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Anti-Tumor Effect of Doxycycline on Glioblastoma Cells

Andrea Wang-Gillam

Eric Siegel

Debra A. Mayes
Wright State University - Main Campus, debra.mayes@wright.edu

Laura F. Hutchins

Yi-Hong Zhou

Research Paper

Anti-Tumor Effect of Doxycycline on Glioblastoma Cells

Andrea Wang-Gillam, Eric Siegel, Debra A. Mayes, Laura F. Hutchins, and Yi-Hong Zhou[1]

Department of Internal Medicine, Washington University in St.Louis, St. Louis, MO, USA [A. Wang-Gillam]; Arkansas Cancer Research Center, University of Arkansas for Medical Sciences, Little Rock, AR, USA [E. Siegel; L. F. Hutchins]; Department of Neuroscience, University of Cincinnati, Cincinnati, OH, USA [D. A. Mayes]; Department of Neurological Surgery, University of California, Irvine, CA, USA [Y.-H. Zhou]

AIM: **Glioblastoma multiforme (GBM) is the most common primary brain tumor in humans, and it is highly invasive. Doxycycline, first identified as an antimicrobial agent, is a nonspecific inhibitor of matrix metalloproteinases (MMPs). Our objective was to investigate the anti-MMP effect of doxycycline at therapeutically acceptable levels on glioma cells *in vitro*.**

METHODS: **The MTT assay was used to determine the anti-proliferative effects of doxycycline. MMP2 activity and expression were determined by gelatinase zymography and real-time quantitative RT-PCR, respectively. Cell invasion was assessed by Matrigel invasion assay.**

RESULTS: **Doxycycline exerted mild anti-proliferative effects on all three glioma cell lines (U251HF, U87 and LN229). In U251HF cells, doxycycline decreased extracellular MMP2 activity and reduced cell invasiveness. Moreover, MMP2 mRNA levels were not altered, suggesting that doxycycline regulates MMP2 activity post-translationally. Alternatively, doxycycline increased the expression and extracellular activity of MMP2 in U87 cells. This may reflect the cellular stress-response related to the cytotoxic effects experienced by U87 cells in response to doxycycline exposure.**

CONCLUSION: **Doxycycline in therapeutic concentrations decreases MMP2 activity and cell invasion in the most aggressive cell line tested, suggesting its potential as a therapeutic MMP inhibitor. The cytotoxic effects of doxycycline, however, can enhance MMP2 expression, and this deserves further exploration.**

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doxycycline

matrix metalloproteinase 2

cell invasion

glioma cell lines

Introduction

Glioblastoma multiforme (GBM[2]) is the most common type of primary brain tumor, with 8,000 to 10,000 cases diagnosed each year in North America. Over the last two decades, GBM has remained one of most malignant cancers. Patients with GBM have a very poor prognosis, with a median survival of ~14 months after surgical resection, chemotherapy and radiation [1]. One of the challenges in the treatment of GBM is the frequency of recurrences. Recurrences often occur adjacent to the resection site, suggesting the presence of residual malignant glioma cells after tumor removal [2]. This clinical phenomenon reflects the aggressive and infiltrative behavior of malignant glioma cells.

Matrix metalloproteinases (MMPs) are an important family of zinc endopeptidases whose members degrade extracellular matrix, providing a space into which cells can migrate [3]. In addition, MMPs can cleave other growth factors and activate signal transduction cascades to promote cell motility and proliferation [4]. Specifically, MMP2 and MMP9 play a pivotal role in enhancing glioma tumor migration, invasion, and angiogenesis [5]. Moreover, increased MMP2 and MMP9 expression in gliomas is associated with poor clinical outcomes [4], and inhibiting these proteinases dramatically reduces the invasiveness of glioma cells *in vitro* and *in vivo* [6]. Therefore, MMP inhibitors are potential candidates to block glioma migration and invasion *in vivo*.

The efficacy of an MMP inhibitor (marimastat) together with an alkylator (temozolomide, TMZ) was previously tested in a phase-II clinical trial in recurrent GBM patients. The combination treatment resulted in a progression-free survival at 6 months that exceeded the literature target by 29% [7]. However, large randomized trials have not been conducted because of multiple factors such as poor oral tolerability, high incidences of musculolskeletal adverse effects with marimastat, and an overall disappointing performance of MMP inhibitors in clinical trials studying other solid tumor types. Therefore, there is a need to explore the efficacy of alternative MMP inhibitors that may have less adverse effects *in vivo*. Doxycycline is one such candidate [8].

