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Original Paper

Study on Therapeutic Action and Mechanism of TMZ Combined with RITA Against Glioblastoma

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Key Words
Apoptosis Signal-regulating Kinase 1 • Central nervous system • Glioblastoma multiforme • Reactivator of p53 and induction of tumor apoptosis • Temozolomide

Abstract
Background/Aims: Glioblastoma multiforme (GBM) is a malignant and aggressive central nervous system (CNS) tumor with high mortality and low survival rate. Effective treatment of GMB is a challenge worldwide. Temozolomide (TMZ) is a drug used to treat GBM, while the survival period of GBM patients with positive treatment remains less than 15 months. Reactivating p53 and Inducing Tumor Apoptosis (RITA) is a novel potential anti-cancer small molecular drug. Thus, it is essential to discover novel targets or develop effective drugs combination strategy to treat GBM. ***Methods:*** The U87 cells and U251 cells (p53 mutated) were treated with DMSO and 1, 5,10, 20 μM RITA, TMZ, RITA+TMA or PFT-α. The cell proliferation was measured using the MTS cell proliferation assay. The cell apoptosis was analyzed by Annexin V-FITC/PI Apoptosis Detection Kit. The key protein expression level was evaluated by WB. Molecular docking and molecular dynamics (MD) simulation methods were applied to simulate the interaction between RITA and ASK1. ***Results:*** Herein, we found that combination RITA and TMZ effectively inhibited the proliferation of U87 cells and promoted the apoptosis of U87 cells. Then the mechanism of RITA and TMZ treating GBM were further studied by detecting the expression of the proteins associating with p53 pathway, such as ASK1, Bax, and so on. RITA bound to the amino acids residues in the activation domain of the ASK1, then induced the conformation change of ASK1 receptor, activated ASK1 and caused a series of signal transduction, further resulted in the physiological effects. ***Conclusion:*** Taken together, the RITA suppressed the cell proliferation in glioblastoma via targeting ASK1.

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Introduction

Glioblastoma multiforme (GBM) is one of the most common central nervous system (CNS) disease, which is a collection of tumors caused by glial cells or their precursors within CNS [1-3]. GBM is a malignant and aggressive tumor [3]. The mortality of the patient with GBM remains high and its overall survival rate below25% [4]. Effective Treatment of GMB is a challenge in the whole world. Currently, maximal surgical resection followed by radiotherapy with concomitant and adjuvant temozolomide (TMZ) are common treatment for GBM. The most effective treatment method for glioblastoma is the maximal safe surgical resection followed by concurrent treatment with TMZ as well as radiation therapy followed by adjuvant TMZ [4-6]. Unfortunately, GBM is prone to drug resistance and recurrence after treating with TMZ, and the treatment efficacy is still suboptimal as two-thirds of patients die by 2 years from diagnosis [5] The survival period of the GBM patients with positive treatment remains less than 15 months [7]. Thus, it is essential to discover novel targets or develop effective drugs combination strategy to treat GBM.

The tumor suppressor protein p53 plays an important role in protecting the organism from cancer [8]. Most of human cancers are caused by mutation or deletions of alleles in p53 [8]. Reactivation of p53 and induction of tumor apoptosis (RITA) is a novel potential anti-cancer small molecular drug [9-11]. RITA induces expression of p53 target genes and massive apoptosis in various tumor cells lines expressing wild-type p53 [9, 12]. RITA may serve as a lead compound for the development of an anticancer drug targets tumors with wild-type p53 [9]. Previous results have found that RITA could efficiently inhibit the growth of tumor and induce the apoptosis of tumor cells [12, 13]. However, the mechanism underlying potential pro-apoptotic effect has not been studied clearly. Previous research has presented that RITA inhibits the cancer via activating the tumor suppressor gene p53 (both p53 wt and p53 mutant (mt) cells) [14]. RITA could also bind to proteins (such as ASK1, HIPK2) relating with the p53 pathway, further cut off the expression of anti-apoptotic proteins Mcl-1, Bcl-2, MAP4 and erythropoietin involving in key oncogenic pathway [11]. RITA could block the Akt pathway and down-regulate c-Myc, cyclin E and β-catenin [11]. Apoptosis signal-regulating kinase 1 (ASK1) is a member of the mitogen-activated proteinkinases (MAP3K), which plays vital roles in regulating the cell apoptosis process [15]. Hong Chang et al. found that ASK1 is the direct receptor protein for RITA, [16, 17] while the mechanism of interaction between RITA and ASK1 needs further investigation. In addition, some study also found that traditional glioma cell lines which did not express a functional p53 were more sensitive to TMZ than the cell lines with functionally intact wild type p53 expression, and the absence of functional p53 could increase the TMZ sensitivity in traditional glioma cell lines. (J Neurooncol. 2011 Mar;102(1):1-7.)

