Original Paper

β-arrestin 1 Overexpression Increases Temozolomide Resistance in Human Malignant Glioma Cells

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ABSTRACT: Many studies highlighted β-arrestins (β-arr) as essential proteins behind the regulation of major cell signaling pathways in different types of cancer. An impaired β-arrestin 1 (β-arr 1) activation/phosphorylation was suggested to be associated with a high malignant phenotype of glioma. Elevated levels of β-arrestin 2 (β-arr 2) mRNA were also found in advanced stages of breast cancer compared to early stages. In addition, β2-arrestin was also linked to a suppressive effect on tumor growth in other types of cancers such as prostate or non-small cell lung cancer. In this study, we analyzed the effect of β-arr 1 overexpression on the cytotoxic effect of Temozolomide (TMZ) in two malignant glioma (MG) cell lines: U-343MGa and Cl2:6. For this purpose, the cells were transected with β-arr 1 and then treated with different concentrations of TMZ for 24, 48 and 72 hours. At the end of the treatment, the cell viability was analyzed by Prestoblue viability assay. Our results showed that TMZ treatment induced cytotoxicity in MG cells while β-arr 1 transfection significantly reduced the TMZ cytotoxic effect in both U-343MGa and Cl2:6 MG cell lines. These results suggest that β-arr 1 overexpression may be a cause of TMZ resistance in MG.

KEYWORDS: *malignant glioma, temozolomide, β-arrestin*

Introduction

Despite the impressive pace at which new cancer treatments are included intro clinical practice each year, bringing markedly improved outcomes and quality of life for patients, progress in the field of neurooncology has been rather disappointing [1-4]. The current standard of care [5] for high grade gliomas (HGG) which involves surgical resection followed by adjuvant temozolomide (TMZ) and radiotherapy (RT) has remained unchanged in the past decades, with 2 and 5 year overall survival (OS) being approximately 25% and 10%, while 2 and 5 year progression-free survival (PFS) are 11.2% and 4.1%, respectively [5,6].

In recent years, attention has shifted from the histology-based classification of brain tumors to a more biomarker orientated division of subgroups, with more and more distinctive markers being linked to the birth, development and treatment response of different types and subtypes of brain tumors. This culminated in 2016 with the new World Health Health (WHO) of brain tumors [7,8] which separated astrocytic tumors based on the isocitrate dehydrogenase (IDH) gene status: wild-type or mutant further underlining the importance of biomarkers in the diagnosis of HGG. Another enzyme, O6-methylguanine-DNA-methyltransferase (MGMT) plays a pivotal role in the mechanisms implicated in DNA repair and subsequently in the way MGs respond to TMZ treatment [6,9] given than TMZ's activity is based on DNA damage via alkylation of the purine bases. MGMT silencing through MGMT gene promoter methylation is directly linked to the tumor's incapacity to repair the DNA damaged by TMZ [9]. Unfortunately, the MGMT-related DNA repair system is not the only mechanism employed by glioma cells in order to escape the cytotoxic effect of TMZ. Several other genetic alterations such DNA Mismatch Repair (MMR) mutations [10], Epithelial Growth Factor Receptor (EGFR) amplification or mutation [11] or conversion of supressor genes such as p53/PTEN [12] from a mutated to a wild-type status can substantially increase resistance to alkylating agents such as TMZ. More recently, miRNAs have also been cited as being implicated in TMZ resistance [13,14]. Furthermore, one study on GBM cell cultures has linked increased TMZ sensitivity to β-arr 1 gene silencing [15].

β-arrestins (β-arr) are a family of ubiquitous multifunctional adapter proteins known for their active role in G protein-protein coupled receptors (GPCRs) regulation by desensitization and internalization [16].

