Research Paper: The Repression of Matrix Metalloproteinases and Cytokine Secretion in Glioblastoma by Targeting K+ Channel

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ABSTRACT

Introduction: Glioblastoma is an aggressive human brain malignancy with poorly understood pathogenesis. Voltage-gated potassium (Kv) channels and Matrix Metalloproteinases (MMPs) are highly expressed in malignant tumors and involved in the progression and metastasis of glioblastoma. This study aimed to determine whether a voltage-dependent potassium channel blocker could modulate astrocytes as a cell involved in the immunopathogenesis of glioblastoma.

Methods: The cytotoxic effect of 4-Aminopyridine (4-AP) at different doses in the cell model of glioblastoma was measured by MTT assay. The ELISA technique and gelatin zymography were used to assess cytokine levels and MMP-9 after 4-AP treatment.

Results: Cytotoxicity analysis data indicated that cell viability reduced by increasing 4-AP level and cell growth decreased gradually by removing 4-AP from the cell medium. 4-AP inhibits the secretion of IL-6 and IL-1 ($P<0.05$). MMP9 activity significantly inhibits with increased 4-AP dose, compared to non-treated cells.

Conclusion: The reduction of cell viability, IL-6 secretion, and MMP-9 activity in an in vitro model of glioblastoma might be assumed 4-AP as an agent for chemoprevention of cancer.

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Highlights

- 4-Aminopyridine, as a K channel blocker, inhibits the secretion of IL-1.
- A voltage-gated potassium channel inhibits the secretion of IL-6.
- MMP9 activity, as a tumor metastasis marker, significantly decreased by 4-AP.

Plain Language Summary

Glioblastoma is the most common primary malignant of the brain, which remains mainly untreatable. A group of enzymes -matrix metalloproteinases- can digest various extracellular matrix macromolecules. They express at a high level and play a role in the glioblastoma invasion. Besides, several substances are secreted by multiple cells and affect cancer metastasis. Among them, cytokines, like interleukin-6, released from glial cells, may contribute to glioblastoma progression. The present study determined whether an agent as a potassium channel blocker could modulate the immunopathogenesis of glioblastoma. We realized the cytotoxic effect of potassium channel blocker at different doses in the U-373 MG glioblastoma astrocytoma cells. Our chosen agent inhibits the secretion of both interleukin and matrix metalloproteinases activity. Overall, we suggest potassium channel blocker as an agent for cancer chemoprevention.

1. Introduction

Glioblastoma Multiforme (GBM) is an aggressive neurological malignancy with non-specific signs and poor prognosis (Yin et al., 2018). Pro-inflammatory cytokines, like Interleukin-6 (IL-6) released from glial cells may contribute to the progression of GBM (Chen et al., 2016; Matias et al., 2018; Wang et al., 2016). Despite immunostaining similarities between glial cells and glioblastoma, studies highlighted the role of astrocytes and oligodendrocytes in tumorigenesis (Krcek, Matschke, Theis, Adamietz, Buhler, & Theiss, 2017; Yin et al., 2018). The etiology of glioblastoma remains unknown; however, the upregulation of FBXL18 in glioma tissues which induces the phosphorylation of Forkhead Box O (FOXO3), was recently reported (Zhang, Yang, Ou, Xia, Zhi, & Cui, 2017). One of the target genes for the stimulated FOXO transcription factors is Matrix Metalloproteinases (MMPs) (Diebold, Petry, Burger, Hess, & Gorlach, 2011).

MMPs are metalloproteinases that are calcium-dependent zinc-containing endopeptidases that can digest various Extracellular Matrix (ECM) macromolecules that play a role in the GBM invasion (Wang, Tong, Jiang, & Yang, 2017). Typically, MMPs are critical mediators of ECM remodeling and regulate normal development and tissue repair. Several studies highlighted the high expression of MMPs and tumor progression in an experimental model and cancer patients (Blaes et al., 2018; Ko et al., 2016). Recently, MMP-9 has been considered a glioma grades biomarker due to its capability to increase capillary permeability (Fan et al., 2018; Li et al., 2016). Thus, MM inhibitors might help control glioblastoma invasiveness.

