NF-κB inhibitor reverses temozolomide resistance in human glioma TR/U251 cells

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Abstract. Glioblastoma multiforme (GBM) demonstrates an unsatisfactory clinical prognosis due to the intrinsic or acquired resistance to temozolomide (TMZ) exhibited by the tumors. One possible cause of TMZ resistance in GBM is the overexpression of O^6-methylguanine-DNA methyltransferase (MGMT), which can repair the TMZ-induced guanine damage in DNA. Additionally, excessive activated NF-κB is reported to be a component of the major inflammatory transcription pathway that is associated with TMZ resistance in GBM. However, the association between the NF-κB pathway and MGMT expression in GBM cells is unknown. Therefore, in the present study, the TMZ resistant (TR) U251 cell line (TR/U251) was successfully constructed to detect how the TR/U251 cell line and the parental U251 cell line each interact with TMZ *in vitro*. The TR/U251 cells were approximately five times more resistant to TMZ compared with the parental cells. Furthermore, it was found that the NF-κB inhibitor BAY 11-7082 suppressed the expression of MGMT in TR/U251 cells and enhanced TMZ-induced cytotoxicity and apoptosis, thereby indicating that the NF-κB pathway and MGMT interact to promote TMZ resistance. The inhibition of NF-κB may be a promising strategy to reverse drug resistance in TR glioma cells. The present results propose a potential mechanism for using the NF-κB inhibitor BAY 11-7082 as a potential therapy for the treatment of TR glioma. Although BAY 11-7082 is a well-known NF- ϰB inhibitor, the present study further investigated its underlying mechanisms through a series of new experiments.

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Introduction

Glioblastoma multiforme (GBM) is one of the most malignant tumors of the central nervous system and exhibits a poor prognosis, despite technological advances in surgical resection radiotherapy and chemotherapy. The average survival time is 8-12 months, due to the high invasiveness and heterogeneity (1,2). Temozolomide (TMZ), an orally administered alkylating agent, has been revealed to provide significant survival benefits for patients with GBM. Although maximal surgical resection combined with TMZ therapy and irradiation has significantly improved the quality of life and prolonged the survival time of patients with GBM, the overall clinical prognosis remains unsatisfactory due to intrinsic or acquired tumor cell resistance to TMZ (3). Previous studies have demonstrated that O^6-methylguanine-DNA methyltransferase (MGMT) is the main factor responsible for chemoresistance to TMZ in GBM cells (4,5). However, the molecular mechanism of TMZ resistance is more complex than a simple dependence on MGMT expression.

Nuclear factor-κB (NF-κB) is a component in the major inflammatory transcription pathway that is associated with glioblastoma and excessively activated by various cytokines and growth factors (6). NF-κB proteins include a family of structurally-similar transcription factors, of which the p65/p50 heterodimer is the key form. Normally, NF-κB exists in the cytosol and is bound to the inhibitory IκBα in an unstimulated state. Classical signaling of the NF-κB activation pathway consists of one of several extracellular ligands, such as TNF-α, IL-1 or LPS, binding to the NF-κB receptor, which in turn activates the IκB kinases (IKKs) IKKα and IKKβ, the latter being particularly important as it phosphorylates IκBα and enables IκBα ubiquitination and degradation, thus releasing and activating the NF-κB protein. Activated NF-κB translocates to the nucleus and binds to DNA, which initiates the transcription of several genes (7,8).

Excessive and aberrant activation of NF-κB has been identified in GBM, and as the oncogene in glioma, the levels of NF-κB activity are much higher in GBM tissue compared with non-GBM tissue, and has also been associated with a worse prognosis in GBM (9) . In addition, NF-κB has been reported to be associated with TMZ resistance primarily due

to the anti-apoptotic activity of NF-κB (10). Therefore, the present study proposed a hypothesis that down-regulation of NF-κB via the IκBα inhibitor reversed the resistance to TMZ, and detected the link between the NF-κB pathway and MGMT expression in a TMZ resistant (TR) cell line.

