# Tramadol attenuates the sensitivity of glioblastoma to temozolomide through the suppression of Cx43-mediated gap junction intercellular communication

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**Abstract.** Analgesics and antineoplastic drugs are often used concurrently for cancer patients. Our previous study reported that gap junctions composed of connexin32 (Cx32) was implicated in the effect of analgesics on cisplatin cytotoxicity. However, the effect of analgesic on the most widely expressed connexin (Cx), connexin43 (Cx43), and whether such effect mediates the influence on chemotherapeutic efficiency remain unknown. By manipulation of Cx43 expression or gap junction function, we found that there were gap junction-dependent and independent effect of Cx43 on temozolomide (TMZ) sensitivity in U87 glioblastoma cells. Studies on survival and apoptosis showed widely used analgesic tramadol significantly reduced TMZ-induced cytotoxicity in control and negative control cells but not shCx43-transfected cells. Proliferation assay demonstrated tramadol suppressed TMZ-induced cytotoxicity only on high density (with gap junction formation) but not on low density (without gap junction formation). Tramadol inhibited dye-coupling through gap junctions between U87 cells. Tramadol treatment for 72 h did not alter Cx43 expression, but decreased Cx43 phosphorylation accompanied with reduced p-ERK and p-JNK. Our results indicated that long-term treatment with tramadol reduced TMZ cytotoxicity in U87 cells by suppressing Cx43-composed gap junctions, suggesting identification and usage of antinociceptive drugs which do not downregulate connexin activity should have beneficial therapeutic consequences.

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*Abbreviations:* GJIC, gap junctional intercellular communication; TMZ, temozolomide; 18-α-GA, 18-α-glycyrrhetinic acid; RA, retinoic acid; MAPKs, mitogen-activated protein kinases; GFP, green fluorescence protein

*Key words:* tramadol, glioblastoma, temozolomide, gap junction, connexin 43

## Introduction

Cancer remains the leading cause of death worldwide. Pain resulting from the abnormal growth of malignant cells is the most common and most feared symptom in cancer patients. Beyond that, the metastatic carcinomas and typically antineoplastic approaches involving chemotherapy, radiotherapy also cause substantial pain that is required to be treated with analgesic drugs (1). For these reasons, analgesics and chemotherapeutic drugs are often used concurrently in antitumor therapy. Thus, there is an urgent need to explore the potential influence of analgesic agents on the antitumor efficacy of antineoplastic drugs and the mechanism of any such effects.

Gap junctions are formed when two hemichannels, in neighboring cells, align and then dock with each other to form a full gap junction channel that spans the space between the two closely apposed cells. Each hemichannel is composed of 6 connexin (Cx) subunits. The six connexin subunits can be homogeneous or heterogeneous. The formation of stable gap junctions allows for direct communication between the cytoplasmic compartments of the two neighbouring cells, as well as providing a conduit for electrical signaling. The channels are large enough to allow for small hydrophilic metabolites and messenger molecules to pass between cells. In humans, 21 members of the Cx family have been identified

and are named by their molecular weights. Among them, Cx43 is the most ubiquitously expressed and is critical for regulating biological and pathological processes in a variety of tissues (2,3).

Gap junction intercellular communication (GJIC) sensitizes cancer cells to chemotherapeutic agents, radiation and other antitumor therapies. Some metabolites or death signal triggered in target cancer cells by those antineoplastic treatments may transfer through gap junctions, thereby substantially increasing their antitumor efficacy (4-6). Such toxicity amplifying phenomenon mediated by GJIC is termed as bystander effect. Specifically, gap junction composed of Cx43 has been shown to increase the cytotoxicity induced by etoposide and cisplatin in several cell lines (7,8). Moreover, Cx43 participate in various essential cellular processes including tumor cell proliferation, migration and chemotherapeutic sensitivity independent of gap junction formation (9,10). Gap junction dependent or independent effects thereby strongly suggest that regulation of Cx43 or gap junctions by pharmacological or biologic approaches would modulate the efficacy of antitumor strategies.

Our research has previously proved that analgesics tramadol and flurbiprofen inhibited gap junction channels in HeLa cells expressing Cx32, and in this case, decreased cisplatin cytotoxicity (11). Although Cx43 is ubiquitously expressed in majority of tissues, there is no evidence of the effect of these analgesic drugs on Cx43 and its constituent gap junctions. Whether Cx43 or Cx43-composed gap junction channels will be affected by analgesics thereby altering chemotherapeutic efficiency has not been investigated.

Temozolomide (TMZ) is used as a first-line antineoplastic drug against Cx43 expressing glioblastoma (GBM) (12). We investigated the interplay between the analgesic tramadol, Cx43 and Cx43-composed gap junction, and the cytotoxicity of TMZ in U87 glioblastoma cells. In the present study, we report that long-term treatment of tramadol attenuated TMZ cytotoxicity through the inhibition of Cx43-composed gap junction.

## Materials and methods

*Materials*. TMZ was purchased from Shanghai Selleck Chemicals Co., Ltd. (Shanghai, China). Tramadol, 18-α-glycyrrhetinic acid (18-α-GA) and retinoic acid (RA) were from Sigma-Aldrich (St. Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), penicillin/streptomycin, calcein-acetoxymethyl ester and CM-DiI were obtained from Invitrogen (Carlsbad, CA, USA). All the antibodies and other reagents were from Sigma-Aldrich unless otherwise indicated.

*Cell lines and cell culture*. U87 cells were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). Cells were maintained in DMEM containing 10% FBS and 1% penicillin/streptomycin at 37˚C in an atmosphere of 5% $CO_2$ in air.

*Drug treatment and gap junction inhibition*. TMZ was reconstituted in dimethyl sulfoxide (DMSO) at the concentration of 50 mM, and then aliquots were stored at -20˚C. Exposures to TMZ were performed for indicated times in the dark with or without tramadol at various concentrations dissolved in phosphate-buffered saline (PBS). Cells were incubated with the gap junction inhibitor 18-α-GA (50 $\mu$M in DMSO) or potentiator RA (20 $\mu$M in DMSO) for 4 h before exposure to TMZ and during TMZ treatment.