The arrestin family is comprised of four members: β-arr1 and 2, arrestin-1 (visual arrestin) and arrestin-4 (cone arrestin). β-arr1 and β-arr2 are two isoforms, which share 78% sequence homology [17] and are also plentifully encountered across different species, with almost 50% shared homology between vertebrate and invertebrate protein isoforms. Through further studies it was discovered that the roles of β-arrs go well beyond that of simple receptor desensitization and internalization, involving activation of cellular signaling pathways such as Mitogen-activated protein

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kinases (MAPK), Wnt, Notch, Hedgehog and Phosphatidylinositol 3-kinase (PI3K), receptor transactivation and recycling, cellular proliferation and differentiation [18-20].

More recently, β-arrs have also been directly linked to tumor development, invasiveness and capacity to metastasize in different types of cancer [21-23]. Furthermore, β-arr expression was shown to correlate to resistance to therapy in different types of cancers such as breast [24,25], ovarian [26,27] and more recently HGG [15].

In this paper, we aim to determine how β-arr 1 expression influences TMZ treatment response on HGG cell lines.

Experimental methods

Cell cultures

MG U343 MG and U343 MGa C1-2:6 cell lines are human malignant glioma cells and were previously described by Nister et al [28].

Both cell lines were maintained in Eagles' minimum essential medium (MEM) supplemented with 10% FBS (vol/vol) and 1% Streptomycin/Penicilin. The cell lines were maintained in a humidified incubator at 95% air /5% carbon dioxide atmosphere and 37^0C in $75cm^2$ flasks.

Plasmids and transfection

Cells were cultured at 80% confluency in six-well plates and transfected with β-arr1 plasmid using using Lipofectamine 2000 (Invitrogen) in accordance to the manufacturer's instructions. Transfected cell were incubated for 24h before being subjected to TMZ treatment.

Temozolomide treatment

For experimental purposes, untrasfected and β-arr1 trasfected cells were seeded in 96-well plates (1.24x104 cells/well) and treated with increasing concentrations of Temozolomide (Sigma-Aldricht) (200μM, 250μM and 300μM). The drug was administered in a single dose at the beginning of the study.

Cell viability assay

Cells were incubated in 96-well plates and viability was measured using a Prestoblue (Life Technologies, Carlsbad, CA, USA) cell viability assay. Cells were incubated for 30 minutes at 37^oC with the Prestoblue reagent. Fluorescence was measured using a Tecan Infinite 1000 plate reader. A standard curve was used to correlate fluorescence to cell number. Each experiment was performed in triplicates.

Statistical analysis

Studies were performed in separate, independent experiments as triplicates. In order to compare the significance between study groups, an analysis of variance (ANOVA) and a Student's t test were performed. The level of statistical significance accepted in our study was $p \leq 0.05$. Experimental data was presented as mean±standard deviation (SD).

Results

The effect of Temozolomide on high glioma cells *in vivo*

Temozolomide is an alkylating agent used in the treatment of HGG. It's cytotoxic activity is based on transferring a methyl group to guanine and subsequent formation of a O6-methylguanine compound resulting in cell cycle arrest during the G2/M phase [29]. This effect can be reversed by MGMT activity which removes the methyl group by transferring it to a cysteine residue, unblocking cellular division [30].

In our experiment, we analyzed the effect of TMZ treatment on two MG cell lines: U343 MGa and CL 2:6. For this propose, the cells were incubated with 200μM, 250μM or 300μM TMZ and the cell viability was determined by Prestoblue assay, 24h, 48h and 72h after the treatment.

Our results show that at 24h, treatment with TMZ 200μM, 250μM or 300μM induced about 20% cell death in U343 MGa cell line and the results were statistically significant ($p \leq 0.05$) (Fig. 1). After 48h, TMZ treatment had a pronounced effect on the untransfected U

343MGa cell line, with proliferation dropping to 63% for the 200 μM dose ($p \leq 0.05$), 57% for the 250μM dose ($p \leq 0.05$) and 54% for the 300μM dose ($p \leq 0.05$) when compared to control (Figure 1). As seen in Fig. 1, 72h exposure to TMZ in the U 343 MGa cell line resulted in a significant decrease in cell proliferation, by 33% for the 200μM TMZ treatment, 36% for the 250μM treatment and 34% for the 300μM treatment ($p \leq 0.05$).