Materials and methods

Drugs and reagents. TMZ was purchased from Tasly Pharmaceutical Co., Ltd. (Tianjin, China). The stock solutions were prepared in dimethyl sulphoxide (DMSO) at a concentration of 10 mM, and the solutions were diluted in the cell culture medium to the respective final concentrations. Sulforhodamine B (SRB) was obtained from Sigma-Aldrich (St. Louis, MO, USA). The IκBα inhibitor BAY 11-7082 was purchased from Beyotime Institute of Biotechnology (Haimen, Jiangsu, China). All antibodies were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA), consisting of primary mouse anti-human polyclonal antibodies against MGMT (cat. no. sc-400720-HDR), NF-κB (p65; cat. no. sc-8008 AC) and β-actin (cat. no. sc-77326-PR), rabbit anti-human polyclonal antibodies against phosphorylated p65 (cat. no. sc-45101), B-cell lymphoma (Bcl)-2 (cat. no. sc-130916) and Bcl-2-associated X protein (cat. no. sc-377576), and secondary HRP-conjugated rabbit anti-goat (cat. no. sc-2768) antibodies.

Cell lines and culture conditions. The human malignant glioma U251 cell line, obtained from the Institute of Biochemistry and Cell Biology (Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai, China), was cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 units/ml of penicillin and 100 μg/ml of streptomycin in a humidified atmosphere containing 5% CO_2 at 37˚C. All media and sera were purchased from Gibco Life Technologies (Carlsbad, CA, USA).

To establish the TR cell line, the U251 cells were initially exposed to 1.25 μM TMZ for two weeks, and the concentration of TMZ was then doubled for 4-8 weeks until it reached 160 μM. After ~10 months, the resistant clones were isolated and confirmed using an SRB assay. The TR variants generated by this method were denoted as TR/U251 cells. The parental U251 cells and the cells treated with DMSO alone were included for parallel analysis.

Chemosensitivity assay. For the chemosensitivity assay, 2,000 U251 or TR/U251 cells were plated in each well of a 96-well plate, at 37˚C in a 5% CO_2 atmosphere. After 24 h, the culture media were replaced with fresh media in the absence or presence of serial concentrations of TMZ. After 96 h, the SRB assay was used to determine the surviving cell numbers using optical density (OD), as measured by absorbance at 490 nm using an ELISA reader (Multiskan MK3; Thermo Fisher Scientific, Waltham, MA, USA). The percentage of cell viability was calculated relative to the untreated cells, which acted as the control. A dose-response curve was plotted, and the 50% inhibiting concentration (IC_{50}) values for TMZ were calculated by the derivation of the best-fit lines using three independent experiments, which were performed in triplicate.

Cell proliferation assay. In total, 5,000 cells/well were seeded in 96-well plates. The cells were serum-starved for 24 h and were treated again with serum in medium prior to the start of the experiment. The relative density of cells was determined using the SRB assay on a daily basis over a 72-h period. Absorbance was measured at 490 nm in three independent experiments performed in triplicate.

Hoechst 33258 nuclear staining. For nuclear staining, ~1x10^5 cells/well were seeded in six-well plates, and treated with TMZ or BAY 11-7082, respectively. The cells were washed with 0.01 M PBS, fixed with 70% ethanol for 2 h at 4˚C, and stained with 5 μg/ml Hoechst 33258. The change in the cell nuclei was then observed using a fluorescence microscope at a magnification of x200 (Olympus, Tokyo, Japan).

Western blot analysis. Western blot analysis was performed using standard techniques, as follows. Briefly, the cells in the culture flasks were lysed in mammalian protein extraction reagent (Thermo Fisher Scientific, Waltham, MA, USA), and the whole protein concentrations were determined using the bicinchoninic acid protein assay kit from Pierce Biotechnology, Inc. (Rockford, IL, USA). The protein was then separated by 10% SDS-PAGE and transferred to polyvinylidene fluoride membranes (EMD Millipore; Billerica, MA, USA). The membranes were blocked using 5% non-fat dry milk in Tris-buffered saline with Tween 20 (TBST; Guidechem, Shanghai, China) for 1 h and then incubated with primary antibodies overnight at 4˚C. Subsequent to three washes with TBST, the membranes were incubated three times with secondary antibodies conjugated to horseradish peroxidase (1:5,000) for 2 h at room temperature. Finally, the immunoreactivity was detected using an enhanced chemiluminescence procedure kit (Thermo Fisher Scientific) and accessed using a quantitative gel and western blotting imaging system (Becton-Dickinson, Franklin Lakes, NJ, USA). The protein expression was quantified subsequent to normalization to β-actin.

