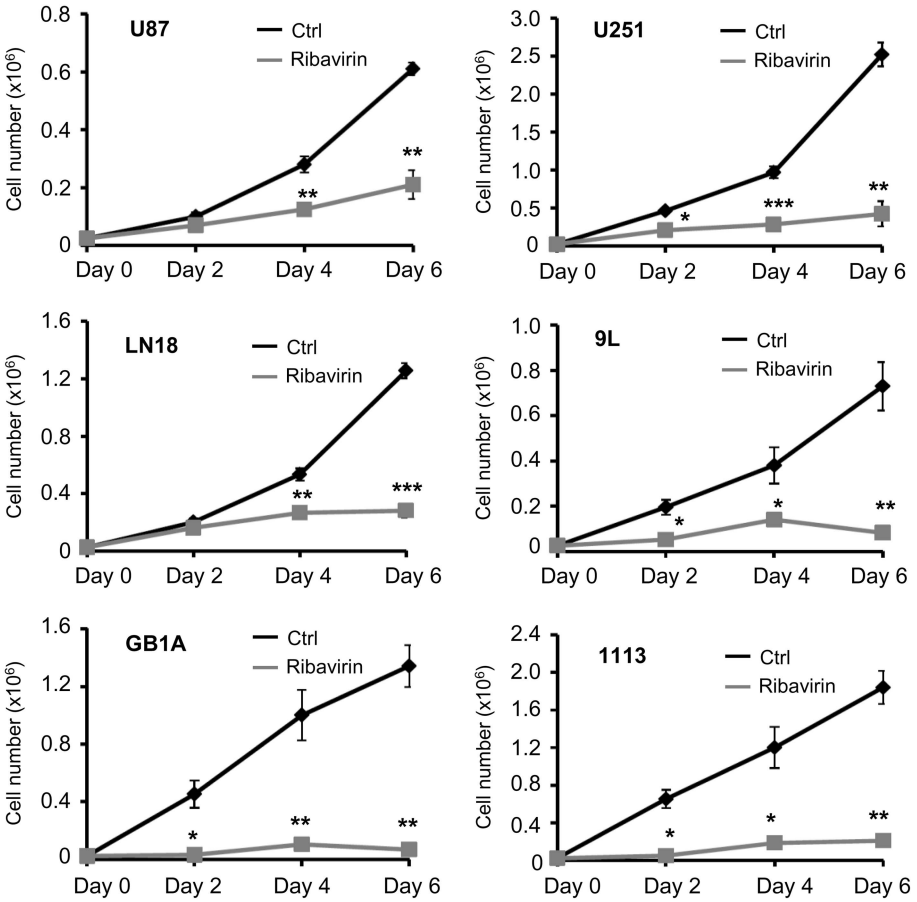

Figure 1. Ribavirin inhibits glioma cell proliferation. Proliferation assays performed with U87, U251, LN18, 9L, GB1A and 1113 cells show a decreased cell number in presence of ribavirin (black curve, Ctrl: PBS vehicle control; gray curve, Ribavirin: Ribavirin 30 μM) (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ Ribavirin vs Ctrl, $n = 3$).