*Transfection*. We used the stable cell lines expressing endogenous shRNA to downregulate Cx43 expression as described (12,13). The PRP.EX3d-MCS1>shCx43>PGK/puro plasmid expressing green fluorescence protein (GFP), shRNA targeting Cx43 (target sequence: 5'-CAATTCTTCTTGCC GCAATTACTCGAGTAATTGCGGCAAGAAGAATTG-3') and the PRP.EX3d-MCS1>negative control>PGK/puro plasmid were purchased from Cyagen Biosciences, Inc. (Santa Clara, CA, USA). Plasmids were separately transfected into U87 cells, and transfected cells were selected on 0.3 mg/ml G418 for 30 days. Before the application of stable transfection cell lines, western blot analysis was used to examine the expression of Cx43 and a 'parachute' dye-coupling assay was performed to examine the dye spread through GJIC.

'*Parachute*' *dye-coupling assay*. To evaluate gap junction function, 'parachute' dye-coupling assay was performed as described by Goldberg *et al* (14) and Koreen *et al* (15). Donor and receiver cells were grown to confluence. The donor cells were double-labeled with 5 $\mu$M CM-DiI, a membrane-permeable dye that does not spread to coupled cells, and with 5 $\mu$M calcein-AM, which is converted intracellularly into the gap junction permeable dye calcein. The donor cells were washed and unincorporated dye was removed. Then, cells were trypsinized and seeded onto the receiver cells at a 1:150 donor/receiver ratio. The donor cells attached to the monolayer of receiver cells and form gap junctions for 4 h at 37˚C. Then examined with a fluorescence microscope. For each experimental condition, the average number of receiver cells containing dye per donor cell was determined and normalized to that of control cultures. To detect functional gap junctions in GFP-expressing cells, we reduced the exposure time to a suitable value to exclude the interference of GFP. The donor and receiver cells were exposed to tramadol during indicated periods in which the donor cells were plated onto the receive cell monolayer.

*Western blotting*. Whole-cell lysates for western blotting were prepared by cell incubation in lysis buffer with protease/phosphatase inhibitors at 4˚C. Then, cell lysates (10 $\mu$g) were separated by SDS/PAGE in 13% Tris-glycine mini-gels and transferred to a PVDF membrane. Membranes were blocked with 5% skim milk-TBST for 30 min. Individual membranes were probed with specifical primary antibody against Cx43 diluted to 1:4,000, p-Cx43 and caspase-3 diluted to 1:2,000, β-tubulin diluted to 1:10,000, ERK, JNK and p38 diluted to 1:1,000 (Cell Signaling Technology, Danvers, MA, USA). After washing with TBST, membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies dissoved in 5% milk-TBST for 30 min at 37˚C. The immunoreactive bands were visualized by ECL plus western blotting detection systerm (GE Healthcare, Piscataway, NJ, USA). All western blot exposures were in the linear range

of detection, and the intensities of the resulting bands were quantified by Quantity One software on GS-800 densitometer (Bio-Rad Laboratories, Hercules, CA, USA).

*CCK-8 assay*. To test the cytotoxicity of TMZ, we used the Cell Counting kit-8 (CCK-8; Dojindo Molecular Technologies, Kumamoto, Japan) which utilizes a highly water-soluble tetrazolium salt for the cell proliferation assay. U87 cells were seeded at a density of $3x10^3$/well in 96-well plates. After treatment with TMZ with or without tramadol for indicated time periods, CCK-8 solution and DMEM at a ratio of 1:9 was added to each well to a total volume of 100 $\mu$l. After 2-h cell incubation, an ultraviolet spectrophotometer was used to determine the optical density at 450 nm (IX51; Beckman Coulter, Brea, CA, USA). Samples for each group were analyzed in triplicate. Cell survival rates with various treatments were calculated using the following formula: Survival rate = Optical density drug/Optical density control.

*Standard colony-forming assay*. Cell survival was assayed by a standard colony-forming assay, adapted for use at high and low cell density, corresponding to conditions in which junctional channel formation was permitted or not, respectively (5). In the high density condition, cells were seeded at 30,000 cells/cm$^2$ so that cultures were 70%-100% confluent during TMZ exposure. Cells were treated with TMZ for 1 week, then washed with PBS, harvested by trypsinization, counted, diluted and seeded into 6-well dishes (500 cells/cm$^2$). Colony formation was assessed 5-7 days later by fixation and staining with crystal violet. Colonies containing 50 or more cells were scored. In the low density condition, cells were directly seeded at the density of 500 cells/cm$^2$ into 6-well plates and treated with TMZ after attachment. They were rinsed and assessed for colony formation as above. Colony formation was normalized to the colony forming efficiency of non-TMZ treated cells. There was no significant difference in plating efficiency between the low- and high-density cultures in the untreated samples (data not shown). Tramadol was added to the culture medium during TMZ treatment.

*Annexin V/PI double-staining assay*. Apoptosis was analyzed using flow cytometry with Annexin V/PI double-staining to detect membrane events. U87 cells ($3.0x10^4$/ml) were seeded into 6-well plates and treated with TMZ with or without 30 $\mu$M tramadol for 1 week. Both floated and attached cells were collected, washed with ice-cold PBS twice, and then incubated at room temperature in the presence of media-binding buffer containing Annexin V (2.5 $\mu$g/ml) and PI (2 ng/ml) for 15 min in the dark. Apoptosis was quantified by flow cytometry (Becton-Dickinson, Franklin Lakes, NJ, USA) immediately and analyzed by the FlowJo software. The cytogram of the four quadrants in the figures was used to distinguish normal (Annexin $V^-/PI^-$), early apoptotic (Annexin $V^+/PI^-$), late apoptotic (Annexin $V^+/PI^+$) and necrotic (Annexin $V^-/PI^+$) cells. Early apoptotic cells appeared in the lower right (Annexin $V^+/PI^-$) quadrant.

