Original Paper

Evidence for the Inhibition by Temozolomide, an Imidazotetrazine Family Alkylator, of Intermediate-Conductance Ca^{2+} -Activated K^{+} Channels in Glioma Cells

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Key Words

Temozolomide • Intermediate-conductance Ca^{2+} -activated K^{+} channel • K^{+} current • Glioma cell • Membrane potential

Abstract

Background: Temozolomide (TMZ), an oral alkylator of the imidazotetrazine family, is used to treat glioma. Whether this drug has any ionic effects in glioma cells remains largely unclear. **Methods:** With the aid of patch-clamp technology, we investigated the effects of TMZ on the ionic currents in U373 glioma cells. The mRNA expression of KCNN4 ($\text{K}_{\text{Ca}}3.1$) in U373 glioma cells and TMZ's effect on K^{+} currents in these KCNN4 siRNA-transfected U373 cells were investigated. **Results:** In whole-cell recordings, TMZ decreased the amplitude of voltage-dependent K^{+} currents (I_{K}) in U373 cells. TMZ-induced I_{K} inhibition was reversed by ionomycin or 1-ethyl-2-benzimidazolinone (1-EBIO). In cell-attached configuration, TMZ concentration-dependently reduced the activity of intermediate-conductance Ca^{2+} -activated K^{+} (IK_{Ca}) channels with an IC_{50} value of 9.2 μM . Chlorzoxazone or 1-EBIO counteracted the TMZ-induced inhibition of IK_{Ca} channels. Although TMZ was unable to modify single-channel conductance, its inhibition of IK_{Ca} channels was weakly voltage-dependent and accompanied by a significant prolongation in the slow component of mean closed time. However, neither large-conductance Ca^{2+} -activated (BK_{Ca}) nor inwardly rectifying K^{+} (Kir) channels were affected by TMZ. In current-clamp mode, TMZ depolarized the cell membrane and 1-EBIO reversed TMZ-induced depolarization. TMZ had no effect on I_{K} in KCNN4 siRNA-transfected U373 cells. **Conclusion:** In addition to the DNA damage it does, its inhibitory effect on IK_{Ca} channels accompanied by membrane depolarization could be an important mechanism underlying TMZ-induced antineoplastic actions.

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Published by S. Karger AG, Basel

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Introduction

Temozolomide (TMZ) (Temodal®) is an oral alkylator of the imidazotetrazine family. It is a small-molecule pro-drug and readily absorbed in the digestive tract, and is considered the most effective pharmacological treatment for gliomas [1-5]. TMZ is cytotoxic because 5-(3-methyltriazene-1-yl)imidazole-4-carboxamide alkylates DNA at the O⁶ and N⁷ positions of guanine [2, 5]. O⁶-methylguanine DNA methyltransferase (MGMT) was thought to be an important factor which influences the cellular response to TMZ [5]. It has been demonstrated that patients without MGMT methylation can also be beneficial to TMZ treatment [6, 7]. It has been suggested that antipsychotics such as clozapine and olanzapine [8], inverse agonists of the histamine H1 receptors, could be combined with TMZ therapy in glioblastoma to increase pro-apoptotic effects. Interestingly, a recent study suggested that TMZ may contribute to a reduction in seizure frequency in patients with low-grade gliomas [9]. Notably, whether this drug interacts with membrane ion currents and membrane potentials relevant to its effects on neoplastic cells remains elusive.

Glioblastoma multiforme is a cancer with a discouraging prognosis, and the relatively short life expectancy for its victims is disappointing despite recent new treatments [3, 4]. Numerous studies have reported that ion channels in malignant glioma cells may contribute to invasive migration and proliferation, thereby producing the malignant behavior of these cells during brain tumor propagation [10]. The magnitude of functional expression in intermediate-conductance Ca^{2+} -activated K^+ (IK_{Ca}) channels in glioma cells has recently been reported to interfere with the progression of malignant tumors [11]. However, whether TMZ interacts with IK_{Ca} channels to affect the behavior of glioma cells is still a mystery.

The intermediate-conductance Ca^{2+} -activated K^+ (IK_{Ca}) channels (also known as K_{Ca} 3.1, SK4, IK_{Ca1} , and KCNN4) are encoded by the *KCNN4* gene. They have been cloned from human, mouse, and rat tissue, and their mechanisms linked to hormonal secretion, cell motility, cell proliferation, and Ca^{2+} influx and K^+ efflux regulation have been extensively studied in many non-excitabile or neoplastic cells [10, 12-19]. These channels have single-channel conductance of 20-60 pS and their pharmacological profiles of IK_{Ca} channels are quite distinguishable from those of large- and small-conductance Ca^{2+} -activated K^+ channels [13, 18-21]. The modulators of IK_{Ca} channels represent a potentially therapeutic approach to a variety of diseases, including malignant gliomas [11, 22].

Our primary objectives were to examine the effect of TMZ, an alkylating agent, on voltage-dependent K^+ current (I_K) in U373 glioma cells, to determine whether this agent affects the activity and gating of IK_{Ca} channels, and to evaluate whether resting membrane potential (V_{rest}) in these cells could be altered by this agent.

Materials and Methods

Drugs and solutions

TMZ (3,4-dihydro-3-methyl-4-oxoimidazo[5,1-d]-as-tetrazine-8-carboxamide; $C_6H_6N_6O_2$), chlorzoxazone, ionomycin, and 9-phenanthrol were purchased from Sigma-Aldrich (St. Louis, MO), 1-ethyl-2-benzimidazolinone (1-EBIO), NS-309 and TRAM-34 (1-((2-chlorophenyl) (diphenyl)methyl)-1H-pyrazole) from Tocris (Bristol, UK), and nifedipine, nimodipine, and paxilline from Enzo Life Science (Plymouth Meeting, PA). Squamocin was kindly donated by Professor Yang-Chang Wu, MD, China Medical University, Taichung City, Taiwan. All culture media, fetal bovine serum, L-glutamine, penicillin-streptomycin, fungizone, and trypsin were purchased from Invitrogen (Carlsbad, CA). All other chemicals including $BaCl_2$ were reagent grade commercial chemicals bought from a variety of vendors. Reagent water was deionized using a Milli-Q water purification system (Millipore, Bedford, MA).