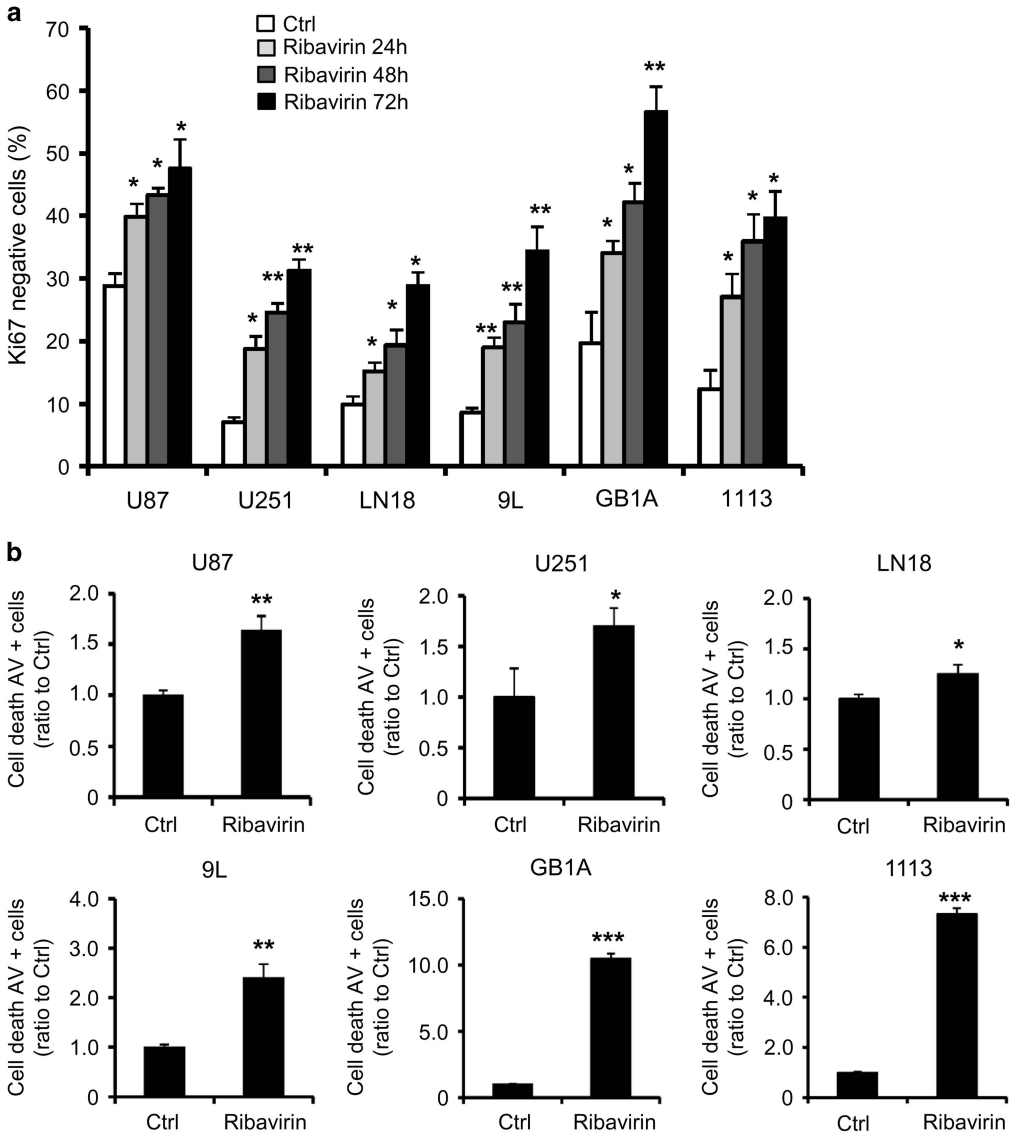

Figure 2. Ribavirin induces GBM cell cycle arrest and cell death. (**a**) Assessment of Ki67-negative GBM cells, using flow cytometry and Ki67/PI staining, 24 h, 48 h and 72 h after ribavirin treatment. Ribavirin significantly increases the number of arrested cells (*$P < 0.05$, **$P < 0.01$ Ribavirin compared with Ctrl, $n = 3$). (**b**) Quantification of cell death for U87, U251, LN18, 9L, GB1A and 1113 GBM cells, using flow cytometry and Annexin-V/PI staining, 72 h after treatment. Ribavirin significantly increases the number of AV positive cells (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ Ribavirin compared with control, $n = 4$).

employed to determine whether ribavirin's effect was related to cell death. Interestingly, the apoptotic cell death rate, reflected by the percentage of Annexin-V positive cells, was significantly increased in all glioma cells in response to 72 h-ribavirin treatment compared with the control cells (Figure 2b, Supplementary Figures S2A and S2B). Again, GB1A and 1113 GBM stem-like cells present an increased sensitivity to ribavirin with a 10.49- and 7.32-fold increase in cell death rate compared with control cells, respectively. These results suggest that ribavirin induces GBM cell cycle arrest, reflected by the increased percentage of Ki67-negative cells (Figure 2a) and cell death, reflected by the increased cell death rate (Figure 2b and Supplementary Figure S2B), which both contribute to the inhibition of glioma cell proliferation.

Ribavirin reduces glioma cell migration and adhesion properties

GBMs are known to be very aggressive and invasive tumors, with high migratory capacities. Interestingly, it has been reported that ribavirin decreases migration and invasion of mammary tumor cells.[22] In light of these findings, we investigated the influence of ribavirin on glioma cell migration (LN18, U251 and 9L) using a scratch wound assay. Of note, U87 cells, as well as GB1A and 1113, do not form a monolayer of cells and we were therefore unable to perform the test on these cells. In addition, we selected time points below 24/28 h after scratch to avoid any implication of the ribavirin effect on proliferation. As shown in Figure 3, 9L, LN18 and U251 glioma cells treated with ribavirin are less efficient in migrating and closing the wound compared with control cells (Figures 3a–c). Consistent with these results, adhesion assays performed on different substrates (plastic, gelatin, laminin, collagen I) reveal that ribavirin significantly impairs glioma cell adhesion, which may indicate therapeutic benefit (Figures 3d–g).

Potential mechanisms of ribavirin action in glioma cells and GBM stem-like cells

We then aimed to investigate the potential molecular mechanisms involved in the response to ribavirin treatment in our glioma cells

