ANTICANCER RESEARCH *44*: 1829-1835 (2024)
doi:10.21873/anticanres.16984

Riluzole Reverses a Number of Undesirable Effects of Dexamethasone in Glioblastoma Cells

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Abstract. *Background/Aim: Glioblastoma multiforme (GBM)-induced oedema is a major cause of morbidity and mortality among patients with GBM. Dexamethasone (Dex) is the most common corticosteroid used pre-operatively to control cerebral oedema in patients with GBM. Dex is associated with many side effects, and shorter overall survival and progression-free survival of patients with GBM. These negative effects of Dex highlight the need for combinational therapy. Riluzole (Ril), a drug used to treat amyotrophic lateral sclerosis (ALS), is thought to have potential as a treatment for various cancers, with clinical trials underway. Here, we investigated whether Ril could reverse some of the undesirable effects of Dex. Materials and Methods: The effect of Dex, Ril, and Ril-Dex treatment on cell migration was monitored using the xCELLigence system. Cell viability assays were performed using 3-(4, 5-dimethylthiazol)-2, 5-diphenyltetrazolium bromide (MTT). The expression of genes involved in migration, glucose metabolism, and stemness was examined using real-time polymerase chain reaction (PCR). Results: Pre-treating GBM cells with Ril reduced Dex-induced cell migration and altered Dex-induced effects on cell invasion, stem cell, and glucose metabolism markers. Furthermore, Ril remained effective in killing GBM cells in combination with Dex. Conclusion: Ril, which acts as an anti-tumorigenic drug, mediates some of the negative effects of Dex; therefore, it could be a potential drug to manage the side effects of Dex therapy in GBM.*

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Key Words: Glioblastoma cells, dexamethasone, riluzole, migration, cell death.

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Among brain tumours, glioblastoma multiforme (GBM) is the most common and most aggressive. Standard therapy consists of surgical resection, radiotherapy and chemotherapy. Cerebral oedema is a major cause of morbidity and mortality in GBM due to the high risk of brain herniation [in up to 60% of GBM patients, reviewed in (1)]. Corticosteroids are commonly used pre-operatively to control cerebral oedema and post-operatively to combat side effects. Dexamethasone (Dex) is the preferred corticosteroid due to its ability to decrease the permeability of the blood-brain barrier. Additional benefits of Dex use in patients with cancer are its ability to control tumor-associated pain, nausea and vomiting and to improve appetite (2). Unfortunately, besides the positive effects of Dex, many side effects, such as abnormal glucose metabolism, gastrointestinal complications, insomnia, and anxiety, have been reported (3). The use of Dex is also associated with shorter overall survival and progression-free survival of patients with GBM (4).

Important correlations between Dex treatment and alterations in gene expression profiles have been identified (5). In a study that included patients with mesenchymal and proneural GBM, Dex-controlled gene network and pathways closely related to proliferation, invasion and angiogenesis were significantly up-regulated in the mesenchymal group. Dex was also shown to increase the invasion and proliferation of cells derived from patients with GBM, highlighting that Dex can increase tumor aggressiveness (5). Furthermore, Dex promoted a glioma stem cell-like phenotype and resistance to chemotherapy in primary tumor cells from human glioblastoma (6). Therefore, the use of Dex in GBM therapy has been questioned. These negative effects of Dex highlight the need for combinational therapy.

Riluzole (Ril) is an approved drug for amyotrophic lateral sclerosis (ALS) (7). Previous research has demonstrated various positive effects of Ril on GBM. For example, in a GBM model using the U87 cell line, Yelskaya *et al.* (8) showed that Ril inhibited cell proliferation by blocking glutamate release. Previously, our lab showed that Ril decreased glucose transporter 3 (GLUT3, gene: *SLC2A3*) in glioblastoma stem-like cells (9). We also showed that Ril was effective in killing brain tumor stem-like cells *in vitro* and inhibiting tumor growth

in vivo. Considering that Dex treatment of GBM cells promotes migration/invasion and a stem-like phenotype and that Ril influences GBM stem-like cells, we investigated whether pre-treatment of GBM cells with Ril would eliminate/reverse some of the negative effects of Dex.