Herein, we evaluated the effect of RITA and TMZ on the proliferation and viability of human glioblastoma U87 cells. The results indicated that RITA effectively inhibited the proliferation of U87 cells and promoted the apoptosis of U87 cells, whose effect was stronger than that of TMZ. Combination RITA and TMZ further decreased the proliferation of U87 cells and enhanced the apoptosis of U87 cells, which suggested that combination of RITA and TMZ was a more effective strategy to treat glioblastoma compared with application of RITA or TMZ single. In addition, we found that RITA and ASK1 could form stable complex with lower energy and better affinity, causing a series of signal transduction, further resulted in the physiological effects. Therefore, our study clarified the mechanism about the function of RITA against human glioblastoma, and further developed a novel therapy model for human glioblastoma, even other cancers.

Materials and Methods

Materials

U87 and U251 cells were obtained from landbiology Technology Co. Ltd (Guangzhou, China). RITA was obtained from CAYMAN (Cat. 10006426), TMZ was obtained from Sigma (Cat. T2577-25MG), DMSO was obtained from Sigma (Cat. D2650-5X5ML), Pifithrin-α (PFT-α) was obtained from Beyotime Biotechnology (Cat. S1816). Fetal bovine serum (FBS), DMEM medium, streptomycin and PBS were obtained from Hyclone.

Cell culture

The U87 cells and U251 cells (p53 mutated) were cultured in DEME high glucose medium supplemented with 10% fetal bovine serum (FBS; Hyclone, USA) and 1% streptomycin according to standard procedures. Cell were incubated in a humidified incubator at 37℃ supplied with 5% CO_2. Cells were treated with DMSO and 1, 5, 10, 20 μM RITA, TMZ, RITA+TMA or PFT-α. All of drugs were dissolved in DMSO.

Cell proliferation assay

Cell proliferation was measured using the MTS cell proliferation assay kit (ab197010). The absorbance at 490 nm (OD490) of all samples were measured. The proliferation rates of cells from day 0 to day 3 were calculated based on OD490 values.

The apoptotic analysis of U87 cells were detected by FACS

The U87 cells treated with DMSO, RITA and RITA+TMZ were harvested and washed after 48 h incubation. The cell apoptosis was analyzed by Annexin V-FITC / PI Apoptosis Detection Kit (Catalog no. KGA106; Nanjing Keygen Biotech Co., Ltd) in the light of the manufacturer's instruction. Annexin V-fluorescein isothiocyanate (FITC) and propidium iodide (PI) were added to fixed cells for 20 min in darkness at room temperature. All samples were immediately measured on FACSort flow cytometry (BD, USA). The data were analyzed using the Cell Quest 3.0 software (BD, USA). The percentage of early apoptosis, late apoptosis and total apoptosis were analyzed.

Western Blotting (WB)

The protein expression level of ASK1, Bax, MDM4, P21, PUMA, P53, MDM2 and Caspase 3/8 in U87 cells treated with RITA, RITA+TMZ or PFT-α were detected by Western Blotting (WB). Protein concentrations were determined using BCA Protein Assay Kit (Keygen Biotech). Proteins extracted from U87 cells treated with RITA, RITA+TMZ or PFT-α were separated in 10% SDS-polyacrylamide gels, electroblotted onto an Immobilon-P transfer polyvinylidene fluoride membrane 20 (Millipore, USA), detected with rabbit anti-human ASK1 antibody (A6274, 1:500, abclonal, USA), Bax (ab32503, 1:1000, abcam, USA), MDM4 (105211, 1:500, GTX), P21 (BS12b9, 1:1000, bioworlcle), PUMA (A2846, 1:1000, abclonal, USA), P53 (2527S, 1:1000, Cell Signaling), MDM2 (A0345, 1:1000, abclonal, USA), Caspase 3 (ab32351, 1:1000, Abcam), Caspase 8 (9496, 1:1000, Cell Signaling), a rabbit anti-human-GAPDH antibody (KC-5G5, 1: 10, 000, KangCheng bio, China) and then visualized by a commercial Immobilon Western HRP Substrate (WBKLS0500, Millipore, USA) under dark conditions.

Molecular docking and molecular dynamics (MD) simulation methods

The crystal structure of N-terminal domains of human ASK1 were extracted from the Protein Data Bank (PDB) (PDB ID: 4BF2), which contains two polypeptide chains and three small molecular legends (ACT, GOL and STU). The three small molecular legends (ACT, GOL and STU) were deleted during pretreatment. The H_2O molecular and metal ions were removed while polar hydrogen and point charge were added by Clean Protein software. The molecular structure of RITA is extracted from the PubChem Compound (CID: 374536). Molecular docking of compounds RITA to active sites of ASK1 was performed by the Docker software package. The CDOCKER-ENERGY, CDOCKER_INTERACTION_ENERGY and RMSD were used to evaluate conformations during docking simulation. The ten optimal conformations were obtained for next step.