The composition of normal Tyrode's solution is (in mM): NaCl 136.5, KCl 5.4, $CaCl_2$ 1.8, $MgCl_2$ 0.53, glucose 5.5, and HEPES-NaOH buffer 5.5 (pH 7.4). To measure K^+ currents and membrane potential, a patch pipette was filled with the following solution (in mM): K-aspartate 130, KCl 20, KH_2PO_4 1, $MgCl_2$ 1, Na_2ATP 3, Na_2GTP 0.1, EGTA 0.1, and HEPES-KOH buffer 5 (pH 7.2). To preclude the possible contamination of Cl^-

currents, Cl^- ions inside the pipette solution were replaced with aspartate. In single-channel recordings, the high K^+ -bathing solution contained (in mM): KCl 145, $MgCl_2$ 0.53, and HEPES-KOH buffer 5 (pH 7.4), and the pipette solution contained (in mM): KCl 145, $MgCl_2$ 2, and HEPES-KOH buffer 5 (pH 7.2).

Cell preparation

The glioblastoma multiforme cell lines U373 and U87MG were purchased from American Type Culture Collection (ATCC, Manassas, VA). They were routinely grown and maintained at a density of 10^6 /ml in high-glucose (4 g/l) Dulbecco's modified Eagle media (Invitrogen) supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml of penicillin and 10 μ g/ml of streptomycin. All cell lines were grown at 37°C in a 5% CO_2 incubator as monolayer cultures and sub-cultured weekly. Fresh media was added every 2-3 days to maintain a healthy cell population. To observe cell growth, a Nikon Eclipse Ti-E inverted microscope (Li Trading Co., Taipei, Taiwan) equipped with a 5-megapixel Coolpix digital camera (Nikon, Kanagawa, Japan) was used. The camera was connected to a personal computer controlled by microscope imaging software (NIS-Elements BR 3.0; Nikon).

U373 cells (2×10^4 per ml) were cultured at 37°C in a 96-well microplate and treated with different concentrations of Trip. After it had been exposed to Trip for 24, 48, or 72 h, 20 μ l of methyl tetrazolium (MTT; 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) solution (5 mg/ml) was added to each well and cells were incubated for another 4 hours. The formazan crystals were dissolved in 150 μ l of dimethylsulfoxide and the absorbance of samples was measured at 490 nm by a microplate reader (model 3550; Bio-Rad Laboratories, CA).

RNA isolation and reverse transcriptase-polymerase chain reaction

A semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) assay was used performed to detect the expression of KCNN4 ($K_{Ca}3.1$) channel mRNA in U373 cells. Total RNA samples were extracted from cells using the Trizol reagent protocol (Invitrogen) and reverse-transcribed into complementary DNA using Superscript II reverse-transcriptase (Invitrogen). The sequences of forward and reverse primers for KCNN4 were:

KCNN4-f: 5'-ACC TTT CAG ACA CAC TTT GG-3'; and

KCNN4-r: 5'-TCT CTG CCT TGT TAA ACT CC-3'.

The PCR cycling conditions were 35 cycles of 95°C for 2 min, 95°C for 20 s, 60°C for 20 s, and 72°C for 10 min. The PCR products were made on 1.5% (w/v) agarose gel containing ethidium bromide and then visualized under ultraviolet trans-illumination. Optical densities of DNA bands were scanned and quantified using an imaging system (AlphaImager 2200; ProteinSimple, Santa Clara, CA).

Transfection with siRNAs

siGENOME SMARTpool RNAi for human KCNN4 (D-004461-01, D-004461-02, D-004461-03, and D-004461-17) was purchased from GE Healthcare Dharmacon (Lafayette, CO). The target sequences for KCNN4 are illustrated in Table 1. U373 cells were transfected with 25 nM of siRNAs using a transfection reagent (Lipofectamine RNAiMAX; Invitrogen). Small concentrations of siRNAs were used to reduce both off-

Table 1. Target sequences of RNAi for human KCNN4

Molecular Weight	External Coefficient
siGENOME SMARTpool siRNA D-004461-01, KCNN4 Target Sequence: CCUGGAUGUUCUACAAACA 13,373.0 (g/mol)	375,046 (L/mol.cm)
siGENOME SMARTpool siRNA D-004461-02, KCNN4 Target Sequence: GGAUAUCCAGUAUACCAAA 13,358.0 (g/mol)	384,391 (L/mol.cm)
siGENOME SMARTpool siRNA D-004461-03, KCNN4 Target Sequence: GGAAGCUGGAGUUUACAA 13,373.0 (g/mol)	372,465 (L/mol.cm)
siGENOME SMARTpool siRNA D-004461-17, KCNN4 Target Sequence: CUGGUUAAAUGCACGAUCA 13,400.8 (g/mol)	374,334 (L/mol.cm)

Fig. 1. KCNN4 siRNA knockdown of the glioma cell line U373. RT-PCR documented the KCNN4 silencing efficiency. Optical densities of DNA bands were scanned and quantified. The mRNA expression at 36hr and 48hr demonstrated significant difference from the mRNA expression at 0 hr.

Fig. 2. The activity of IK_{Ca} channels recorded from U373 cells transfected with (Ab) or without (Aa) KCNN4 siRNAs. In this set of experiments, cells were immersed in high K^+ solution (145 mM) that contained 1.8 mM $CaCl_2$, the patch pipette was filled with K^+ -containing solution, cell-attached configuration was performed, and the potential was constantly held at -70 mV. (B) Bar graph showing summary of IK_{Ca} -channel activity between control cells and KCNN4 siRNA-transfected cells. Each bar indicates mean $\pm$ SEM of data from 5 cells. *Significantly different from control ($P < 0.05$).

target effects and any nonspecific responses to them. Gene-specific siRNA oligomers mixed from four target sequences were diluted in reduced serum medium (Opti-MEM I; Invitrogen) and mixed with transfection reagent. After they had been incubated at room temperature for 20 min, the complexes were added to the cells. Electrophysiological recordings were used to evaluate the siRNA knockdown efficiency. The KCNN4 siRNA knockdown efficiency were shown in the Figure 1. The activity of IK_{Ca} channels recorded from U373 cells transfected with (Ab) or without (Aa) KCNN4 siRNAs were shown in the Figure 2.

Electrophysiological measurements

Shortly before the experiments, cells were dissociated and an aliquot of cell suspension was transferred to a recording chamber mounted on the stage of an inverted fluorescent microscope (CKX-41;

Olympus, Tokyo, Japan) [23, 24]. Cells were put into a normal Tyrode's bathing solution (see "Drugs and solutions", page 7, paragraph 2 above) at room temperature (20–25°C). After cells were left to adhere to the bottom for several minutes, the experiments were performed. Patch electrodes were made of Kimax-51 glass capillaries (Kimax-51; Kimble, Vineland, NJ) using a PP-830 electrode puller (Narishige, Tokyo, Japan) or a P-97 Flaming/Brown micropipette puller (Sutter, Novato, CA), and their tips were fire-polished with an MF-83 microforge (Narishige). The pipettes used had a resistance of 3–5 MΩ when filled with the different intracellular solutions described above. Currents were recorded using a patch-clamp amplifier (RK-400; Biologic, Claix, France) [10, 25]. The offset potential between the pipette and bathing solution was compensated for with amplifier after the pipette had been placed in the bathing solution but immediately before a seal was made. The experiments were designed to evaluate the possible effects of TMZ or other related compounds on ionic currents and membrane potential in U373 and U87MG glioma cells. To measure the resting membrane potential (V_{rest}), current-clamp recordings were made in K^+ -containing pipette solution.