In the CL 2:6 cell line, exposure to the 200μM, 250μM and 300μM TMZ doses determined a progressive drop in proliferation by 14%, 21% and 28%, respectively ($p \leq 0.05$) (Fig. 2). TMZ treatment resulted in a significant cytotoxic effect in the Cl 2:6 cell line after 48h, with cell proliferation decreasing by 22% for 200μM TMZ ($p \leq 0.05$), 31% for 250μM TMZ ($p \leq 0.05$) and 33% for 300μM TMZ ($p \leq 0.05$). Finally, at 72h, proliferation after treatment with

the 200μM TMZ dose decreased by only 2% ($p>0.05$), while exposure to 250μM and 300μM TMZ inhibited proliferation by 22% ($p\leq0.05$) and 34% ($p\leq0.05$) (Fig. 2).

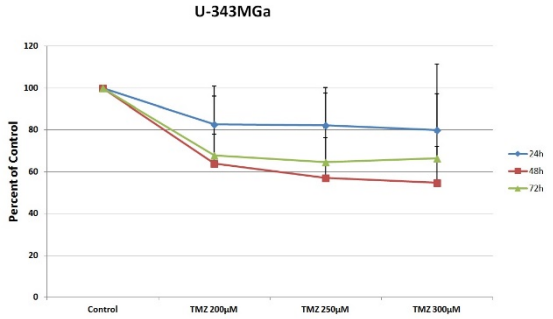

Fig. 1. TMZ effect on viability of U343 MGa cells. The cells were exposed to 200μM, 250μM or 300μM of TMZ for 24 hours. The cytotoxic effect of the TMZ effect was evaluated by Prestoblue assay. Results are expressed as percentage of control. Appropriate control groups with diluents only were included. Data are mean and standard error of three separate experiments

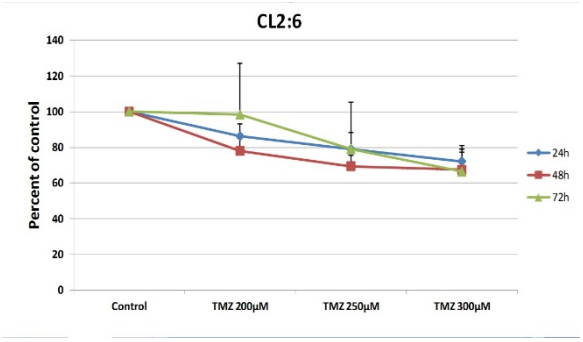

Fig. 2. TMZ effect on viability of U343 MGa cells. The cells were exposed to 200μM, 250μM or 300μM TMZ for 24 hours. The cytotoxic effect of the TMZ effect was evaluated by Prestoblue assay. Results are expressed as percentage of control. Appropriate control groups with diluents only were included. Data are mean and standard error of three separate experiments

β-arrestin 1 transfection counteracted the TMZ effect in malignant glioma cells *in vitro*

According to recent studies, β-arr 1 is strongly linked to signal modulation through many pathways such as Wnt, Hedgehog, Notch and MAPK [16,18,31]. In addition, β-arr 1 activity seems to be involved in the proliferation and progression of different types of cancer [32-34]. In our experiment, we determined the effect of β-arr 1 overexpression, induced by plasmid transfection on the TMZ treatment response of two MG cell lines (U343 MGa and CL 2:6), at 24h, 48h and 72h.

After 24h, β-arr1 transfection slightly counteracted the cytotoxic effect of 200μM TMZ by 4% but the results were not statistically significant ($p>0.05$). The cytotoxic effect induced by 250μM or 300μM TMZ treatment at 24h was not affected by β-arr 1 transfection (Fig. 3A).