Based on the results of molecular docking, the protein-ligand complex model with optimal energy was introduced, the force filed and solvent environment was provided by CHARMM. Then, the molecular dynamic calculation was performed as previous research [18]. The trajectory analysis was performed to calculate the system energy and interaction energy.

Results

RITA combined with temozolomide (TMZ) inhibited the proliferation of human glioblastoma U87 cells

To study the effect of RITA and TMZ on the proliferation and viability of U87 cells, MTS assay were performed (Fig. 1). Compared with the control group (DMSO treated U87 cells), the results showed that 1, 5, 10 and 20 μM RITA potently inhibited the viability at 72 h with inhibitory rates of 25.94%, 31.58%, 38.16% and 41.38%, respectively (Fig. 1A). However, the inhibitory rates of TMZ aren't so high as RITA (Fig. 1B). The inhibition effects of RITA on viability of U87 cells were time-dependent and dose-dependent. Total concentration of RITA and TMZ were 1, 5, 10 and 20 μM, the corresponding inhibition rates of U87 cells were 29.21%, 38.59, 44.67 and 52.11%, respectively (Fig. 1C). RITA combined with TMZ induced a more potently inhibition effect on the proliferation of U87 cells. Moreover, the efficacy of RITA and TMZ were positive correlation, including concentration and experimental time.

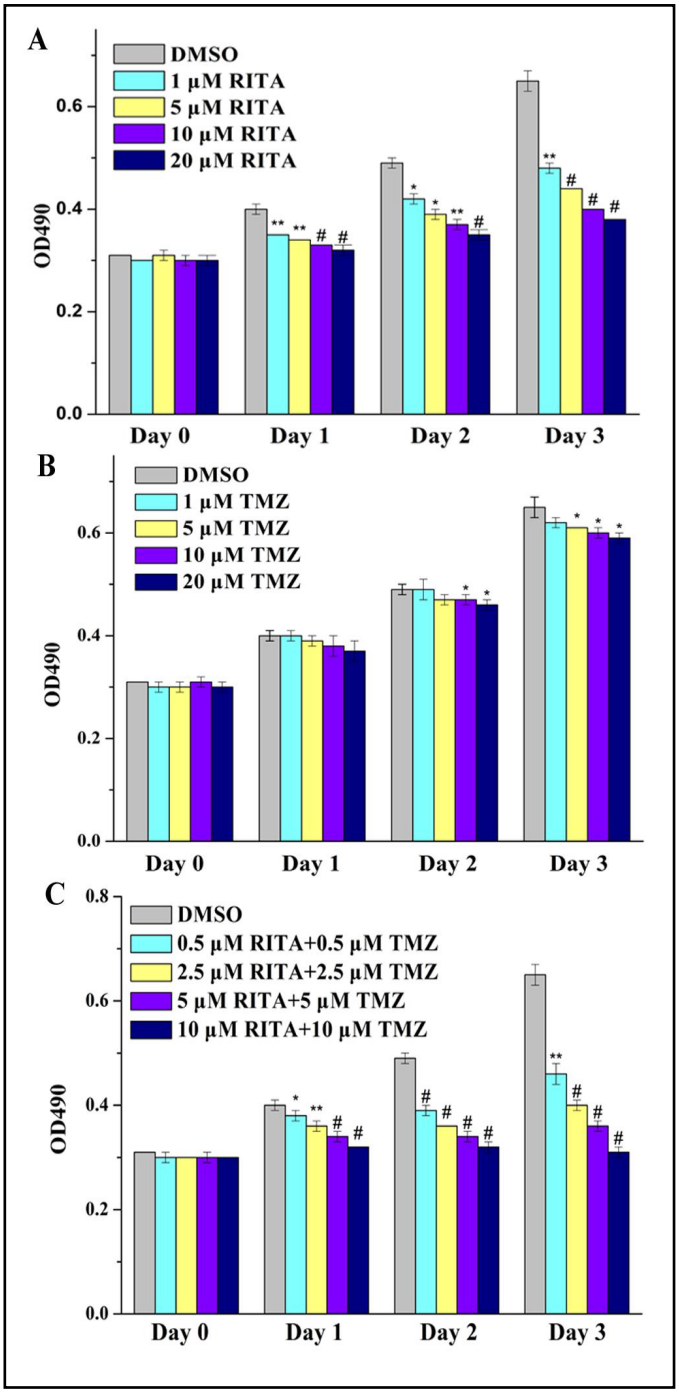

Fig. 1. Effect of RITA and TMZ on the proliferation of U87 cells. (A) effect of RITA on the proliferation of U87 cells. (B) effect of TMZ on the proliferation of U87 cells. (C) effect of RITA combined with TMZ on the proliferation of U87 cells. The OD490 values were determined at various experimental time. The data were presented as Mean ± SD, n=3, *p<0.05, **p<0.005, #p<0.001 vs DMSO group.