Data recordings

The signals comprising both voltage and current tracings were stored online in a laptop computer at 10 kHz using a data acquisition system (Digidata-1322A; Molecular Devices, Sunnyvale, CA) equipped with a SCSI card (SlimSCSI 1460; Adaptec Milpitas, CA) and data acquisition software (pCLAMP 9.2; Molecular Devices). Current signals were low-pass filtered at 1 or 3 kHz. The pCLAMP-created profiles for either rectangular or ramp-voltage pulses were used to determine the current-voltage (I - V) relationships for ion currents. For further analyses, the data were exported to graphing software (Origin 8.0; OriginLab, Northampton, MA) or a spreadsheet (MS Excel 2013; Microsoft, Redmond, WA).

Data analyses

To calculate the concentration-dependent inhibition of TMZ on the probability of IK_{Ca} -channel openings, U373 cells were placed in high K^+ bathing solution containing 1.8 mM of $CaCl_2$ with a constant holding potential was of -70 mV. The concentration-response relationship for TMZ-induced inhibition of IK_{Ca} channels was then determined using a non-linear regression analysis with the Hill equation:

$$\text{percentage inhibition} = \frac{E_{\max} \times [C]^{n_H}}{IC_{50}^{n_H} + [C]^{n_H}}$$

where $[C]$ is the TMZ concentration; IC_{50} and n_H are the concentration required for a 50% inhibition and the Hill coefficient, respectively; and $E_{\max}$ is the maximal inhibition of IK_{Ca} -channel activity induced by TMZ. Single-channel currents in glioma cells were analyzed using pClamp 9.2. Single-channel amplitudes were determined by fitting Gaussian distributions to the amplitude histograms of the closed and open states. Channel activity was defined as $N \cdot P_o$, which is estimated using the following equation:

$$N \cdot P_o = \frac{A_1 + 2A_2 + 3A_3 + \dots + nA_n}{A_0 + A_1 + A_2 + A_3 + A_4 + \dots + A_n}$$

where N is the number of active channels in the patch, A_0 is the area under the curve of an all-points histogram corresponding to the closed state, and $A_1 \dots A_n$ represents the histogram areas that indicate the levels of the distinct open state for 1 to n channels in the patch examined. The single-channel conductance was calculated using a linear regression with the mean values of current amplitudes measured at different holding potentials. Open or closed lifetime distributions were fitted with logarithmically scaled bin width. To evaluate the voltage dependence of the inhibitory effect of TMZ on the activity of IK_{Ca} channels, the data were evaluated and fit using the Woodhull equation [22]:

$$\frac{I}{I_0} = 1 + \frac{B}{K_0 \exp\left(\frac{F\delta V}{RT}\right)}$$

where I_0 is the current through the unblocked IK_{Ca} channel at a given membrane potential, K_0 is the equilibrium constant at 0 mV, B is the blocker concentration, V is the membrane potential in mV, F is Faraday's constant, R is the universal gas constant, T is absolute temperature, and δ is the fraction of the membrane voltage sensed at the binding site (i.e., the effective valence for a monovalent blocker). Reported fits were commonly made with nonlinear, least-squares, curve-fitting algorithms.

Table 2. Comparison of the energy levels of TRAM-34, clotrimazole, and TMZ calculated using the ChemBio3D program. Data are Kcal/mol

Types of energy	TRAM-34	Clotrimazole	TMZ
Torsion	12.32	19.00	70.75
1,4-Van der Waals	13.13	7.75	0
Dipole/Dipole	-3.08	-1.09	-20.53
Bend	25.96	30.63	26.83
Stretch-Bend	-3.11	-2.56	-1.91
Stretch	50.57	52.28	45.73

Using the Chem3D and ChemDraw programs embedded in ChemBio3D Ultra 14 (PerkinElmer, Waltham, MA), the different parameters for TRAM-34, clotrimazole, and TMZ involved in regulating IK_{Ca} channels (torsion energy, 1,4-Van der Waals force, and bend, stretch, and stretch-bend energies) were calculated and the result was shown in the Table 2 in the supplementary material.

Statistical analyses

The data of macroscopic and single-channel currents are presented as means $\pm$ standard error of the mean (SEM) with sample sizes (n) indicating the number of cells from which the data were obtained. The paired Student's *t* test was used for the analyses. In addition, Dunnett's test for multiple comparisons was used. SPSS 16.2 (SPSS Inc., Chicago, IL) and statistical analysis modules locally written in the R programming language were used for all analyses. Significance was set at $P < 0.05$.

Results

Changes in I_K amplitude in TMZ-treated U373 cells

In an initial set of experiments, the whole-cell mode of the patch-clamp technique was used to evaluate the effect of TMZ on I_K in U373 cells. Cells were bathed in normal Tyrode's solution containing 1.8 mM of $CaCl_2$, and the recording pipette was filled with a solution

Fig. 3. Inhibitory effect of TMZ on voltage-dependent K^+ current (I_K) in U373 cells. The cells were bathed in normal Tyrode's solution containing 1.8 mM $CaCl_2$. (A) Superimposed current traces in control (upper panel) and during exposure to 10 μ M of TMZ (lower panel). The cell examined was held at -50 mV, and voltage pulses from -100 to +40 mV in 10-mV increments were applied. The uppermost part in (A) indicates the voltage protocol used. (B) Averaged *I-V* relationships of I_K measured in control (■) and after adding 10 μ M of TMZ (□) (mean $\pm$ SEM; n = 6-9 for each point). (C) Time course of TMZ-induced inhibitory effect on I_K measured at the level of -100 mV. The cell examined was hyperpolarized from a holding potential of -50 mV to -100 mV at a rate of 0.1 Hz. Horizontal bar at top labeled "TMZ" indicates when 10 μ M of TMZ was added to the bath. (D) Summary of the data (mean $\pm$ SEM; n = 7-9 for each bar). Current amplitude was measured when each cell was hyperpolarized from -50 to -100 mV. TMZ: 10 μ M of TMZ; CTZ: 3 μ M of clotrimazole; TRAM: 3 μ M of TRAM-34; Iono: 10 μ M of ionomycin. *Significantly different from the control group ($P < 0.05$). **Significantly different from the TMZ-only group (red bar) ($P < 0.05$).