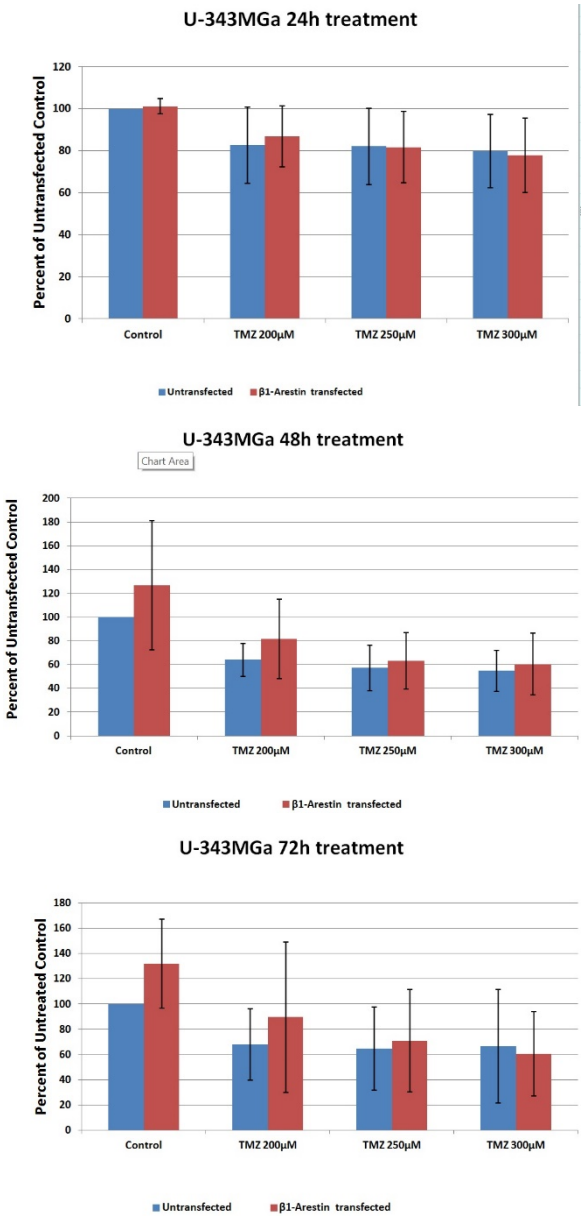

Fig. 3. The effect of β-arr 1 transection on U343 MGa cells response to TMZ treatment. U343 MGa cells (blue bars) and β-arr 1 transected MGa cells (red bars) were treated with 200μM, 250μM or 300μM of TMZ for 24 hours (A), 48 hours (B) and 72 hours (C). The cytotoxic effect of the TMZ effect was evaluated by Prestoblue assay at the end of the experiment. Results are expressed as percentage of control. Appropriate control groups with diluents only were included. Data are mean and standard error of three separate experiments

Our results show that after 48h, β-arr1 transfection significantly increased the proliferation of U 343MGa cells by 26% in comparison to control cells ($p\leq0.05$) (Figure

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3B). β-arr1 transfection prevented the toxic effect induced by 200μM TMZ by 18%, 48h after the treatment ($p \leq 0.05$) but no significant prevention of the cytotoxicity induced by 250 and 300μM TMZ doses were observed ($p > 0.05$) (Fig. 3B).

After β-arr1 transfection, a noticeable increase in cell proliferation (31%) was observed for the transfected cells when compared to control. Transfection with β-arr1 also counteracted the cytotoxic effect induced by 200μM TMZ by 12% when compared to untrasfected cells ($p \leq 0.05$). β-arr1 transfection also seemed to heavily block the cytotoxic effect of 250μM TMZ in untrasfected cells but the result was not statistically significant ($p > 0.05$). β-arr1 transfection did not prevent cytotoxicity induced by treatment with 300 μM TMZ at 72h ($p \geq 0.05$) (Fig. 3C).