*Statistical analysis*. Statistical package for social sciences (SPSS) version 13.0 was used and differences with $P<0.05$ are considered statistically significant. Two-group comparisons were analyzed using an unpaired Student's t-test. A one-way ANOVA with Tukey's post hoc test was used to analyze the multiple group comparisons. All analyses were plotted using SigmaPlot (Jandel Scientific, Inc., San Rafael, CA, USA).

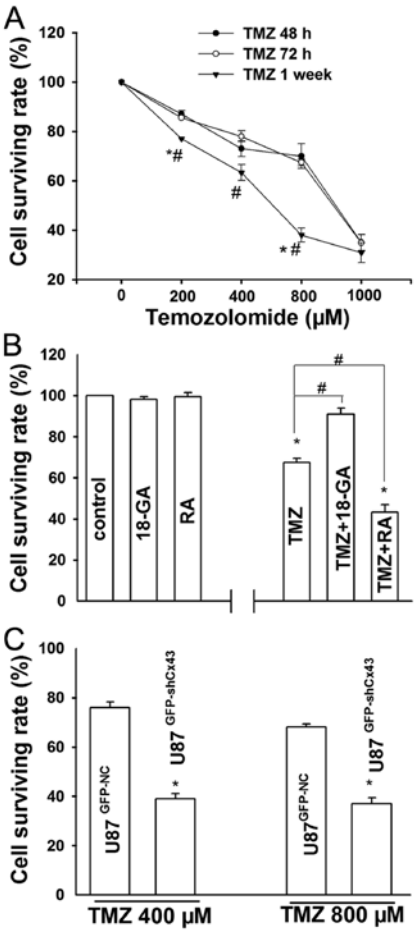


Figure 1. The effects of Cx43 and gap junction intercellular communication on temozolomide cytotoxicity. (A) As detected by CCK-8 assay described in Materials and methods, temozolomide at different concentrations reduced U87 cell survival rates at the indicated times. Points, mean for four experiments; bars, SEM. *Significantly different from 24-h group; #significantly different from 48-h group. (B) Survival rates of U87 cells in the presence of 800 $\mu$M temozolomide for 72 h with or without gap junction inhibitor 18-α-GA (50 $\mu$M) and gap junction enhancer RA (20 $\mu$M). (C) Survival rates of U87 cells in the presence of 800 $\mu$M TMZ for 72 h in both $U87^{GFP-NC}$ and $U87^{GFP-shCx43}$ cells. Columns, mean for four experiments; bars, SEM. *$P<0.05$, significantly different from control in (B) and from the negative control in (C); #$P<0.05$, significantly different from temozolomide group in (B). TMZ, temozolomide.

## Results

*The effect of Cx43 and Cx43-composed gap junctions on TMZ-induced cytotoxicity in U87 cells*. To evaluate TMZ cytotoxicity, U87 cells were treated with TMZ for indicated time periods and then the cell viability was assessed using CCK-8 assays (Fig. 1). The cell optical density after TMZ treatment relative to the optical density of control is shown as cell survival rates. Fig. 1A illustrated that TMZ treatment (from 0 to 1,000 $\mu$M) for 48, 72 h and 1 week significantly reduced U87 cell survival in a concentration-dependent manner. The survival rates of U87 cells after 48 h TMZ treatment was

