Rapamycin inhibits human glioma cell proliferation through down-regulating mammalian target of rapamycin pathway and up-regulating microRNA-143

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Abstract
The objective of this study was to test the hypothesis that rapamycin regulates cell proliferation, apoptosis and glycolysis in human glioma cells and to investigate the underlying mechanisms. Human malignant glioma cell line U251 cells were treated with 100 mM rapamycin for 6, 12 and 24 h. Cell proliferation and apoptosis were assayed by methylthiazol tetrazolium assay and flow cytometric analyses. RNA and protein expression levels were then measured by real-time polymerase chain reaction and Western blotting, respectively. The administration of rapamycin inhibited the proliferation and induced the apoptosis of U251 cells. Prolonged treatment with rapamycin gradually decreased the mammalian target of rapamycin signalling in U251 cells. Moreover, our data showed rapamycin up-regulated miR-143 and down-regulated hexokinase 2—a key enzyme in glycolysis—in the glioma U251 cells. Collectively, our data suggest a new anti-tumour role of rapamycin in gliomas and indicates that rapamycin-mediated glioma cell proliferation might be through inhibiting the mammalian target of the rapamycin pathway and up-regulating tumour suppressing miR-143. These data suggest a promising, novel rapamycin- and miR-143-based therapy to treat malignant glioma.

Introduction
Malignant gliomas are the most common brain tumours, accounting for about 80% of the malignant tumours that develop in the central nervous system, resistant to many pro-apoptotic treatments such as radiotherapy, chemotherapy and adjuvant therapies. Despite combined therapies including surgery, radiotherapy and chemotherapy, the prognosis of glioma patients remains poor. Over the past 10 years, the prognosis for glioblastoma multiform (GBM), the most malignant type of glioma, has not improved with a median survival of approximately 1 year and a 5-year survival rate of only 2%. A more effective therapeutic strategy is urgently needed.

Cancer cells preferentially metabolize glucose through glycolysis, even in the presence of adequate levels of oxygen—a phenomenon known as the Warburg Effect. In fact, the Warburg Effect reflects the anomalous characteristic of glucose metabolism in malignant tumours. Cancer cells could survive if provided with adequate energy and molecules like nucleotides, fatty acids and adenosine tri-phosphate (ATP), which are necessary for the rapid proliferation of cancer cells. In addition, the Warburg Effect also makes the cells less dependent on oxygen, ensuring their survival under hypoxic and anoxic conditions. As a result, the development of therapeutic agents targeting this anomalous glucose metabolism may provide alternative therapeutic strategies. In various cancers, the high rate of glycolysis is mainly attributable to the abnormal over-expression of key enzymes responsible in glycolysis, such as hexokinase 2 (HK2). The over-expression of HK2 provides cancer cells with adequate glycolytic flux by promoting the first step of glycolysis and thus the shift towards aerobic glycolysis. Moreover, HK2 has been reported to be involved in the maintenance of the malignant state of tumours.

MicroRNAs (miRNAs) are small (21–24 nucleotides) endogenous non-coding RNA molecules that regulate gene expression at the post-transcriptional level by binding to the 3’ untranslated region (UTR) of target messenger RNAs (mRNAs), resulting in either mRNA degradation or translational repression. Growing evidence has suggested that miRNAs play important roles in regulating multiple biological processes, including tumourigenesis and progression, by repressing the expression of key oncogenes. Recent studies have shown that the deregulation or dysfunction of miRNAs is associated with poor clinical outcomes of patients with malignant tumours and that miRNAs have the potential to be used for cancer therapeutics. miR-143 is located at a fragile site on chromosome 5 that is frequently deleted in cancer and has been reported to be down-regulated in several cancers. Moreover, decreased expression of miR-143 in various tumours has been shown to be associated with poor prognostic features as well as lower survival rates.

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Rapamycin was initially isolated from the soil bacterium Streptomyces hygroscopicus as a fungicide, when it was discovered to have potential anti-tumour properties. Through FK-binding protein 12, rapamycin interacts with mammalian target of rapamycin (mTOR), a serine/threonine protein kinase that is critical for cell survival, metabolism and proliferation. By negatively regulating mTOR, rapamycin can trigger multiple biochemical signalling pathways in mammalian cells. Most importantly, its anti-proliferative effect makes rapamycin a promising agent for suppressing tumour growth. As a result, understanding the molecular signalling mechanisms of rapamycin-induced apoptosis may facilitate the development of strategies for the prevention and treatment of glioma. However, whether or not rapamycin has an impact on miRNAs and glycolysis in malignant gliomas remains unclear.

