

## Effects of Cinnamaldehyde on the Viability and Expression of Chemokine Receptor Genes in Temozolomide-treated Glioma Cells

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**Abstract.** *Background/Aim:* The effects of cinnamaldehyde on glioma are still unclear. We aimed to investigate the effects of cinnamaldehyde on the viability and expression of chemokine receptors CXCR4 and CXCR7 in temozolomide (TMZ)-treated glioma cells. *Materials and Methods:* Cell viability and CXCR4 and CXCR7 expression were measured by western blotting at 72 h after treatment with various concentrations of cinnamaldehyde and TMZ. *Results:* Cell viability was significantly lower after treatment with 300  $\mu$ M TMZ, 50  $\mu$ M cinnamaldehyde, 75  $\mu$ M cinnamaldehyde, or combined treatment with 300  $\mu$ M TMZ plus 50  $\mu$ M or 75  $\mu$ M cinnamaldehyde than after no treatment (i.e., without TMZ or cinnamaldehyde); and significantly lower after combined treatment with 300  $\mu$ M TMZ plus 75  $\mu$ M cinnamaldehyde but not 50  $\mu$ M cinnamaldehyde, than treatment with 300  $\mu$ M TMZ alone. Western blotting showed that either single treatments or combined treatments had lower CXCR4 expression (compared to the no-treatment control). Compared to 300  $\mu$ M TMZ alone, both combined treatment of 300  $\mu$ M TMZ plus 50  $\mu$ M

*cinnamaldehyde or 75  $\mu$ M cinnamaldehyde had significantly lowered CXCR4 expression. However, CXCR7 expression was not significantly different in all groups. Conclusion:* Cinnamaldehyde, acting with TMZ, reduces glioma cell viability possibly via decreasing CXCR4 expression.

Patients with malignant glioma have a short life expectancy on currently available treatment, which is surgical resection followed by adjuvant radiotherapy plus temozolomide (TMZ), an alkylating drug. Despite recent advances in basic research and clinical treatment protocols, the prognosis of malignant glioma remains dismal (1). Therefore, it is urgent to search for new pathways of cancer treatment and new agents and/or combined therapies that use unconventional mechanisms of action. For example, caffeine can increase glioma cell death by decreasing histone deacetylase 1 activity and/or by increasing histone acetyltransferase (p300) activity (2). Tetrandrine and caffeine can induce glioma cell death possibly via increasing eukaryotic translation initiation factor-2  $\alpha$  phosphorylation, decreasing cyclin-D1 expression, increasing p300 expression, and increasing caspase-dependent and -independent apoptosis (3, 4).

Chemokine receptors (CXCR) are G protein-coupled receptors that regulate the migration of many different cancer cell types. One of 19 known human chemokine receptors, CXCR4 is activated exclusively by the chemokine CXCL12. CXCR4 and CXCR7 are key regulators in central nervous system development, and involved in neuromodulation, neuroprotection, and interactions between neurons, microglia, and astrocytes in adult brain (5). Also, CXCR4 and CXCR7 are frequently found in brain tumors including

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gliomas, meningiomas, and even pituitary adenomas (6, 7). CXCR4 and CXCR7 are overexpressed in a subpopulation of chemo- and radio-therapy resistant tumorigenic cancer stem-like cells with the invasive potential of glioblastoma cells (8). Recurrence of glioblastoma after chemo-radiation may be associated with a switch in angiogenic pattern from vascular endothelial growth factor receptor 2 (VEGFR2)-hypoxia-induced factor-1 $\alpha$  (HIF-1 $\alpha$ ) to the CXCL12-CXCR4 pathway (9). The CXCL12-CXCR7 axis mediates apoptosis resistance and promotes glioma progression in rat C6 glioma cells (10). Thus, agents against CXCR4 and CXCR7 might possibly be helpful for glioma treatment.

Cinnamaldehyde, molecular formula  $C_9H_8O$ , is a naturally-occurring aldehyde and the main mediator of mast cell inhibition in cinnamon extract (11). It has anti-bacterial, anti-fungal, anti-diabetic, and anti-cancerous activity (12). Cinnamaldehyde has been shown to inhibit inflammation and brain damage in mice with permanent cerebral ischemia (13), and inhibit angiogenesis through inhibition of VEGF receptor type 2 (VEGFR2) via suppressing HIF-1 $\alpha$  gene expression (14). Also, cinnamaldehyde can induce apoptosis in several human tumor cells, including human promyelocytic leukemia HL-60 cells, human oral squamous cell carcinoma HSC-3 cells, and tumors such as non-small cell lung cancer (15-17). It also reverses epithelial-mesenchymal transition through inhibition of the Wnt/ $\beta$ -catenin pathway (16) and has been used to treat various types of cancer, including breast, prostate, and colon cancers, leukemia, hepatocellular carcinoma, and oral cancer (18).

