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Original Article

Aggravated DNA damage as a basis for enhanced glioma cell killing by MJ-66 in combination with minocycline

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Abstract: Despite recent advances in the treatment of malignant glomas, the prognosis of patients remains very poor and more efficient therapeutic approaches are urgently needed. In the present study, we investigated whether 2-(naphthalene-1-yl)-6-pyrrolidinyl-4-quinazolinone (MJ-66), a synthetic quinazolinone analog, induces glioma cell death through DNA damage. Treatment of C6 glioma cells with MJ-66 resulted in a time-dependent increase in γ-H2AX and increased the appearance of nuclear γ-H2AX foci. MJ-66 interfered with G2/M DNA damage checkpoint through increasing phosphorylated levels of Chk1 and Cdc25C. UCN-01, a Chk1 inhibitor, reversed MJ-66-induced activation of Cdc25C and caspase 3. MJ-66 inhibited tumor growth and prolonged survival time in intracranial glioma xenograft model. The combination of MJ-66 and Mino enhanced DNA damage and synergistically inhibited tumor growth and prolonged survival time in intracranial glioma xenograft model. These results suggest that the combination of MJ-66 and Mino may be developed as a new therapeutic strategy against malignant gliomas.

Keywords: Gliomas, DNA damage, quinazolinone, minocycline, brain tumor

Introduction

Malignant gliomas are the most common primary brain tumors. Although the prevalence of malignant gliomas is relatively low compared with other cancers such as lung, liver, colorectal, breast and prostate cancers, it has disproportionately high mortality [1]. For patients with the most severe, aggressive form of malignant glioma (grade IV glioma or glioblastoma multiform, GBM), median survival time from the time of diagnosis without any treatment is 3 months and is around 1-2 year with treatment [2-4]. The current treatment regimen for GBM patients is surgical resection, radiotherapy and chemotherapy [5, 6]. However, the infiltration and location of GBM in the brain makes it very difficulty or impossible to completely remove GBM by surgery [7]. In a majority of cases, with or without surgical excision, combination radiation treatment and chemotherapy are used to combat the malignancy. Despite improvements provided by cytoreductive surgery and primary chemotherapy, the prognosis of patients with malignant gliomas remains very poor. Therefore, development of novel strategies and the identification of more efficient therapeutic approaches are urgently needed.

Minocycline (Mino) is a member of the tetracycline family with broad-spectrum antibiotic activity against acne and rosacea [8, 9]. It is a small, highly lipophilic molecule that can be readily absorbed from the gut after oral ingestion and capable of crossing the blood-brain barrier [10]. We have previously shown that Mino induced nonapoptotic cell death in glioma cells which were associated with the presence of autophagic vacuoles in the cytoplasm. Pretreatment with autophagy inhibitor 3-methyladenine (3-MA) suppressed the induction of acidic vesicular organelles and the accumulation of LC3-II to the autophagosome membrane in glioma cells treated with Mino. Mino effectively inhibited tumor growth and induced autophagy in the xenograft tumor model of C6