In malignant tumors, voltage-gated potassium (Kv) channels are highly expressed and involved in the progression of various cancer types (Aissaoui et al., 2018; Martinez et al., 2015). Furthermore, 4-Aminopyridine (4-AP) is a potent inhibitor of the voltage-dependent potassium channel. In vitro studies revealed that it can be improved action potential conduction in poorly myelinated nerve fiber (Rabadi, Kreymborg, & Vincent, 2013). Moreover, 4-AP enhanced apoptotic properties in various cancer cells and passed through the blood-brain barrier (Hassan et al., 2018; Luo et al., 2018; Wang et al., 2014).

The high-grade glioma comprised a cell collection of astrocytoma and others; therefore, using the U-373 MG glioblastoma astrocytoma cells should be an appropriate model to resemble its aggressive behavior. Moreover, these cells can produce pro-inflammatory cytokines. In this study, the cultured U-373 MG was treated by 4-AP, and its cytotoxic and anti-invasive effect was evaluated. Moreover, the impact of 4-AP in the repression of IL-6, IL-1, and Tumor necrosis factor (TNF) levels in the samples was qualitatively determined by ELISA. Reduced cell viability, IL-6 secretion, and MMP-9 activity in an experimental model of GBM might be assumed 4-AP as adjuvant therapy in cancer.

2. Methods

Cell culture

The U-373 MG (human glioblastoma astrocytoma cell line) was obtained from the National Cell Bank of Iran (NCBI), Pasteur Institute of Iran, Tehran, Iran. U-373 MG was seeded at an initial density of 2x104cells/well in 96-well tissue culture plates containing RPMI-1640 medium with 5% fetal calf serum, penicillin at 100 U/mL, and streptomycin at 100 µg/mL (Gibco BRL, Life Technologies, NY, USA). In this experiment, U-373 MG cells were cultured in the presence of 50ng/mL Lipopolysaccharide (LPS) to stimulate and then were pretreated for 4 h with 4-AP at selected concentrations.

Cytotoxicity analysis

Triplicate, twofold dilutions of 4-AP (Merck, Frankfurter, Germany) preparations at concentrations of 0.1–10 mM were added to cultured cells. After overnight incubation under 5% CO2, 37oC, and saturated humidity, cells were subjected to a colorimetric assay using 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide (MTT) (Sigma-Aldrich, St. Louis, MO, USA) assay. MTT (5mg/mL PBS) was added to cell culture, incubated for 4 h at 37 °C, then solubilized with acidic isopropanol. The absorbance was measured on a SPECTRA max PLUS384 reader (Molecular Devices Corp., Sunnyvale, CA, USA) at 570 nm. Based on MTT results, cytotoxicity as the percentage of the viable cell at different concentrations of samples and IC50 as the dose at which 50% of the cell death was calculated. Moreover, viability was evaluated after removing 4-AP from the cell medium.

Zymoanalysis

This technique detected MMP-9 in corresponding supernatants of cells conditioned-media with some modifications (Khorramizadeh et al., 2005). Briefly, the aliquots of conditioned media were subjected to electrophoresis in a polyacrylamide gel containing gelatin (2mg/mL) (Sigma-Aldrich, St. Louis, MO, USA) for 3 hours (80 volts). The gel was washed (2.5% Triton X100), incubated at 37oC overnight (0.1 M Tris HCl, pH 7.4), and afterward stained (0.5% Coomassie Blue). After de-staining, using a UVI Pro Gel Documentation system (GDS-8000 System), proteolysis bands were quantitatively evaluated and expressed as the "relative expression" of gelatinolytic activity.