Doxycycline is an antimicrobial agent, and has been widely used in treating malaria, anthrax, tick-born diseases and other bacterial infections [9]. No major toxicity has been

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[1]**Correspondence:** Dr. Yi-Hong Zhou, Department of Neurological Surgery, University of California, Irvine, No. 101, The City Drive, Building 56, Suit 400, Zot 5397, Orange, CA 92868, USA. Phone: 1-949-8245767. Fax: 1-714-4568284. E-mail: yihongz@uci.edu

[2]**Abbreviations:** GBM, glioblastoma multiforme; MMP2, matrix metalloproteinase 2; QRT-PCR, quantitative reverse transcriptase-polymerase chain reaction; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

reported with long-term use, and doxycycline exhibits a variety of anti-tumor properties, such as inhibition of mitochondrial protein synthesis [10,11], anti-invasion effects via inhibition of MMPs [12-14], and anti-angiogenesis effects [15]. It works as a non-specific MMP inhibitor in a variety of tumor cell lines, including breast, colon, mesothelioma, osteosarcoma, renal, prostate, and melanoma [13,14,16-20]. Although the mechanism of MMP inhibition is not entirely clear, chelation of a zinc ion by doxycycline at the active site of the enzyme has been reported [21]. This MMP inhibition by doxycycline can occur within the normal therapeutic dose range (less than 10 μg/ml)[22,23], which makes it a very attractive agent for further examination as a potential therapy targeting tumor migration and invasion [24].

To investigate the potential of doxycycline as a therapeutic agent for GBM, we determined its effects on GBM cell growth in three GBM cell lines and observed its effects on MMP2 activity and invasion capacity *in vitro*. Our data showed that doxycycline, either as a cytotoxic agent or an MMP inhibitor, is a reasonable candidate to further explore as a pharmaceutical intervention for GBM.

Materials and Methods

Chemical reagent

Doxycycline was purchased from Sigma-Aldrich (St Louis, MO, USA) and dissolved in PBS to prepare a stock solution of 10 mg/ml. The stock solution was stored at -20°C.

Cell culture

U87, LN229 and U251HF were obtained from the Department of Neuro-Oncology, University of Texas M. D. Anderson Cancer Center. LN229 has wild-type PTEN and a *p53* gene mutation, while U87 has wild-type p53 with a PTEN mutation [25]. U251HF has the *p53* gene mutation and a *PTEN* gene deletion [26]. All cell lines were cultured in DMEM/F12 medium supplemented with 5% calf serum, penicillin (100 U/ml), and streptomycin (100 μg/ml) and incubated in a humidified atmosphere containing 5% CO_2 at 37°C.

Cell proliferation assay

To assay cell viability after doxycycline treatment, we conducted the MTT assay as described previously [27]. Briefly, 2000 cells/well were seeded in 96-well plates and allowed to adhere after overnight culture. The cells were then treated with various concentrations of doxycycline for 24, 48 and 72 h in quadruplicate. The media was decanted, 100 μl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) in DMEM/F12 medium (1 mg/ml) was added to each well, and cells were incubated at 37°C for 1 h. Formazan crystals in the wells were solubilized in 100 μl of DMSO by shaking at room temperature for 1 h. Absorbance was measured at a wavelength of 570 nm by a spectrophotometer. The assay was repeated in 4-5 independent experiment replications. In each replication, the MTT values of treatment groups for each day were normalized to, and expressed as percents of, the mean MTT values of untreated cells at day 1.

Gelatinase zymography

To determine the effects of doxycycline on the activity of secreted gelatinase from the glioma cells, quadruplicate sets of cells (1×10^5) were seeded in 24-well plates, allowed to attach, then treated with doxycycline at 5, 10 and 15 μg/ml for 48 h under serum-free conditions. The conditioned media were collected from glioma cells after they had been treated with doxycycline for 48 h. We felt that conditioned media obtained from continuous incubation with the drug closely reflected what happened in the clinical setting as patients were treated with doxycycline once to twice daily to maintain a steady level of drug concentration. Conditioned media secreted from one set of glioma cells were collected, and RNA was extracted from the same cells for analysis of gene expression. MTT assays were performed on the remaining three sets of glioma cells as described above but with triplicate amounts of reagents.