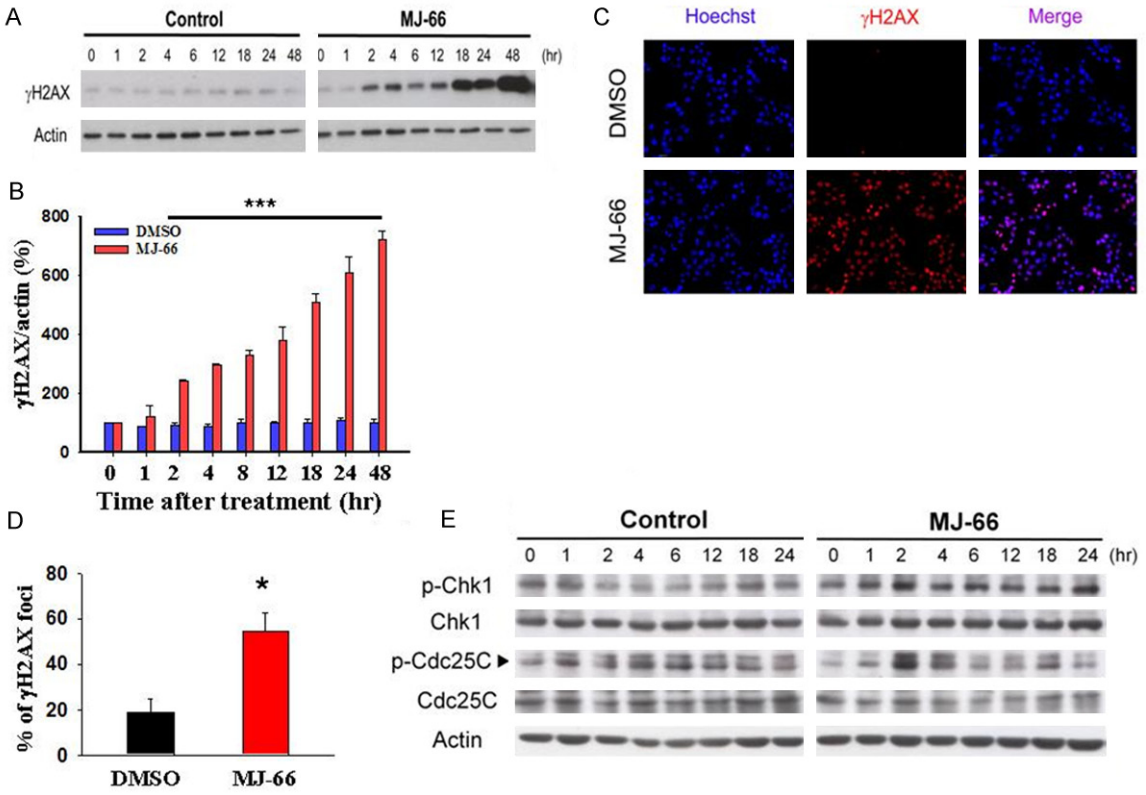

Figure 1. MJ-66 increases γH2AX expression in C6 glioma cells. A. C6 glioma cells were treated with MJ-66 (60 nM) or vehicle (DMSO) for indicated times and cell lysates were blotted with antibody for γH2AX. B. Quantitative analysis of γH2AX expression after treatment with MJ-66 for the indicated times. *p<0.05, **p<0.01 vs. DMSO. C. Glioma cells were treated with MJ-66 (60 nM) for 48 hrs and morphology was analyzed with Hoechst 33342 and γH2AX staining. D. Quantification of γH2AX/Hoechst 33342-positive cells after treatment with MJ-66. *p<0.05 vs. DMSO. E. MJ-66 increases Chk1 and Cdc25C phosphorylation in C6 glioma cells. C6 glioma cells were treated with MJ-66 (60 nM) or vehicle (DMSO) for indicated times and cell lysates were blotted with antibodies for p-Chk1, Chk1, p-Cdc25C and Cdc25C.

glioma cells. These results suggest that Mino may kill glioma cells by inducing autophagic cell death [11].

We previously investigated the effect of 2-(naphthalene-1-yl)-6-pyrrolidinyl-4-quinazolinone (MJ-66) on malignant glioma cells. We found that MJ-66-induced cell death was associated with multinucleated phenotype and multipolar spindles that are typical characteristics of mitotic catastrophe [12]. Since Mino and MJ-66 induced glioma cell death through different mechanism, in the present study, we set out to explore whether the combination of these two agents would potentiate the antitumor potency. In this report, we show that this is indeed the case. The addition of MJ-66 together with Mino to glioma cells in culture severely triggered DNA damage and caused substantially more cell death than either drug alone. The synergistic effect could also be observed in xenograft tumor model. Together, our study establishes MJ-66 and Mino as a novel drug combination that should be evaluated further as a potentially effective anticancer therapy.

Materials and methods

Cell culture and regents

The human glioma cell lines U87 provided by Dr. Michael Hsiao (Genomics Research Center, Academia Sinica, Taiwan) was cultured in Dulbecco's Modified Eagle medium (DMEM, Caisson) supplemented with 10% fetal bovine serum (FBS, Sigma-Aldrich), 2 mM L-glutamine (Caisson), 100 U/ml penicillin, and 0.1 mg/ml streptomycin (Caisson). The rat glioma C6 cell line provided by Dr. Shun-Fen Tzeng (National