The quantification of cytokine levels

The levels of IL-1, IL-6, and TNF in the samples were assessed using the ELISA kits (R&D Systems, Minneapolis, USA). The cytokine in the samples is bound to an immobilized antibody as directed by the manufacturer, followed by incubation with an enzyme-linked monoclonal antibody specific to the cytokine provided. After washing, a substrate was applied, and the color changed in proportion to the cytokine quantity. The response was stopped, and the absorption was measured with an ELx800brand ELISA system at 450 nm (BioTek Instruments, Inc., Winooski, VT). The effects of the examined agent on cell proliferation, cytokine secretion, and gelatinase inhibition were analyzed using the Student's t-test in SPSS. The obtained data were presented as Mean±SD with n=3 for in vitro experiments. Besides, the differences were considered significant at $P<0.05$.

3. Results

4-AP decreased the proliferation of human glioma cell lines

The efficacy of 4-Ap therapy is presented on the U-373 MG cell line at various doses (0.1-10µM) in Figure 1. The administration of 4-AP at various concentrations indicates no noticeable low-dose cytotoxicity. The results of Student's t-test indicated that mean % viability value at 0.1 µM of 4-Ap was significantly higher than one at 2 µM ($P<0.05$), 4 µM, and 10µM of it ($P<0.001$). The mean % viability value at 2 µM of 4-Ap was significantly different to one at 4 µM ($P<0.05$) and 10 µM of 4-Ap ($P<0.001$). The difference in mean % viability value between concentrations 4 µM and 10 µM of 4-Ap was also significant ($P<0.001$). Thus, the biocompatibility and tolerability of U-373 MG against higher amounts of 4-AP were significantly less ($P<0.001$).The calculated median lethal dose (LD_{50}) for 4-AP was at concentration of 0.48µM according to equation $y= -4.5x+10.3\ x^2 + 94.8$. The inhibitory effect of 4-Ap on cell proliferation at 48 hours is similar to 24 hours in a concentration below 5 µM (data not presented). Moreover, cell culture changed after 24 hours of drug intervention showed a static effect on cell proliferation at doses over 5 µM after two overnight.

4-AP inhibited matrix metalloproteinase activity in human glioma cell lines

The dose-response study of 4-Ap on MMP-9 activity is depicted in Figure 2. Comparison of the densitometric analysis of gelatinase activity at different doses demonstrated that the inhibitory efficacy of 4-Ap at all doses

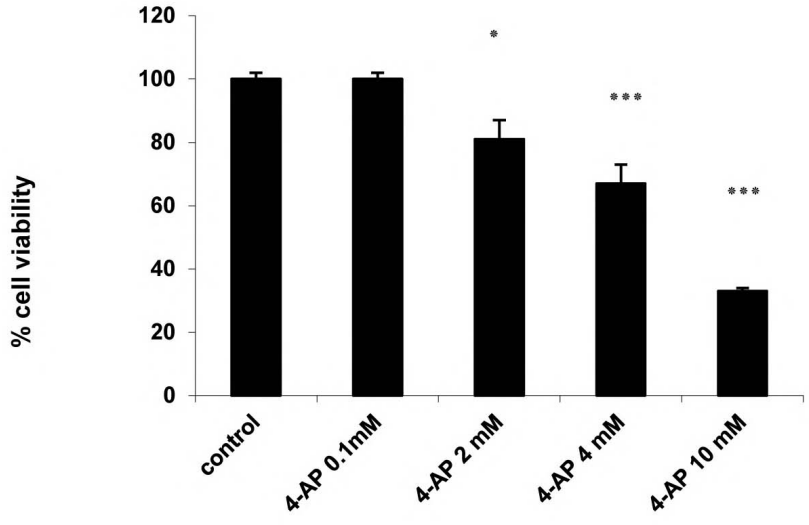

Figure 1. Relative cytotoxic effect

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For the astrocytoma [U373-MG] cell line induced by 4-Ap (* P<0.05, ** P<0.01, *** P<0.001).

was statistically significant compared with non-treated cells (P<0.05). However, the mean % gelatinase activity at 0.1 µM of 4-Ap was not significantly higher than one at 2 µM, 4 µM, and 10µM of 4-Ap.