Statistical analysis. Each study was replicated in at least three independent experiments. SPSS 13.0 statistical software (SPSS, Inc., Chicago, IL, USA) was used to perform the statistical analysis. The data are presented as the mean ± standard deviation. One-way analysis of variance (ANOVA) and Student's *t*-test were used to analyze the significance of the differences between study groups. The least significant difference method for multiple comparisons was used to compare the parental and control groups when ANOVA indicated a statistical significance between the groups. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Generation of the TR/U251 cell line. The TR/U251 cell line was generated from the parental U251 cell line by initially exposing the cells to 1.25 μM TMZ for two weeks, and then doubling the TMZ concentration every two passages over a period of 4-8 weeks, with the doses ranging between 12.5 and 160 μM. The IC_{50} of TMZ in the TR/U251 cells

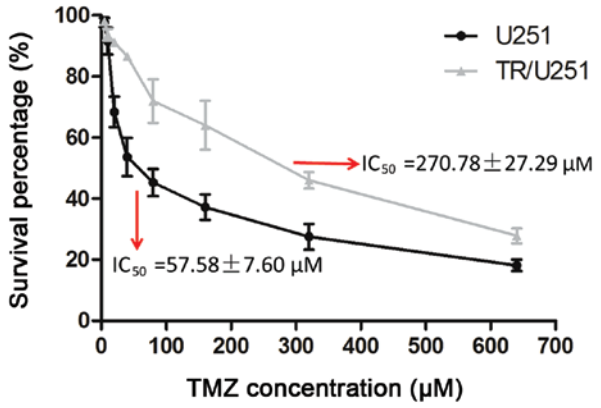

Figure 1. Survival rate of U251 and TR/U251 cells treated with various concentrations of TMZ. A dose-dependent association between the survival rate of U251 and TR/U251 cells and TMZ concentration can be observed. The survival of U251 cells was significantly lower compared with TR/U251 cells following treatment with various concentrations of TMZ. The IC_{50} of TMZ in U251 cells is 57.58±7.60 μM, while the IC_{50} of TMZ in TR/U251 cells is 270.78±27.29 μM. TMZ, temozolomide; TR, TMZ-resistant; IC_{50}, 50% inhibiting concentration.

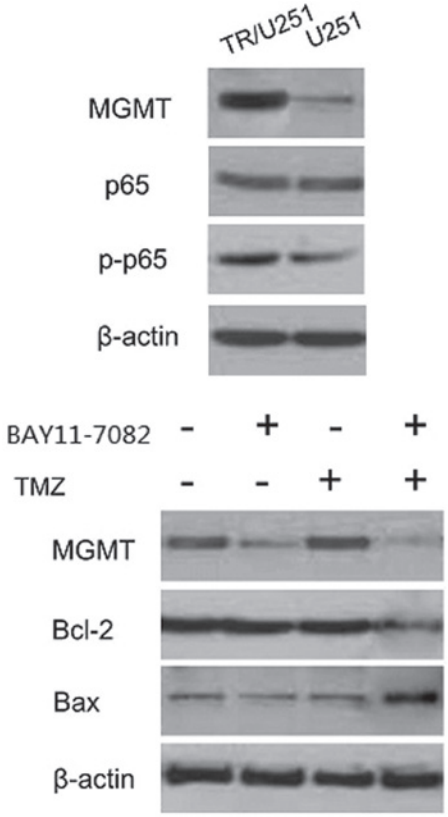

Figure 2. Expression of MGMT, Bcl-2 and p65 in the BAY 11-7082, TMZ and combination treatment groups. A high expression of MGMT was found in the TR/U251 cells, while expression was hardly detected in the U251 cells. Similarly, the levels of phosphorylated-P65 were increased in the TR/U251 cells compared with the U251 cells. No statistical difference was found in the NF-κB expression, assessed through the detection of the p65 subunit, between the two cell types. The TR/U251 cells demonstrated a ~5.4-fold higher level of phosphorylated-P65 compared with the U251 cells. MGMT was hardly detected when cells were cultured with a combination of TMZ and BAY 11-7082. A higher level of MGMT was detected in the cells treated with TMZ or BAY 11-7082 separately. The expression of Bcl-2 and Bax was reversed only in the cells treated with a combination of TMZ and BAY 11-7082. TMZ, temozolomide; TR, TMZ-resistant; MGMT, O^6-methylguanine-DNA methyltransferase; Bcl-2, B-cell lymphoma-2; Bax, Bcl-2-associated X protein; p-p65, phosphorylated p65.