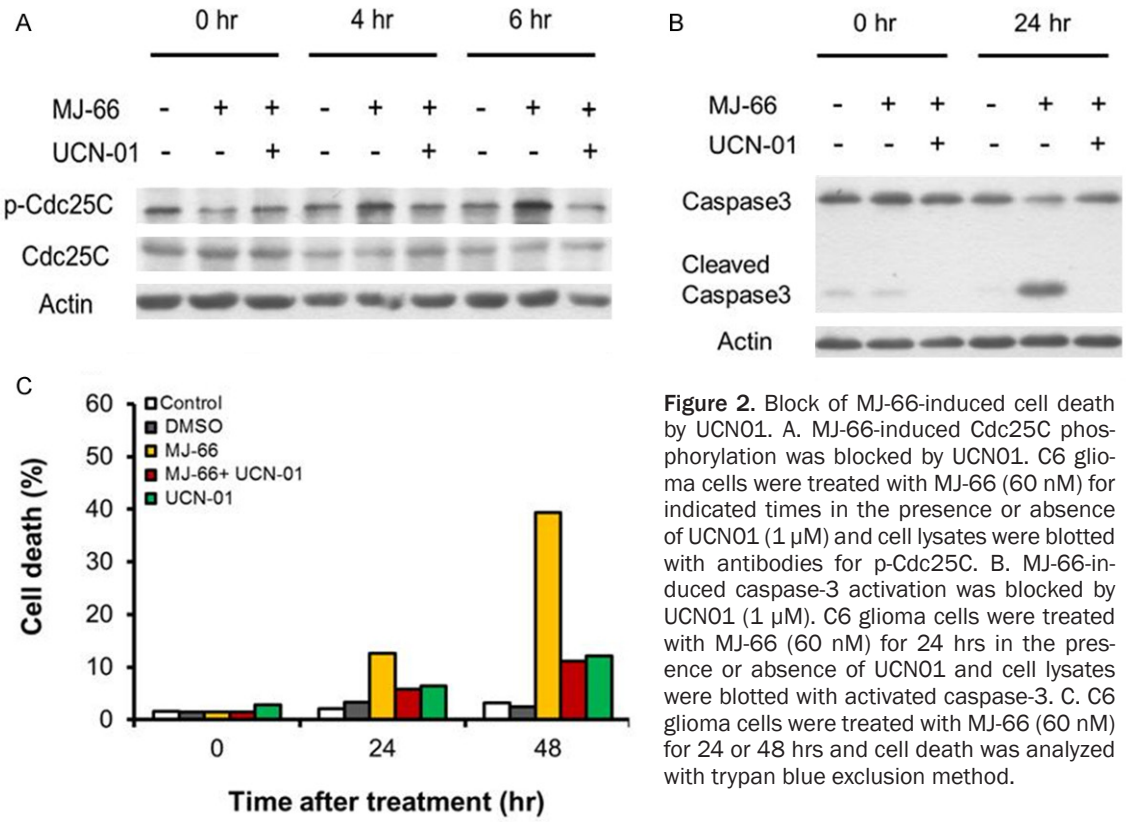

Figure 2. Block of MJ-66-induced cell death by UCN01. A. MJ-66-induced Cdc25C phosphorylation was blocked by UCN01. C6 glioma cells were treated with MJ-66 (60 nM) for indicated times in the presence or absence of UCN01 (1 μM) and cell lysates were blotted with antibodies for p-Cdc25C. B. MJ-66-induced caspase-3 activation was blocked by UCN01 (1 μM). C6 glioma cells were treated with MJ-66 (60 nM) for 24 hrs in the presence or absence of UCN01 and cell lysates were blotted with activated caspase-3. C. C6 glioma cells were treated with MJ-66 (60 nM) for 24 or 48 hrs and cell death was analyzed with trypan blue exclusion method.

Cheng Kung University, Taiwan) was cultured in DMEM/F12 (Caisson) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin, and 0.1 mg/ml streptomycin. All cells were maintained in a humidified incubator at 37°C and 5% CO_2/95% air. MJ-66 was dissolved in dimethylsulfoxide (DMSO) as stock solution at concentration of 1 mM. UCN-01 (7-hydroxystaurosporine, Sigma-Aldrich) was dissolved in DMSO as stock solution at concentration of 10 mM.

Cell proliferation and viability assay

WST-1 assay: Cell viability was determined by WST-1 (2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2, 4-disulfophenyl)-2H-tetrazolium) assay. WST-1 assay is a colorimetric assay for determining cell viability [13]. The WST-1 tetrazolium compound is bio-reduced by NADH or NAD+ produced by dehydrogenase in live cells into a colored formazan product. Cells were seeded in 96-well plates ($2x10^3$/well) for WST-1 assay and were incubated for 24 hrs at 37°C. Culture medium containing MJ-66, UCN-01 (1 μM), minocycline (Mino) or vehicle was added to each well, and cells were incubated at 37°C for the indicated time points (0, 24, 48, 72 hrs). At the indicated time points, medium was removed, and then fresh culture medium (100 μl/well) with WST-1 solution (10 μl/well) was added, and cells were incubated at 37°C for 1-4 hrs. The absorbance of soluble formazan was measured at 440 nm with microplate reader (Molecular device). The cell viability was determined by the percentage of the absorption relative to the vehicle-treated control culture.

Trypan blue exclusion assay

Cell viability was also determined by trypan-blue exclusion assay (Sigma). Cells were seeded in 6-well plates ($2x10^4$/well) for trypan blue exclusion assay, and incubated for 24 hrs at 37°C. Culture medium containing MJ-66, minocycline or DMSO (0.006%) was added to each well, and cells were incubated at 37°C for indicated time points. At indicated time points, cells were suspended with 0.05% trypsin-EDTA, and stained with trypan-blue dye (0.4%). The cell viability was evaluated by the percentage of