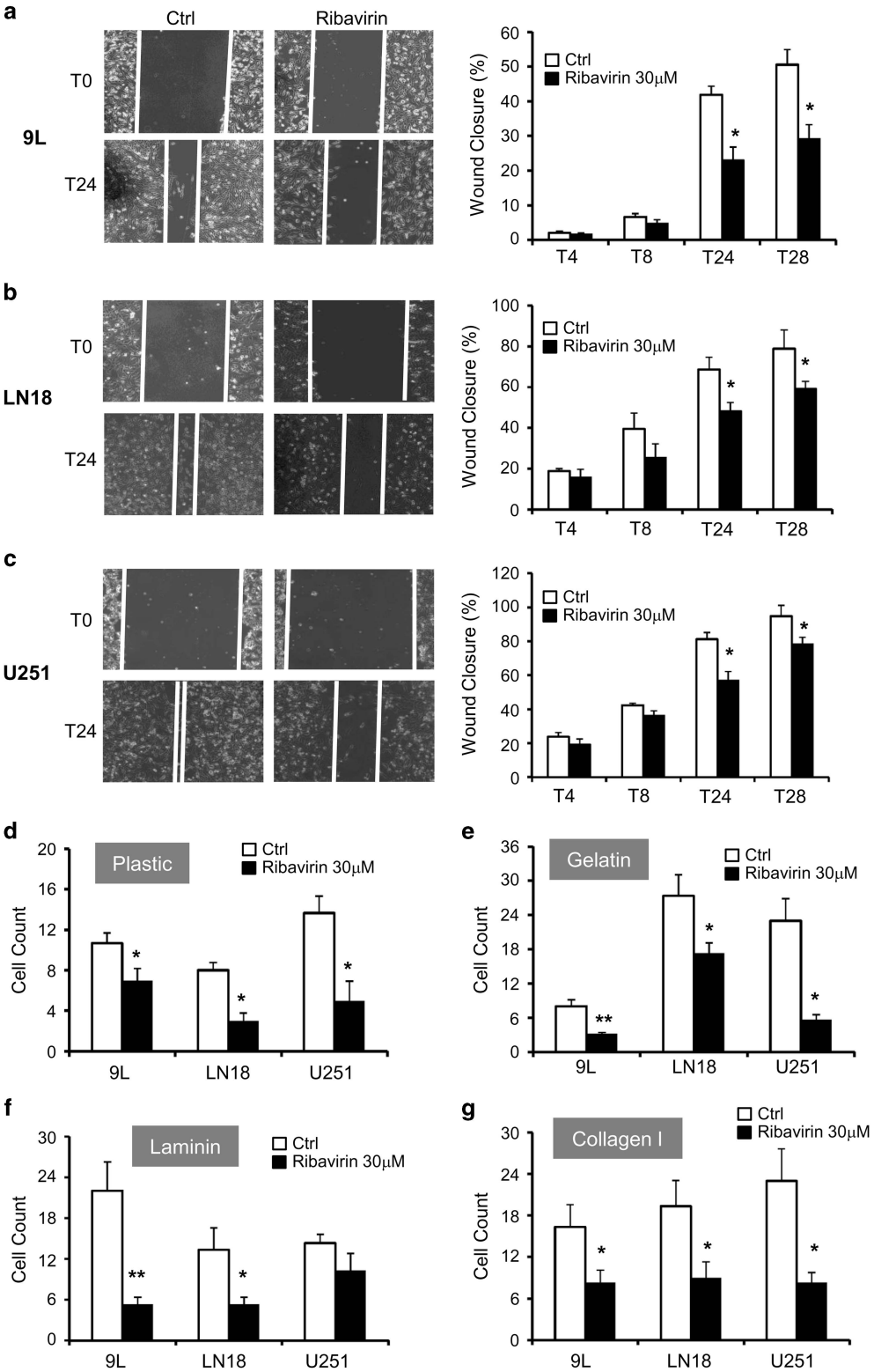

Figure 3. Ribavirin affects human glioma cell migration and adhesion properties. (**a**–**c**) Migration of control cells (Ctrl) and Ribavirin-treated cells (Ribavirin 30 μM) was assessed using a scratch wound assay. Test was performed with 9L (**a**), LN18 (**b**), and U251 (**c**) cell lines. Of note, U87, GB1A and 1113 cells do not form a monolayer of cells and we were therefore unable to perform the test for these cells. Left panels show representative photographs taken after the scratch (T0), and 24 h later (T24). Right panels show the quantification of the percentage of wound closure determined at 4 h (T4), 8 h (T8), 24 h (T24) and 28 h (T28) after wound, at consistent locations. Wound closure was significantly decreased for ribavirin-treated cells compared with vehicle-treated control cells at 24 and 28 h ($*P < 0.05$, $n = 3$). (**d**–**g**) Adhesion capacities of 9L, LN18 and U251 GBM cells either treated with ribavirin or untreated, on plastic (**d**), gelatin (**e**), laminin (**f**) and collagen I (**g**) was determined using an adhesion assay. Ribavirin treatment significantly decreases the number of adherent cells on each substrate ($*P < 0.05$, $**P < 0.01$, Ribavirin compared with control, $n = 4$).

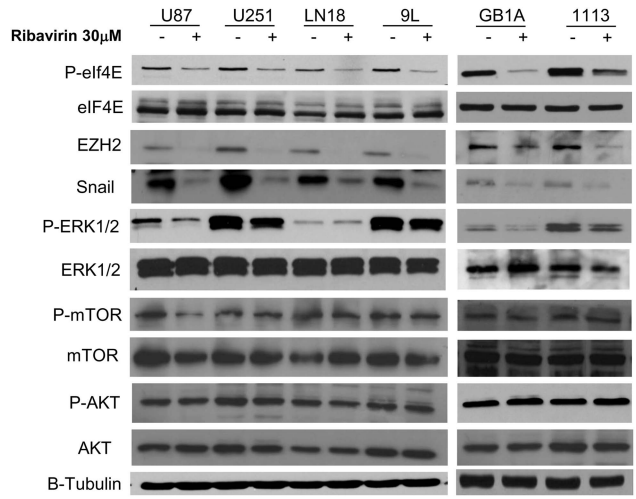

Figure 4. EIF4E, EZH2 and ERK are modulated in response to ribavirin in GBM cells. Western Blot analyses of eIF4E, EZH2, Snail, ERK, AKT and mTOR expression in U87, U251, LN18, 9L, GB1A and 1113 cells 72 h after ribavirin treatment. Ribavirin decreases eIF4E and ERK phosphorylation as well as EZH2 and Snail expression without affecting AKT or mTOR levels.

(Figure 4). Several works have previously demonstrated that ribavirin inhibits several pathways in cancer cells, such as the *de novo* synthesis of purines through the inosine 5′-monophosphate dehydrogenase pathway or the activity of the mTOR and ERK signaling.[6,11,28] Ribavirin can also downregulate the epigenetic controller, EZH2 enzyme,[6] and the eukaryotic translation initiation factor eIF4E, leading to inhibition of methyl-7-guanosine mRNA cap binding and protein synthesis.[12,13] Ribavirin is the only approved drug with documented activity against eIF4E.[4,21,22] As eIF4E levels are known to be elevated in GBM,[15] we first assessed eIF4e expression in our cells after 72 h of ribavirin treatment. Interestingly, ribavirin did not affect total eIF4E expression. However, we observed that all glioma cells exhibited a significant decrease in eIF4E activation characterized by decreased phosphorylation at S209 (Figure 4a and Supplementary Figure S3A). In addition to eIF4E, we also looked at the AKT/mTOR and ERK pathways. Although we noticed a significant decrease in ERK phosphorylation, no significant differences were observed in AKT/mTOR expression or phosphorylation levels suggesting that ribavirin treatment does not affect these pathways in GBM cells (Figure 4a and Supplementary Figure S3A). Finally, evaluation of EZH2 and its partner, Snail, reveals that ribavirin decreases their expression in all tested glioma cells (Figure 4a and Supplementary Figure S3A), suggesting a possible role for EZH2 in ribavirin anti-cancer effects in human glioma cells.