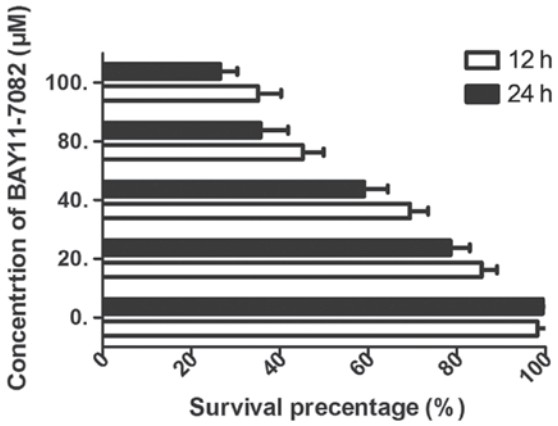

Figure 3. Survival rate at various concentrations of BAY 11-7082. A dose- and time-dependent association between the survival rate of the TR/U251 cells and BAY 11-7082 concentration. A longer incubation time with BAY 11-7082 significantly decreased the survival rate of TR/U251 cells, and the same was found for treatment with a higher concentration of BAY 11-7082. TMZ, temozolomide.

was 270.78±27.29 μM and the IC_{50} of TMZ in the parental U251 cells was 57.58±7.60 μM. Therefore, the TR/U251 cells were approximately five times more resistant to TMZ compared with the parental cells (Fig. 1). The TMZ resistance of the TR/U251 cells was maintained with the absence of drugs for approximately eight weeks. In addition, the TR cells exhibited constant resistance subsequent to TMZ withdrawal, which was maintained for an almost three-month period of time (data not shown).

High expression of MGMT and NF-κB in TR/U251 cells compared with the parental cells. MGMT, the DNA repair protein, contributed to drug resistance through the demethylation of the methyl added to O^6-methylguanine by TMZ (11). Additionally, the inflammatory transcription factor NF-κB has been demonstrated to be highly expressed and activated in the GBM cells and can be induced by anticancer drugs, including the alkylating agent TMZ (12,13). To investigate whether the TMZ resistance that occurred in TR cells was due to the expression of MGMT or NF-κB, the expression levels of MGMT, NF-κB and phosphorylated NF-κB were investigated by western blot analysis of the parental and TR/U251 cells. Western blot analysis detected high MGMT expression in the TR/U251 cells, but an extremely low or hardly-detected expression in the parental U251 cells. Similarly, the levels of phosphorylated-P65, a subunit of NF-κB, were increased in the TR/U251 cells compared with the parental cells. No evident difference in NF-κB (P65) expression was found between the two cell lines. The TR/U251 cells exhibited an ~5.4-fold increase in the level of phosphorylated-P65 (Fig. 2). Accordingly, the results indicated that TMZ resistance acquired in TR cells may be associated with MGMT or NF-κB.

BAY 11-7082 enhances TMZ-induced cytotoxicity in TR/U251 cells. Since a high expression of NF-κB was detected in the TR/U251 cells compared with the parental cells, the IκBα inhibitor BAY 11-7082 was used to determine whether inhibition of NF-κB sensitizes TR/U251 cells to TMZ-induced cell death. The morphological and cell proliferation assays for TMZ

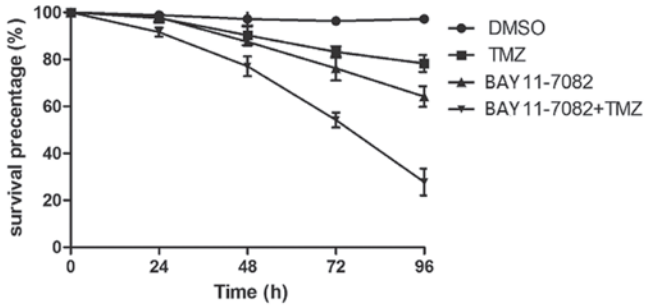

Figure 4. The survival rate of TR/U251 cells in each group. The survival rate of the TR/U251 cells slowly decreased between 97.6±1.4 and 78.3±3.6% when exposed to 80 μM TMZ for 24-96 h. While exposed to 50 μM BAY 11-7082, the viability of the cells decreased between 97.9±2.3 and 64.2±4.4%. The survival rate markedly decreased between 91.6±1.9 and 27.8±5.7% when the cells were exposed to 80 μM TMZ in combination with 50 μM BAY 11-7082 (P<0.01). The data are presented as the mean±standard deviation. TMZ, temozolomide; TR, TMZ-resistant; DMSO, dimethyl sulphoxide.