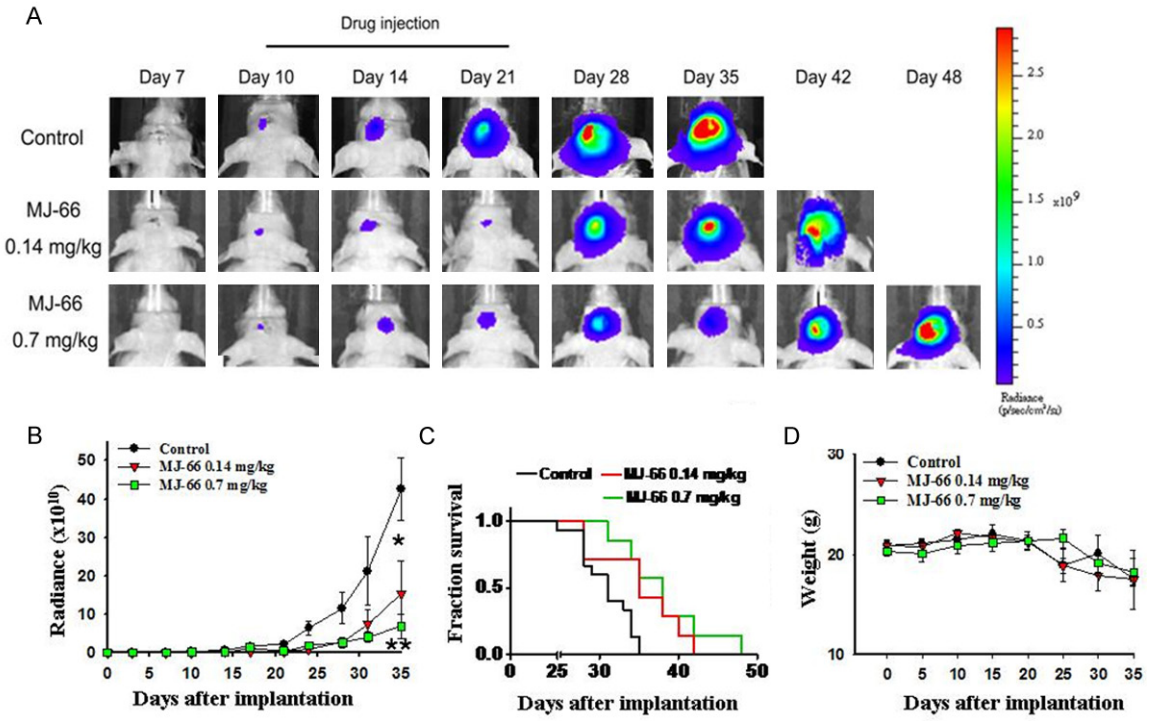

Figure 3. MJ-66 inhibits intracranial tumor growth. A. U87 glioma cells were injected intracranially into athymic mice and tumor growth was studied using the IVIS-200 imaging system. At Day 10 after intracranial injection of tumor cells, MJ-66 (0.14 or 0.7 mg/kg) or vehicle were administered intraperitoneally once per day for 10 days and tumor growth was observed for 10 days after the cessation of treatment. B. MJ-66 significantly inhibited tumor growth. C. Kaplan-Meier plot of survival in MJ-66-treated animals. Animals on higher dose of MJ-66 survived significantly longer. D. Weight measurements were taken every 5 days. There were no difference among control and 2 MJ-66-treated groups.

death relative to the total cell, and cell growth curve was determined by live cell relative to the total number of cells.

Immunofluorescent staining

$2x10^4$ cells were seeded on the PDL-coating 12 mm glass coverslips in 24-well plate and allowed to attach for 24 hrs at 37°C. Culture medium containing MJ-66 or DMSO (0.008%) was added, and cells were incubated at 37°C. At indicated time points, medium were removed, and then cells were fixed in 4% paraformaldehyde (PFA) in PBS for 30 min. After permeabilized by 0.2% Triton X-100 in 0.1 M PBS for 10 min and 10% methanol containing 0.2% Triton X-100 in 0.1 M PBS for 5 min, cells were blocked in 3% normal goat serum (NGS, Jackson ImmunoResearch Lab., USA) for 1 hr. The cells were immunostained for DNA damage with rabbit monoclonal H2AX phospho S139 (γH2AX, Epitomics), and then stained with rabbit secondary antibody conjugated with Texas Red for 1 hr. Nuclei were stained with Hoechst 33342 for 10 min (0.5 μg/ml, Sigma-Aldrich, B2261). Fluorescence images were detected by a fluorescence microscope (DM2500, Leica).

Western blotting assay

$3x10^5$ cells were seeded in 10 cm plate and incubated at least 24 hrs at 37°C. Glioma cells were treated with medium containing MJ-66, UCN-01 (1 μM), minocycline or vehicle, and incubated at 37°C for the indicated time points. Cell pellets were collected and centrifuged at 4,000 rpm for 5 min and stored at -80°C. Cell pellets were lysed in a RIPA lysis buffer containing 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 0.25% sodium deoxycholate, and 0.1% sodium dodecyl sulfate (SDS) with complete protease inhibitor cocktail (Roche). Lysates were shaken at 40 rpm on ice for 1 hr and then centrifuged at 13,000 rpm for 30 min. Supernatants were collected and then protein concentration was measured by Bradford assay. The protein was resuspended in 5X sam-