We followed the gelatinase zymography method described previously [28]. Briefly 1-10 μl of each conditioned medium was loaded into a 10% SDS-polyacrylamide gel containing 0.1% gelatin with a layer of stacking gel. Proteins were fractioned by electrophoresis at 100 volts for 90 min. The gel was washed by 0.25% Triton X-100 for 30 min, then incubated in protease reactivation buffer [50 mM Tris-HCl (pH 7.5), 150 mM NaCl and 10 mM $CaCl_2$] for 16 h at 37°C with gentle horizontal shaking. Gelatinase activities were visualized as clear bands after the gel was stained with 0.1% Coomassie blue in 10% acetic acid and 10% isopropanol followed by de-staining with 10% acetic acid and 30% methanol. The band sizes were determined by parallel loading of a protein marker. After wrapping the gel in a cellophane sheet and allowing it to dry in air, the bands were quantified using ChemiImager 5500 and Alpha Ease FC software (Alpha Innotech Corp, San Leandro, CA, USA). The assay was repeated using materials from three independent experiment replications with 3 repeats per treatment. To adjust for doxycycline dose effects on cell proliferation, MMP2 activities from each treatment group were converted into ratios relative to the mean MTT value for that treatment group. To normalize the different replications to a common reference, the MMP2/MTT ratios were expressed as percentages of the mean of the no-treatment control prior to statistical analysis.

RNA isolation, cDNA synthesis, and real-time quantitative RT-PCR (QRT-PCR)

RNA was extracted from cell cultures using Ultraspec (Biotecx Laboratories, Houston, TX, USA) following the manufacturer's protocol and converted to cDNA by SuperScript reverse transcriptase II (Invitrogen, Carlsbad, CA, USA) with a poly-d(T) primer as described previously [29]. Gene expressions were quantified using a Light Cycle (LC) instrument and LC Faststart DNA Master SYBR Green I enzyme mix (Roche, Indianapolis, IN, USA) with primers and gene-specific standards for *GAPDH*, *enolase-α*, and *MMP2* as described previously [29]. The real time QRT-PCR of each cDNA sample was repeated in two individual runs, and the data from two separate experiments was taken for statistical analysis.

In vitro invasion assay

The Matrigel Basement Membrane Matrix (Becton Dickson Labware, Bedford, MA, USA) was used to perform the *in vitro* cell invasion assays. U251HF cells (1.0×10^5 in 0.7 ml DMEM/F12 with and without 5 or 10 μg/ml of doxycycline) were seeded in the upper chamber of a Costar Transwell (12-mm diameter, 12-μm pore size), which was coated with 200 μl of Matrigel (1 mg/ml). Medium (1 ml) supplemented with 0.05% calf serum was added to the lower compartment of the chamber to provide chemo-attractants. After incubation for 16 h, the cells that had invaded through the Matrigel and migrated to the lower surface of the filter were fixed and stained using a HEMA3 staining kit (Fisher Scientific, Houston, TX, USA). A total of ten pictures of randomly picked microscope fields at 200× magnification were taken for each filter, and cell numbers were counted. Each experiment was performed in triplicate and repeated twice.

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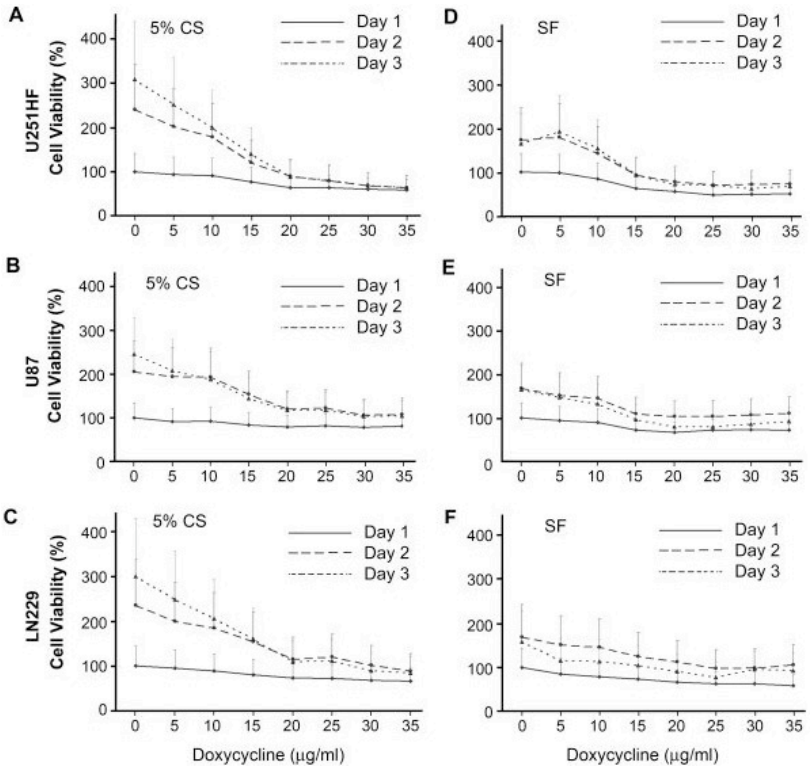