Materials and Methods

Materials and cell culture. This study used the U87MG (RRID: CVCL_0022) (ATTC, Manassas, VI, USA) GBM cell line, which have been authenticated using STR profiling within the last three years. All experiments were performed with mycoplasma-free cells. Cell culture media, fetal bovine sera and other supplements were purchased from Gibco Life Technologies (Carlsbad, CA, USA). Dex was purchased from Sigma-Aldrich (St. Louis, MO, USA). Ril was purchased from TOCRIS (Bristol, UK), and temozolomide (TMZ) was purchased from MSD Sharp & Dohme GmbH (Haar, Germany). Ril and Dex were dissolved in double-distilled water. TMZ was dissolved in dimethyl sulfoxide (DMSO) (Sigma-Aldrich). The cells were grown in the presence of penicillin/streptomycin (Thermo Fisher Scientific, Waltham, MA, USA).

Experimental setup. The experiments in this study were designed as follows: U87MG cells were pretreated with 25 μM Ril for 72 h in all Ril groups. Simultaneously, all control (Co) and single Dex treatment groups received vehicle fetal bovine serum (FBS)-free medium (Figure 1). After 72 h, the Co group continued to receive FBS-free medium, the Ril groups received an additional 25 μM Ril, the Dex group received 10 μM Dex, and the Ril+Dex group received 25 μM Ril and 10 μM Dex. In addition, for cell viability analysis, TMZ or irradiation was added to the treatment described above to simulate standard of care therapy. For the radiation treatment, Co, Ril, Dex and Ril+Dex groups were irradiated with 10 Gy on day 5 (96 h). To simulate chemotherapy, 100 μM TMZ was added to the corresponding treatment on day 5 (96 h). In the treatment with TMZ groups the vehicle was DMSO, therefore all groups had DMSO added and the groups were described as follows: Co_DMSO, Ril_DMSO, Dex_DMSO, TMZ (dissolved in DMSO), Ril+TMZ, Dex+TMZ and Ril+Dex_DMSO. For cell death and gene expression analysis, cells were harvested after a further 72 h (144 h in total). For the migration assay, cells were monitored/recorded over a 6 h timeframe (72 h pre-treatment + 6 h co-treatment). A schematic representation of the experimental setup is shown in Figure 1.

Cell viability assay. Cell viability assays were performed using 3-(4, 5-dimethylthiazol)-2, 5-diphenyltetrazolium bromide (MTT) (Sigma-Aldrich). The cells were seeded in 96-well plates at a final density of 10×10^3 cells/well. The cells were given 24 h to attach and then treated, as presented in Figure 1, with Dex (10 μM) or Ril (25 μM) only or a combination of Dex (10 μM) and Ril (25 μM). As a control treatment (Co), cells received FBS-free medium. The drug concentrations were within a wide range previously described for *in vitro* studies (10, 11). The drugs were diluted in 100 μl of FBS-free medium. Cell viability was also tested after standard treatment with 100 μM TMZ or/and radiation with one dose of 10 Gy on day 5 (96 h) as described in the Experimental setup. After 144 h, MTT was added, and the cell cultures were incubated for a further 4 h at 37°C. The sample absorption was measured using a spectrophotometer reader (Bio-TEK, Winooski, VT, USA) at 562 nm wavelength.