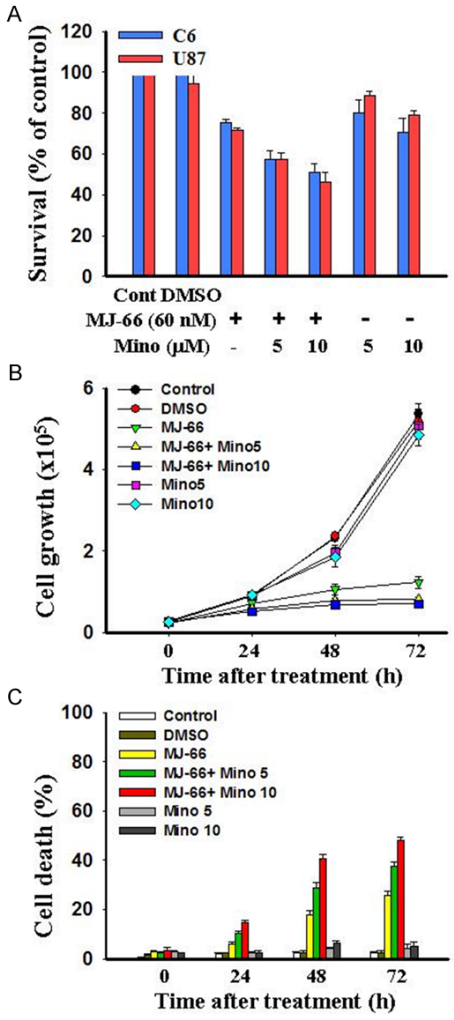

Figure 4. Effects of MJ-66 and Mino alone or in combination on cell survival. A. C6 and U87 glioma cells were treated with MJ-66 (60 nM) and Mino (5 or 10 μM) alone or in combination and cell survival was assessed 48 hrs after the treatment using MTS assay. B. MJ-66 plus Mino synergistically inhibited cell growth. C. C6 glioma cells were treated with MJ-66 (60 nM) and Mino (5 or 10 μM) alone or in combination and cell death was assessed using trypan blue exclusion assay. Combination drug index (CDI) for MJ-66 and 5 μM Mino was 0.90 at 48 hrs and 0.88 at 72 hrs after treatment; for MJ-66 and 10 μM Mino was 0.77 at 48 hrs and 0.73 at 72 hrs after treatment.

ple buffer (12.5 mM Tris, 25% glycerol, 4% SDS, 1.54% DTT and 0.02% Bromophenol blue) and boiled at boiled-water for 10 min. Protein electrophoresis on 15%, 10% or 9% SDS-polyacrylamide gel under 100 volt, and the separated protein was transferred to a PVDF membrane (Immunobilon transfer membranes, Millipore) by semi-dry transfer system (BIO-RAD) under 400 mA, 20 volt for 2 hrs. The membrane was then immersed in 5% nonfat milk or 3% bovine serum albumin (BSA, Sigma) for 1 hr at room temperature to perform non-specific blocking, and then reacted with the following primary antibodies: rabbit monoclonal H2AX phospho S139 (γH2AX, 1:1000, Epitomics), mouse monoclonal β-actin (1:100000, Millipore), rabbit monoclonal Chk1 phospho S345 (1:1000, Cell signaling), mouse monoclonal Chk1 (1:1000, Gentex), rabbit monoclonal Cdc25C phospho S216 (1:1000, Epitomics), rabbit monoclonal Cdc25C (1:2500, Epitomics), rabbit polyclonal Caspase-3 (1:2000, Cell Signaling), or mouse monoclonal microtubule-associated protein 1 LC3 (1:1000, MBL International) at 4°C overnight. HRP-conjugated secondary antibody (Jackson ImmunoResearch Lab., USA) was used and incubated at room temperature for 1 hr. After washed with TBST for three times on shaker at 50 rpm, the ECL-plus chemical reagents (PerkinElmer) were added to the membrane and incubated for 1 min. Films (Fuji, Japan) were exposed at different time points to ensure the optimum density, but not saturated. The results of western blotting were analyzed for densitometry by using ImageJ software. The protein levels in all groups were expressed as a percentage of those in controls.

In vivo intracranial xenograft animal model and bioluminescence imaging

U87 glioma cells were transduced with lentiviral vector expressing GFP and firefly luciferase. GFP overexpression infected cells were sorted out for further passages (FACS-Aria, BD Biosciences). For tumorigenesis, luciferase-expressing glioma cells ($1x10^5$ cells per 1 μl PBS) were inoculated intracranially into the 8 to 10-week-old male nude mice (BALB/cAnN-Foxnlnu/CrlNarl mice, National Laboratory Animal Center). Nude mice were anesthetized with chlorohydrate and placed on a stereotaxic device. Subsequently, a hamilton syringe with 30-gauge needle was mounted on a stereotaxic device, and injected U87 luciferase-expressing glioma cells into the left side of the brains, 1.5 mm caudal and lateral to the bregma, and at a depth of 3.5 to 4 mm. Ten days after tumor implantation, MJ-66 (0.14 mg/kg or 0.7 mg/kg in saline), Mino (20 mg/kg in saline) or vehicle control (saline or DMSO) were injected intraper-