However, the effects of cinnamaldehyde on glioma are still unclear. In this study, our aim was to investigate the effect of cinnamaldehyde on the viability and expression of CXCR4 and CXCR7 in TMZ-treated glioma cells.

## Materials and Methods

**Cell line.** T98G, a human Caucasian glioblastoma cell line, was provided by the National Taiwan University Hospital (Taipei, Taiwan, ROC) and maintained in Modified Eagle's Medium (MEM; Seromed, Berlin, Germany) supplemented with 10% fetal calf serum (FCS; Biological Industries, Bet-Haemek, Israel), 2 mM L-glutamate, 100 U/ml penicillin, and 100 lg/ml streptomycin. All cells were cultured at 37°C in a 5% CO<sub>2</sub> incubator.

**Cell viability.** Cell viability was measured at 72 hours after treatment with various concentrations of TMZ (300  $\mu$ M), 50  $\mu$ M cinnamaldehyde, 75  $\mu$ M cinnamaldehyde, or combined treatment using an MTT (3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazoliumbromide)-based colorimetric assay. For this assay,  $5 \times 10^3$  cells were seeded in triplicate wells of a flat-bottomed 24-well microtiter plate and cultured overnight before treatment.

**Western blot analysis.** The total protein concentrations in cell extracts were measured using a BCA protein assay kit (Pierce, Rockford, IL, USA). Cellular protein (20-50  $\mu$ g) was loaded onto 10% SDS polyacrylamide gels using bovine serum albumin for a

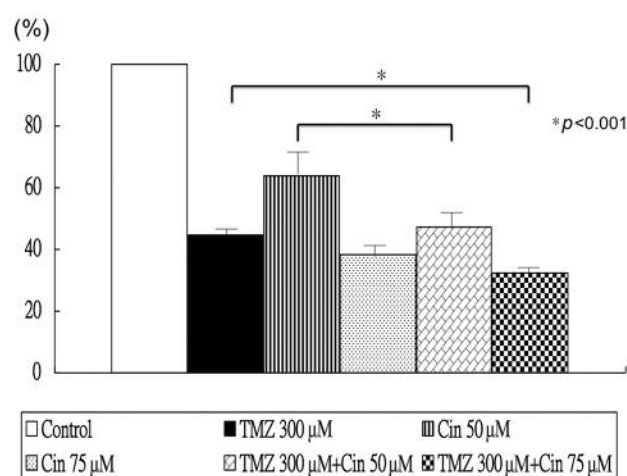


Figure 1. Effects of TMZ and/or cinnamaldehyde treatment on the viability of T98G cells at 72 h. Cell viability was significantly lower in cells than in the control (untreated) cells, regardless of treatment. In addition, viability was significantly lower in cells treated with TMZ 300  $\mu$ M and cinnamaldehyde 75  $\mu$ M than in cells treated with TMZ 300  $\mu$ M alone.

reference standard. Protein bands were then transferred electrophoretically to PVDF membranes (Micron Separations, Westborough, MA, USA), and the membranes were treated with various antibodies (anti-CXCR4, -CXCR7, - $\beta$ -actin), followed by a horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and Western blotting reagent ECL (Santa Cruz Biotechnology) to visualize the antigen-antibody reactions.

**Statistical analysis.** The expression levels of CXCR4 and CXCR7 relative to those of  $\beta$ -actin were compared separately between all groups by one-way analysis of variance (ANOVA) with post-hoc Bonferroni correction. All analyses were performed using the commercialized software STATA10, and  $p < 0.05$  was considered statistically significant.