Figure 1: Determination of the effect of doxycycline on glioma cell growth by MTT assay, showing the growth of U251HF, U87 and LN229 glioma cells in the presence of various concentrations of doxycycline in medium supplemented with 5% calf serum (CS)(A-C, respectively) or in serum-free (SF) medium (D-F, respectively). Cell viabilities of the treatment groups were normalized to the mean MTT values of untreated cells at Day 1 (assigned as 100%) for each cell line. The data represent mean ± SD of 12-15 repeats from 4-5 independent experiments from the Least-Square Means (LS Means) and ANOVA SD analyzed via mixed-models ANOVA.

Statistical Analysis

Mixed-model ANOVAs, with either Bonferroni's or Dunnett's procedures for the *post hoc* comparisons, were used to examine the statistical differences between groups in the cell proliferation assays, gelatinase zymography, and real-time QRT-PCR of *MMP2* expression. Matrigel cell counts were analyzed via Poisson regression, with generalized estimating equations to model the correlation between different microscope fields of view from the same culture and with cell counts normalized to the percent of control by means of the offset variable. The statistical significance was set at $P < 0.05$.

Results

Doxycycline affects glioma cell proliferation

Though there have been reports of doxycycline's cytotoxic effects in multiple tumor cell lines, no studies have been done using malignant glioma cell lines. We studied the effect of doxycycline on glioma cell proliferation by exposing U251HF, U87, and LN229 cells to increasing concentrations of doxycycline (5, 10, 15, 20, 25, 30 and 35 μg/ml) for 1, 2, and 3 days in DMEM/F12 medium containing 0 or 5% serum. Cell viability was determined by MTT assay. Comparison of treated cells to untreated control cells at Day 1 revealed that doxycycline had no significant immediate effect on cell viability when applied at low concentrations (5-15 μg/ml) to all three glioma cell lines in the presence or absence of serum ($P > 0.05$, Figure 1 and Table 1). Moderate suppression from doxycycline at higher concentrations (20-35 μg/ml) in serum-free conditions was observed at Day 1 in U251HF and LN229 cells ($P < 0.05$), with a similar trend shown by U87 cells ($P = 0.06$).

In culture conditions supplemented with 5% calf serum, a concentration-dependent growth-inhibitory effect was observed in all three glioma cell lines starting from Day 2 after treatment, as shown by the trend of change on cell viability (Figure 1). Significant *P* values were observed at Days 2 and 3 when comparing high concentration groups with the untreated control, while comparison of low concentration groups with the control revealed no significant *P* value (Table 1). When a low concentration of doxycycline was used, cells continued to proliferate for 2 days, as shown by the comparison of Day 2 to Day 1 ($P < 0.0001$ or less). Although there was a trend for MTT values to increase from Day 2 to Day 3 under low concentration conditions, the *P* values were not significant (Table 1), including comparison with no-treatment controls. This suggests that cell proliferation slowed considerably by Day 3 in this assay condition. Thus there was a growth-inhibitory but not a cytotoxic effect from doxycycline on all three glioma cell lines treated in medium containing serum.

Under serum-free culture conditions, cells that were exposed to low concentrations of doxycycline displayed a similar behavior to those cultured in 5% serum, as shown by similar *P* values in Table 1. However, viability of U87 and LN229 cells tended to be reduced in response to high concentrations of doxycycline treatments. A similar, but less obvious trend was observed with lower concentrations as well (Figure 1E and 1F). For example, the cell viability of U87 cells at Day 3 was reduced to 79% compared to that at Day 2 in the high concentration group ($P < 0.05$), indicating a cytotoxic effect of doxycycline on U87 cells in serum-free conditions. In U251HF cells, the curve for cell viability was essentially the same between Day 2 and Day 3 for all treatment groups including the untreated control cells (Figure 1D). Thus in serum-free condition, U251HF cells were more resistant to the cytotoxic effect of doxycycline than U87 and LN229 cells. Based on these observations, we chose 2 days as the treatment duration for the zymography and invasion studies, and normalized zymography data by MTT value of the cells.