4-AP diminished cytokine secretion in human glioma cell lines

Our cytokine assay for IL-1 didn't show any statistical differences between treated and non-treated cells (P=0.05). The mean differences between non-treated and treated with 0.1µM were 13.3+ 1.24 vs. 12.8+0.8, respectively. As per Figure 3, the IL-1 level for 4-Ap at a unique concentration of 10µM was statistically significant, compared with non-treated cells (5.5+0.5 vs. 13.3+1.24, P<0.05). Using the ELISA method for measuring TNF, we determined that 4-Ap-treated cells produced little TNF than non-treated cells (122.5+4.9 pg/mL). The TNF decrement due to 4-AP was not statistically significant (Figure 4). Furthermore, administrating 4-AP suggested a significant reduction of IL-6 secretion compared with the control group (P<0.001) (Figure 5). The mean differences of IL-6 between non-treated (320+8.0 pg/mL) and treated with 0.1µM, 2 µM, 4 µM, and 10 µM (209+5.1, 155+8.7, 113+3.0 & 14+1.2), respectively.

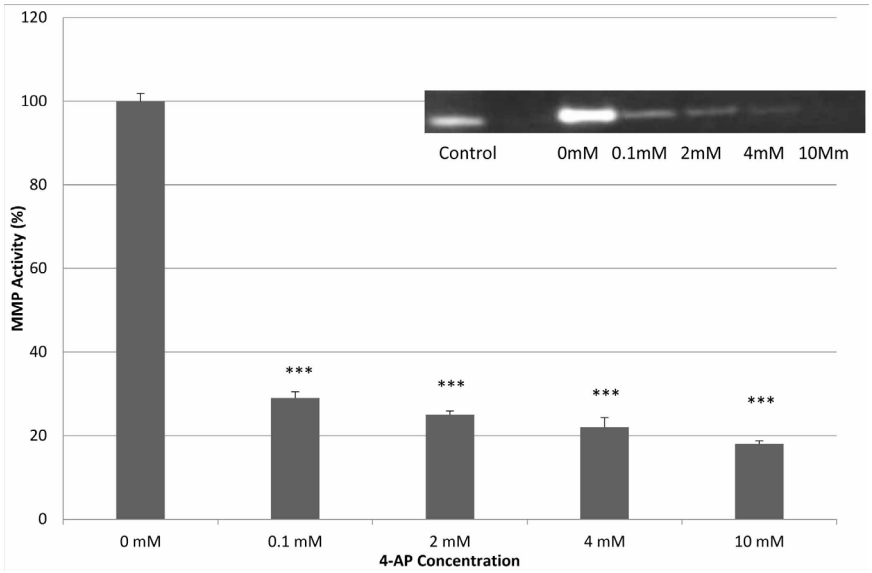

Figure 2. The relative expression of gelatinolytic activity

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For the astrocytoma cell line induced by 4-Ap, compared to the untreated controls (*P<0.05).