Ribavirin significantly inhibits tumor growth *in vivo*

Inhibition of tumor growth *in vivo* by ribavirin has been demonstrated in several cancer types [12,22] but never in glioma. To investigate the anti-neoplastic effect of ribavirin in glioma *in vivo*, we orthotopically implanted rat 9L gliosarcoma tumors into the brains of immune-competent rats, and human GBM stem-like GB1A cells into the brains of immunodeficient mice. Previous *in vivo* studies conducted using ribavirin as well as toxicity studies performed on rats and mice (Supplementary Figures S4A and B) helped us to determine the ideal ribavirin dosage for both models. Subsequently, rats and mice were intraperitoneally injected daily with either vehicle (H_2O) or 10 and 100 mg/kg ribavirin, respectively throughout the experiment (Figure 5a). As shown in Figure 5 and Supplementary Figure S4, ribavirin significantly increased survival compared with vehicle-treated animals. More specifically, the median survival of control rats implanted with 9L tumors was 12 days, whereas the median survival of rats treated with ribavirin (10 mg/kg) was significantly extended to 15 days (Figure 5b and Supplementary Figure S4C). Similarly, median survival of vehicle-treated mice implanted with human GBM stem-like GB1A cells was 12 days but the median survival of mice treated with ribavirin (100 mg/kg) was extended to 19 days (Figure 5e and Supplementary Figure S4D). In addition, we harvested rat and mouse brains from control and treated groups at day 7 after the orthotopic graft to perform H&E staining and assess tumor size. Images and quantifications, shown in Figures 5c, d, f and g and Supplementary Figure S5, reveal that 9L and GB1A tumors were significantly smaller in the ribavirin-treated group compared with the respective controls at day 7, which potentially explains the subsequent extended median survival. These data demonstrate that ribavirin affects tumor growth in an *in vivo* setting.

Temozolomide and irradiation treatments are enhanced by ribavirin

TMZ and IR, with surgery when possible, are part of the standard treatment for patients with GBM.[18,19] On the basis of our findings, we investigated whether or not ribavirin could enhance the cytotoxic effects of TMZ and/or radiation *in vitro* and *in vivo*. We performed flow cytometry experiments, using Annexin-V/PI, to analyse the effects of ribavirin used in combination with TMZ and/or radiation on glioma cell death (Figure 6a and Supplementary Figure S6). U87, U251, LN18, 9L, GB1A and 1113 cells were treated with either TMZ (100 μM), Ribavirin (30 μM)+TMZ (100 μM), IR (5 Gy), Ribavirin (30 μM)+IR (5 Gy) or TMZ (100 μM)+IR (5 Gy)+Ribavirin (30 μM) for 48 h. As expected, treated cells show an increase in cell death compared with control vehicle-treated/non-irradiated cells (Figure 6a and Supplementary Figure S6). Interestingly, when we combined TMZ or radiation with ribavirin, we were able to enhance cell death compared with individual treatment alone in U87 (Rib+TMZ: 5.29% of Annexin-V+ cells vs TMZ: 4.25%; Rib+IR: 4.53% vs IR: 2.51%), U251 (Rib+TMZ: 19.05% vs TMZ: 11.51%; Rib +IR: 18.9% vs IR: 15.48%), 9L (Rib+TMZ: 18.27% vs TMZ: 8.12%; Rib +IR: 39.02% vs IR: 28.42%), GB1A (Rib+TMZ: 34.51% vs TMZ: 4.79%; Rib+IR: 48.2% vs IR: 10.31%) and 1113 cells (Rib+TMZ: 24.63% vs TMZ: 9.91%; Rib+IR: 37.8% vs IR: 12.2%) (Figure 6a and Supplementary Figure S6). Moreover, the combination of ribavirin with IR and TMZ (Rib+TMZ+IR) clearly induced more cell death than the respective single treatments or dual combination (Figure 6a and Supplementary Figure S6B). Finally and most importantly, we compared the efficacy of TMZ (50 mg/kg)/IR (10 Gy)/ribavirin (10 mg/kg) combination vs the standard TMZ (50 mg/kg)/IR (10 Gy) combination (Figure 6b) *in vivo* using our aggressive 9L model. As expected, ribavirin, IR or TMZ as single agents significantly increased survival compared with vehicle-treated animals (Figure 6c), but importantly, we noticed that ribavirin treatment significantly potentiated the effect of TMZ and IR extending the median survival from 25 days (TMZ/IR) to 29 days (TMZ/IR/Ribavirin, $P = 0.0151$) (Figure 6c). These results strongly suggest that the combination of TMZ or IR with ribavirin could potentially enhance the efficacy of these therapies.

DISCUSSION

The growing cost of medical care worldwide, particularly in oncology, has incentivized scientists and clinicians to repurpose drugs to alleviate the financial burden of drug development,[27,29,30] and recent evidence shows that ribavirin, an anti-viral drug, has an unexpected role in inhibiting tumor cell growth.[5,7–9,23,31] In our present study, we clearly demonstrate that ribavirin treatment leads to a significant decrease in all tested GBM cell growth (Figure 1, Supplementary Figure S1 and data not shown). Of note, glioma stem-like cells (GB1A and 1113) show an increased sensitivity to ribavirin with a stronger anti-proliferative effect