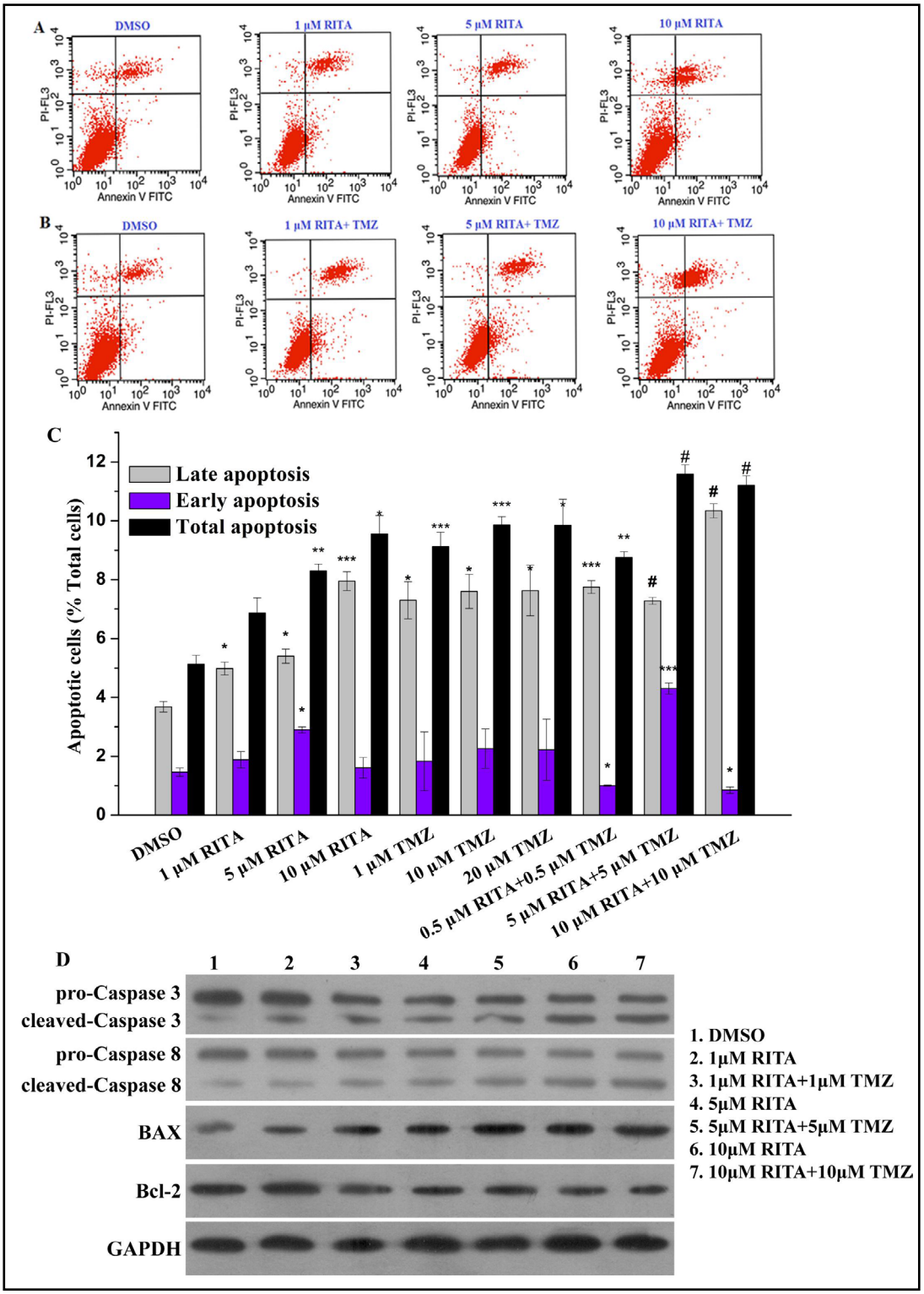

Fig. 2. RITA and TMZ promoted the apoptosis of U87 cells. (A) the effect of RITA on the apoptosis of U87 cells detected by FITC. (B) the effect of RITA and TMZ on the apoptosis of U87 cells detected by FITC. (C) the percentage of early apoptosis, late apoptosis and total apoptosis in U87 cells treated with DMSO, various concentration RITA, various concentration RITA+TMZ. The data was presented as Mean ± SD, n=3, *p<0.05, **p<0.01, ***p<0.005, #p<0.001 vs DMSO group. (D) the effect of RITA and TMZ on the apoptosis of U87 cells evaluated by western blot.