In this study, we have demonstrated that administration of rapamycin inhibited the proliferation—and induced the apoptosis of—GBM U251 cells by inhibiting mTOR signalling. In addition, treatment with rapamycin resulted in an increased expression of miR-143 and deregulation of HK2, which plays a crucial role in glucose metabolism. These findings provide novel insights into the molecular mechanisms of rapamycin as a therapeutic agent for glioma. In addition, our study also helps to further understand the functions of miRNAs in brain tumour pathogenesis.

Materials and methods

Cell culture

U251, a human glioma cancer cell line, was purchased from American Type Culture Collection. U251 cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% foetal bovine serum and 1% penicillin/streptomycin at 37°C with 5% CO₂. Rapamycin (obtained from Fluka) was dissolved in dimethyl sulphoxide (DMSO) and used in cell culture at a final concentration of 100 mM. The cells were treated for 6, 12 or 24 h.

Methythiazol tetrazolium (MTT) assay

Cells were incubated at 37°C for 3 h in medium supplemented with MTT (0.5 mg/ml, Promega). The medium was removed and 100 µl of DMSO was added into each well. The plate was gently rotated on an orbital shaker for 10 min to completely dissolve the precipitation. The absorbance was measured at 570 nm with a Microplate Reader (Bio-Rad).

Flow cytometric analyses

Apoptosis of U251 cells was analyzed by flow cytometry (FACSCalibur, Beckman Coulter). Annexin V-fluorescein isothiocyanate (Annexin V-FITC) and propidium iodide (PI) staining was used to visualise apoptotic cells, according to the manufacturer’s instructions. In brief, 2 × 10⁵ cells were seeded in 6-well plates and treated with rapamycin for 6, 12 or 24 h. Cells were then collected and washed twice with phosphate buffered saline (PBS) and resuspended in 400 µl of 1× binding buffer. Subsequently, 5 µl of the Annexin V-FITC and PI solution were added. Samples were incubated for 15 min at room temperature and analyzed by flow cytometry.

Western blotting

Equal numbers of cells were plated in a 6-well culture plate for 6, 12 or 24 h prior to treatment. Cells were solubilised in cold radio-immunoprecipitation assay lysis buffer and then separated with 5% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). After SDS-PAGE, proteins were transferred from the gel to a polyvinylidene fluoride membrane. Membranes were blocked in 5% non-fat dried milk in PBS-Tween 20 (PBST) for 2 h, and then incubated overnight with specific primary antibodies (Abcam) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a control. After incubation with the appropriate secondary antibody, immune complexes were detected using an enhanced chemiluminescence (ECL) kit. Results were visualised by autoradiography using preflashed Kodak XAR film.

Real-time PCR (RT-PCR)

Total RNA was extracted from cultured cells using an RNeasy RNA isolation kit (Qiagen). RNA was then reverse transcribed into cDNA using miScript II RT Kit. cDNA was then amplified by RT-PCR using SYBR Green dye universal master mix with miScript primers for miR-143 and HK2 gene on a Light Cycler 480 instrument for 40 cycles. The relative amount of mRNA to GAPDH was calculated using crossing point values and scaled relative to control samples set at a value of 1. Gene expression results obtained from experimental samples were plotted and compared with those obtained from the control.

Statistical analysis

All data are expressed as mean ± SD of triplicate experiments and all experiments were repeated at least 3 times. The data were analysed by one-way analysis of variance (ANOVA) and Student’s t-test. A p value of <0.05 was considered to be significant. Statistical analysis was performed using SPSS 17.0 statistical software.

Results

Rapamycin inhibits cell proliferation and increases apoptosis in glioma cells

We first examined the proliferation and apoptosis of U251 cells after treatment with rapamycin. MTT assay was used to detect the relative cell proliferation of cells treated with rapamycin for different durations (Figure 1). We found that rapamycin...
significantly inhibited the proliferation of U251 cells as compared with the controls (p < 0.05).