RNA purification, reverse transcription and real-time polymerase chain reaction (PCR) for the analysis of gene expression. Total cellular RNA was isolated using an RNeasy kit (Qiagen, Hilden, Germany) and reverse-transcribed into cDNA using a SuperScript III first-strand synthesis kit (Life Technologies, Waltham, MA, USA). For real-time PCR amplification, cDNA (100 ng) was used. Real-time PCR was performed on a BioRAD CFX384 cycler using iTaq universal SYBR-Green Mastermix (Bio-rad, Hercules, CA, USA) and gene-specific primers for the following: CDH2 (N-cadherin) forward: 5' CTCCATGTGCCGGATAGC 3', 5' reverse: CGATTTCACCAGAAG CCTCTAC 3'; tissue factor pathway inhibitor-2 (TFPi2) forward: 5' GTCGATTCTGCTGCTTTTCC 3', reverse: 5' CAGCTCTGCGTGT ACCTGTC 3'; cluster of differentiation 90 (CD90) forward: 5' CACCCTCTCCGCACACCT 3', reverse: 5' CCCCACCATCCCA CTACC 3'; SLC2A1 (glucose transporter 1, GLUT1), forward: 5' ATCGTGGCCATCTTTGGCTTTGTG 3', reverse: 5' CTGGAAGC ACATGCCCACAATGAA 3'; SLC2A3 (glucose transporter 3, GLUT3) forward: 5' AGCTCTCTGGGATCAATGCTGTGT 3', reverse: 5' ATGGTGGCATAGATGGGCTCTTGA 3'; S100 calcium binding protein A10 (S100A10), forward: 5' AACAAAGG AGGACCTGAGAGTAC 3', reverse: 5' CTTTGCCAT CTCTAC ACTGGTCC 3'; and β-actin forward: 5' TCCTTCCTGGGCAT GGAG 3', reverse: 5' AGGAGGAGCAATGATCTTGATCTT 3'.

Migration assay. The rate of cell migration was monitored in real-time using the xCELLigence system (ACEA Biosciences Inc., San Diego, CA, USA) with CIM plates. Cells were pre-treated with 25 μM Ril for 72 h. After 72 h, 50,000 of these cells were seeded in each well of the upper chamber of the CIM plate in serum-free media, including the drugs and combinations thereof. The lower chamber of the same CIM plate contained culture medium, including 5% FBS (which acts as a chemoattractant) and the corresponding treatment drugs. The electrode impedance value of each well was automatically monitored by the xCELLigence system (ACEA Biosciences Inc.) for 6 h and expressed as the cell index.

Statistical analysis. All cell migration, RT-PCR, and MTT analysis were performed at least thrice. Statistically significant differences were analyzed using two-sided *t*-tests for two-group comparisons. Calculations were performed using the statistics software GraphPad Prism 5 (GraphPad Software, La Jolla, CA, USA). A probability of $p<0.05$ was considered statistically significant.

Results

Ril decreased the Dex-induced increase in cell migration. Reports on the involvement of Dex in the migration of GBM cells are conflicting (5, 12). In our previous study, we showed the effect of Ril on U87MG GBM cells (11), where the strong inhibitory effect of Ril on these cells was shown after 72 h. Therefore, in this study, we pre-treated the cells with Ril (Ril and Ril/Dex groups) or vehicle [control (Co) and Dex groups] for 72 h. After 72 h pretreatment, the respective groups were further treated with either vehicle (Co group), Ril (Ril and Ril+Dex groups) or/and Dex (Dex and Ril+Dex groups). Migration was monitored at 6 h (Figure 1A). Dex alone significantly increased migration, whereas Ril alone had no effect on the migration of these cells