RITA and TMZ promoted the apoptosis of U87 cells

To investigate the effect of RITA and TMZ on the apoptosis of U87 cells, the apoptotic analysis of U87 cells were detected by flow cytometry (Fig. 2). The percentage of early apoptosis, late apoptosis and total apoptosis in U87 cells treated with DMSO, various concentration RITA, various concentration RITA+TMZ were showed in Fig. 2A-2C. The percentage of late apoptosis and total apoptosis were increased with the increasing concentration of RITA, which suggested that the apoptotic effect of RITA on U87 cells were dose-dependent. The results also indicated that the strongest effect of RITA concentration on the early apoptosis of U87 cells was 5 μM. The percentage of total apoptosis (early + late apoptosis) in 5 μM RITA+TMZ group are similar to that in 10 μM RITA+TMZ group, and higher than that in 10 μM RITA group, which suggested that TMZ enhanced the apoptotic effect of RITA in U87 cells. Statistical analysis suggested that the difference between the DMSO group and the RITA or RITA+TMZ group were significant (*p<0.05, **p<0.01 vs DMSO group). The apoptosis results were further confirmed with western blot, and immunoblot results indicated that the combination of RITA and TMZ increased the level of apoptosis genes (cleaved-Caspase 8 and BAX) and decreased the level of anti-apoptosis genes (Bcl-2), indicating the better therapeutic action of combined group (Fig. 2D). However, the combination didn't show any obvious effect on the level of either pro-Caspase 3 or cleaved-Caspase 3 compared with signal treatment group.

RITA inhibited the proliferation of U87 cells via promoting the p53

The Pifithrin-α (PFT-α) is an inhibitor of p53 and can protect against various p53-mediated genotoxic agents [19, 20]. To determine the effect of p53 and PFT-α on the cell proliferation of U87, PFT-α was applied to treat the U87 cells. Compared with the DMSO group, PFT-α promoted the cell proliferation of U87 and the effect of PFT-α was time-