Fig. 4. Characterization of $I_{K_{Ca}}$ channels in U373 cells. In cell-attached current recordings, cells were bathed in a high-concentration K^+ (145 mM) solution that contained 1.8 mM of $CaCl_2$ with the holding potential set at -70 mV. (A) Original current traces of $I_{K_{Ca}}$ channels obtained in the control and during exposure to $3 \mu M$ of clotrimazole (CTZ), $3 \mu M$ of TRAM-34 (TRAM), and $3 \mu M$ of TRAM-34 plus $30 \mu M$ of 1-EBIO. (B) Time course of the inhibitory effect of TRAM-34 on the likelihood that $I_{K_{Ca}}$ channels at -70 mV would be open. Horizontal bar at top labeled "TRAM" indicates when $3 \mu M$ of TRAM-34 was added to the bath. (C) Summary of the data (mean $\pm$ SEM; $n = 8-11$ for each bar). *Significantly different from the control group ($P < 0.05$). **Significantly different from the TRAM-34-only group ($P < 0.05$). CZ: $30 \mu M$ chlorzoxazone; Iono: $10 \mu M$ ionomycin; 9-PA: $20 \mu M$ 9-phenanthrol; NS-309: $1 \mu M$ NS-309.

which contained a low concentration ($0.1 \mu M$) of EGTA and 3 mM of ATP. To measure I_K , each cell was held at -50 mV, and different voltage pulses ranging from -100 to $+40$ mV in 10 -mV increments were applied. Interestingly, when a 10 - μM concentration of TMZ was applied, the I_K amplitude was significantly lower throughout the entire range of the voltage-clamp step. For example, at the level of -100 mV, the I_K amplitude in TMZ-treated U373 cells fell from 62.3 ± 7.9 to 23.7 ± 4.1 pA ($n = 9$, $P < 0.05$) (Fig. 3C). After the TMZ had been removed by washing the cells, the I_K amplitude rose to 49.2 ± 6.1 pA ($n = 6$). Similar results occurred in U87MG cells. Figure 3B depicts the I - V (current versus voltage) curves of I_K obtained in cells treated and not treated with TMZ; Figure 3C illustrates the time course of the inhibition of I_K in TMZ-treated U373 cells.

To determine whether the inhibitory effect of TMZ on I_K in U373 cells can be shared by the regulators of $I_{K_{Ca}}$ channels, the effects of clotrimazole, TRAM-34, and TRAM-34 plus ionomycin were also investigated. Clotrimazole and TRAM-34 are potent inhibitors of $I_{K_{Ca}}$ channels, whereas ionomycin is a Ca^{2+} ionophore [10, 12, 13]. At -100 mV, the I_K amplitude

Fig. 5. TMZ did not affect BK_{Ca} -channel activity in U373 cells. Cell-attached current recordings were made in these experiments. Cells were bathed in high-concentration- K^+ solution that contained no Ca^{2+} . (A) Original current traces without added TMZ (control), with 30 μM of added TMZ, and with 30 μM of added TMZ plus 1 μM of paxilline (TMZ + Pax). Each cell was held at +60 mV. Upward deflections in current indicate open channels. (B) Time course of TMZ effect on the open probability of BK_{Ca} channels at +60 mV. Labeled horizontal bars indicate when TMZ and Pax were added. (C) Summary of the data showing (mean $\pm$ SEM; $n = 9-10$ for each bar). *Significantly different from the control group ($P < 0.05$).

was significantly lower in cells treated with 3 μM of clotrimazole and in cells treated with 3 μM of TRAM-34 (3 μM) (62.1 ± 7.8 pA pretreatment to 22.2 ± 3.5 pA posttreatment [$n = 8$, $P < 0.05$]) (Fig. 3D). The I_K amplitude of TMZ-treated cells to which 10 μM of ionomycin was added was significantly higher (45.1 ± 4.9 pA [$n = 7$, $P < 0.05$]).

Pharmacological properties of IK_{Ca} channels in U373 cells

The results from whole-cell experiments suggested that I_K could arise from K^+ flux through the intermediate-conductance subtype of Ca^{2+} -activated K^+ channels, because TRAM-34-induced decrease in I_K amplitude was higher in cells treated with 1-EBIO or ionomycin. Therefore, to determine how TMZ affects I_K , we investigated the effects of IK_{Ca} -channel regulators in U373 cells. In the first set of single-channel recordings, a cell-attached configuration was used and, under symmetrical K^+ (145 mM) conditions, the activity of IK_{Ca} channels was readily detectable (Fig. 4A and 4B). However, after 3 μM of either clotrimazole or TRAM-34 had been added to the bath, the IK_{Ca} channels were significantly less likely to be open (Fig. 4C). IK_{Ca} channels that were closed in TRAM-34-treated cells were reopened after the cells had been treated with 1-EBIO, chlorzoxazone, ionomycin, 9-phenanthrol (a potent activator of $KCa3.1$), and NS-309 (a selective opener of $KCa3.1$). In addition, IK_{Ca} -

Fig. 6. TMZ did not affect Kir-channel activity in U373 cells bathed in Tyrode's solution without Ca^{2+} . Cell-attached current recordings were made; the cells were held at 0 mV relative to the bath. (A) Original current traces control cells without TMZ, from cells with 30 μ M of added TMZ, and from cells with 30 μ M of added TMZ plus 1 mM of $BaCl_2$. Downward deflections in current indicate open channels. (B) Time course of TMZ-induced inhibition of the likelihood that Kir channels will be open at 0 mV relative to the bath. Labeled horizontal bars indicate when TMZ and $BaCl_2$ were added. (C) Summary of the data (mean $\pm$ SEM; $n = 9-10$ for each bar). *Significantly different from the TMZ-only group ($P < 0.05$).

channels in nifedipine-treated (3 μ M) and nimodipine-treated (3 μ M) U373 cells were closed (data not shown).

TMZ and large-conductance Ca^{2+} -activated K^+ (BK_{Ca}) channels in U373 cells.

The unitary conductance of cells bathed in 145 mM K^+ solution and recorded in cell-attached configuration was 212 ± 13 pS ($n = 12$) at depolarized potentials (Fig. 5). The likelihood of IK_{Ca} -channels being open in cells treated with ionomycin (10 μ M) or squamocin (10 μ M) significantly higher. However, the likelihood of BK_{Ca} -channels being open in cells treated with 30 μ M of TMZ (0.018 ± 0.006 [in the absence of TMZ], $n = 10$ versus 0.019 ± 0.005 [in the presence of TMZ], $n = 10$) was not significantly different. The likelihood of BK_{Ca} -channels being open in cells treated with paxilline (1 μ M) was significantly lower: 0.002 ± 0.0008 ($n = 9$, $P < 0.05$), but there was no significant change in single-channel conductance.