## Results

Figure 1 shows the effects of TMZ and/or cinnamaldehyde on the cell viability of T98G cells at 72 h. Compared to control (untreated)-cell viability, treated cell viability was significantly lower regardless of treatment (one way ANOVA,  $p < 0.0001$ ) or treatment with 300  $\mu$ M TMZ alone ( $44.7 \pm 1.7\%$ ,  $p < 0.001$ ); 50  $\mu$ M cinnamaldehyde alone ( $63.8 \pm 9.3\%$ ,  $p < 0.001$ ); 75  $\mu$ M cinnamaldehyde alone ( $38.1 \pm 2.0\%$ ,  $p < 0.001$ ); combined 300  $\mu$ M TMZ and 50  $\mu$ M cinnamaldehyde ( $47.2 \pm 4.7\%$ ,  $p < 0.001$ ); and combined 300  $\mu$ M TMZ and 75  $\mu$ M cinnamaldehyde ( $32.4 \pm 1.4\%$ ,  $p < 0.001$ ).

Cells receiving combined treatment [*i.e.*, 300  $\mu$ M TMZ plus 75  $\mu$ M cinnamaldehyde ( $p = 0.001$ ), but not 300  $\mu$ M TMZ plus 50  $\mu$ M cinnamaldehyde] had a significantly lower viability than cells treated with 300  $\mu$ M TMZ alone.

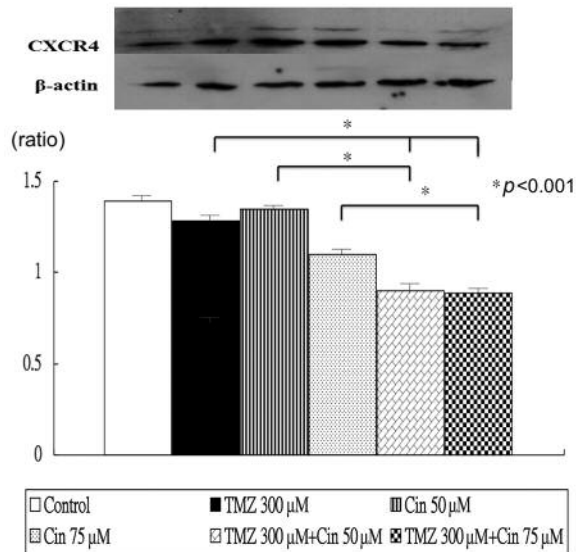


Figure 2. Western blot results for CXCR4 expression in T98G glioma 72 h after TMZ and/or cinnamaldehyde treatment. In addition, cells receiving the combined treatment (300 μM TMZ plus 50 μM cinnamaldehyde and 75 μM cinnamaldehyde) had a significantly lower protein expression of CXCR4 relative to β-actin than cells treated with TMZ 300 μM alone.

Figure 2 shows the western blot results for CXCR4 expression of T98G cells 72 h after TMZ and/or cinnamaldehyde treatment. There was a significant difference of CXCR4 expression (relative to β-actin) between different treatment groups (one-way ANOVA,  $p < 0.0001$ ). Compared to CXCR4 expression (relative to β-actin) in control cells ( $1.39 \pm 0.01$ ), CXCR4 expression was significantly lower in cells receiving 300 μM TMZ alone ( $1.28 \pm 0.01$ ,  $p < 0.001$ ), 50 μM cinnamaldehyde alone ( $1.35 \pm 0.01$ ,  $p = 0.015$ ), 75 μM cinnamaldehyde alone ( $1.10 \pm 0.00$ ,  $p < 0.001$ ), the combined treatment (300 μM TMZ plus 75 μM cinnamaldehyde;  $0.90 \pm 0.01$ ,  $p < 0.001$ ), and the combined 300 μM TMZ plus 50 μM cinnamaldehyde ( $0.89 \pm 0.03$ ,  $p < 0.001$ ). Furthermore, cells receiving combined treatment [*i.e.*, 300 μM TMZ plus 50 μM cinnamaldehyde ( $p < 0.001$ ) and 300 μM TMZ plus 50 μM cinnamaldehyde ( $p < 0.001$ )] had significantly lower CXCR4 expression than cells treated with 300 μM TMZ alone.

Figure 3 shows the western blot results for CXCR7 expression of T98G cells 72 hours after TMZ and/or cinnamaldehyde treatment. The expressions of CXCR7 *versus* β-actin were not significantly different regardless of treatment (one-way ANOVA,  $p = 0.1511$ ).