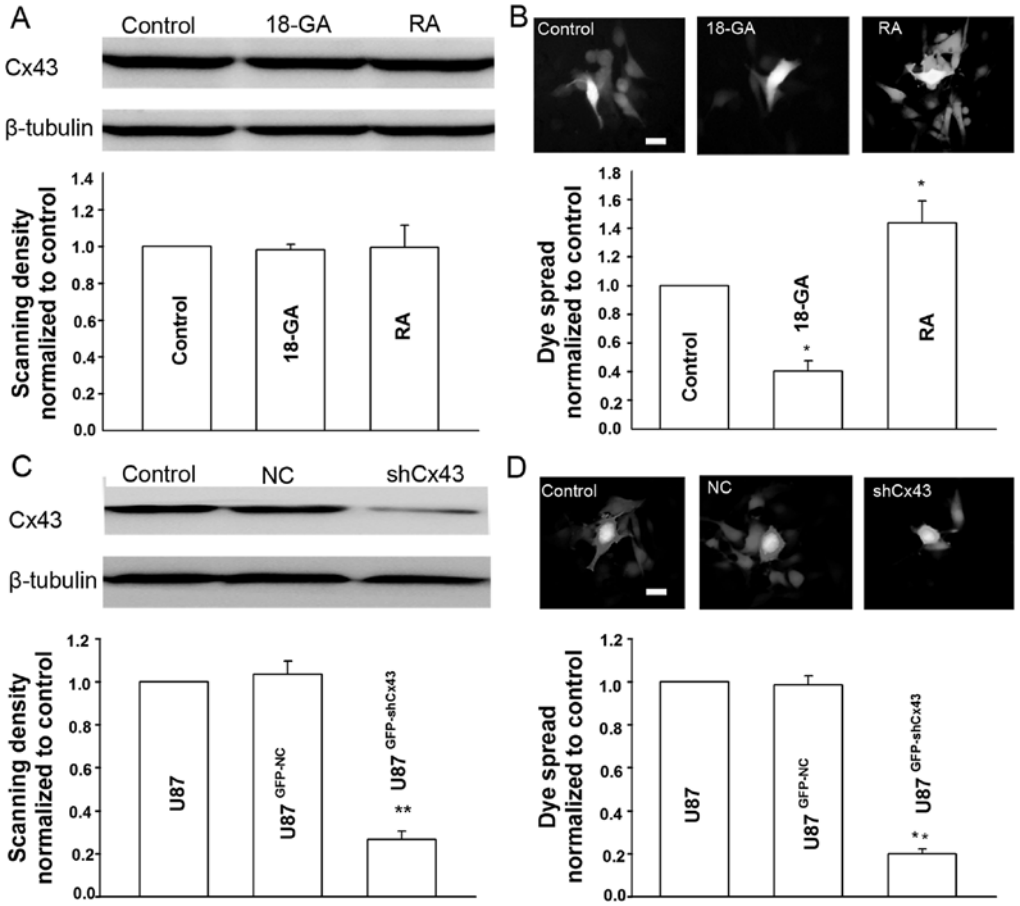


Figure 2. Modulation of Cx43 expression and gap junction intercellular communication by shCx43 and pharmacological inhibitor 18-α-GA or enhancer RA. (A) Western blot analysis shows no change was detected for Cx43 expression in cells treated with 50 $\mu$M 18-α-GA and 20 $\mu$M RA for 72 h. (B) As measured by parachute dye-coupling, treatment of 18-α-GA for 72 h reduced the dye coupling through gap junctions in U87 cells, while treatment of RA for 72 h enhanced the dye spread between U87 cells (magnification, x100). (C) Western blot analyses shows shRNA-mediated knockdown of Cx43 expression in U87$^{GFP\text{-}NC}$ and U87G$^{GFP\text{-}shCx43}$ cells. (D) The dye spreading rate in Cx43-deficient cells (U87$^{GFP\text{-}shCx43}$) is lower than that in U87 cells and U87$^{GFP\text{-}NC}$ cells (magnification, x100). Columns, the means of three to four experiments; bars, SEM. $^{**}$P<0.01 significantly different from control cells in (C and D).

similar with that of 72 h. Though cell surival of U87 cells after 0-800 $\mu$M TMZ exposure for 1 week was much lower than that in both 48- and 72-h treatment groups, similar inhibition of the cell survival was observed after 1,000 $\mu$M TMZ treatment for all indicated times.

To investigate the effect of intercellular communication by gap junction on cytotoxicity, cells exposed to TMZ were concurrently treated with the gap junction inhibitor 18-α-GA or a gap junction enhancer RA. Western blot analysis showed that neither 20 $\mu$M RA nor 50 $\mu$M 18-α-GA affected Cx43 expression (Fig. 2A). Data from a parachute assay is shown in Fig. 2B: 18-α-GA inhibited dye transfer between U87 cells while RA enhanced dye transfer. In this case, dye transfer is used as a surrogate of cell coupling via gap junctions. Additionally, stable cell lines expressing endogenous Cx43-shRNA were used to evaluate the effect of Cx43 on TMZ cytotoxicity. As shown in Fig. 2C and D, both Cx43 expression and gap junction communication were drastically downregulated in Cx43-shRNA transfected U87 (U87$^{GFP\text{-}Cx43shRNA}$) cells.

As shown in Fig. 1B, neither 18-a-GA nor RA had any effect by themselves on U87 cell survival. In contrast, TMZ by itself significantly reduced cell survival in U87 cells (800 $\mu$M TMZ for 72 h). RA (20 $\mu$M) significantly enhanced TMZ-induced cytotoxicity, resulting in a lower survival of U87 cells when the drugs were applied concurrently. Conversely, TMZ-induced cytotoxicity was significantly suppressed by 18-α-GA (50 $\mu$M), resulting in higher survival when U87 cells were treated with both drugs. These results suggest an important role for gap junctions in determining U87 cell survival following exposure to TMZ. In Fig. 1C, U87$^{GFP\text{-}Cx43shRNA}$ cells were much more sensitive to TMZ, yielding a reduced survival fraction when treated with 400 or 800 $\mu$M TMZ as compared with U87$^{GFP\text{-}NC}$ cells. This indicated Cx43 may act as a single protein to decrease the sensitivity of U87 cells to TMZ.

*Tramadol attenuates TMZ-induced cytotoxicity in U87 cells in the presence of Cx43, but not in its absence*. Fig. 3 indicates the effect of tramadol on cell response to TMZ using CCK-8 assay. Treatment with different doses of tramadol alone for as long as 1 week did not alter cell survival in U87 cells (Fig. 3A). Tramadol alleviated TMZ cytotoxicity in a dose-dependent manner, resulting in substantially higher U87 cell survival rates when U87 cells were treated with both drugs for 48, 72 h and 1 week (Fig. 3B). However, tramadol treatment for 72 h had no effect on TMZ cytotoxicity in U87$^{GFP\text{-}Cx43shRNA}$ cells. No alteration in TMZ cytotoxicity was observed even with prolonged tramadol treatment for 1 week (Fig. 3C). Our results here strongly suggest that the

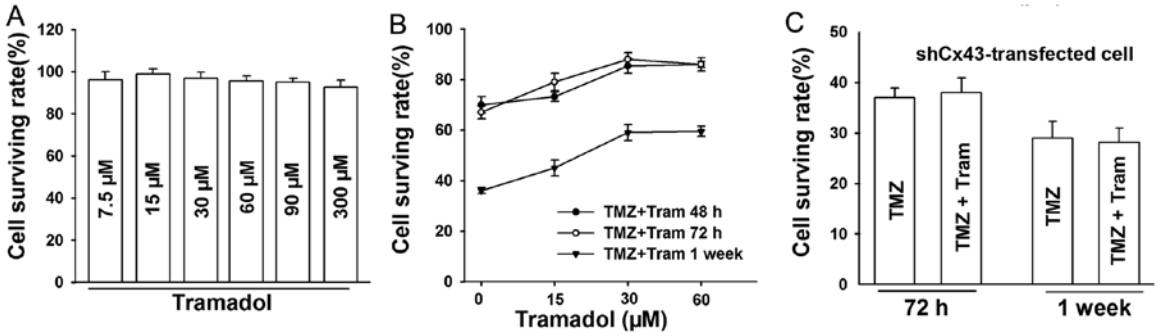


Figure 3. Tramadol inhibited TMZ cytotoxicity in the presence of Cx43. (A) Treatment with different concentrations of tramadol for 1 week had no effect on U87 cell survival rates. (B) Survival rates of U87 cells in the presence of 800 $\mu$M TMZ with or without different concentrations of tramadol treatment for 48, 72 h and 1 week. Points, mean for four experiments; bars, SEM. (C) Survival of U87$^{GFP\text{-}shCx43}$ cells in the presence of 800 $\mu$M with or without 30 $\mu$M tramadol for 72 h or 1 week. Columns, the means of three to four experiments; bars, SEM. TMZ, temozolomide.