TMZ and inwardly rectifying K^+ (Kir) channels in U373 cells

U373 cells were bathed in Ca^{2+} -Tyrode's solution and single-channel current was recorded. When the cell was held at 0 mV relative to the bath, the activity of Kir channels was clearly observed (Fig. 6). However, after TMZ (30 μ M) had been added to the bath, there was no significant change in Kir-channel activity. In the group treated with TMZ plus 1 mM of $BaCl_2$, however, the likelihood of Kir-channels being open was significantly lower: it fell from 0.45 to 0.03 ± 0.01 ($n = 9$, $P < 0.05$).

TMZ and the activity of IK_{Ca} channels in U373 cells

Experiments to evaluate the effect of TMZ on IK_{Ca} -channel activity were also done. After TMZ had been added to the bath medium, the IK_{Ca} -channels were significantly less likely to be open (Fig. 7A and 7B). At -70 mV in the controls, the probability of open IK_{Ca} channels was 0.014 ± 0.003 ($n = 9$). One minute after TMZ (10 μ M) had been added to the bath, the probability was significantly ($P < 0.05$) lower: 0.0084 ± 0.0009 ($n = 9$). After the cells had been washed to eliminate the added TMZ, channel activity rose to 0.011 ± 0.003 ($n = 6$). In our experiments, no significantly difference between control and washout of TMZ was found. The amplitudes of the single-channel currents were unaltered after TMZ treatment.

Fig. 7. Concentration-response relationship for TMZ-induced inhibition of IK_{Ca} channels in U373 cells. In these experiments, cells were bathed in a high-concentration K^+ (145 mM) solution that contained 1.8 mM of $CaCl_2$ with the holding potential set at -70 mV. Cell-attached current recordings were made. Channel activity at 3 M and 10 μ M concentrations of TMZ was measured. (A) Original current traces showing changes in the activity of IK_{Ca} channels obtained with and without added TMZ. Downward deflections in current indicate open channels. (B) The time course of the inhibitory effect of TMZ on the likelihood that IK_{Ca} channels would be open at -70 mV. Horizontal bars labeled "TMZ" indicate when the TMZ was added. (C) Concentration-response curve for TMZ-induced inhibition of IK_{Ca} channels (mean $\pm$ SEM; $n = 9-13$ for each

point). Various concentrations of TMZ (1-100 μ M) were added to the bath. The smooth line represents the best fit to the modified Hill equation described in MATERIALS AND METHODS. The IC_{50} value was 9.2 μ M (data not shown), the maximal percentage of inhibition was 100%, and the Hill coefficient for TMZ-mediated inhibition of IK_{Ca} channels was 1.2 (data not shown). (D) I - V relationships of IK_{Ca} channels without ($\blacksquare$) and with ($\square$) 10 μ M of added TMZ (mean $\pm$ SEM; $n = 9-12$ for each point). Adding TMZ had no significant effect on the conductance of single IK_{Ca} channels in these cells.

Fig. 8. Voltage dependence of TMZ-stimulated IK_{Ca} channels in U373 cells. The likelihood that IK_{Ca} channels would be open measured at different levels of membrane potential when the cells were exposed to 10 μ M of TMZ (mean $\pm$ SEM; $n = 5-8$ for each point). The data were fitted using the Woodhull equation described under MATERIALS AND METHODS. The equilibrium constant at 0 mV (K_D) and the fraction of membrane potential sensed by TMZ at its binding site within the channel (δ) required for TMZ to inhibit open IK_{Ca} channels were 8.7 μ M and 0.21, respectively.

After TMZ treatment (1-100 μ M), the probability of open IK_{Ca} -channels was concentration-dependent (Fig. 7C). The half-maximal concentration needed for TMZ to inhibit IK_{Ca} -channel activity was 9.2 μ M, and the Hill coefficient was 1.3.

Fig. 9. Mean closed time of IK_{Ca} channels in the absence and presence of TMZ recorded from U373 cells. Closed-time histograms obtained in cells without TMZ (upper) and with 10 μ M of added TMZ (lower). The abscissa and ordinate represent the logarithm of closed time (msec) and the square root of the event number ($\sqrt{n}$), respectively. The smooth red line shown in each panel was fitted using a two-exponent function. The dashed vertical green lines show the values of the time constant in the closed state. Adding TMZ prolonged the slow component of the mean closed time, but there was no discernible change in mean open time.

Fig. 10. Effect of TMZ on the resting membrane potential (V_{rest}) in U373 cells. In these experiments, the cells were bathed in normal Tyrode's solution containing 1.8 mM of $CaCl_2$ and current-clamp recordings were made. The recording pipette was filled with a solution that contained K^+ . (A) Original potential trace showing the effect of TMZ (10 and 30 μ M) and TMZ (30 μ M) plus 1-EBIO (30 μ M) on V_{rest} . The horizontal bars indicate when TMZ and TMZ plus 1-EBIO were added. (B) Summary of the data (mean $\pm$ SEM; $n = 6-9$ for each bar). *Significantly different from the control group ($P < 0.05$). **Significantly different from TMZ-only group ($P < 0.05$).

TMZ and single-channel conductance of IK_{Ca} channels in U373 cells

How TMZ treatment affected IK_{Ca} channels at different membrane potentials was evaluated. The plots of current amplitude as a function of holding potential were constructed. The single-channel conductance of IK_{Ca} channels calculated from linear $I-V$ relationship in control was 32 ± 4 pS ($n = 9$) with a reversal potential of 0 ± 2 mV ($n = 9$) (Fig. 7D). However, single-channel slope conductance (31 ± 4 pS; $n = 9$, $P > 0.05$) of IK_{Ca} channels was unchanged after TMZ (10 μ M) treatment.

Voltage and TMZ-mediated inhibition of IK_{Ca} channels in U373 cells

Changes in the probability of IK_{Ca} -channel openings in the absence and presence of TMZ were measured in different levels of membrane potentials. The relations were determined

Fig. 11. TMZ did not affect I_K in U373 cells transfected with KCNN4 siRNAs. Cells were bathed in normal Tyrode's solution that contained 1.8 mM of $CaCl_2$. The cells were held at -50 mV and voltages ranging from -100 to $+40$ mV were applied in 20-mV increments. Averaged $I-V$ relationships of I_K measured in the control group (■), after adding 10 μ M of TMZ (□) and further addition of $BaCl_2$ (1 mM) (○) (mean $\pm$ SEM; $n = 5-8$ for each point).

using the Woodhull equation described in MATERIALS AND METHODS (Dunn, 1998 [22]). The values of K_0 and δ required for the inhibitory effect of TMZ on IK_{Ca} channels in these cells were 8.7 μ M and 0.21, respectively (Fig. 8). The results indicate that the inhibitory effect of this drug on the open probability of IK_{Ca} channels observed in these cells is dependent on the TMZ concentration as well as on changes in membrane voltage.