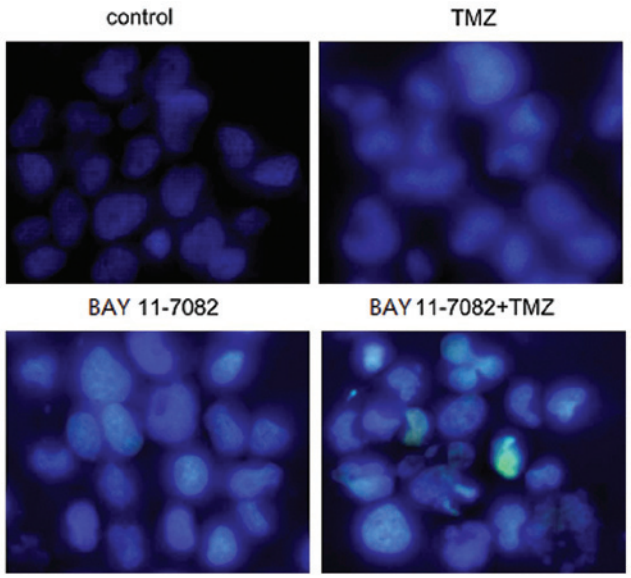

Figure 5. Changes in the morphology of cells treated as a control, or with TMZ, BAY 11-7082 or a combination of the two, as shown by immunity fluorescence. This revealed that the cells in the control group demonstrated a stable morphology, while in the BAY 11-7082 + TMZ-treated group, the cells were completely lysed; the cells had burst and the normal shape was lost. TMZ, temozolomide.

were performed in the presence and absence of BAY 11-7082. To evaluate the inhibition efficiency of BAY 11-7082, the TR/U251 cells were treated with various concentrations of BAY 11-7082. The cell survival rate was found to be inhibited in a dose- and time-dependent manner by BAY 11-7082 and the efficacy was maximized at 100 μM (Fig. 3). Therefore, the IC_{50} of 50 μM was selected to combine with TMZ. In comparison to treatment with TMZ or BAY 11-7082 alone, there was significant damage to the morphology of cells subsequent to treatment with TMZ in combination with BAY 11-7082. When exposed to 80 μM TMZ for 24-96 h, the survival rate for the TR/U251cells slowly decreased between 97.6±1.4 and 78.3±3.6%, while exposure to 50 μM BAY 11-7082 decreased the cell viability between 97.9±2.3 and 64.2±4.4%. By contrast, the survival rate markedly decreased between 91.6±1.9 and 27.8±5.7% when the cells were exposed to 80 μM TMZ in combination with 50 μM BAY 11-7082 (P<0.01; Fig. 4). These results revealed that combination treatment with BAY 11-7082 and TMZ resulted in synergistic decreases in cell viability as compared to each treatment alone.

BAY 11-7082 suppresses the expression of MGMT and enhances the TMZ-induced apoptosis in TR/U251 cells. To further explore the molecular mechanism underlying the effect of BAY 11-7082 on TR cells, an experiment was performed to assess the hypothesis that inhibition of proliferation may be due to changes in the expression of MGMT and pro-apoptotic proteins. First, the expression of NF-κB was detected at various concentrations of BAY 11-7082 for 24 h in TR/U251 cells. As Fig. 4 shows, the optimal concentration of BAY 11-7082 was 20 μM and the optimal concentration of TMZ was 100 μM, which was one-third of the IC_{50} of TMZ in the TR/U251 cells. Based on these conditions, the TR/U251 cells were incubated with 100 μM TMZ or 20 μM BAY 11-7082 alone, or co-treated with BAY 11-7082 and TMZ. Western blot analysis revealed that exposure to BAY 11-7082 alone significantly decreased the level of MGMT in TR/U251 cells compared with the DMSO treatment. However, combination treatment with TMZ completely suppressed the level of MGMT. In addition, the expression of Bcl-2 was elevated following incubation with BAY 11-7082, alone and in combination with TMZ, whereas the decreased level of caspase 3 was observed following treatment with BAY 11-7082 alone or in combination with TMZ (Fig. 5). Therefore, this result was consistent with the association between NF-κB and TMZ resistance, indicating that the inhibition of NF-κB is able to reverse TMZ resistance in TR cells.