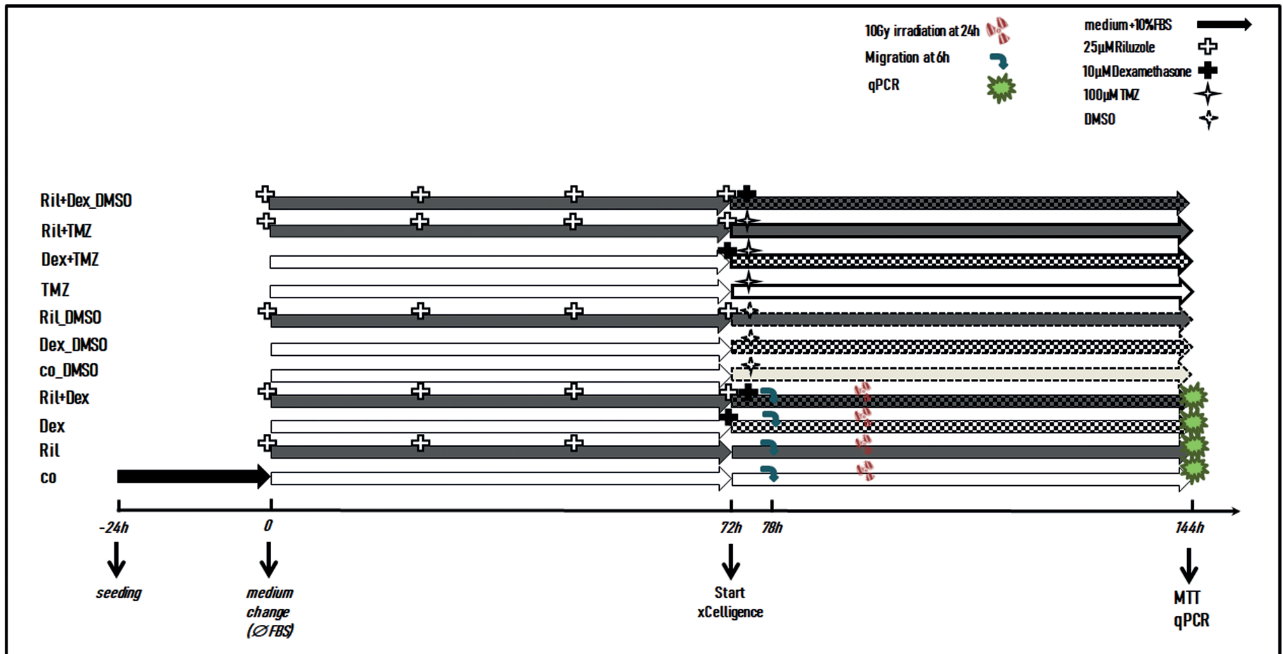

Figure 1. *Schematic representation of the experimental setup used to analyze migration, gene expression and cell viability.*