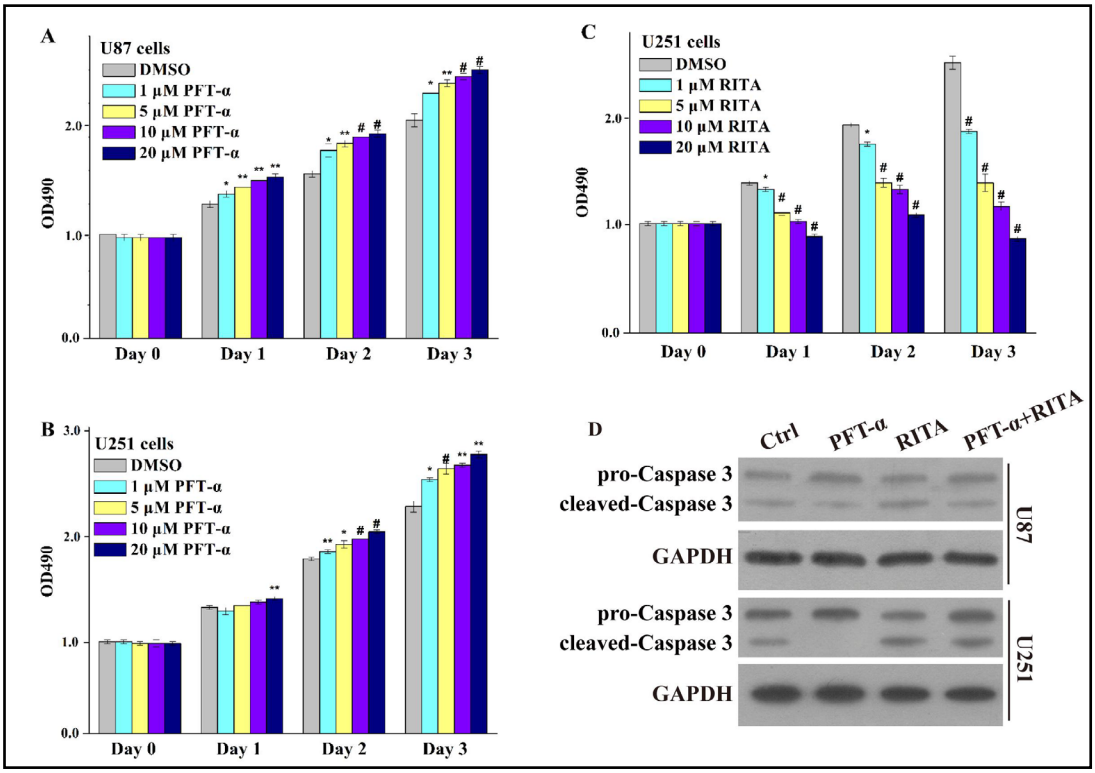

Fig. 3. RITA inhibited the proliferation of U87 cells via promoting the p53. (A) the effect of PFT-α on the proliferation of U87 cells. (B) the effect of PFT-α on the proliferation of U251 cells. (C) comparing the effects of RITA on the proliferation of U87 cells and U251 cells. The data were presented as Mean ± SD, n=3, *p<0.05, **p<0.005, #p<0.001 vs DMSO group. (D) Effect of PFT-α and RITA on cell apoptosis.