Effect of doxycycline on the MMP2 activity of glioma cell lines

Table 1: Bonferroni *P* values for comparisons of cell viability among grouped high or low concentrations of doxycycline treatment per cell line and medium

	Comparison	U251HF		U87		LN229	
		5% serum	Serum free	5% serum	Serum free	5% serum	Serum free
Day 1	Low dose[1] *vs.* Ctrl[2]	1.0000	1.0000	1.0000	1.0000	0.4146	1.0000
Day 2	Low dose *vs.* Ctrl	0.2081	1.0000	1.0000	1.0000	1.0000	1.0000
Day 3	Low dose *vs.* Ctrl	0.0378	1.0000	0.0694	0.3269	0.1658	0.3464
Low dose	Day 2 *vs.* Day 1	**< .0001**	0.0007	**< .0001**	**< .0001**	**< .0001**	**< .0001**
Low dose	Day 3 *vs.* Day 2	1.0000	1.0000	1.0000	1.0000	1.0000	0.4941
Day 1	High dose[3] *vs.* Ctrl	0.0243	0.0010	0.5625	0.0622	1.0000	0.0325
Day 2	High dose *vs.* Ctrl	**< .0001**	**< .0001**	**< .0001**	0.0026	**< .0001**	0.0201
Day 3	High dose *vs.* Ctrl	**< .0001**	**< .0001**	**< .0001**	**< .0001**	**< .0001**	0.0028
High dose	Day 2 *vs.* Day 1	0.6062	0.0088	**< .0001**	**< .0001**	0.0003	**< .0001**
High dose	Day 3 *vs.* Day 2	1.0000	1.0000	1.0000	0.0373	1.0000	1.0000

[1]5-15 μg/ml doxycycline; [2]control, no doxycycline; [3]20-35 μg/ml doxycycline.

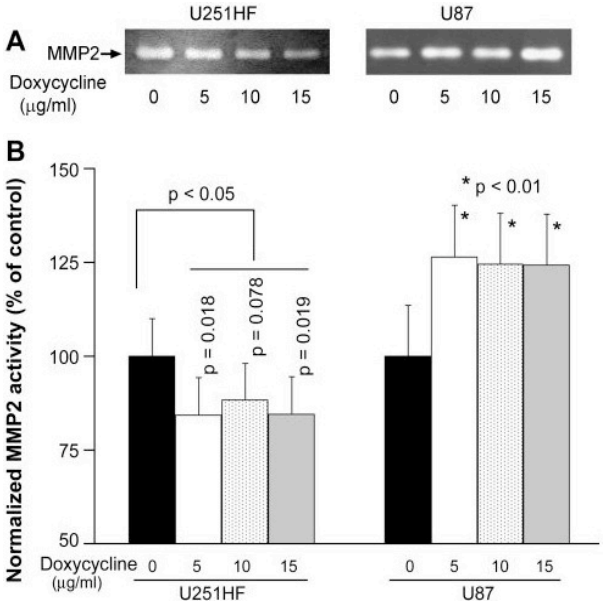

Figure 2: Determination of the effect of doxycycline on MMP2 activity of glioma cells by zymography assay. A, Representative zymography gels for U251HF and U87 cells after treatment with doxycycline (5, 10, and 15 μg/ml) for 48 h. B, Normalized extracellular active MMP2 levels of U251HF and U87 cells after doxycycline treatment. The data represent mean ± SD of 9 repeats from 3 independent experiments from the LSMeans and ANOVA SD analyzed via mixed-models ANOVA. Horizontal lines above bars indicate treatment groupings, with Dunnett-adjusted *P* values for comparisons to the untreated grouping.

MMP2 and MMP9 are two proteinases responsible for degradation of extracellular matrix that is necessary for GBM tumor invasion [5]. MMP2 activities were easily detected in both U87 and U251HF cells, but MMP9 activity was too low to be detected in either cell line. In Figure 2A, representative zymography gels of U251HF and U87 cells following doxycycline treatment are shown. Decreased MMP2 activity was observed as the concentration of doxycycline increased (5, 10 and 15 μg/ml) in U251HF cells, while no change or slightly increased MMP2 activity was observed in U87 cells. Cell viability studies from the same experiments showed more inhibition of cell viability in U87 cells than U251HF cells (data not shown), consistent with results based on cells treated in 96-well plates (Figure 1D and 1E).

Because of the occurrence of cytotoxic effects of doxycycline in U87 cells, we normalized quantified MMP2 activity to the mean of the MTT values of cells in paralleled groups. As shown in Figure 2B, the extracellular active MMP2 level of U251HF cells was decreased by exposure to low concentrations of doxycycline (5, 10 and 15 μg/ml) to 84.4%, 88.3%, and 84.5%, respectively (ANOVA pooled SD = 9.9%; $P < 0.05$ in all three cases). However, doxycycline unexpectedly increased MMP2 activity of U87 cells by 126.5%, 124.5%, and 124.2%, respectively, after the same treatment (ANOVA pooled SD = 13.6%; $P < 0.01$ in all three cases), a result in accordance with an increase in *MMP2* expression as described below.