## Discussion

This study showed that cinnamaldehyde and TMZ exerted synergistic cytotoxic effects on glioma cells. Both TMZ and cinnamaldehyde decreased CXCR4, but not CXCR7, protein

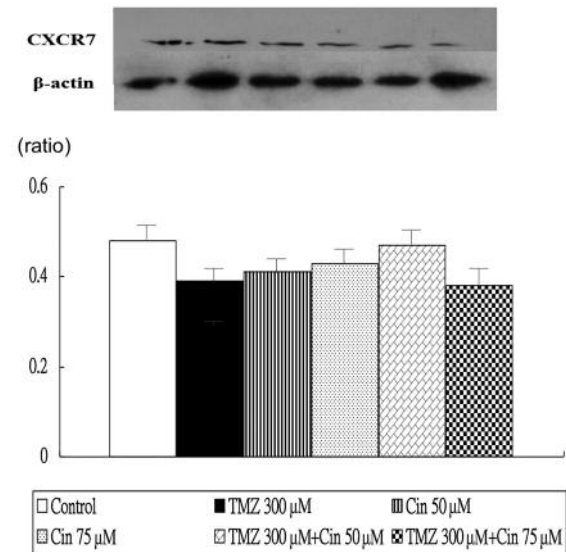


Figure 3. Western blot results for CXCR7 expression in T98G glioma 72 h after TMZ and/or cinnamaldehyde treatment. CXCR7 expression relative to β-actin was not significantly different in all studied groups.

expression in glioma cells. Also, cinnamaldehyde significantly enhanced TMZ-induced decrease in cell viability and CXCR4 expression of glioma cells.

Previous studies have shown that CXCR might be involved in the carcinogenesis of glioma. For example, there is a relatively large CXCR4-positive subset of glioma stem cells (19). CXCL12 and its receptor CXCR4 promote glioma growth and angiogenesis from glioma stem-like cells by stimulating VEGF production (20). As for CXCR7, it is strongly expressed in many tumor types, but weakly expressed in most normal tissues. The chemokine receptor CXCR7 is highly expressed in human glioma cells and mediates anti-apoptotic effects (21). CXCR7 is also involved in cell survival, cell adhesion, tumor development, and metastasis. Expression of CXCR7 has been associated with the more aggressive character of cancers, such as prostate cancer, breast cancer, glioma, and hepatocellular carcinoma (22).

CXCR4 and CXCR7 might play different roles on cancer formation and progression. Meanwhile, our results showed that both TMZ and cinnamaldehyde decreased CXCR4, but not CXCR7, protein expression in glioma cells. Thus, it seems that both TMZ and cinnamaldehyde might exert their cytotoxicity on glioma *via* inhibiting the CXCR4-related angiogenesis pathway mainly (9, 20), but not *via* inhibiting CXCR7-related apoptosis resistance or metastasis pathway (10, 21, 22).

Furthermore, resistance to long-term TMZ treatment is a big challenge in the treatment of glioma. And, this resistance was reported to be correlated with the up-regulation of

CXCL2, CXCL3, and CXCL8 expression in the U373 and T98G cells (23). Transcription of CXCL12 and its receptors were also significantly induced by sub-lethal doses of TMZ (10). In the present study, we showed that 300  $\mu$ M TMZ treatment for 72 h could decrease glioma cell viability as well as CXCR4 expression in glioma cells. And, TMZ-induced changes on glioma cells could be enhanced by cinnamaldehyde.

A previous study showed that recurrence of glioblastoma after chemo-radiation could be associated with a switch in angiogenic pathway from VEGFR2 HIF-1 $\alpha$  to CXCL12-CXCR4 (9). Zhang *et al.* reported that different isomers of cinnamaldehyde can inhibit angiogenesis through inhibition of VEGFR2 *via* suppressing *HIF-1 $\alpha$*  gene expression (14). Herein, we showed that cinnamaldehyde decreased CXCR4 protein expression in glioma cells. Thus, we surmise that cinnamaldehyde might be used to decrease the recurrence of glioblastoma after chemo-radiation.

## Conclusion

Both TMZ and cinnamaldehyde were cytotoxic and decreased CXCR4 protein expression in glioma cells. Cinnamaldehyde also enhanced TMZ-induced glioma cell. These findings might be helpful in clinical trial design in the future.

## Conflicts of Interest

The Authors declare no conflicts of interest. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

## Authors' Contributions

JCC performed the treatment and wrote the manuscript. PSH and SMC performed the study. JHH designed the study, performed the treatment, analyzed data, wrote manuscript and supervised the study.

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