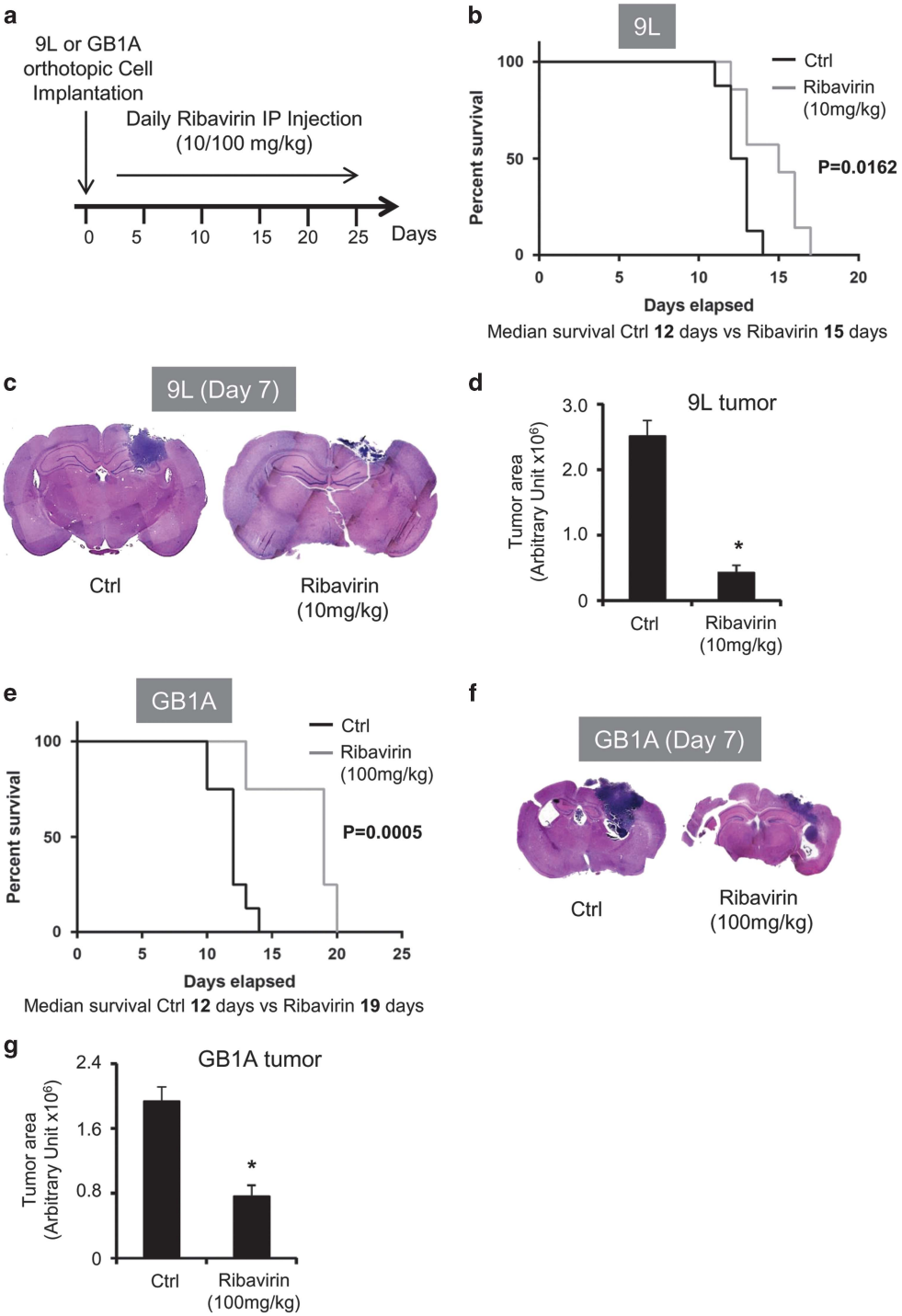

Figure 5. Ribavirin improves the survival of treated animals in two *in vivo* GBM rodent models. (**a**) After orthotopical 9L tumor or GB1A cell implantation, rats and mice were treated daily with IP injection of ribavirin 10 mg/kg and 100 mg/kg, respectively. (**b**) Kaplan–Meier survival curves following intracranial implantation of 9L tumors in rats and treatment with vehicle control (water, black curve, $n=8$) or ribavirin (10 mg/kg, gray curve, $n=8$). Ribavirin-treated animals exhibit a significant increased median survival (15 days) compared with controls (12 days) ($P=0.0162$). Data are representative of at least three independent experiments. (**c** and **d**) Representative photographs of sections of rat brain 7 days after 9L tumor implantation and ribavirin treatment (10 mg/kg) or not. H&E staining (**c**) and quantification of tumor area (**d**) reveals a smaller tumor size at day 7 after implantation in the ribavirin-treated group. (**e**) Kaplan–Meier survival curves following intracranial implantation of GB1A cells in immunodeficient Nude mice and treatment with vehicle control (water, black curve, $n=8$) or ribavirin (100 mg/kg, gray curve, $n=8$). Ribavirin-treated animals exhibit a significant increased median survival (19 days) compared with controls (12 days) ($P=0.0005$). Data are representative of at least three independent experiments. (**f** and **g**) Representative photographs of sections of mouse brain 7 days after GB1A cell implantation and ribavirin treatment (100 mg/kg) or not. H&E staining (**f**) and quantification of tumor area (**g**) reveals a smaller tumor size at day 7 after implantation in the ribavirin-treated group.