Effect of doxycycline on regulation of MMP2 gene transcription in glioma cell lines

To determine if doxycycline regulates MMP2 at the level of transcription, real-time QRT-PCR for *MMP2*, *enolase-α* and *GAPDH* were performed on cDNA extracted from U25HF and U87 glioma cells with and without exposure to doxycycline. As shown in Figure 3A, the levels of *MMP2* gene expression were normalized to either *enolase-α* or *GAPDH* in U251HF cells. Treatment with low concentrations of doxycycline did not change *MMP2* expression levels from that from the control group ($P > 0.05$ after both pairwise comparison and linear trend analysis).

For U87 cells, however, the trend with increasing concentrations of doxycycline is statistically significant whether normalized to *enolase-α* or to *GAPDH* (Figure 3B). Pairwise comparisons to 0 μg/ml were not so significant once the results were adjusted for the multiple comparisons, but the test for trend was significant ($P = 0.012$ for *MMP2/enolase-a*; $P = 0.020$ for *MMP2/GAPDH*), indicating that doxycycline has an enhancing effect on *MMP2* transcription in U87 cells.

Effect of doxycycline on glioma cell invasion

Since MMP2 is a proinvasive protein, the doxycycline-mediated reduction of extracellular active MMP2 levels in U251HF cells would be expected to result in decreased cell invasiveness. The Matrigel invasion assay was performed to confirm this hypothesis. As shown in Figure 4, treatment with doxycycline brought about a concentration-dependent decrease in the number of cells that invaded through the filter following treatment with doxycycline. U251HF cells exposed to doxycycline at 5 μg/ml and 10 μg/ml showed respective reductions (95% confidence intervals) of 24.8% (12.6%-35.3%) and 43.4% (30.4%-53.9%) compared to untreated control cells ($P < 0.001$ in both cases). Thus the

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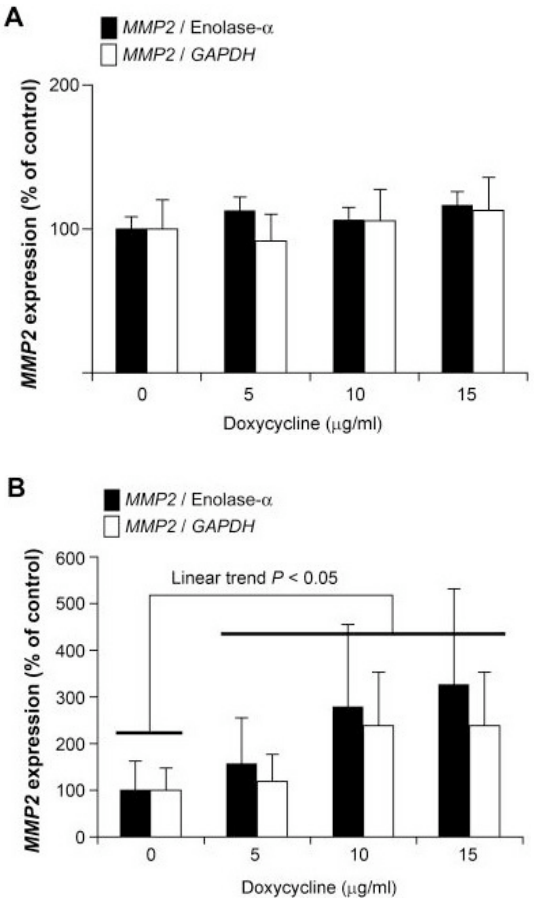

Figure 3: Determination of the effect of doxycycline on MMP2 expression in glioma cells by real-time QRT-PCR. The level of MMP2 expression in (A) U251HF and (B) U87 cells 48 h after treatment with doxycycline at various concentrations was normalized to enolase-a and GAPDH expression, and was expressed as percent of zero-concentration control in each of two replicate experiments, then log-transformed to stabilize variance for analysis via mixed-models ANOVA. The data represent mean ± SD, which were calculated by back-transformation from ANOVA results. Post-hoc contrasts for linear trend with concentration yielded the following results for U251HF cells: F = 2.13, DFs = (1, 3), P = 0.24 for MMP2/enolase-a, and F = 0.87, DFs = (1, 3), P = 0.42 for MMP2/GAPDH. For U87 cells: F = 12.49, DFs = (1, 6), P = 0.012 for MMP2/enolase-a, and F = 9.89, DFs = (1, 6), P = 0.020 for MMP2/GAPDH.

reduction of MMP2 activity by doxycycline in U251HF cells was correlated with reduction of the cell invasiveness.