Discussion

TMZ chemotherapy combined with surgery and radiotherapy is currently regarded as the standard treatment in glioma. Although oral administration of TMZ is the most effective strategy for the chemotherapy of glioma, its clinical efficacy is limited in the majority of cases by the recurrence or development of intrinsic or acquired TMZ resistance (14). Since the current understanding of mechanism behind TMZ resistance remains unclear, the present study aimed to establish a resistant cell line model to explore the molecular mechanism of TMZ resistance in glioma.

As a DNA repair enzyme, MGMT is a protein that can transfer the alkylated group of methylguanine from the O^6 position to its own cysteine residue (4,5,15), so that the TMZ-induced guanine damage in DNA can be repaired. Previous studies have demonstrated that the levels of MGMT vary considerably in glioma cells, which can be divided into the MGMT repair-deficient cells and cells with a sufficient MGMT repair (16). Provided that the cytotoxicity effects of the alkylating agent can be reduced in cells with a high level of endogenous MGMT activity, the MGMT expression may be considered to be the most important molecular predictor of TMZ resistance and prognosis in glioma. In the present study, a TR glioma cell model was successfully generated by exposure of parental U251 cells to a gradually increasing TMZ

concentration. The resulting TR/U251 cells exhibited a strong resistance to TMZ, demonstrating an IC_{50} that was six times higher than the IC_{50} of the parental cells, and also demonstrated a good stability for almost three months subsequent to TMZ withdrawal. Notably, the TR/U251 cell line exhibited high levels of MGMT and also demonstrated a more stable morphology and more vigorous cell proliferation compared with the parental U251 cells. Accordingly, it was hypothesized that this may be associated with the high expression of MGMT in the TR/U251 cells, as MGMT-positive cells selectively survive during the stepwise screening process. However, MGMT-negative cells are eliminated, which is in agreement with the earlier stepwise inducing method that established the anticancer drug-resistant cells.

Constitutively activated NF-κB is one of the major transcription factors associated with GBM and it may be responsible for aspects of cancer development that include the inhibition of apoptosis, invasion, immune response and inflammation (9). Previous studies have demonstrated that GBM tissues possess constitutive NF-κB activation, which is also associated with the grade of GBM and a worse prognosis (10,11). Additionally, increasing evidence demonstrates the key role of the NF-κB signaling pathway in the drug resistance of GBM. NF-κB is activated in response to treatment with cytotoxic drugs, such as taxanes, vinca alkaloids and topoisomerase inhibitors. Similarly, alkylating agents, including carmustine and TMZ, can also activate NF-κB through DNA damage pathway activation (10,17). In the present study, stepwise exposure to TMZ was found to result in a marked increase in the NF-κB subunit P65 and phosphorylated-P65 compared with the parental cell. Inactivated NF-κB usually locates to the cytoplasm. Upon DNA damage caused by alkylating agents, activated ataxia telangiectasia mutated kinase triggers multiple events to promote cell survival and facilitate repair, including the activation of NF-κB (18). Therefore, the vigorous cell proliferation may also be due to the constant self-repair of DNA damage and NF-κB activation in GBM.

Taking into account that MGMT and NF-κB are each highly expressed in the TR/U251 cells, then the association between the two may be associated with TMZ resistance. The present study revealed that the IκBα inhibitor BAY 11-7082 combined with TMZ markedly suppressed the level of MGMT in TR/U251 cells and promoted the initiation of TMZ-induced apoptosis (Fig. 2), indicating the key role of NF-κB in the regulation of MGMT expression. Previous studies have also demonstrated that forced expression of the NF-κB p65 subunit in HEK293 cells induced an increase in MGMT expression, whereas a lack of NF-κB completely abrogated the induction of apoptosis, independent of MGMT promoter methylation (19). Furthermore, resveratrol reverses TMZ resistance by regulation of MGMT in T98 GBM cells in a NF-κB-independent manner (20). The aforementioned studies revealed a significant correlation between the extent of NF-κB activation and MGMT expression in glioma cells. Provided that NF-κB and MGMT are each involved in the regulation of TMZ resistance, the two can also be considered to be promising targets for novel therapeutic approaches in glioma. Therefore, the inhibition of NF-κB can decrease MGMT activity and combine with TMZ treatment to possibly offer clinical benefits for glioma patients exhibiting TMZ resistance.

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