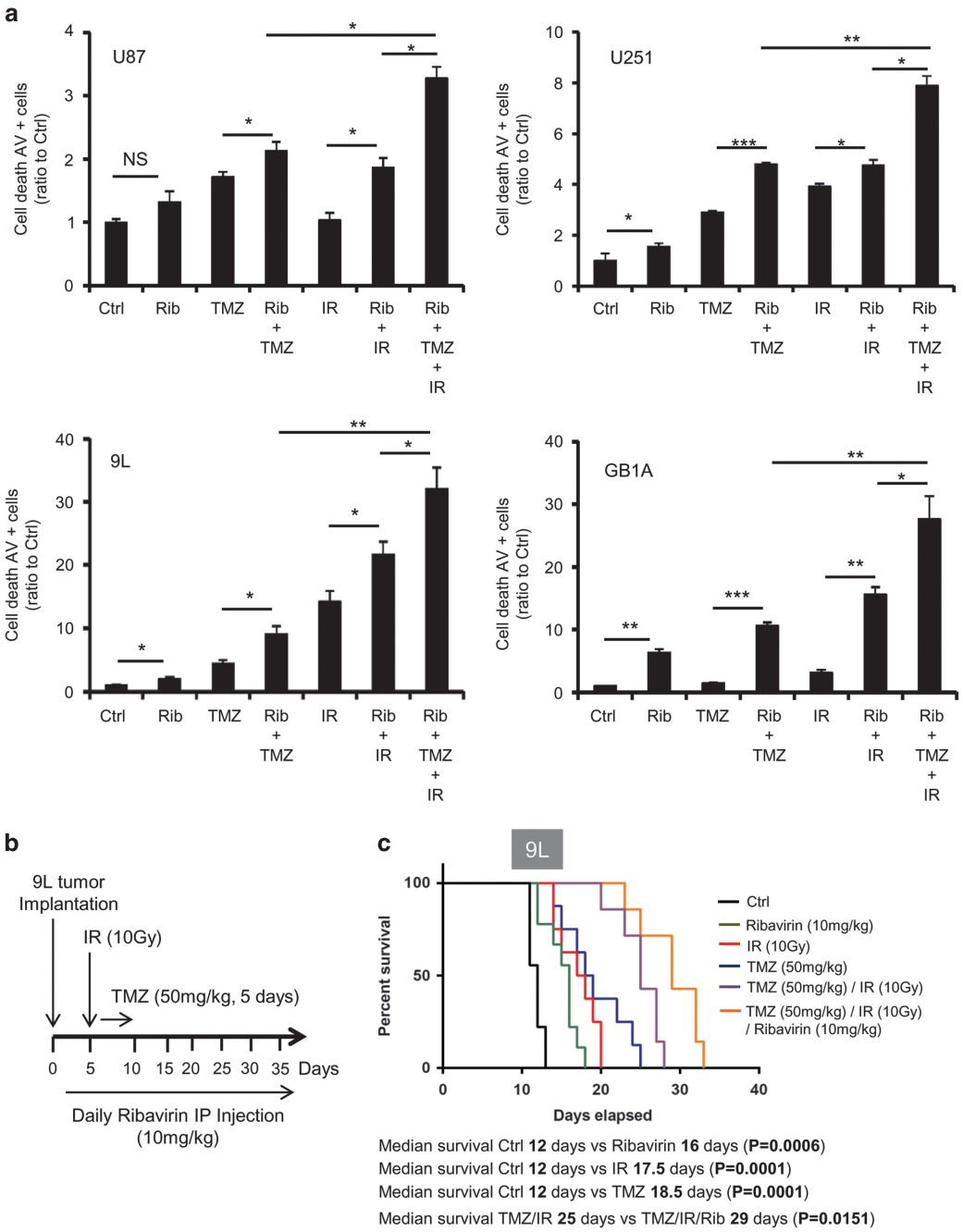

Figure 6. Ribavirin enhances the effect of TMZ and IR on GBM cells. (**a**) Quantification of cell death for U87, U251, 9L and GB1A cells, using flow cytometry and AV/PI staining, 48 h after ribavirin treatment (30 μM) and TMZ (100 μM) and/or IR (5 Gy). TMZ and/or IR combined with ribavirin present a stronger effect on cell death than TMZ or IR alone, particularly in U251 and 9L GBM cells (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, Ribavirin compared with control, $n = 3$). (**b**) After orthotopic 9L tumor implantation, rats were treated daily with IP injection of ribavirin 10 mg/kg. Five days after implantation, rats received one session of IR treatment (10 Gy) and oral TMZ for 5 days. (**c**) Kaplan–Meier survival curves following intracranial implantation of 9L tumors in rats and treatment with vehicle control (water, black curve, $n = 9$), ribavirin (10 mg/kg, green curve, $n = 9$), IR (10 Gy, red curve, $n = 8$), TMZ (50 mg/kg, blue curve, $n = 8$), TMZ and IR (TMZ 50 mg/kg, IR 10 Gy, purple curve, $n = 7$), TMZ and IR and ribavirin (TMZ 50 mg/kg, IR 10 Gy, ribavirin 10 mg/kg, orange curve, $n = 7$). Ribavirin-treated animals exhibit a significant increased median survival (16 days) compared with controls (12 days) ($P = 0.0006$). Animals treated with combination of ribavirin and IR and TMZ show a significant increase in median survival (29 days) compared with IR- and TMZ-treated animals (25 days) ($P = 0.0151$).

compared with the other cell lines. Interestingly, it is known that there is a positive correlation between Sox2, a master transcription factor involved in the proliferation and self-renewal of glioma-initiating cells, and the onco-protein, eIF4E, in glioma tissues.[32] Accordingly, it has been shown that eIF4E modulates Sox2 expression and its inhibition leads to decreased Sox2 protein levels, which could impact glioma stem-like cell proliferation and survival. Although further investigations are clearly needed, the eIF4E-Sox2 axis could potentially explain the higher sensitivity of ribavirin in our human GBM stem-like cells. In addition, Ribavirin treatment reduces the number of GBM cells undergoing cell division and increases cell cycle arrest, reflected by the increase in

Ki67-negative cells. These data confirm findings from a previous study, which showed that a melanoma cell line treated with ribavirin arrested in the G_0/G_1 phase.[12,33]

The molecular mechanisms underlying the anti-cancer activity of ribavirin are as of yet, not clearly understood, particularly in glioma. Only one study reported that ribavirin might be effective against GBM cell growth. In this study, the authors show that ribavirin inhibits U87 glioma cell survival and correlate the mRNA expression of *Pdgfr-A* with U87 cell sensitivity to ribavirin, suggesting that PDGFR-A may potentially have a role in the anti-tumor efficacy of ribavirin.[23]

In the present study, we demonstrate in glioma and glioma stem-like cells that ribavirin efficiently decreases eIF4E phosphorylation on serine 209 (S209). Furthermore, it has been shown in GBM that inhibition of eIF4E phosphorylation and modulation of its association with eIF4E-binding protein 1 led to inhibition of protein synthesis, consequently leading to inhibition of glioma cell proliferation *in vitro* and tumor growth *in vivo* in an orthotopic GBM mouse model.[34] In addition, it has been demonstrated that eIF4E phosphorylation is required for translational control of numerous pro-tumorigenic functions, such as remodeling of the extracellular matrix (matrix metalloproteinase, MMP), inhibition of apoptosis (baculoviral IAP repeat-containing protein 2) and cell proliferation (vascular endothelial growth factor).[35] Thus, ribavirin-dependent modulation of eIF4E phosphorylation likely influences GBM cell growth and death processes as well as invasive properties and therefore these established mechanisms in part likely explain the effect we observed in glioma cells in response to ribavirin treatment.[36,37]

Since eIF4E phosphorylation is also regulated by the MEK/ERK pathway,[11] we investigated ERK levels and our data show that ribavirin significantly reduces ERK phosphorylation, suggesting that this pathway is modulated by ribavirin. Interestingly, it is known that ERK inhibition leads to anti-glioma effects,[38,39] and more recently, Chen *et al.*[40] demonstrated that expression of PDGFR-A on GBM cell surface was negatively regulated in an ERK-dependent fashion, ultimately impacting glioma cell proliferation and survival. Although these observations could be related to the expression and role of PDGFR-A in the anti-GBM efficacy of ribavirin as mentioned above,[23] further investigation is required to assess how ribavirin-induced ERK modulation regulates PDGFR-A and the anti-tumor efficacy of ribavirin.