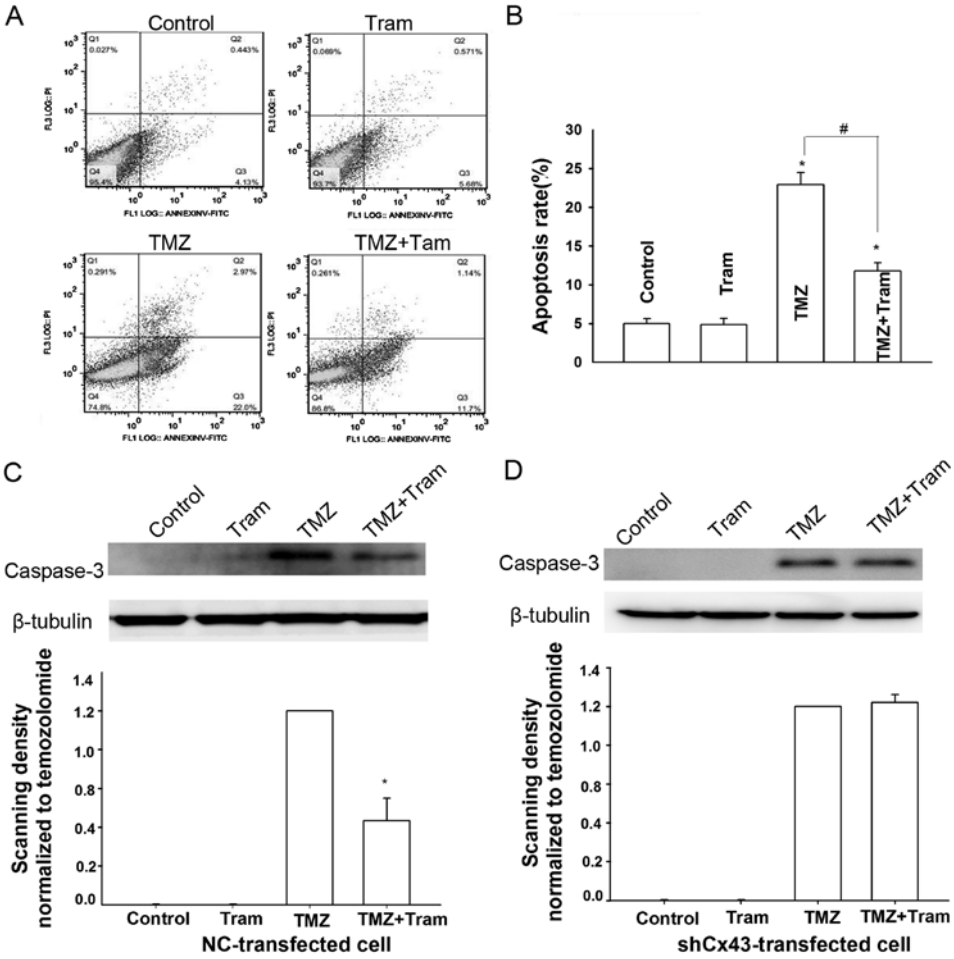


Figure 4. Tramadol inhibites TMZ induced apoptosis in the presence of Cx43 assayed by flow cytometry and cleaved caspase-3 expression. (A and B) Cytograms from flow cytometric analysis labeled with Annexin V/PI showing the proportion of early apoptotic cells (positive for only Annexin V) after 800 $\mu$M TMZ treatment for 1 week with or without 30 $\mu$M tramadol. Columns, means from three independent experiments; bars, SEM. $^{*}$P<0.05, significantly different from control group; $^{\#}$P<0.05, significantly different from TMZ group. (C) The expression of cleaved caspase-3 in U87$^{GFP\text{-}NC}$ cells treated with TMZ with or without tramadol. (D) The expression of cleaved caspase-3 in U87$^{GFP\text{-}shCx43}$ cells treated with TMZ with or without tramadol. Columns, means from three independent experiments; bars, SEM. $^{*}$P<0.05, significantly different from TMZ group in (C and D). TMZ, temozolomide.

reduction of TMZ cytotoxicity by tramadol was dependent on Cx43.

*Tramadol attenuates TMZ-induced apoptosis in U87 cells in the presence of Cx43, but not in its absence*. Flow cytometry with Annexin V and PI double labeling was used to assess the effect of tramadol on TMZ-induced early stage apoptosis in U87 cells. TMZ treatment for 1 week induced early apoptosis in U87 cells. In the absence of any drug, the apoptosis rate in U87 cells was ~5% and was not changed following exposure to tramadol. In contrast, tramadol significantly reduced TMZ-induced apoptosis by almost 50%. The apoptosis rate

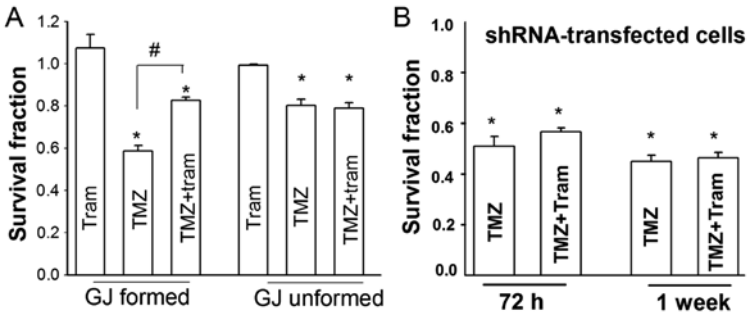


Figure 5. Tramadol attenuates TMZ-induced proliferation suppression and this was Cx43-composed gap junction-dependent. (A) Clonogenic survival of cells treated with 800 $\mu$M TMZ and co-incubated with tramadol for 1 week at high (gap junction formation) and low cell density (no gap junction formation). (B) Clonogenic survival of siRNA-transfected cells treated with 800 $\mu$M TMZ for 72 h and 1 week with or without tramadol at high density (30,000 cells/ml). Columns, mean for three and four experiments; bars, SEM. *P<0.05, significantly different from control; #P<0.05, significantly different from the TMZ bar. TMZ, temozolomide.