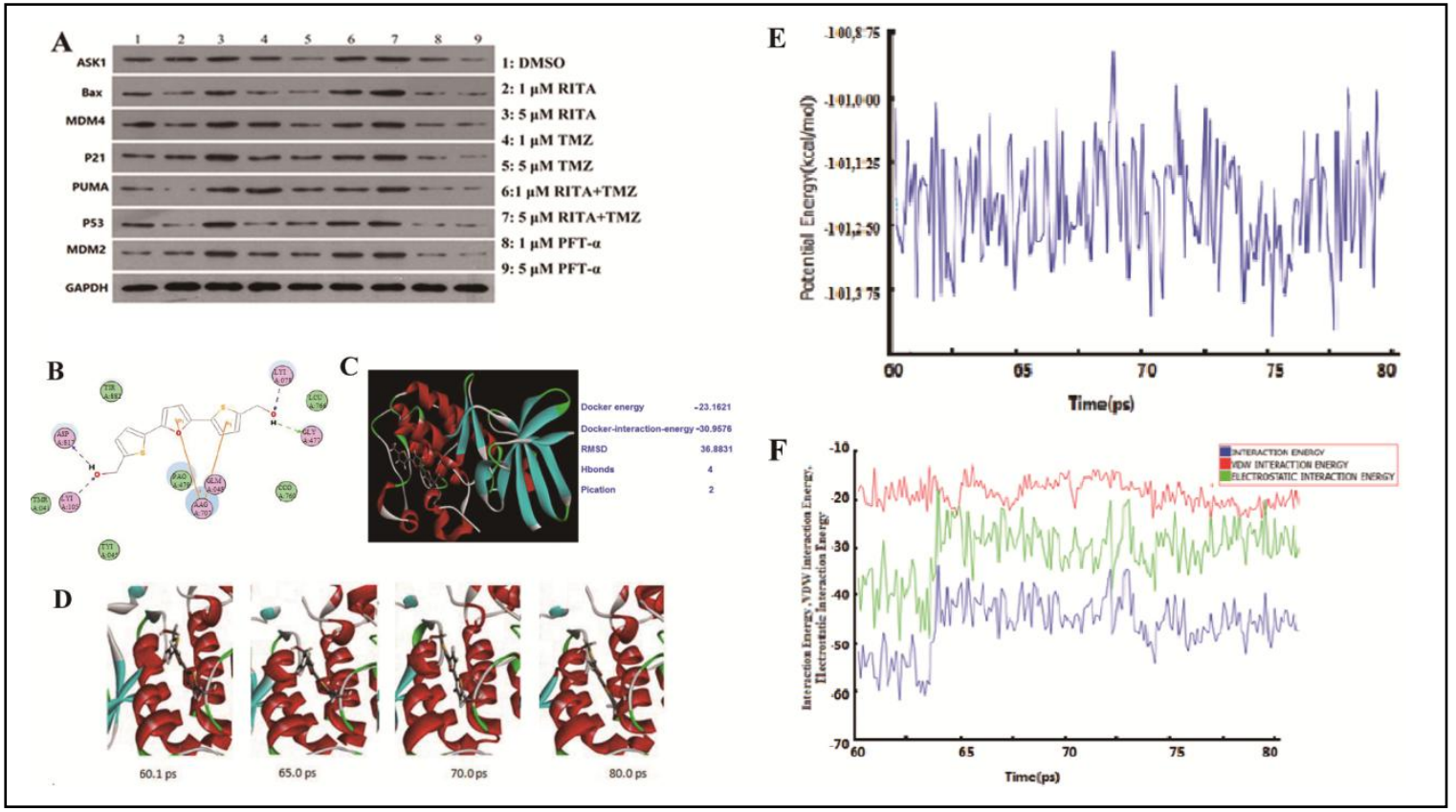

Fig. 4. The targeting molecular of RITA. (A). detecting the protein expression of ASK1, Bax, MDM4, P53, MDM2, P21, and PUMA by WB. (B) The dynamic molecular conformations of the backbone atoms of the average structure of ASK1-RITA complex in active site 3 in the period of 60.1-80.0ps. (C) The time dependence of Potential Energy of ASK1-RITA complexes in the period of 60-80ps MD simulations for complex sits 3 energy curve. (D) The time dependence of Interaction Energy/Van der Waals Energy/Electrostatic Energy of ASK1-RITA complexes in the period of 60-80ps MD simulations. complex Site3 energy curve. Blue line represents Interaction energy, Red line represents VdW Interaction Energy, Green line represents Electrostatic Interaction Energy. Herein, molecular docking of compounds RITA to active sites of ASK1 was performed by the Docker software package. The CDOCKER-ENERGY, CDOCKER_INTERACTION_ENERGY and RMSD were used to evaluate conformations during docking simulation. F. The time dependence of Interaction Energy/Van der Waals Energy/ Electrostatic Energy of ASK1-RITA complexes in the period of 60-80ps MD simulations. Blue line represents Interaction energy, Red line represents VdW Interaction Energy, Green line represents Electrostatic Interaction Energy.

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dependent and dose-dependent. U251 cells was p53 mutated human glioblastoma cells while U87 was p53 wild type glioblastoma cells. Comparing the effects of RITA on the proliferation and apoptosis of U87 cells and U251 cells (Fig. 1A and Fig. 3, the raw quantitative data were shown in Supplementary Table 1 - For all supplemental material see www.karger.com/10.1159/000495923), the inhibitory effect of RITA on U251 cells was stronger than that on U87 cells. To further confirm this conclusion, both U87 cells and U251 cells were treated with PFT-α, RITA and PFT-α+RITA respectively, and the apoptosis was analyzed with immunoblot. The results showed that RITA could promote cell apoptosis obviously, which was rescued by the co-treatment of PFT-α. In addition, the immunoblot result also indicated that the promoting apoptosis effect of RITA on U251 cells was stronger than that on U87 cells as well. The results suggested that RITA inhibiting the proliferation and promoting cell apoptosis of glioblastoma cells is dependent with p53.

Detecting the targeting molecules of RITA

To uncover the targeting molecular of RITA, the protein expression of ASK1, Bax, MDM4, P53, MDM2, P21 and PUMA in U87 cells treated with DMSO, RITA, TMZ and RITA+TMZ were determined by WB, respectively (Fig. 4). Compared with DMSO group, 5 μM RITA and 5 μM RITA+TMZ obviously increased the expression of ASK1, Bax, MDM4, P53, MDM2, P21 and PUMA. PFT-α decreased the expression of ASK1, Bax, MDM4, P53, MDM2, P21 and PUMA, compared with the DMSO group, which is consistent with the PFT-α promoting the proliferation of U87 cells (Fig. 4A).

To further investigate the interaction between ASK1 and RITA, molecular docking and molecular dynamics (MD) simulation methods were performed (Fig. 4B-4D). To optimize the docking energy and results and better stimulate the particle motion in human solvated environment, themolecular dynamic calculations between RITAand the active pocket 3 of ASK1 were performed (Fig. 4B). The active pockets 3 was formed between 2 peptides α-helix. The molecular trajectory remains stable after 70.0 ps. The conformation between 70.0 ps and 80.0 ps were substantially identical. The time dependence of Potential Energy of ASK1-RITA complexes in the period of 60-80ps MD simulations for complex pocket 3 energy curve was showed in Fig. 4C. The stable state was reached after the short-term stimulation. Energy fluctuated at about -101175 kcal/mol, the internal energy of the system had no obvious fluctuation, lower energy value, stable extension, which indicated that the complex system was very stable. To further calculate the interaction energy between any atom in the RITA-4BF2 pocket 3 complex system, the time dependence of interaction energy/Van der Waals Energy/ Electrostatic Energy of ASK1-RITA complexes in the period of 60-80ps were calculated. The interaction energy/Van der Waals Energy/ Electrostatic Energy of ASK1-RITA complexes distributed between -60 and -10 (Fig. 4D-4F). Theoretically, we speculated that RITA bound to the amino acids residues in the activation domain of the ASK1, then induced the conformation change of ASK1 receptor, activated ASK1 and caused series of signal transduction, further resulted in the physiological effects. Taken together, the RITA suppressed the cell proliferation in glioblastoma via targeting ASK1.