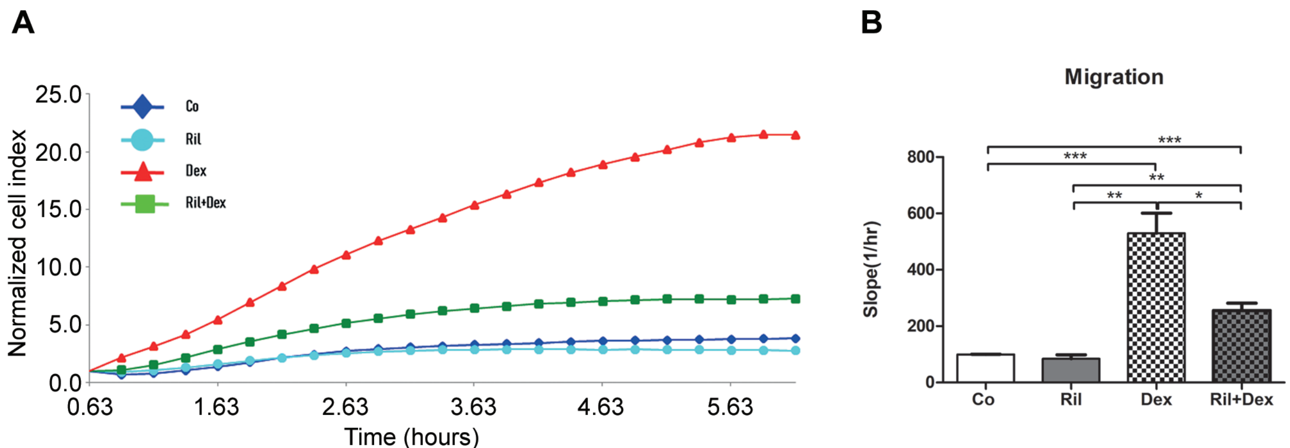

Figure 2. *Cell migration analysis, (A) a representative image of real-time monitoring of cell migration in single and riluzole (Ril)-Dex co-treated cells. The rate of migration of the cells treated with Dexamethasone (Dex), Ril, and Ril+Dex was monitored using the xCELLigence system, analyzing the slope (B) of the line between 1- and 6- h intervals. The bars depict the mean±SEM from three independent experiments. Significantly different at: *p<0.05, **p<0.01, and ***p<0.001.*

(Figure 2A and B). Co-administration of Ril and Dex significantly reduced cell migration (Figure 2A and B).

Dex induced changes in gene expression and attenuation of these changes by Ril *co-administration*. Dex drives changes in gene expression in GBM cells (6). We analyzed the expression of *TFPi2*, *CDH2* (N- Cadherin), CD90, *SLC2A1* (GLUT1), *SLC2A3* (GLUT3) and *S100A10* genes involved in the migration, glucose metabolism, and stemness of GBM cells. Treatment groups were as follows: 1) Ril, only Ril treatment, 2) Dex, only Dex and 3) Ril+Dex, co-administration of Ril and Dex, as previously described. The single treatment with Dex had no effect on the expression of TFPi2 mRNA (Figure 3A). However, we detected a significant