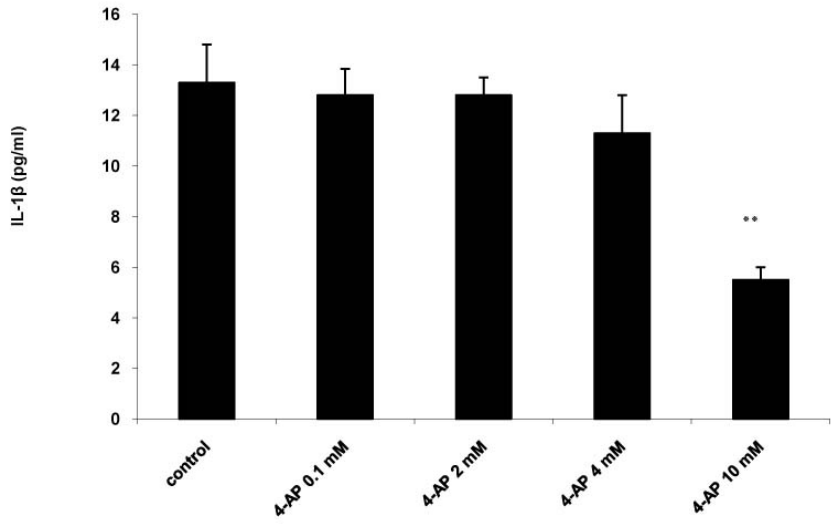

Figure 3. IL-1 concentration

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For the astrocytoma cell line treated with indicated the concentrations of 4-Ap, compared to untreated control (*P<0.01).

4. Discussion

Immune system dysregulation is among the critical mechanisms in the pathophysiology of numerous tumors (Voutsadakis, 2018a, 2018b). Moreover, selective and non-selective ion channels are necessary for the physiopathology of several cancers, including GBM (Morelli et al., 2016). This study attempted to clarify the influence of 4-AP, a broad-spectrum blocker of potassium channels, on MMP-9 activity, various types of cytokines, and its cytotoxic effects on the experimental cell model U373-MG.

Cytotoxicity test for treated cells revealed that 4-AP could exert its toxicity at 2uM up to 10µM. Furthermore, our data and other investigations showed that this agent elicited more minor cytotoxic characteristics at doses below Its LD_{50} (Wang et al., 2014). Moreover, after removing 4-Ap in cell culture, the cell proliferates, suggesting potassium channel blocking should be considered a probable mechanism for 4-Ap inhibitory function. The mechanism by which potassium gate inhibitors, such as 4-AP, exert antitumor effects are not entirely defined but are postulated to involve up-regulation of phosphatase and tensin homolog, modulation of protein kinase B signaling pathway, and apoptosis enhancement (Hassan et al., 2018; Luo et al., 2018; Wang et al., 2014).

The degradation of ECM, primarily by MMPs, is one of the fundamental stages of tumor metastasis (Sharifta-brizi et al., 2005; Zhong et al., 2018). The upregulation

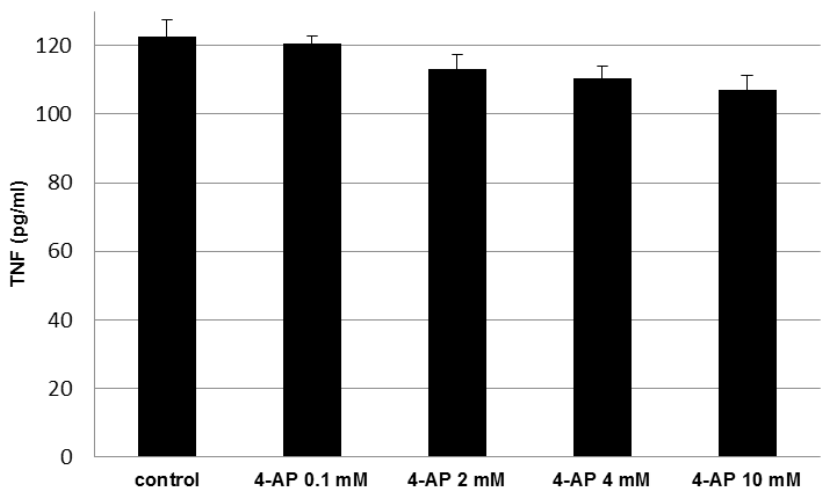

Figure 4. TNF concentration

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For the astrocytoma cell line treated with indicated concentrations of 4-Ap.