Discussion

GBM is considered as one of the most aggressive primary malignant brain tumor in the world [3]. However, the treatment of GBM is still a challenge so far. Some groups have indicated that the TMZ is an effective drug to treat GBM, but the survival period remains below 15 months and the recurrence is high [1, 7]. Therefore, developing novel treatment methods are very necessary. RITA could efficiently suppress the growth of tumor and might act as apotentialanti-cancer molecule. In this study, we mainly speculated that the combination RITA and TMZ probably was a novel strategy to treat GBM.

In this paper, we found that the inhibitory effect of combination RITA and TMZ on the proliferation and viability of U87 cells was stronger than that of RITA or TMZ single. Compared with the RITA or TMZ group, RITA+TMZ obviously promoted the apoptosis of U87 cells.

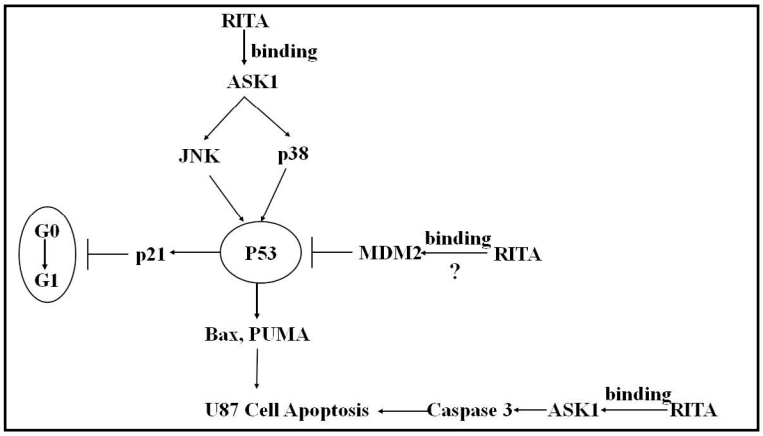

Fig. 5. The mechanism of RITA inducing the apoptosis of U87 cells.

The results suggested that combination RITA and TMZ indeed a more effective strategy to treat GBM. RITA could inhibit the proliferation of U87 cells and U251 cells, which is in accord with RITA activating p53 (wt) and p53 (mt) [21]. The mechanism of RITA + TMZ treating U87 cells was further studied by experiments. The experimental results showed that RITA+TMZ upregulated the expression of ASK1, Bax, Caspase 3, MDM4, P53, P21 and PUMA, which is consistent with the inhibitory effect of RITA+TMZ on the proliferation of U87 cells and previous result [16]. Previous results has found that ASK1 is the direct receptor protein for RITA [17]. While the mechanism of interaction between RITA and ASK1 needs further investigation. Herein, the conformation of ASK1+RITA complex and their interaction were simulated. The results showed that stable ASK1-RITA complex was formed with better kinetic balance, lower potential energy and total energy as well as higher binding flexibility. To further confirm the regulating relationship between RITA and ASK1, ASK1-siRNA knockdown experiment was further performed herein. Even though the ASK1-siRNA showed some effect to downregulate the level of ASK1 in U87 cells, the upregulation of ASK1 as well as the cleaved-Caspase 3 could be induced by the treatment of RITA, which is similar to the control siRNA group (Supplementary Fig. 1). Therefore, a total ASK1-knockout cell line is still necessary for such evaluation.

MDM2 is oncogenic proteins and it form the protein-protein interaction with the N-terminal trans-activation domain of P53, thereby blocking the P53 transcriptional activity [22, 23]. RITA could bind with P53 and inhibit interaction of P53 and MDM2, which effect the negative regulation of P53 downstream genes and restore the function of apoptosis induced by P53 in tumor cells [16, 24]. But other research indicates that RITA does not block P53-MDM2 binding *in vitro* [25]. The relationship between p53, RITA and MDM2 needs further investigation. In this paper, we speculated that the mechanism of RITA inducing the apoptosis of U87 cells based on our results and precious reports (Fig. 5, a summary of both previous published results and the results reported in this paper). RITA binds with ASK1and form a stable complex, then change the conformation of the active domain of ASK1; ASK1 activating P53 via modulating JNK or p38, or upregulating the expression of Caspase 3 to induce the apoptosis of U87 cells. Activated P53 also affected the p21 and caused the cell cycle arrest. However, we also noticed the similar effect of RITA on the two different cell lines, which were differed by the expression of wild type p53 and mutated p53. This means RITA still have other function to inhibit cell proliferation and promote cell apoptosis. However, the mechanism still need our further exploration.

Acknowledgements

Wei Zhao, Bao Zhang and Qinghua Wu designed the study, performed the primary experiments and wrote the draft; Zhongxu Cao and Weiwei Xiao collected and analyzed the data; LiZhu, Qian Xie and Ling Li did the duplicated experiments and editing the manuscript. This work was financially supported by the National Natural Science Foundation of China (81301911).

Disclosure Statement

The authors declare that they have no conflict of interest.

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