Prior studies have also shown ribavirin to be an epigenetic regulator by downregulating EZH2 enzyme activity and H3K27 histone methylation.[6] Recent evidence suggests that EZH2 inhibition may alter cell cycle progression and cell survival, as well as induce radio-sensitivity in GBM.[41–45] Thus, it is possible that ribavirin-mediated loss of EZH2 expression promotes cell death in glioma cells. Our data ultimately suggest that ribavirin may have potential as a therapeutic agent in the treatment of malignant gliomas through the modulation of several critical pathways, including eIF4E, ERK and EZH2 (Figure 7a). However, it is clear that additional studies are needed to determine precisely the direct or indirect mechanisms of action of ribavirin on these pathways and the contribution of each pathway to the cellular phenotypes we observed.

GBMs are very invasive tumors and inhibiting these invasive capacities is particularly important. Ribavirin decreases migration and invasion of mammary tumor cells through reduced level of MMP-3 and MMP-9,[22] therefore we assessed whether ribavirin could reduce GBM cell migration and adhesion. Surprisingly, we observed that ribavirin treatment significantly decreased migration/adhesion properties of human and rat glioma cells, which may be associated with the observed concomitant decrease in Snail expression. This modulation of Snail expression may explain ribavirin's anti-migratory/adhesive activity as it has been reported that Snail promotes epithelial to mesenchymal transition in GBM and impacts GBM cell invasiveness.[46,47] Specifically, it has been

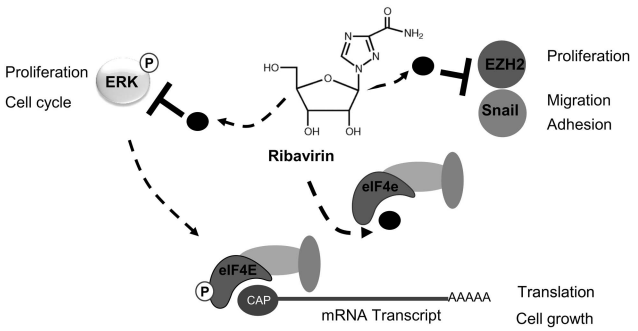

Figure 7. Ribavirin, an anti-GBM agent. Schematic representing the potential mechanisms involved in GBM cells in response to ribavirin treatment. Ribavirin could regulate glioma cell proliferation, cell cycle, cell death, migration and adhesion properties through modulation of eIF4E, EZH2 and ERK pathways.

shown that Snail knockdown can suppress migration and invasion of KNS42, U87 and U373 cells and that Snail inhibition also suppresses proliferation and viability of these GBM cells.[46] Our data suggest that Snail inhibition may be partly responsible for reducing the migratory behavior of glioma cells following ribavirin treatment, but it is likely that modulation of additional factors also has a role.

Our *in vitro* data combined with the fact that ribavirin is known to cross the blood-brain barrier [48,49] led us to assess the efficiency of ribavirin *in vivo* in rat and mouse models of GBM. We clearly demonstrate that daily ribavirin treatment reduces both 9L and GB1A tumor growth, significantly extending animal survival (Figure 5). Furthermore, pharmacokinetic and dosing studies in mice, rats and humans are well documented,[50–53] and our data (Supplementary Figures S4A and B) as well as recent studies using ribavirin in breast cancer [22] helped us to determine that 10 mg/kg/day of ribavirin in rats and 100 mg/kg/day in mice were appropriate doses. These doses did not induce any toxicity and should be easily achievable in humans by the prescribed clinical oral administration of ribavirin, which patients with hepatitis C tolerate well at 800–1500 mg/day. Indeed, in a Phase1 clinical trial for patients with AML, ribavirin was used in combination with cytarabine at a dosage as high as 2200 mg/day suggesting that ribavirin could potentially be used at higher dosage than 800–1500 mg/day.[7] In addition, ribavirin treatment, especially for hepatitis C patients, is a long-term therapy running from 24 to 48 weeks. In a cancer treatment setting, ribavirin could be used for short time periods but at higher concentrations, which might maximize its effect on tumor cells.

A previous study reported that ribavirin enhances human breast adenocarcinoma cell (MDA-MB-231) radio-sensitivity, supporting the use of ribavirin in combination with radiotherapy as a valid strategy for breast cancer patients.[54] However, thus far no study has highlighted the efficacy of ribavirin and its use in combination with radio/chemotherapy as an anti-glioma agent. With this idea in mind, we determined the effect of ribavirin in combination with the standard treatment for GBM, TMZ and IR, and we observed that combining ribavirin with TMZ and radiotherapy could potentially enhance the efficacy of these therapies *in vitro* and most importantly *in vivo* (Figure 6), reinforcing the potential benefits of using ribavirin for patients with high-grade glioma. An important determinant of chemotherapy failure and resistance to TMZ therapy for GBM patient is the presence of the O-6-methylguanine-DNA methyltransferase (MGMT) enzyme, which removes the alkyl groups formed by the TMZ and allows DNA damage repair. A high degree of unmethylated *MGMT* promoter, resulting in MGMT expression, is associated with resistance to TMZ and poor patient outcome. Interestingly, in 2014, Zekri *et al.*

evaluated the impact of *MGMT* promoter methylation in 53 patients with hepatitis C virus induced hepatocellular carcinoma (HCC) treated with interferon and ribavirin (ClinicalTrials.gov, NCT01758939).[55] Their findings highlight that patients with HCC possessing an unmethylated *MGMT* promoter had better responses to therapy and suggest that ribavirin may be useful in the treatment of resistant GBM.

In conclusion, we provide evidence that the FDA-approved drug ribavirin may be effective against GBM. The clinical efficacy of ribavirin against cancer is currently being investigated in the treatment of AML, oropharyngeal squamous cell carcinoma (ClinicalTrials.gov, NCT01721525) and metastatic breast cancer (ClinicalTrials.gov, NCT01056757). The published phase1 clinical trial of ribavirin used with low-dose cytarabine (cytosine analog) in treating AML was promising despite reductions in plasma levels of ribavirin possibly due to impaired absorption in the presence of cytarabine.[7] Given the encouraging results in both clinical and laboratory settings and the need for new GBM treatments, our *in vitro* and *in vivo* pre-clinical results could represent valuable translational evidence for evaluating ribavirin as a therapeutic agent in a clinical trial for patients with aggressive GBM.