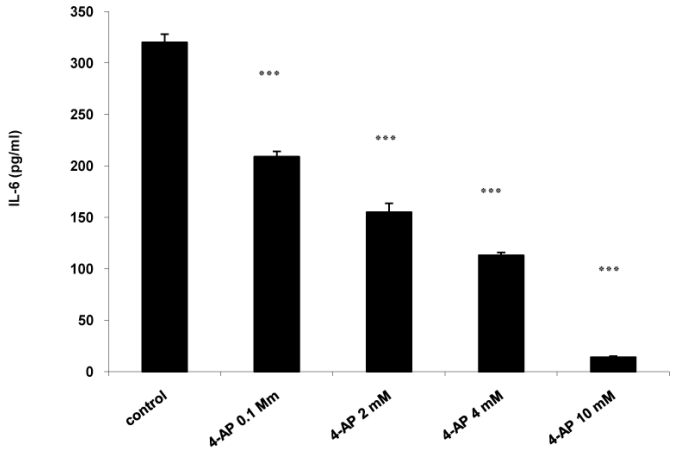

Figure 5. IL-6 concentration

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For the astrocytoma cell line treated with indicated concentrations of 4-Ap, compared to untreated control (*** P<0.001).

of collagen XVI can induce MMP-9 expression by certain glioma cell lines, like U-373 MG; thus, the inhibition of MMPs can prevent tumor metastasis (Bedal, Grassel, Oefner, Reinders, Reichert, & Bauer, 2014; Senner, Ratzinger, Mertsch, Grassel, & Paulus, 2008). Few records are available on the impact of voltage-dependent potassium channels in regulating the action and biosynthesis of MMPs. Some studies reported no TLR4 expression on astrocytes and oligodendroglial cells; however, others demonstrated its presence on glioma cell lines like U-373 MG (Che et al., 2017). LPS stimulation activated TLR 4 signaling, which facilities immune evasion of glioma cells through MMP secretion. As per zymography results, there was a significant reduction of MMP-9 activity in these LPS induced cells; it might indicate various signaling pathways blockage. Our finding revealed that the tested drug inhibited MMP activity in a dose-response fashion (Figure 2). Therefore, this agent's ability to inhibit matrix metalloproteinase could be valuable to prevent tumor invasion and metastasis.

Among different cell subpopulations in the brain, astrocytes play their role as an Antigen-Presenting Cell (APC). These cells produce a collection of cytokines such as IL-6 and TNF in response to provocation. Evidence signified that the inflammatory response that adjusts cytokines pattern occurs in the tumor microenvironment (Roato & Ferracini, 2018; Worzfeld et al., 2017). The effect of 4-AP in modulation cytokine secretion was examined. According to our finding among several cytokines, IL-6 was significantly reduced in astrocytoma cells by treatment with 4-AP compared with the control group. Our data agreed with experiments that demonstrated that GBM cells are up-regulated, and secreted IL-6 and anti-IL6 directed therapies are potential utility for anti-cancer treatments (Trikha, Corringham, Klein, & Rossi, 2003; West et al., 2018).

The strategy for GBM treatment is chemotherapy, radiotherapy, and surgery. Because of cellular complexity, undefined pathology, and the short survival rate of patients with GBM, new promising medication was considered. The meaningful MMP and IL-6 inhibition and the capability to pass through the blood-brain barrier with mild side effects might be recommended 4-AP for chemoprevention of GBM patients. However, the clinical consequence of these findings must be further studied.

5. Conclusion

In summary, our study provides evidence that repression in the expression of MMP and cytokine secretion is an important aspect in glioblastoma. Targeting K+ channel induces cell viability reduction, IL-6 and MMP-9 activity decrement in an in vitro model of glioblastoma. However, more studies are required to confirm 4-AP significant role as chemopreventive agent aginst glioblastoma.

Ethical Considerations

Compliance with ethical guidelines

The research protocol was approved by the local Ethics Committee of the Tehran University of Medical Sciences.

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Authors' contributions

All authors equally contributed to preparing this article.

Conflict of interest

The authors declared no conflict of interest.

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