Discussion

Doxycycline belongs to the tetracycline family of drugs, members of which function as anti-microbial agents by preventing the binding of aminoacyl tRNA to the ribosome, thereby inhibiting protein synthesis [30]. It has been known for years that members of the tetracycline family, such as doxycycline and minocycline, inhibit the growth of various tumor cells *in vitro* [31]. Doxycycline was found to be the most effective analogue of tetracycline at inhibiting cell survival in a human adenocarcinoma cell line [21]. The mechanisms for doxycycline anti-proliferative effects have been reported, and include impairment of mitochondrial protein synthesis [10,11], proliferation arrest in the G_1 phase of cell cycle [32], and induction of apoptosis by caspase-3

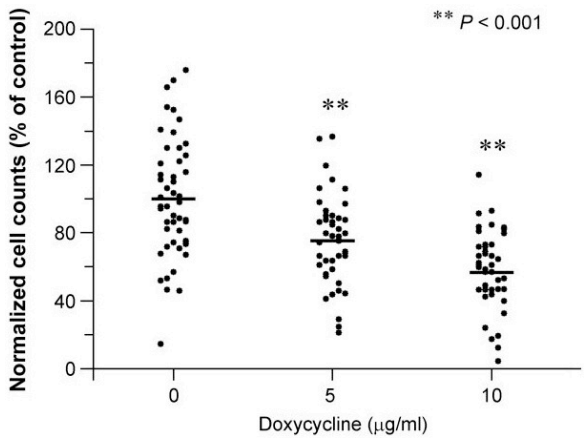

Figure 4: Determination of the effect of doxycycline on the invasiveness of U251HF cells by Matrigel invasion assay. The position of the bar is the mean of the normalized cell counts from triplicate invasion assays of 2 independent experiments. Matrigel cell counts were analyzed via Poisson Regression with Generalized Estimating Equation (GEE) methodology to model the correlation among different microscope fields of view from the same culture. Cell counts were normalized to the percent of the control by means of the offset variable.

activation in human T-lymphoblastic leukemia cells [33]. However, the serum concentrations of doxycycline required to achieve these cytotoxic effects are generally higher (20 to 50 μg/ml) than the typical therapeutic serum concentration used to treat infections, generally from 0 to 5 μg/ml and occasionally as high as 10 μg/ml [22], making doxycycline less desirable as a cytotoxic agent.

There are reports showing that different cancer cell lines have different sensitivities to doxycycline. Doxycycline at 10 μg/ml was sufficient to induce G_0/G_1 arrest, and a concentration of 20 μg/ml was able to provoke mitochondrion-mediated apoptosis in HT 29 cells [17,34]. In prostate cancer cell culture, necrosis and apoptosis were not apparent until doxycycline concentrations exceeded 20 μg/ml [13]. In our study, when three glioma cell lines were cultured in 5% serum, the anti-proliferative effects of doxycycline were also observed only following high-concentration treatments (20, 25, 30, and 35 μg/ml). In clinical settings, GBM is highly resistant to cytotoxic intervention as compared to other solid tumors, and this may be a clinical reflection of our observation that glioma cells require higher concentrations of doxycycline *in vitro* to achieve its effect on growth inhibition.

We also investigated the anti-proliferative effects of doxycycline when cells were exposed to serum-free conditions in an attempt to avoid the effects of various growth hormones and cytokines in the serum that may not exist around the tumor bed due to brain-blood barrier. We chose 24-48 h as the length of the treatment and serum-free medium as the culture condition to investigate any effect of doxycycline on cell proliferation, MMP2 activity and cell invasion. In this study, we applied a new concept, termed normalized MMP2 activity (MMP2 activity/MTT value) in order to minimize confounding by the anti-proliferative effect from doxycycline on the measurement of extracellular active MMP2 level in the treatment group.