MATERIALS AND METHODS

Cell lines and culture conditions

Human GBM cell lines, U87 (ATCC HTB-14), T98G (ATCC CRL-1690) (obtained from the American Type Culture Collection, ATCC, Manassas, VA, USA), U251, LN18 and SF767 (obtained from C. Simon's laboratory, UPENN, Philadelphia, PA, USA) and rat glioma cell lines, C6 (ATCC CCL-107, obtained from ATCC), F98 (obtained from R. Barth laboratory, Ohio State University, Columbus, OH, USA), 9L gliosarcoma (obtained from the Brain Tumor Research Center, UCSF, CA, USA) were used and routinely maintained in Dulbecco's Modified Eagle Medium (Lonza, Portsmouth, NH, USA) supplemented with 10% fetal calf serum (Lonza) at 37C° in 5% CO_2-humidified incubators as previously described [27,56] and monthly tested for mycoplasma contamination using Look Out Kit (Sigma-Aldrich, St Louis, MO, USA). Human primary neurosphere culture, 0913 (GB1A), was originally derived by Vescovi *et al.* and cultured as previously described.[57,58] Human primary brain tumor stem-like cell line, JHU-1113 (1113), was generated within the department of Neurosurgery of the JHU (Baltimore, MD, USA) within compliance of JHU regulations and profiled by whole genome sequencing as part of GBM genome project, and were grown in NeuroCult NS-A basal medium containing NeuroCult NS-A proliferation supplements (Stem Cell Technologies, Vancouver, Canada). When indicated, cells were treated or not with the following compounds: phosphate buffered saline (PBS) or water as vehicle, 30 and 50 μM ribavirin (1-β-D-ribofuranosy-1,2,4-triazole-3-carboxamide, Sigma-Aldrich), 100 μM TMZ (Temodar, Schering-Plough, a subsidiary of Merck & Co., Inc., NJ, USA), and irradiated or not at 5 Gy using the Gammacell 40 Exactor irradiator (Nordion, Ottawa, ON, Canada).

Cell growth-viability assay

Glioma cells (2.5×10^4) were treated or not with 30–50 μM ribavirin (Sigma-Aldrich) or PBS (Lonza) as vehicle. Cells were then collected and counted using Malassez slide (Invitrogen, Carlsbad, CA, USA) as previously described.[27]

Flow cytometry

72 or 48 h after treatment with ribavirin (30 μM), TMZ (100 μM) and/or ionizing radiations (5 Gy), cells were collected, washed in PBS and prepared for flow cytometry.[59] For cell death, we used reagents from the APC-Annexin-V/Dead Cell Apoptosis Kit (Invitrogen). For cell cycle analysis, cells were labeled with FITC-anti-Ki67 antibody (Abcam, Cambridge, MA, USA) and run on FACS Calibur (Becton-Dickinson, Franklin Lakes, NJ, USA). Subsequent analyses were performed using FlowJo software (FlowJo LLC, Ashland, OR, USA).

Migration/adhesion assay

Migration and Adhesion assays were performed as described.[60,61] For the adhesion assay, ribavirin-pre-treated (48 h) and control cells, were seeded into 24-well plates coated or not with Collagen I (1 μg/ml, Chemicon Millipore, Billerica, MA, USA), gelatin (2 mg/ml, Sigma-Aldrich) or laminin (10 μg/ml, Sigma-Aldrich) and incubated for 1 h at 37 °C. Cells were then carefully washed 3 times with PBS, fixed and counted. The number of remaining adherent cells was counted in at least four different wells for each condition using a Zeiss Observer Z.1 AX10 microscope (Zeiss, Thornwood, NY, USA) and and ImageJ software (Windows 1.47, Research Services Branch, NIH, Bethesda, MD, USA). The data are representative of three independent experiments.

Western blot analysis

Cells treated or not with ribavirin (30 μM), were lysed and western blots were performed as previously described [27,56] using monoclonal rabbit antibodies, anti-phospho (S209) eIF4E (1/1000, #9742, Cell Signaling, Beverly, MA, USA), anti-eIF4E (1/1000, #9741, Cell Signaling), anti-EZH2 (1/1000, #ab3748, Abcam), anti-Snail (1/1000, #ab180714, Abcam), anti-phospho (T202/Y204) ERK (1/1000, #4370, Cell Signaling), anti-ERK (1/1000, #9102, Cell Signaling), anti-phospho (S2448) mTOR (1/1000, #2971, Cell Signaling), anti-mTOR (1/1000, #2972, Cell Signaling), anti-phospho (S473) AKT (1/1000, #9271, Cell Signaling), anti-AKT (1/1000, #9272, Cell Signaling), and were normalized using a rabbit polyclonal antibody anti-β-tubulin (1/1000, #2146, Cell Signaling). Gel quantification was performed using ImageJ software.

Orthotopic xenograft experiments

Female rats (F344) and mice (NU/NU athymic) were purchased from Charles River (Cambridge, MA, USA) and intracranially implanted with 9L gliosarcoma tumors or 5×10^5 GB1A cells as previously described.[62,63] Animals were given intraperitoneal injections of pharmaceutical grade anesthetic, analgesia, and study agents (water, ribavirin, rats: 10 mg/kg/day, mice: 100 mg/kg/day, TMZ: 50 mg/kg, IR: 10 Gy). All procedures were performed in accordance with the guidelines set forth by the Johns Hopkins University Animal Care and Use Committee.

Statistical analysis

Unpaired *t*-test was used to calculate final *P*-values. Data are representative of at least three independent experiments and significance is represented by * in which $*P < 0.05$, $**P < 0.01$, $***P < 0.001$. For the Kaplan–Meier survival analysis, we compared vehicle-treated and ribavirin-treated groups using the log rank test.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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