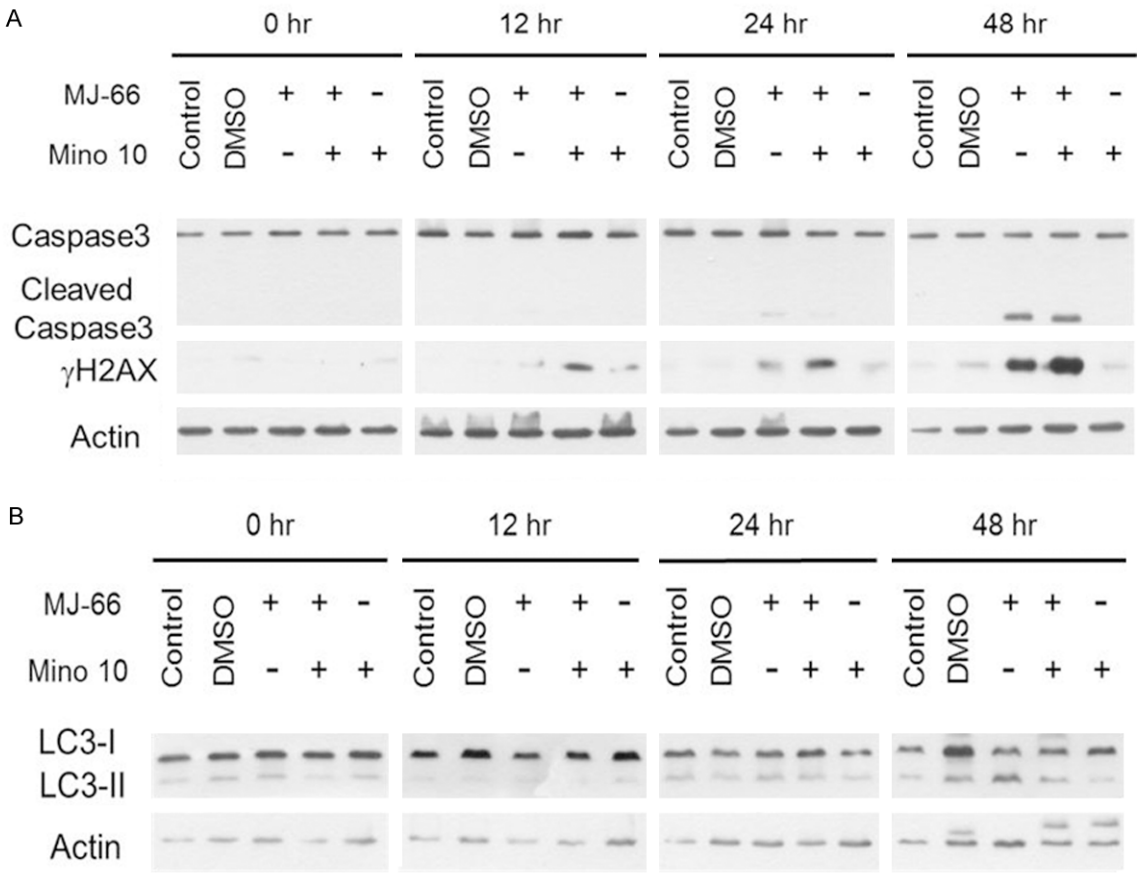

Figure 5. MJ-66 in combination with Mino increases glioma cell death through enhancing DNA damage. A. U87 glioma cells were treated with MJ-66 (60 nM) and Mino (10 μM) alone or in combination for indicated times and cell lysates were blotted with antibodies against caspase 3 and γH2AX. B. U87 glioma cells were treated with MJ-66 (60 nM) and Mino (10 μM) alone or in combination for indicated times and cell lysates were blotted with antibodies against LC3.

itoneally once per day for 10 times. Tumors were allowed to develop and monitored by the IVIS spectrum Live Imaging System (IVIS-200, Xenogen) twice per week. Before monitoring, mice were injected with 100 μg luciferin (Caliper), and simultaneously anesthetized with isoflurane. The results of luciferase radiance were quantitated by Live Imaging Software (Xenogen) and the results of survival time were analyzed by using GraphPad Prism software.

Statistical analysis

Experiments were performed at least in triplicate. All results were expressed as the mean ± standard error of the mean. Independent experiments were analyzed by unpaired t test. Survival data were presented using Kaplan-Meier plots. Levels of $p<0.05$ were considered to be of statistical significance.

Results

MJ-66 induces γ-H2AX phosphorylation and increases nuclear γ-H2AX foci

In the presence of DNA damage, H2AX is phosphorylated on the 139th serine residue termed γ-H2AX [14]. Hence, γ-H2AX is a sensitive biomarker for DNA double-strand breaks (DSBs) [15]. We first determined whether MJ-66 induced γ-H2AX expression. C6 glioma cells were treated with MJ-66 (60 nM) or vehicle for indicated times and whole cell lysates were blotted with antibody for γH2AX. **Figure 1A** and **1B** show a time-dependent increase in γ-H2AX after treatment with MJ-66 ($F_{(8, 27)}=96.71$, $p<0.001$). In addition, we found that MJ-66 increased the appearance of nuclear γ-H2AX foci ($t_{(4)}=3.718$, $p<0.05$ vs. vehicle) that have been taken as an indicator for the presence of DSBs [16, 17] (**Figure 1C** and **1D**).