Our data revealed two different responses to doxycycline in two glioma cell lines. In the U251HF cell line, cell proliferation was not altered while the extracellular active MMP2 level was decreased with low-concentration doxy-

cycline treatment (5, 10, and 15 μg/ml). By real-time QRT-PCR, we demonstrated unchanged MMP2 mRNA levels in response to doxycycline treatments, which suggested that the effects of doxycycline on MMP2 were at the post-translational level. This is consistent with a known mechanism, that doxycycline inhibits MMP2 through chelation of the zinc ion at the active site of the enzyme [21], rather than at the level of mRNA by reducing MMP2 mRNA stability as reported in one study [24]. Interestingly, there was a 20% decrease in normalized MMP2 activity by a low-concentration doxycycline treatment (5 μg/ml), and the effect of inhibition was not strengthened upon increasing the concentration. This suggests that 5 μg/ml of doxycycline has reached to the maximal inhibition on MMP2 activity in U251HF cells. Conversely, in U87 cells, low-concentration doxycycline slightly inhibited cell proliferation while enhanced extracellular MMP2 activity. One possible explanation is that mild cytotoxic effects exerted by low-concentration doxycycline in U87 cells triggered cellular stress response which activated MMP2 gene transcription, as shown by real-time QRT-PCR. Although not directly observed, there was a concentration-dependent inhibition on MMP2 activity in U87 by doxycycline which masked a concentration-dependent increase of MMP2 secretion. The different responses toward doxycycline in the above two cell lines are striking, which may be related to their genetic difference. A wild-type p53 phenotype may render U87 cells more susceptible to cytotoxic agents than the more aggressive U251HF cell line which has a mutated p53 phenotype.

Emerging evidence suggests a balance between glioma cell migration/invasion and proliferation [35]. Biopsies obtained from glioma specimens and their migratory tracts have demonstrated that highly invasive glioma cells have a slower proliferation rate [36], which renders the invasive glioma cell phenotype relatively resistant to cytotoxic interventions, such as radiation and chemotherapy. It has been reported that the integrin signal transduction pathways are often shared by cell invasion, proliferation and survival [37]. Our study in U251HF and U87 cells provides a good model for further exploration of the regulation mechanism behind the balance between migration/invasion and proliferation. Such a model will enable us to have a better rationale in designing clinical trials with MMP inhibitors and cytotoxic agents.

Our observation of doxycycline's effect in U251HF cells on cell invasion is comparable to data published in the current literature. Doxycycline at 6 μg/ml inhibited osteosarcoma cell invasion by 28.8% [38]. Doxycycline at 5 μg/ml reduced active and latent forms of MMP2 secreted from cultured abdominal aorta aneurysm tissue by 50% and 30%, and cultured smooth muscle cells by 37% [24], suggesting that doxycycline may be a promising anti-aneurysm and anti-atherosclerotic plaque agent for clinical trials [39,40].

Doxycycline is a non-specific MMP inhibitor. Although we were unable to examine the effect of doxycycline on MMP9 due to relatively low MMP9 activities in all three glioma cell cultures, it has been reported that doxycycline inhibits TGF-β1-induced MMP9 via the Smad and MAPK pathways in human corneal epithelial cells [41].

The complexity of the MMP family makes studying MMP inhibitors challenging. MMPs have multiple signaling pathways which might serve as targets for anticancer intervention; however, the inhibition of some MMPs could potentially have pro-tumorigenic effect [42]. So far, two main classes of MMP inhibitors have been investigated. One class represents a family of small molecules that bind to the enzymatic site. Members in the other class are pseudopeptides that act as competitive inhibitors for MMPs [44]. With emerging evidence suggesting that tetracycline family members can function as MMP inhibitors, there is a renewed interest in studying tetracycline analogues, such as doxycycline, minocycline, and especially non-antimicrobial tetracycline analogs. Application of the anti-MMP properties of tetracycline family members has extended into multiple medical fields, such as neurological disease, inflammatory diseases and tumor metastasis [9].

MMP2 has been validated as an anticancer drug target in some aggressive tumor types [42]. Our study is the first to show that doxycycline in acceptable therapeutic concentrations functions as a MMP2 inhibitor of glioma cells, and this is manifested by a decrease in MMP2 activity. Interestingly, doxycycline inhibits U251HF cell migration and invasion in a concentration-dependent fashion which suggests that mechanisms other than a simple decrease in MMP activity are involved. It has been reported that doxycycline exerts an anti-angiogenesis effect independent of being an MMP inhibitor [44].

Doxycycline has a long track record as being a safe drug, and it is the only FDA approved MMP inhibitor for the treatment of periodontitis [8]. Our study revealed that doxycycline at the clinical therapeutic level can decrease MMP2 activity at the post-translational level, and moreover can hinder invasion by one of the most aggressive glioma cell lines. However, in U87 cells, the cytotoxic effects of doxycycline may trigger MMP2 expression indirectly, and this warrants further investigation. With the drug's long and safe history doxycycline clearly deserves more attention as a therapeutic intervention in GBM, especially considering that few efficacious agents are currently available.

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