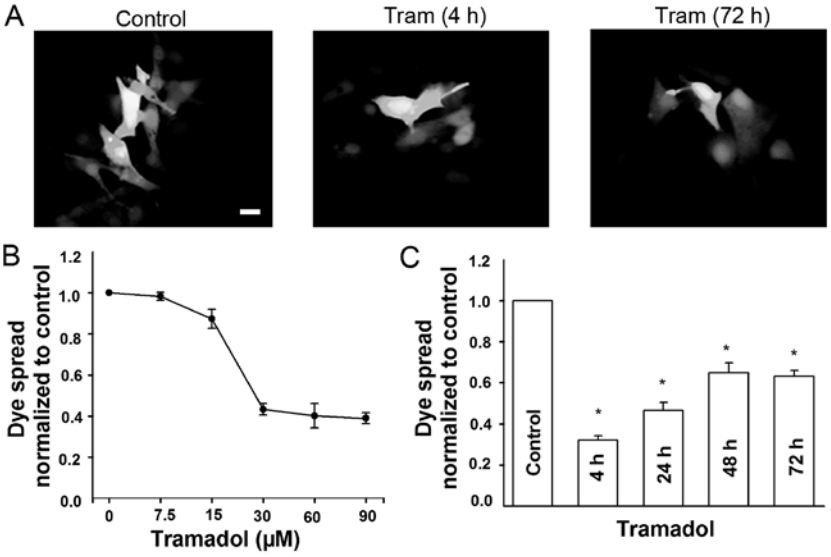


Figure 6. Effect of tramadol on the dye coupling through gap junctions composed of Cx43. (A) The dye spread image of U87 cells with 30 $\mu$M tramadol treatment for 4, 24, 48 and 72 h. (B) The dye spread of U87 cells exposed to different concentrations of tramadol for 4 h. Points, mean for five experiments; bars, SEM. (C) The dye spread of U87 cells exposed to 30 $\mu$M tramadol for 4 and 72 h. Columns, means from four experiments; bars, SEM; *P<0.05, significantly different from control group.

was reduced from 23% when cells were treated with TMZ alone to 12% when simultaneously treated with tramadol and TMZ (Fig. 4A and B).

Caspase cascade systems always play an important role in the induction, transduction and amplification of intracellular apoptotic signals. There are two classic caspase pathways including the participation of mitochondria or the interaction of death receptors with its ligands. Both of them may finally lead to caspase-3 activation, which is considered to be the most important of the executioner caspases. Therefore, cleaved caspase-3 expression (active caspase-3) was determinated after treatment with TMZ with or without tramadol. In $U87^{GFP\text{-}NC}$ and $U87^{GFP\text{-}Cx43shRNA}$ cells, tramadol was unable to induce cleaved caspase-3 expression. TMZ induced apparent expression of cleaved caspase-3. In $U87^{GFP\text{-}NC}$ cells, tramadol significantly reduced caspase-3 expression induced by TMZ. On the contrary, tramadol showed no effect on TMZ-induced caspase-3 expression in $U87^{GFP\text{-}Cx43shRNA}$ cells (Fig. 4C and D). These findings indicated tramadol suppressed TMZ-induced apoptosis in the presence of Cx43.

*Effect of tramadol on TMZ-induced cytotoxicity is dependent on gap junction composed of Cx43*. To investigate whether Cx43-dependence influences tramadol on TMZ toxicity is associated with gap junctions or Cx43 protein itself, cell proliferation after TMZ treatment with or without tramadol were assessed by standard colony formation assay under conditions where gap junction formation was not possible (low density; 500 cells/$cm^2$; cells not in direct contact with each other) or possible (at high density 30,000 cells/$cm^2$) (Fig. 5A). A total of 800 $\mu$M TMZ treatment for 1 week reduced the clonogenic survival of U87 cells at both gap junction-formed and unformed cells. However, the clonogenic survival of gap junction-formed cells exposed to TMZ was substantially less than gap junction unformed cells. Tramadol reduced the suppression of clonogenic survival induced by TMZ in gap junction-formed cells, while tramadol could not affect TMZ-induced suvival suppression in gap junction unformed cells. Additionally, neither tramadol treatment for 72 h nor 1 week exert effect on TMZ-induced proliferation inhibition in shRNA-transfected cells (Fig. 5B). Thus, our results here