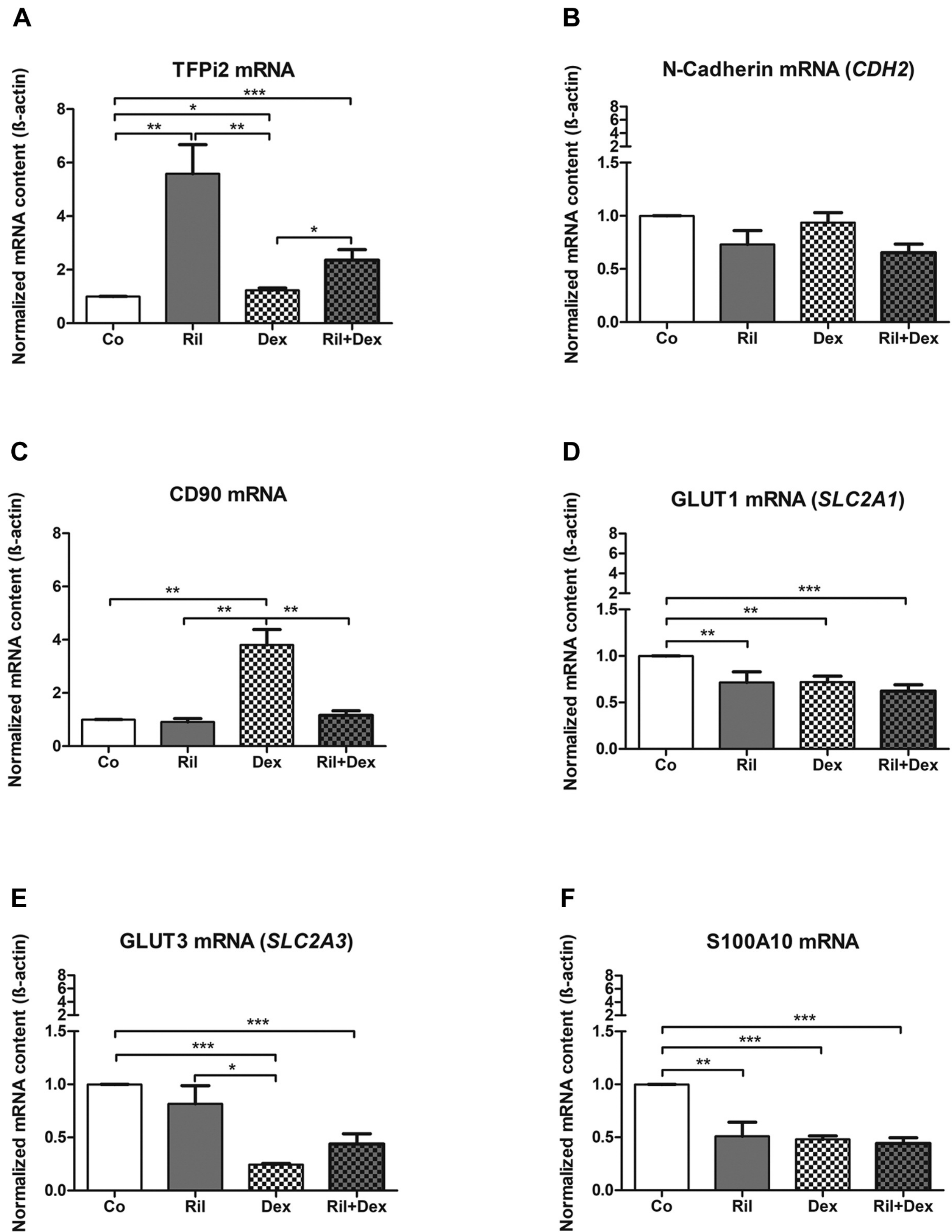

Figure 3. *RT-PCR analysis of the U87MG cells treated with Riluzole (Ril), Dexamethasone (Dex) and Ril-Dex co-treatment. Expression analysis of following mRNA (A) TFPi2, (B) N- Cadherin (CDH2), (C) CD90, (D) GLUT1 (SLC2A1), (E) GLUT3 (SLC2A3), and (F) S100A10 in U87MG GBM cells after 144 h.*

increase in the expression level of TFPi2 mRNA in Ril alone and Ril+Dex groups. Gene expression analysis revealed no significant changes in the expression of N-cadherin mRNA, in Dex, Ril or co-treatment groups (Figure 3B). Furthermore, Dex significantly increased the expression of CD90 mRNA, a stem cell and neural to mesenchymal transition marker, whereas the co-treatment with Ril significantly reversed this effect (Figure 3C). Finally, the single Dex treatment significantly reduced the expression of genes involved in glucose metabolism [*SLC2A1* (GLUT1), *SLC2A3* (GLUT3) and *S100A10*]. The positive effect of Dex on the expression of glucose metabolism genes remained even after it was combined with Ril (Figure 3D-F). Ril single treatment significantly reduced GLUT1 and S100A10 gene expression.

Dex does not interfere with Ril-*induced GBM cell death*. As pre-treatment with Ril had a positive effect, reversing the negative effects of Dex on cell migration and gene expression, we investigated whether Ril would still influence cell viability (11) after co-application with Dex. Compared to the control, Ril alone had a significant effect on cell death whereas Dex did not (Figure 4A). The effect of Ril on cell viability remained significant after co-application of Dex compared to control and Dex alone (Figure 4A) confirming that Dex did not interfere with Ril -induced GBM cell death. There was no additional effect on cell viability of any treatment, neither after 10 Gy irradiation (Figure 4B) nor under TMZ treatment (Figure 4C).

Discussion

Peri-tumoral oedema associated with GBM influences the clinical course and prognosis of GBM (13). Dex is the preferred steroid used to reduce brain tumor oedema (14) and post-operative side effects (2). However, side effects of Dex, including promoting GBM aggressiveness, have emerged from a number of clinical studies (3) and *in vitro* and *in vivo* studies (5, 6), with these studies reporting that Dex facilitates C6 cell migration (15), significantly increases the invasiveness of GBM stem-like cells and promotes proliferation and angiogenesis *in vivo* (5). However, inhibitory effects of Dex on migration of glioma cell lines have also been reported (12).