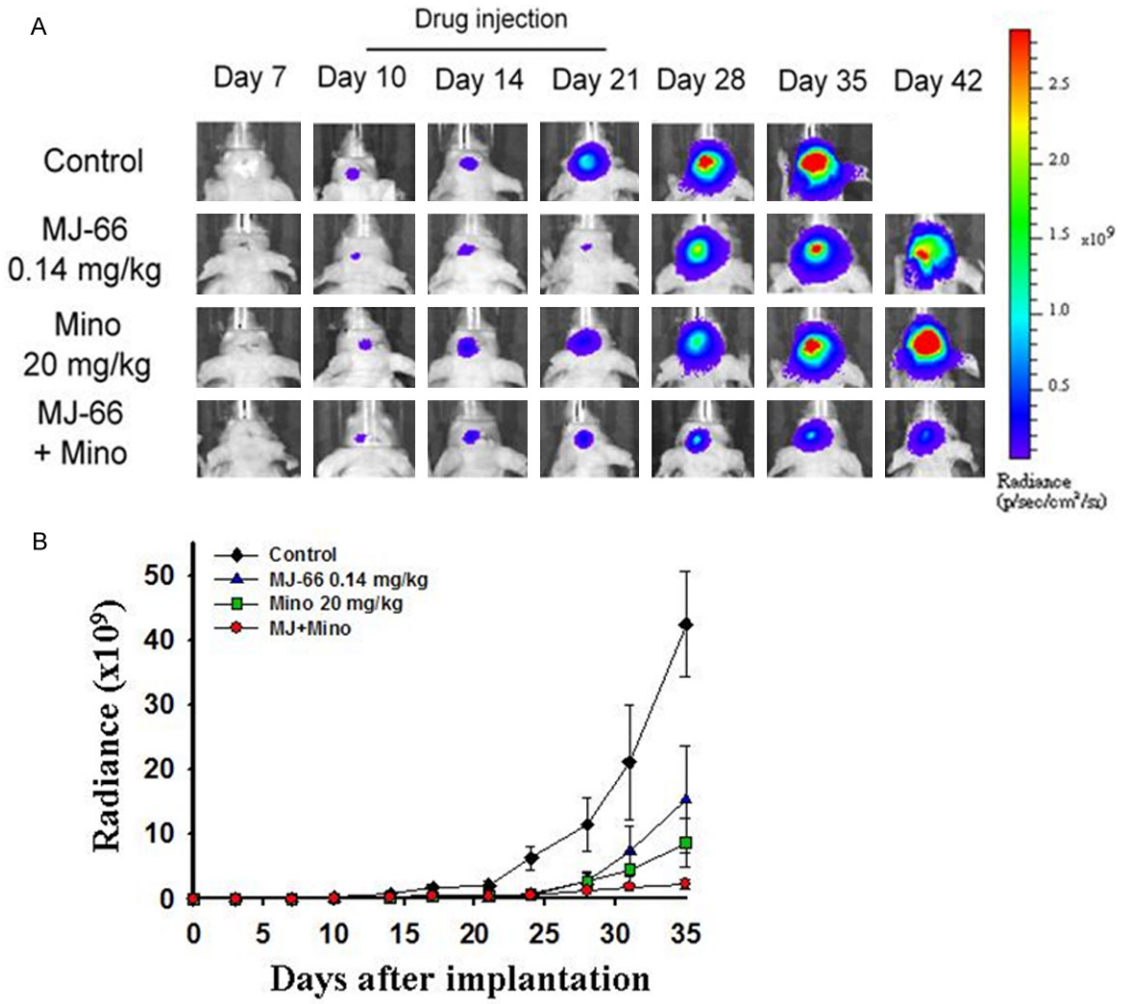

Figure 6. MJ-66 and Mino in combination synergistically inhibits intracranial tumor growth. A. U87 glioma cells were injected intracranially into athymic mice and tumor growth was studied using the IVIS-200 imaging system. At Day 10 after intracranial injection of tumor cells, MJ-66 (0.14 mg/kg), Mino (20 mg/kg) or in combination were administered intraperitoneally once per day for 10 days and tumor growth was observed for 10 days after the cessation of treatment. B. MJ-66 and Mino in combination synergistically inhibited intracranial tumor growth.

MJ-66 induces phosphorylation of Chk1 and Cdc25C

Mammalian DNA damage checkpoints are controlled by ataxia-telangiectasia, mutated and ataxia-telangiectasia, mutated and Rad3-related kinases and their downstream effector kinases Chk1 and Chk2 [18]. We determined the phosphorylation status of Chk1 and Chk2 kinases by using phosphorylation specific antibody against Chk1 as a measurement of their activation. C6 glioma cells were treated with MJ-66 (60 nM) or vehicle for indicated times and cell lysates were blotted with antibodies for p-Chk1, Chk1, p-Cdc25C and Cdc25C (**Figure 1E**). The increase in Chk1 phosphorylation was transient which peaked at 4 hrs after treatment of MJ-66 and subsided within 12 hrs ($F_{(7,32)}$=8.45, p<0.001). Similarly, MJ-66 treatment led to transient Cdc25C phosphorylation which peaked at 4 hrs after treatment and subsided within 6 hrs ($F_{(7,24)}$=4.569, p<0.01).

Block of MJ-66-induced Cdc25C phosphorylation and cell death by UCN-01

UCN-01 is a protein kinase inhibitor currently undergoing clinical trials for cancer treatment. UCN-01 potently inhibited the ability of Chk1 to phosphorylate Cdc25C resulting in the abrogation of cell cycle checkpoint function in the G2 phase of cell cycle [19, 20]. We determined

whether UCN-01 affected MJ-66-induced Cdc25C phosphorylation. C6 glioma cells were treated with MJ-66 (60 nM) in the presence or absence of UCN-01 (1 mM) for indicated times and cell lysates were blotted with antibodies for p-Cdc25C and Cdc25C. **Figure 2A** shows that MJ-66-induced Cdc25C phosphorylation was blocked by UCN-01. In addition, UCN-01 abrogated MJ-66-induced caspase-3 activation (**Figurea 2B**). These effects were paralleled by the reduction of MJ-66-mediated cell death (**Figure 2C**).