After 24h, B-arr1 transfection had a noticeable effect on Cl2:6 proliferation, as seen in Fig. 4A. Transfected cells presented an 19% uptick in growth when compared to control ($p \leq 0.05$). The effect of TMZ treatment was also hindered by transfection, resulting in a reduction in cytotoxicity by 8% for 200μM TMZ ($p \leq 0.05$), by 9% for 250μM TMZ ($p \geq 0.05$) and by 5% for 300μM TMZ ($p \geq 0.05$) (Fig. 4A).

As shown from our results, after 48h, Cl 2:6 cells transfected with β-arr1 presented a 11% increase in proliferation compared to untransfected control.

As for Cl 2:6 cells exposed to TMZ, β-arr1 transfection strongly counteracted the cytotoxic effect of 200μM TMZ by 25% ($p \leq 0.05$), by 14% for the 250μM TMZ dose ($p \leq 0.05$) and by 8% for the 300μM TMZ dose ($p > 0.05$).

72h after β-arr1 transfection, a noticeable increase in proliferation (11%) was observed in the Cl 2:6 cell population when compared to control. Transfection with β-arr1 also counteracted the alkylating agent's effect with a 20% drop in cytotoxicity, observed for the 200μM TMZ dose ($p \leq 0.05$), a markedly reduced cytotoxic effect, by almost 20%, for the 250μM dose ($p \leq 0.05$) and a 8% drop in cytotoxicity for the 300μM TMZ dose ($p \leq 0.05$) (Fig. 4C).