TMZ and the kinetic behavior of IK_{Ca} channels in U373 cells

In cell-attached patches, open- and closed-time histograms of IK_{Ca} channels at the level of -70 mV can be fitted using a one- or two-exponential curve. The time constant for the open-time histogram was 4.5 ± 0.7 msec, but those for the fast and slow components of the closed-time histogram were 13.5 ± 1.4 and 67.3 ± 7.9 msec, respectively ($n = 8$). As illustrated in Figure 9, in TMZ-treated (10 M) cells, the mean closed time of the slow component was significantly higher: 337.8 ± 10.6 msec ($n = 8$, $P < 0.05$). The mean closed time of the fast component, however, was not significantly different (14.4 ± 1.5 msec; $n = 8$). In TMZ-treated cells, the mean open time (3.2 ± 0.5 msec [in the absence of TMZ], $n = 8$) was not significantly different (3.1 ± 0.6 msec [in the presence of TMZ], $n = 8$).

TMZ and resting membrane potential (V_{rest}) in U373 cells

To explore changes in membrane potential, TMZ-treated U373 cells were bathed in normal Tyrode's solution containing 1.8 mM of $CaCl_2$ and the recording pipettes were filled with solution that contained K^+ . The V_{rest} in this current-clamp configuration was -66 ± 4 mV ($n = 12$). The membrane potentials of TMZ- and TRAM-34-treated cells were significantly depolarized: V_{rest} rose from -66 ± 4 mV to -41 ± 2 mV ($n = 9$, $P < 0.05$) and -40 ± 2 mV ($n = 7$, $P < 0.05$), respectively. Cells treated with TMZ (30 μ M) and then 1-EBIO (30 μ M) were significantly repolarized: from -41 ± 2 mV to -54 ± 3 mV ($n = 6$, $P < 0.05$) (Fig. 10A). Using ionomycin (10 μ M) instead of 1-EBIO yielded similar results in U373 and U87MG cells (data not shown).

TMZ and I_K in U373 cells transfected with KCNN4 siRNAs

The effect of TMZ on I_K was assessed in U373 cells transfected with KCNN4 siRNAs. The average $I-V$ relationship of I_K (membrane potential polarization) obtained with or without TMZ treatment (10 μ M) was not significantly different: in untreated cells, the I_K amplitude was -50 mV and in treated cells it was $+20$ mV (Fig. 11). Further addition of $BaCl_2$ (1 mM) was effective in suppressing residual current amplitude.

Discussion

We found that, in U373 cells, TMZ (1) effectively suppressed the amplitude of I_K ; (2) dose-dependently reduced the probability that IK_{Ca} channels would be open, (3) Its reduction

in channel activity is weakly voltage-dependent and associated with an increase in mean closed time, despite inability to modify single-channel conductance of IK_{Ca} channels; (4) did not affect the activity of BK_{Ca} or Kir channels; (5) significantly depolarized cell membrane potential, which was reversed by 1-EBIO; and (6) had no effect on I_K in U373 cells transfected with KCNN4 siRNAs. Therefore, in addition to depleting MGMT [5, 7], TMZ regulates IK_{Ca} channels in glioma cells.

The single-channel conductance of IK_{Ca} channels in U373 cells was calculated to be 32 pS, a value similar to the prototypical IK_{Ca} channels present in other types of cells [10, 11, 13, 26], but apparently less than that of BK_{Ca} channels [9, 27]. Moreover, clotrimazole and TRAM-34 inhibited, but chlorzoxazone and 1-EBIO stimulated the activity of these channels in U373 cells. TMZ-mediated inhibition of IK_{Ca} -channel activity weakly depends on membrane voltage and is believed to be via a direct interaction with the $K_{Ca3.1}$ channel protein in glioma cells.

TMZ treatment did not significantly affect the amount of $K_{Ca3.1}$ mRNA expressed in U373 cells. The I_K seen in U373 cells is conducted primarily by IK_{Ca} (i.e., KCNN4-encoded) channels. The IC_{50} value required for TMZ-induced inhibition of IK_{Ca} channels was about 9.2 μ M. Peak plasma concentrations of TMZ range from 3 to 15 μ g/ml (15–77 μ M) [5, 28], and those in cerebrospinal fluid from 30% to 40% of plasma concentrations.² TMZ inhibits IK_{Ca} channels in humans at these low concentrations because it is highly lipophilic and crosses the blood-brain barrier [1, 5, 28, 29]. Because of TMZ's high potency, our findings might be important in determining TMZ's *in vivo* mechanism.

Different types of kinetic behaviors might facilitate TMZ's inhibition of IK_{Ca} -channel activity. TMZ has no significant effect on IK_{Ca} single-channel conductance; therefore, it is unlikely that it acts within the channel's central pore. However, the slow component of mean closed time of the channel was significantly prolonged. Based on the kinetic analyses, TMZ-mediated inhibition of IK_{Ca} channels may be characterized by a greater affinity for the IK_{Ca} channel in the closed state. IK_{Ca} channels regulate the proliferation of prostate cancer cells by controlling Ca^{2+} entry into these cells [14]. However, we saw no significant changes in BK_{Ca} -channel activity in U373 cells that led us to suggest that TMZ's ability to inhibit IK_{Ca} channels does not arise secondarily from the reduction of intracellular Ca^{2+} [17]. Regardless of the detailed mechanism of its actions, there should be a relevant link between how TMZ affects glioma cells and its inhibition of IK_{Ca} -channel activity [2, 7, 11, 18–20, 30]. Moreover, in our study, TMZ inhibited the I_K amplitude and IK_{Ca} -channel activity within a few minutes in U373 cells. Because the onset of inhibition was rapid and because that is inconsistent with binding to nuclear DNAs, the mechanism that TMZ uses to interact with IK_{Ca} channels should be direct and not genomic.

According to the Uniprot KB database (<http://www.uniprot.org/uniprot>), there are four cysteine residues (at 267, 269, 276, and 278) in the amino-acid sequence of human KCNN4 (O15554, KCNN4_HUMAN), all of which are in the 6th transmembrane region (S6). It is thus possible that, in addition to methylation at the O⁶ position of guanine in DNA [2, 5], the TMZ molecule may transfer methyl groups to the cysteine residues in the $K_{Ca3.1}$ -channel subunit. As glioma cells are exposed to TMZ, the tethering of certain amino acid residues, possibly at the slide-helices of the IK_{Ca} channel, may occur and produce voltage-dependent decreases in channel opening and in channel closure accompanied by a lengthening of mean closed time. Consequently, the TMZ-induced alkylated cysteines of IK_{Ca} channels are likely to inhibit I_K amplitude and membrane potentials. TMZ and other structurally related compounds might provide a structural basis for investigating IK_{Ca} channels in different types of cells.