To further investigate whether rapamycin could also induce the apoptosis of U251 cells, cells of different groups were analysed using Annexin V/PI double-staining and flow cytometry analysis (Figure 2). Our data suggests that rapamycin could induce the apoptosis of U251 cells. The histogram plots indicate that the percentage of apoptotic cells increased more than 3 times in the group treated with rapamycin for 24 h compared with the control group (p < 0.05). This data suggests that rapamycin is able to inhibit cell proliferation and induce cell apoptosis in U251 cells.

Rapamycin inhibits the mTOR pathway
To examine the molecular mechanism of rapamycin-induced cell proliferation inhibition and apoptosis in U251 cells, we investigated the effect of rapamycin on the activity of mTOR signalling (a key signalling pathway in glioma pathogenesis) using Western blots. We found that the phosphorylation of S6 kinase, a direct substrate of mTOR, was significantly reduced by rapamycin in a time-dependent manner in U251 cells (Figure 3). This data suggests that rapamycin may inhibit glioma cell proliferation by repressing the mTOR pathway.

Rapamycin regulates miR-143 and HK2
miRNAs play important roles in regulating protein expression, cell proliferation, apoptosis and cancer progression. miR-143 has been found to act as a suppressor in several malignant tumours14,15. Recently, mTOR was reported to regulate the expression of miR-143 in lung adenocarcinoma cell lines19. Hence, to further understand the molecular mechanisms of rapamycin/mTOR-mediated growth inhibition of glioma cells, we determined the expression of

![Figure 1](image1.png)

**Figure 1:** Rapamycin inhibits cell proliferation in glioma cells. MTT assay was used to determine the proliferation of cells after U251 cells were treated with 100 mM rapamycin for 6, 12 or 24 h. With prolonged treatment with rapamycin, the relative cell proliferation rate gradually decreased. The difference between each group was statistically significant (p < 0.05).

![Figure 2](image2.png)

**Figure 2:** Rapamycin was found to increase apoptosis in glioma cells. After treatment of U251 cells with control PBS (a), or 100 mM rapamycin for 6 h (b), 12 h (c), or 24 h (d), apoptotic cells were detected by Annexin V/PI double staining. There was a significant difference between all groups (p < 0.05). The insert (e) is the quantitative data.

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Rapamycin inhibits human glioma cell proliferation through down-regulating mammalian target of rapamycin pathway and up-regulating microRNA-143. The up-regulation of miR-143 by rapamycin was observed to be time-dependent, with a maximal increase (3.5-fold) achieved at 24 h treatment (Figure 4). In various malignant tumours, the glycolysis level is aberrantly up-regulated, which is necessary for the rapid proliferation of cancer cells. Hence, HK2, a predicted target of miR-143, was chosen for further analysis based on its critical function in glycolysis. As shown in Figure 4, during the treatment of U251 cells with rapamycin, HK2 expression was down-regulated and conversely correlated with miR-143, suggesting that rapamycin might inhibit glioma cell proliferation by up-regulating the tumour suppressing miR-143 at least partially by repressing the downstream target gene HK2 (Figure 5).

Discussion
Rapamycin has been suggested to impact multiple cellular responses, including the inhibition of proliferation and induction of apoptosis in various cancer cell lines derived from different tumour types\textsuperscript{20–22}. In the current study, we found that with prolonged treatment of human glioma U251 cells with 100 mM of rapamycin, cell proliferation was significantly decreased, while the apoptotic rate gradually increased. Rapamycin-mediated glioma cell proliferation inhibition could result from a down-regulation of the mTOR pathway and up-regulation of tumour suppressing miR-143. Our data further confirmed that rapamycin could be used as a promising therapeutic agent for the treatment of human malignant gliomas.

In multiple types of malignant tumours, the phosphatidylinositol 3′-kinase (PI3K)/Akt/mTOR signalling pathway is aberrantly activated. mTOR acts as one of the major downstream signalling targets of

References

Abbreviations list
ATP, adenosine tri-phosphate; Annexin V-FITC, Annexin V-fluorescein isothiocyanate; DMSO, dimethyl sulphoxide; ECL, enhanced chemiluminescence; GBM, glioblastoma multiform; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HK, hexokinase 2; mTOR, mammalian target of rapamycin; mRNA, messenger RNA; MTT assay, methylthiazol tetrazolium assay; miRNA, micro-RNAs; PBS, phosphate buffered saline; RT-PCR, real-time polymerase chain reaction; SDS-PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis; UTR, untranslated region.