In this study, we investigated whether Ril would reduce some of the negative effects of Dex in cell culture experiments. Our results showed that Dex increased the migration of U87MG cells and that Ril co-treatment decreased the migration of these cells (Figure 2B). Ril treatment alone did not have an effect on cell migration (11). To clarify the mechanism underlying the beneficial effect of Ril on Dex-induced increased cell migration, we conducted expression analysis of two genes known for their roles in GBM migration/invasion. As reported previously, TFPi2 down-regulation is associated with a poor prognosis in GBM

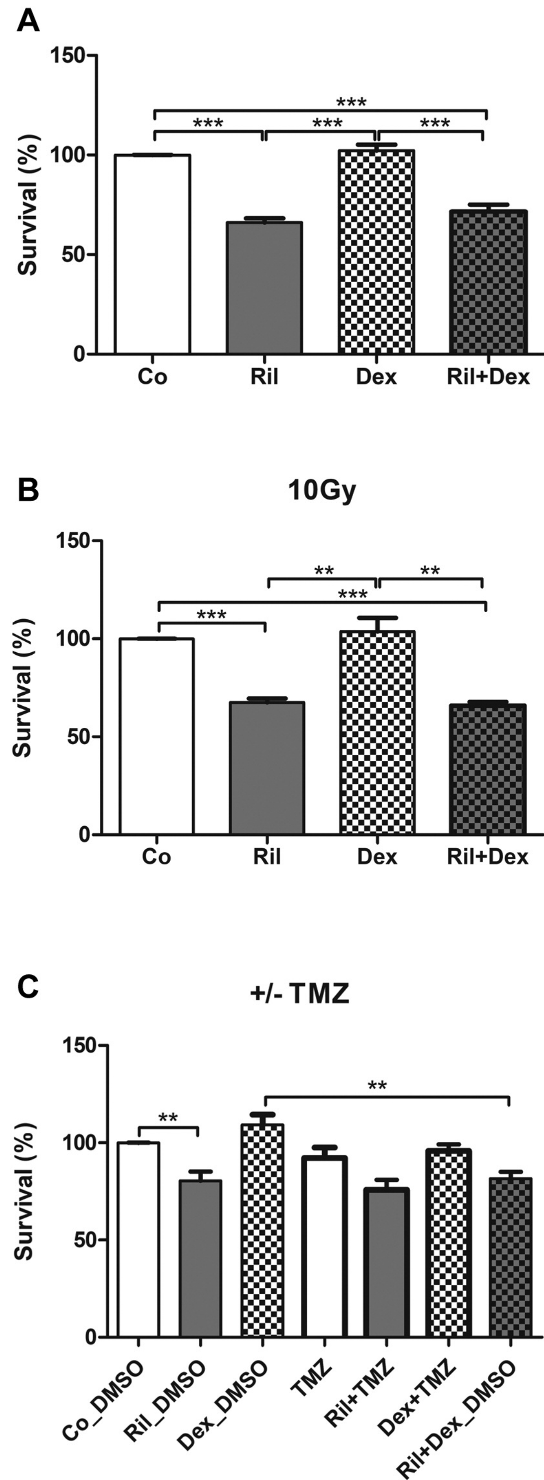

Figure 4. *Dex did not influence the death of glioblastoma (GBM) cells. (A) Riluzole (Ril) significantly increased GBM cell death, and Dexamethasone (Dex) did not interfere with GMB cell death induced by Ril. (B) The effect of Co, Ril, Dex and Ril-Dex after radiotherapy or (C) after chemotherapy (where all groups contained DMSO since TMZ is dissolved in DMSO). The groups were described as follows: Co_DMSO, Ril_DMSO, Dex_DMSO, TMZ, Ril+TMZ, Dex+TMZ and Ril+Dex_DMSO.*

(16), and knockdown of this gene in GBM leads to an increase in cell migration/invasion (17). Previously, we showed that Ril can significantly increase the expression of this gene (11). In this study, co-administration of Ril and Dex had positive effects on gene expression related to migration/invasion in GBM cells. Changes in TFPi2 mRNA expression are probably not the reason for the decrease in migration, as Dex alone causes up-regulation of this gene. N-cadherin is expressed in glioma cells (18). The Ril -Dex treatment had no effect on N-Cadherin mRNA expression. Thus, the positive effect of co-administration of Dex and Ril on cell migration is likely not attributed to changes in TFPi2 and N-cadherin mRNA expression.