ChemBio3D analysis was used to evaluate the energy levels TRAM-34 (95.08 kcal/mol), clotrimazole (109.22 kcal/mol), and TMZ (118.64 kcal/mol), which suggested that the degree of freedom of each of these molecules is similar. Except for the significantly greater energy level of the dipole/dipole-force in the TMZ molecule, the parameters of these substances are not significantly different (Table 2). Additionally, ChemMine Tools (<http://chemmine.ucr.edu>) were used to calculate the maximum and minimum scores for similarity of maximum common substructure between TRAM-34 (0.2000) and TMZ (0.3571). Therefore, the results

also imply that, similar to TRAM-34 or clotrimazole, TMZ has a similar chemical structure and is able to effectively suppress IK_{Ca} channels.

In current-clamp recordings from U373 cells, TMZ depolarized the cell membrane and 1-EBIO reversed the TMZ-induced depolarization. One may argue that other types of K^+ channels can account for TMZ-mediated inhibition of whole-cell I_K in U373 cells [18, 30]. However, TMZ had no significant effect on the activity of BK_{Ca} or Kir channels in these cells, despite being able to depolarize V_{rest} . Therefore, in spite of the alkylating effects of TMZ on DNA damage, the IK_{Ca} channel functionally expressed in glioma cells could be considered to be one of the major molecular targets of TMZ structurally related compounds to reduce the spread of glioblastoma multiforme.

It has been reported that TMZ is actively involving in AMP-activated protein kinase (AMPK) activation, through O6-methylguanine production [31]. Activation of AMPA by TMZ contributes to glioblastoma cell apoptosis, probably by promoting p53 activation and inhibiting mTOR complex 1 signaling [31, 32]. Interestingly, inhibition of the $K_{Ca}3.1$ channels by AMPK has also been studied [33]. A downregulation of $K_{Ca}3.1$ by AMPK transport was suggested as a role of preserving the intracellular ionic environment in the face of metabolic stress, as well as determine cell survival in pathological conditions [34]. Whether TMZ's inhibition on $K_{Ca}3.1$ is actively directly associated with AMPK activation is worth further investigation.

A recent study with immunolabelling $K_{Ca}3.1$ channels reported that IK_{Ca} channels tended to be differentially expressed in excitatory and inhibitory neurons of the central nervous system [26]. Different isoforms of $K_{Ca}3.1$ might be present in various types of body tissue, including gliomas, but whether TMZ can inhibit different types of IK_{Ca} channels is still unknown. Interestingly, the diminishing effects of some antipsychotics such as olanzapine on $K_{Ca}3.1$ channels could be potentially useful in TMZ therapy [35, 36]. Clinical and basic studies investigating to what extent TMZ-induced effects on glioma cells are attributable to a direct inhibition of IK_{Ca} channels are thus warranted.

Acknowledgments

We thank Ching-An Kao for helping with cell preparation and William Franke for English editing.

This research was funded by grants from the Taiwan National Science Council (NSC-101-2320-B-006-009, NSC-101-2815-C-006-082-B, and NSC-102-2314-B-006-051-MY3), National Cheng Kung University Hospital (NCKUH-10407024), Chi-Mei Medical Center (CMNCKU10205), and the Program for Promoting Academic Excellence and Developing World Class Research Centers, Ministry of Education, Taiwan.

Disclosure Statement

None declared.

References

- 1 Brandes AA, Pasetto LM, Vastola F, Monfardini S: Temozolomide in patients with high grade gliomas. *Oncology* 2000;59:181-186.
- 2 Villano JL, Seery TE, Bressler LR: Temozolomide in malignant gliomas: Current use and future targets. *Cancer Chemother Pharmacol* 2009;64:647-655.
- 3 Omuro A, DeAngelis LM: Glioblastoma and other malignant gliomas: A clinical review. *JAMA* 2013;310:1842-1850.
- 4 Jovcevska I, Kocevar N, Komel R: Glioma and glioblastoma - how much do we (not) know? *Mol Clin Oncol* 2013;1:935-941.