MJ-66 inhibits intracranial growth of glioma cells

We determined whether MJ-66 exhibited antitumor effect under in vivo conditions using intracranial tumor model. Transduced glioma cells were injected intracranially into athymic mice and tumor growth was studied using the IVIS-200 imaging system. At Day 10 after intracranial injection of tumor cells, MJ-66 (0.14 or 0.7 mg/kg) or saline were administered intraperitoneally once per day for 10 days and tumor growth was observed for 10 days after the cessation of treatment (**Figure 3A**). Ten days after the cessation of drug injection, MJ-66 significantly inhibited tumor growth (**Figure 3B**) and increased the survival of the experimental mice (**Figure 3C**). Kaplan-Meier analysis of the survival data demonstrated a statistically significant difference ($P<0.01$) in median survival between MJ-66- and saline-treated mice. Control mice receiving saline succumbed to disease around 35 days. MJ-66 treatment significantly increased survival to between 46 (0.14 mg/kg) and 48 (0.7 mg/kg) days. MJ-66, however, did not significantly affect body weight of the mice (**Figure 3D**).

MJ-66 in combination with Mino induces synergistic cytotoxicity

C6 and U87 glioma cells were treated with MJ-66 (60 nM) and Mino (10 mM) alone or in combination and cell survival was assessed using MTS assay (**Figure 4A**). MJ-66 plus Mino synergistically inhibited cell growth (**Figure 4B**) and increased cell death assessed using trypan blue exclusion assay (**Figure 4C**). Combination drug index (CDI) for MJ-66 and 5 mM Mino was 0.90 at 48 h and 0.88 at 72 h after treatment; for MJ-66 and 10 mM Mino was 0.77 at 48 h and 0.73 at 72 h after treatment.

Figure 5 showed that after the treatment with MJ-66 (60 nM) and Mino (10 mM), the expression of cleaved fragments of caspase 3 (**Figure 5A**) and LC3-II (**Figure 5B**) were not different from that treatment with MJ-66 alone. By contrast, the level of γH2AX was higher in the combination group compared with MJ-66 alone or Mino alone (**Figure 5A**). These results suggest that synergistical effect on the inhibition of tumor growth is likely mediated through enhancement of DNA damage in glioma cells.

MJ-66 in combination with Mino synergistically inhibits intracranial growth of glioma cells

We determined whether combination of MJ-66 and Mino synergistically inhibited tumor growth in vivo using intracranial tumor model. Transduced glioma cells were injected intracranially into athymic mice. At Day 10 after intracranial injection of tumor cells, MJ-66 (0.14 mg/kg), Mino (20 mg/kg) or in combination were administered intraperitoneally once per day for 10 days and tumor growth was observed for 10 days after the cessation of treatment (**Figure 6A**). As shown in **Figure 6B**, MJ-66 and Mino combination synergistically inhibits intracranial growth of C6 glioma cells.

Discussion

Quinazolinone alkaloids which were isolated from Rutaceae family possessed diverse pharmacological properties including antifungal, anti-malarial, anti-inflammatory and anti-cancer activities [21-23]. Recently, we have designed and synthesized a series of 4-quinazolinone analogs as potential anti-glioma agents. We found MJ-66-induced cell death was associated with multinucleated phenotype and multipolar spindles that were typical characteristics of mitotic catastrophe. Flow cytometry analysis revealed that MJ-66 caused glioma cell cycle arrest at G2/M phase and increased the proportion of polypoidy cells [12]. However, the upstream pathway that led to cell cycle arrest at G2/M phase remains to be defined. Here, we demonstrated that γH2AX was expressed and co-localized with Hoechst staining in glioma cells as early as 2 hr after the treatment with MJ-66. UCN-01 which potently inhibited the ability of Chk1 to phosphorylate Cdc25C reversed MJ-66-induced caspase 3 activation and glioma cell death suggesting that Chk1/Cdc25C pathway is essential for

MJ-66-induced mitotic catastrophic cell death. Consistent with our results, a recent study has demonstrated that quinazolinone derivative induced oxidative stress and up-regulated ATM/p53 signaling pathway in human osteogenic sarcoma cells [24].

Mino is a lipophilic molecule that is readily absorbed from the gut after oral ingestion and capable of crossing the blood-brain barrier [10]. We have previously shown that minocycline induced cell death in glioma cells which were associated with the presence of autophagic vacuoles in the cytoplasm [11]. Monotherapies have proven largely ineffective for the treatment of glioblastomas suggesting that increased patient benefit may be achieved by combining therapies. In the present study, we demonstrated that Mino significantly enhanced MJ-66-mediated inhibition of cell growth and induction of cell death. In intracranial glioma xenograft, Mino also potentiated MJ-66-induced inhibition of tumor growth. Interestingly, we found that Mino in combination with MJ-66 increased γ-H2AX activation without affect MJ-66-induced caspase 3 activation and Mino-induced LC3-II conversion. Thus, Mino potentiated MJ-66's effect on the inhibition of tumor growth is likely mediated through enhancement of DNA damage in glioma cells. These results coupled to clinical availability and safe track record makes Mino a promising agent for the adjuvant treatment of malignant gliomas.

In summary, we have shown that MJ-66 induced γH2AX expression which was co-localized with Hoechst staining in glioma cells. UCN-01, an inhibitor of Chk1 to phosphorylate Cdc25C, reversed MJ-66-induced caspase 3 activation and glioma cell death suggesting that Chk1/Cdc25C pathway is essential for MJ-66-induced mitotic catastrophic cell death. Mino significantly enhanced MJ-66-mediated inhibition of cell growth and induction of cell death. In intracranial glioma xenograft, Mino also potentiated MJ-66-induced inhibition of tumor growth. These results suggest Mino a promising agent for the adjuvant treatment of malignant gliomas.

Acknowledgements

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