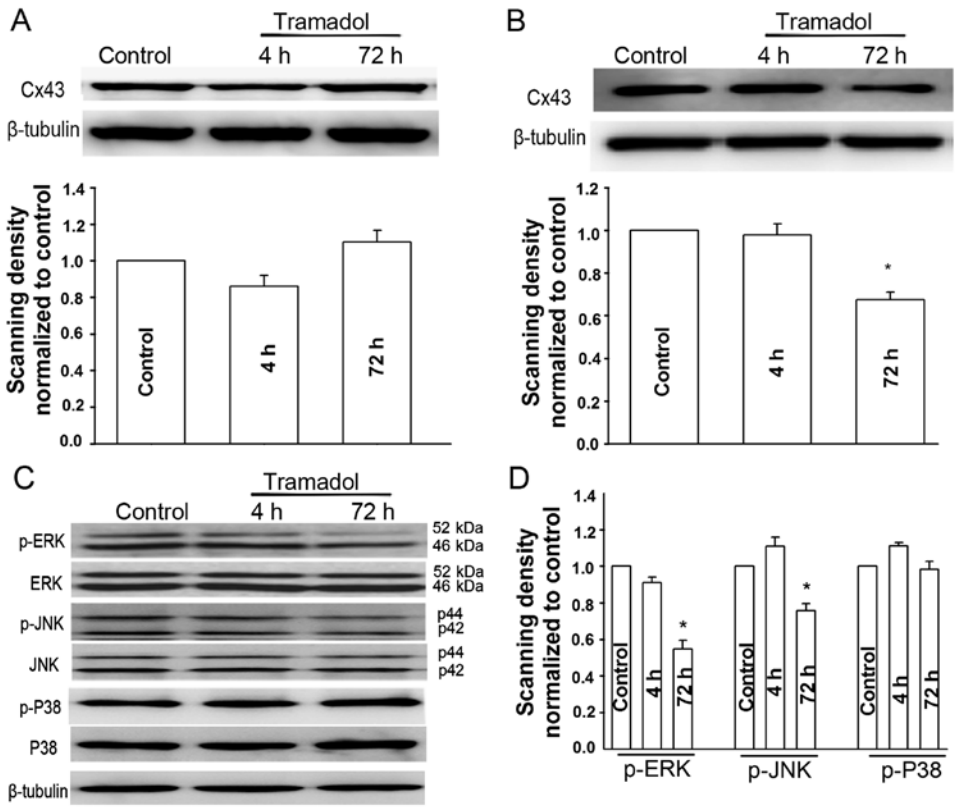


Figure 7. Effects of tramadol on Cx43 and p-Cx43 expression and the involvement of MAPKs on Cx43 phosphorylation. (A) Cx43 expression in U87 cells with 30 $\mu$M tramadol treatment for 4 and 72 h. (B) p-Cx43 expression in U87 cells with 30 $\mu$M tramadol treatment for 4 and 72 h. (C and D) p-ERK, p-JNK, p-P38 expression in U87 cells with tramadol treatment for 4 and 72 h. Bar graph is derived from densitometric scanning of the blots. Columns, mean for three to four experiments; bars, SEM. *P<0.05, significantly different from control.

suggest the inhibition of tramadol on TMZ cytotoxicity is dependent on gap junctions composed of Cx43.

*Tramadol inhibited dye coupling through gap junction composed of Cx43 in U87 cells.* We then assessed the effect of tramadol on gap junction function by parachute assay. In Fig. 6, tramadol inhibited the gap junction mediated dye spread at 4 h and continued to 72 h. Fig. 6A shows micrographs to illustrate the spreading of dye between cells. Fig. 6B plots the inhibition of dye coupling as a function of tramadol concentration. The inhibition of dye coupling was gradually augmented with increasing concentration of tramadol from 7.5 to 30 $\mu$M for 4 h and maintained to be the highest until 90 $\mu$M. The dye spread after 30 $\mu$M tramadol treatment for 48 and 72 h were decreased compared with that for 4 h, and it was maintained at 62 and 60% of control, respectively (Fig. 6C).

*Effects of tramadol on Cx43 and p-Cx43 expression in U87 cells.* Not only the expression of Cxs affect gap junction formation and its function, but Cx43 phosphorylation also plays an important role in regulation of gap junction functions through direct action or by indirectly modulating Cx43 disassembly and internalization from the cell surface. We then determined whether the inhibitory effect of tramadol on gap junctional communication was the result of regulation on Cx43 or by phosphorylation (Fig. 7). Western blotting demonstrated that 30 $\mu$M tramadol treatment slightly reduced total Cx43 expression for 4 h, but not for 72 h. Phosphorylated Cx43 expression was altered by tramadol exposure for only 72 h. Moreover, both phosphorylated JNK and ERK were significantly reduced after tramadol exposure for 72 h but not 4 h. Phosphorylated p38 expression was unchanged by tramadol treatment for 4 and 72 h. These results indicated that decreased gap junctional intercellular communication between U87 cells by long-term tramadol treatment may likely relate to the reduction of Cx43 phosphorylation via ERK and JNK.

## Discussion

The data outlined in the present study suggest that TMZ toxicity is strongly dependent on the presence of functional gap junctions in U87 cells. In addition, Cx43 by itself, in a gap junction independent manner, appears to also modify TMZ toxicity. Moreover, we observed that long-term treatment with tramadol, at an appropriate analgesic concentration, reduced TMZ toxicity through depressing Cx43-mediated gap junctional intercellular communication that was probably associated with downregulation of Cx43 phosphorylation via MAPKs. The present study highlights the importance of making a rational choice for the analgesic that would be routinely provided concurrently with antineoplastic agents.

In the central nervous system, GBM is the most common malignancy and accounts for more than 45% of all malignant brain tumors. The typical therapies for GBM involves surgery, chemotherapy, radiotherapy or combination therapy. TMZ is a front-line chemo-alkylating agent used to treat

glioblastoma (16). TMZ causes DNA damage through the DNA alkylation, which induces mismatches in the DNA repair pathway and finally leads to cell death. The concentrations of TMZ we used are similar to those employed in other clinically relevant studies (17). Using this concentration paradigm make the GJIC-mechanism observed in this study. Nevertheless, a much higher concentration than that may result in extensive cell death and no GJIC-mediated effects will be identified. Of course, at higher concentration TMZ will also have significantly larger effects on non-cancerous cells and will thereby produce even greater side-effects.

The present study establishes the role for functional gap junctions in TMZ toxicity in U87 cells. Other studies have previously established roles for gap junctions composed of Cx43 on the cytotoxicity induced by radiotherapy and different antineoplastic drugs including cisplatin, etoposide, 5-FU and others (8,18-20). Glioblastoma is a heterogeneous disease in which Cx43 expression is still present. It has been reported that thymidine kinase/ganciclovir suicide gene therapy targeting glioblastoma was strongly inhibited by gap junction inhibitors (12). The bystander effect of gap junctions composed of Cx43 was shown to enhance the antitumor effect of miR-124-3p in glioblastoma cell lines and xenograft models (13). A different study showed an enhancing effect of gap junctions on cisplatin cytotoxicity in U87 cells (21). Our results showing the involvement of functional Cx43-containing gap junctions in TMZ cytotoxicity, provide key insight into the importance of the bystander effect in glioblastoma cells treated with TMZ. Further research will be needed to determine whether this bystander effect is observed ubiquitously for antineoplastic therapies in glioblastoma or not.