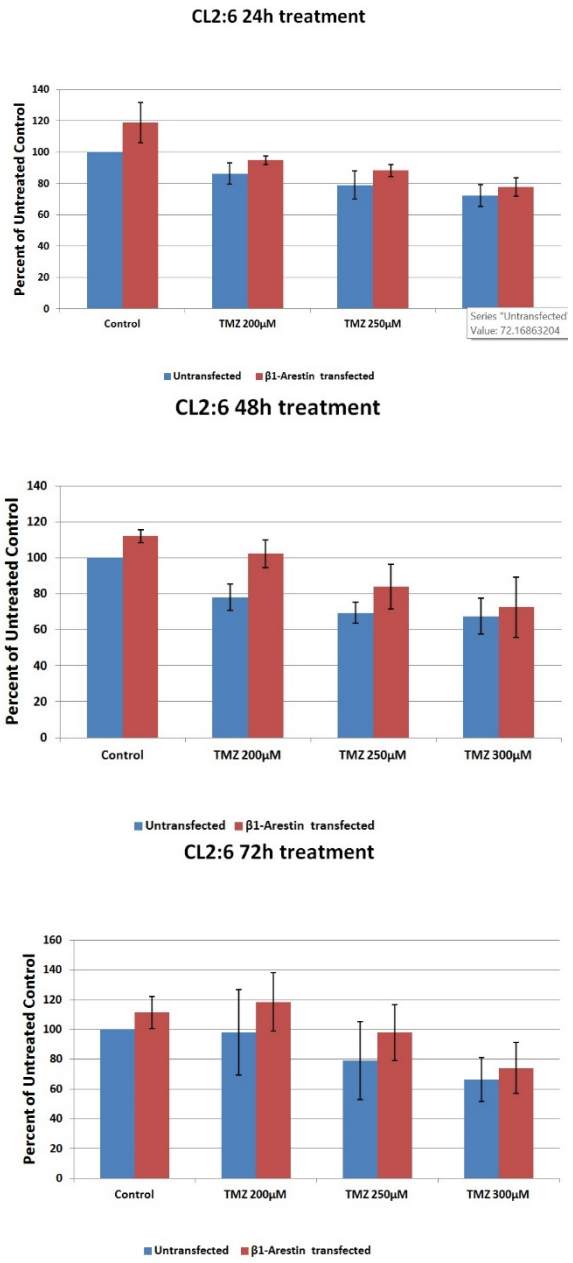

Fig. 4. The effect of β-arr 1 transection on Cl 2:6 cells response to TMZ treatment. Cl 2:6 cells (blue bars) and β-arr 1 transected Cl 2:6 cells (red bars) were treated with 200 μM, 250 μM or 300 μM of TMZ for 24 hours (A), 48 hours (B) and 72 hours (C). The cytotoxic effect of the TMZ effect was evaluated by Prestoblue assay at the end of the experiment. Results are expressed as percentage of control. Appropriate control groups with diluents only were included. Data are mean and standard error of three separate experiments

Discussion

Apart from the traditional role in receptor desensitization, β-arrs activity has been linked to different types of cancers, mostly through the activation of cancer-specific signaling pathways [32]. In a study by Dasgupta et al [33], results have shown that nicotine induces β-arr1 translocation to the nucleus in human non-small

cell lung cancer, which in turn promoted the transcription of pro-proliferative and anti-apoptosis genes, accelerating tumor growth [33]. Interesting enough, another study on murine models has shown that β-arr2 gene knockdown promotes cell growth and accelerates the processes behind metastasis [35]. This theory is backed up by several other studies which link β-arr1 activity to an increased malignant phenotype in other types of cancer, such as ovarian [26], colorectal [34] or breast [25] while β-arr2 is mentioned as having an opposite, suppressive effect in prostate cancer [36]. This strange dual and opposite nature of two proteins, which share an almost identical sequence homology (78%) and similar physiological roles, requires further research given their ubiquitous nature and the intricacies between them and many molecular mechanisms.

High grade gliomas (HGGs) harbor an impressive number of gene mutations and chromosome alterations, which results in simultaneous recruitment of several signaling pathways [37-39]. So far, little information is available on the role β-arrs play in gliomagenesis, proliferation or therapeutic response. A tendency for β-arr1 expression and dephosphorylation to be elevated in higher grade tumors compared to low grade gliomas was suggested in a study published by Mandel et al in 2009 [40]. In addition, a link between β-arr1 and tumor proliferation in GBMs was also reported [41].

A recent study by Lan T et al. has shown that β-arrestin 1 gene knockdown reduces proliferation, glycolysis and the metastatic potential in GBM cells [15]. Even more interestingly, the author demonstrated that β-arr 1 gene downregulation increased TMZ efficacy in GBM cells [15]. In accordance with these results we found that β-arr1 overexpression increased resistance to TMZ treatment in two MG cell lines (U 343 MGa and Cl 2:6). However, the effect was more pronounced in the Cl 2:6 cell line. One explanation for this difference could be related to the phenotypical variations between the two cell lines. In a study by Nister at al it was discovered that the U343 MGa Cl 2:6 cell line produces higher levels of PDGF-like growth factor in comparison to the U343 which acts as an autocrine growth stimulator [28]. However, no definitive information is yet available on the existence of crosstalk between PDGFR and β-arrs. Altogether, more information is required to link β-arr 1 and 2 activity to the major events and mechanisms that constitute the driving forces behind the birth, progression, invasion and metastasis of cancer.

Conclusions

In our study, we have shown that β-arr1 overexpression influences both proliferation and treatment resistance in MG preclinical models. However, more research is needed in order to better understand the mechanisms by which β-arr1 expression regulates cellular growth and drug resistance in cancer cells. Additionally, further investigations are required to correlate β-arr1 levels to treatment response to other agents such as monoclonal antibodies or tyrosine kinase receptor inhibitors, which have so far demonstrated a modest effect in clinical trials. In the future, β-arrs might emerge as possible biomarkers for the diagnosis of HGGs and also as attractive therapeutic targets, their inactivation resulting in enhanced sensitivity to other agents.

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