Aberrant changes in GLUT1, GLUT3, and S100A10 have been found in glioblastoma, as well as in several other forms of cancer, as reviewed previously (19). In this study, Dex had a positive effect when administered alone and this remained even after when co-administered with Ril. By significantly reducing the expression of GLUT1, GLUT3 and S100A10 mRNA, the treatment had positive effect by possibly reducing glucose metabolism in GBM cells (Figure 3).

As reported previously, Dex treatment of GBM cells promotes the development of a GBM stem cell phenotype and confers resistance to physiological stress and chemotherapy (6). In this study, Dex treatment significantly increased expression level CD90 mRNA (Figure 3C), which was previously identified as a marker for cancer stem cells in high-grade gliomas (20) and as having high tumorigenic and metastatic potential in esophageal squamous cell carcinoma (21). In our study, the pre-treatment with Ril significantly decreased the expression level of CD90 mRNA (Figure 3C). To elucidate the effects of drugs (single or co-administered) on specific pathways, knockdown of relevant genes could be performed, followed by an analysis of their influence on these pathways. The results could then be compared with drug treatment effects to confirm the influence of specific genes on particular pathways.

The effects of Ril on GBM cell death and GBM stem-like cells have previously been described (9, 11). As Dex did not interfere with GBM cell death induced by Ril (Figure 4), combined treatment with Ril and Dex could be beneficial for patients receiving Dex treatment for GBM oedema. Although previous research reported that Dex protects GBM cells from TMZ-induced apoptosis (22) and irradiation-induced autophagy (23), we detected no such negative effect of Dex in this study. This variable effect of Dex is potentially due to different experimental settings or cell lines.

Ril attenuates cytotoxic brain oedema, as assessed by T2-weighted magnetic resonance imaging following focal cerebral ischemia (24). Stover *et al*. (25) showed that Ril substantially decreased brain oedema formation, which might be due to anti-sodium or anti-glutamate effects of Ril or a combination of these effects.

Conclusion

Pre-treatment of U87MG GBM cells with Ril reduced the Dex-induced increase in cell migration. Ril also reversed the effect of Dex on CD90 mRNA expression. Furthermore, co-treatment with Ril and Dex could have beneficial effects in terms of increasing GBM cell death. We suggest that the use of Ril, which acts as an anti-tumorigenic drug, could mediate some of the negative effects of Dex and contribute to the anti-edematous effect of Dex. However, further studies including more GBM cell lines and *in vivo* studies, as well as studies on the possible use of Ril as an anti-edematous drug in GBM patients are needed.

Conflicts of Interest

The Author(s) declare no conflicts of interest with respect to the research, authorship and/or publication of this article. They also do not have relevant or non-financial interests to disclose.

Authors' Contributions

The work reported in this article has been performed by the authors, unless clearly specified in the text. Jonathan Keul: Conceptualization, Methodology, Formal Analysis, Investigation, Writing – Original Draft. Swetlana Sperling: Conceptualization, Methodology, Validation, Writing – Review & Editing. Veit Rohde: Funding, Writing-Review & Editing. Dorothee Mielke: Writing – Review & Editing. Milena Ninkovic: Conceptualization, Methodology, Investigation, Writing – Review & Editing, Supervision.

Funding

The Authors declare that no funds, grants or other support were received during the preparation of this manuscript.

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Received February 8, 2024
Revised March 18, 2024
Accepted March 19, 2024