We also found that Cx43 expression was inversely correlated to TMZ sensitivity in a gap junction-dependent as well as gap junction independent manner. If the entire Cx43 effect on TMZ toxicity was mediated by gap junctions, then inhibiting gap junctions with a drug, 18-α-GA, should have produced a change in chemotherapeutic sensitivity equivalent to disrupting gap junctions by downregulating Cx43 with a shRNA construct. It did not, which suggests that there is an important component of TMZ toxicity that relates to a completely different function of Cx43. This finding is consistent with the results of others (22,23). The carboxyl tail of Cx43 can be post-translationally modified and contains the largest part number of potential binding sites for other proteins. These sites are thought to be the basis for Cx43 forming signaling complexes through direct or indirect interaction with other proteins known to participate in chemotherapeutic resistance, potentially leading to the modulation of the sensitivity of cells to antineoplastic agents. Alternatively, and more related to our study, 'toxic' or 'death' signals triggered by antitumor agents are supposed to pass through gap junctions and enhance cell death and apoptosis in adjacent cells (4,5,13). Enhanced gap junction function would therefore increase the cytotoxicity induced by chemotherapeutic. Our results show that the gap junction mediated effect is quite different from the gap junction independent effect.

Cx43 appears to have the ability to bi-directionally alter TMZ sensitivity. Alteration in gap junction function without affecting protein expression decreases TMZ toxicity, while decreasing all Cx43 expression increases toxicity. Such Cx-mediated effects similarly exist in other cell settings and in different stages of tumorigenesis (9,24,25). However, on confluent U87 cells where Cx43 was expressed normally and functional gap junctions between neighboring cells were formed, neither 18-α-GA and RA nor prolonged treatment with tramadol induced any change in Cx43 expression, which excludes changes in Cx43 protein from producing gap-junction independent alterations in TMZ sensitivity. However, more work needs to be done to better elucidate the interplay between the two pathways.

Because of its limited opioid induced side-effects and because it blocks serotonin and norepinephrine re-uptake in a manner similar to antidepressant medications, tramadol is widely used in treatment of moderate to moderately severe pain including post-operative pain, chronic neuropathic pain and cancer pain (26). We showed prolonged tramadol treatment by itself produced no toxicity, but when co-administered with TMZ attenuated apoptosis and toxicity only in the presence of Cx43. More importantly, tramadol attenuates TMZ cytotoxicity only under high density condition where gap junction formation is possible. Given that tramadol treatment reduced gap junction function, our results strongly suggest that the underlying mechanism associated with the inhibitory effect on TMZ cytotoxicity is from the suppression of gap junctions. The concentration of tramadol we used is higher than the reported plasma concentration (2467±540 ng/ml) in patients given a single dose of tramadol (27). Nonetheless, the dosage used in this study are similar and even lower relative to those given in other *in vitro* and *in vivo* experiments (28-30). Much higher doses of tramadol have been administered clinically over months in cancer patients with an expected linear increase of the serum concentration (31,32). Furthermore, a wide range of actual serum concentrations of tramadol is expected due to the large differences in liver metabolic enzyme activity.

Tramadol which may rapidly penetrate the blood-brain barrier (30), exerts antinociceptive action as a weak agonist of the μ opioid receptor and inhibits synaptic norepinephrine and serotonin re-uptake. There is no evidence that noradrenaline and 5-HT transporters exist in glioblastoma cells and the relationship between the pathway of noradrenaline and 5-HT and gap junction function have not been documented. Even though μ opioid receptor was expressed in some glioblastoma cells (33), tramodol is recognized to exhibit relatively weak activity on opioid receptor. No direct evidence has verified its existance in U87 cells. Phosphorylation participates in the regulation of gap junction function by direct action on channel activity or by triggering Cx disassembly, degradation and internalization. Cx43 is easily phosphorylated by a multiplicity of phosphorylation protein kinases such as MAPKs (34). In the present study, tramadol treatment for 4 h decreased Cx43 expression without changing phosphorylated Cx43, indicating the suppression effect of gap junction function by short-term treatment of tramadol was due to the reduced Cx43 level. Tramadol treatment for 72 h showed no effect on Cx43 expression, but reduced Cx43 phosphorylation accompanied with the downregulation of p-JNK and p-ERK expression. Similar effect of tramadol on pERK expression was also shown in breast cancer cells (29). Since other studies have shown that the phosphorylation of Cx43 may increase gap junctional intercellular communication and the suppression of Cx43

phosphorylation induced the reduction of gap junction functions in some cell lines (35,36). We suppose that the deceasing GJIC induced by prolonged tramadol exposure is likely associated with the downregulation of Cx43 phosphorylation through the ERK and JNK molecular pathway.

Gap junctions, formed by Cxs, have important roles in maintenance of tissue homeostasis and regulation of cell growth and differentiation (3,37). Reduced expression and disruption of gap junction function is usually correlated with tumorigenic phenotypes in various cancer cells. However, studies have shown that increased levels of Cxs including Cx43 and constituent gap junctions are involved in metastasis, invasiveness and extravasation (38,39). Primary tumors that initially exhibit impaired intercellular communication via gap junction exhibit functional gap junctions at the metastatic stage (40). In addition to glioblastoma cells, suppression of gap junction function by tramadol here is a clue to its possible therapeutic effect on advanced tumors expressing Cx43. Since increased Cx43 and GJIC in spinal cord astrocytes are closely associated with neuropathic pain (41), our finding also suggests a probable Cx43-related mechanism for the anti-nociception actions of tramadol in the spinal cord.

The present study reveals that a previously unknown consequence of long-term treatment with tramadol is to reduce Cx43 containing gap junction function thereby counteracting TMZ-induced toxicity in glioblastoma cells. Our results indicate a profitable strategy whereby augmenting gap junction function could increase the sensitivity of TMZ in glioblastoma and perhaps other TMZ-resistant cancers. Moreover, this study has clinical implication in optimizing the selection of analgesic for glioblastoma patients concurrently receiving chemotherapy agents. That would be for example, where possible, analgesics with no effect on gap junctions other than tramadol might be preferable to use to control cancer pain in glioblastoma patients. Otherwise, if tramadol should be used, it may be advisable to increase the dose of antitumor drugs to preserve their effectiveness. Finally, it is important to investigate the possible interplay between analgesics with different mechanism of anti-nociceptive action, various gap junctions and chemotherapeutic agents in specific cancers to develop an effective combination strategy in antitumor treatment.

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