- 5 Chang L, Su J, Jia X, Ren H: Treating malignant glioma in chinese patients: Update on temozolomide. *Onco Targets Ther* 2014;7:235-244.
- 6 von Deimling A, Korshunov A, Hartmann C: The next generation of glioma biomarkers: Mgmt methylation, braf fusions and idh1 mutations. *Brain Pathol* 2011;21:74-87.
- 7 Sze CI, Su WP, Chiang MF, Lu CY, Chen YA, Chang NS: Assessing current therapeutic approaches to decode potential resistance mechanisms in glioblastomas. *Front Oncol* 2013;3:59.
- 8 Kast RE, Karpel-Massler G, Halatsch ME: Can the therapeutic effects of temozolomide be potentiated by stimulating amp-activated protein kinase with olanzepine and metformin? *Br J Pharmacol* 2011;164:1393-1396.
- 9 Koekkoek JA, Dirven L, Heimans JJ, Postma TJ, Vos MJ, Reijneveld JC, Taphoorn MJ: Seizure reduction in a low-grade glioma: More than a beneficial side effect of temozolomide. *J Neurol Neurosurg Psychiatry* 2014
- 10 Huang MH, Huang YM, Wu SN: The inhibition by oxaliplatin, a platinum-based anti-neoplastic agent, of the activity of intermediate-conductance $ca(2)(+)$ -activated $k(+)$ channels in human glioma cells. *Cell Physiol Biochem* 2015;37:1390-1406.
- 11 Wulff H, Kolski-Andreaco A, Sankaranarayanan A, Sabatier JM, Shakkottai V: Modulators of small- and intermediate-conductance calcium-activated potassium channels and their therapeutic indications. *Curr Med Chem* 2007;14:1437-1457.
- 12 Wulff H, Miller MJ, Hansel W, Grissmer S, Cahalan MD, Chandy KG: Design of a potent and selective inhibitor of the intermediate-conductance $ca2+$ -activated $k+$ channel, $ikca1$: A potential immunosuppressant. *Proc Natl Acad Sci U S A* 2000;97:8151-8156.
- 13 Jensen BS, Strobaek D, Olesen SP, Christophersen P: The $ca2+$ -activated $k+$ channel of intermediate conductance: A molecular target for novel treatments? *Curr Drug Targets* 2001;2:401-422.
- 14 Lallet-Daher H, Roudbaraki M, Bavencoffe A, Mariot P, Gackiere F, Bidaux G, Urbain R, Gosset P, Delcourt P, Fleurisse L, Slomianny C, Dewailly E, Mauroy B, Bonnal JL, Skryma R, Prevarskaya N: Intermediate-conductance $ca2+$ -activated $k+$ channels ($ikca1$) regulate human prostate cancer cell proliferation through a close control of calcium entry. *Oncogene* 2009;28:1792-1806.
- 15 Liang Z, Chen L, McClafferty H, Lukowski R, MacGregor D, King JT, Rizzi S, Sausbier M, McCobb DP, Knaus HG, Ruth P, Shipston MJ: Control of hypothalamic-pituitary-adrenal stress axis activity by the intermediate conductance calcium-activated potassium channel, $sk4$. *J Physiol* 2011;589:5965-5986.
- 16 Ohya S, Niwa S, Kojima Y, Sasaki S, Sakuragi M, Kohri K, Imaizumi Y: Intermediate-conductance $ca2+$ -activated $k+$ channel, $kca3.1$, as a novel therapeutic target for benign prostatic hyperplasia. *J Pharmacol Exp Ther* 2011;338:528-536.
- 17 McFerrin MB, Turner KL, Cuddapah VA, Sontheimer H: Differential role of ik and bk potassium channels as mediators of intrinsic and extrinsic apoptotic cell death. *Am J Physiol Cell Physiol* 2012;303:C1070-1078.
- 18 Schwab A, Fabian A, Hanley PJ, Stock C: Role of ion channels and transporters in cell migration. *Physiol Rev* 2012;92:1865-1913.
- 19 Leanza L, O'Reilly P, Doyle A, Venturini E, Zoratti M, Szegezdi E, Szabo I: Correlation between potassium channel expression and sensitivity to drug-induced cell death in tumor cell lines. *Curr Pharm Des* 2014;20:189-200.
- 20 Wu SN, Chiang HT, Chang FR, Liaw CC, Wu YC: Stimulatory effects of squamocin, an annonaceous acetogenin, on $ca(2+)$ -activated $k+$ current in cultured smooth muscle cells of human coronary artery. *Chem Res Toxicol* 2003;16:15-22.
- 21 Maezawa I, Zimin PI, Wulff H, Jin LW: Amyloid-beta protein oligomer at low nanomolar concentrations activates microglia and induces microglial neurotoxicity. *J Biol Chem* 2011;286:3693-3706.
- 22 Dunn PM: The action of blocking agents applied to the inner face of $ca(2+)$ -activated $k+$ channels from human erythrocytes. *J Membr Biol* 1998;165:133-143.
- 23 Huang CW, Hung TY, Liao YK, Hsu MC, Wu SN: Underlying mechanism of regulatory actions of diclofenac, a nonsteroidal anti-inflammatory agent, on neuronal potassium channels and firing: An experimental and theoretical study. *J Physiol Pharmacol* 2013;64:269-280.
- 24 Huang CW, Hung TY, Wu SN: The inhibitory actions by lacosamide, a functionalized amino acid, on voltage-gated $na+$ currents. *Neuroscience* 2015;287:125-136.
- 25 Huang CW, Huang CC, Lin MW, Tsai JJ, Wu SN: The synergistic inhibitory actions of oxcarbazepine on voltage-gated sodium and potassium currents in differentiated ng108-15 neuronal cells and model neurons. *Int J Neuropsychopharmacol* 2008;11:597-610.

- 26 Turner RW, Kruskic M, Teves M, Scheidl-Yee T, Hameed S, Zamponi GW: Neuronal expression of the intermediate conductance calcium-activated potassium channel $kca3.1$ in the mammalian central nervous system. *Pflugers Arch* 2015;467:311-328.
- 27 Wu SN, Peng H, Chen BS, Wang YJ, Wu PY, Lin MW: Potent activation of large-conductance $ca2+$ -activated $k+$ channels by the diphenylurea 1,3-bis-[2-hydroxy-5-(trifluoromethyl)phenyl]urea (ns1643) in pituitary tumor (gh3) cells. *Mol Pharmacol* 2008;74:1696-1704.
- 28 Huang G, Zhang N, Bi X, Dou M: Solid lipid nanoparticles of temozolomide: Potential reduction of cardiac and nephric toxicity. *Int J Pharm* 2008;355:314-320.
- 29 Goldwirt L, Beccaria K, Carpentier A, Farinotti R, Fernandez C: Irinotecan and temozolomide brain distribution: A focus on abcb1. *Cancer Chemother Pharmacol* 2014;74:185-193.
- 30 Maljevic S, Lerche H: Potassium channels: A review of broadening therapeutic possibilities for neurological diseases. *J Neurol* 2013;260:2201-2211.
- 31 Zhang WB, Wang Z, Shu F, Jin YH, Liu HY, Wang QJ, Yang Y: Activation of amp-activated protein kinase by temozolomide contributes to apoptosis in glioblastoma cells via p53 activation and mtorc1 inhibition. *J Biol Chem* 2010;285:40461-40471.
- 32 Cheng SW, Fryer LG, Carling D, Shepherd PR: Thr2446 is a novel mammalian target of rapamycin (mTOR) phosphorylation site regulated by nutrient status. *J Biol Chem* 2004;279:15719-15722.
- 33 Klein H, Garneau L, Trinh NT, Prive A, Dionne F, Goupil E, Thuringer D, Parent L, Brochiero E, Sauve R: Inhibition of the $kca3.1$ channels by amp-activated protein kinase in human airway epithelial cells. *Am J Physiol Cell Physiol* 2009;296:C285-295.
- 34 Bhalla V, Oyster NM, Fitch AC, Wijngaarden MA, Neumann D, Schlattner U, Pearce D, Hallows KR: Amp-activated kinase inhibits the epithelial $na+$ channel through functional regulation of the ubiquitin ligase nedd4-2. *J Biol Chem* 2006;281:26159-26169.
- 35 Kast RE: Profound blockage of $cxc4$ signaling at multiple points using the synergy between plerixafor, mirtazapine, and clotrimazole as a new glioblastoma treatment adjunct. *Turk Neurosurg* 2010;20:425-429.
- 36 Sciacaluga M, Fioretti B, Catacuzzeno L, Pagani F, Bertollini C, Rosito M, Catalano M, D'Alessandro G, Santoro A, Cantore G, Ragozzino D, Castigli E, Franciolini F, Limatola C: Cxcl12-induced glioblastoma cell migration requires intermediate conductance $ca2+$ -activated $k+$ channel activity. *Am J Physiol Cell Physiol* 2010;299:C175-184.