Transient receptor potential melastatin 7 signaling in U251 cell migration and invasion involves calcineurin

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Abstract

Transient receptor potential melastatin 7 (TRPM7) is a divalent cation channel that has crucial functions in glioblastoma (GBM), which remains the most prevalent and lethal primary brain tumor in adults. Altering TRPM7 activity has previously been reported to affect GBM cell function (i.e., migration, invasion, and proliferation), thus elucidating the TRPM7-mediated signaling pathway in GBM could reveal novel therapeutic targets. Calcineurin, a Ca²⁺-dependent phosphatase, also influences GBM cell survival and migration. However, the role or the relationship between TRPM7 and calcineurin in GBM signaling has not previously been investigated. In this study, we provide evidence that there is a possible interaction between TRPM7 and calcineurin in the GBM cell line U251. Moreover, we employed pharmacological approaches to show that TRPM7 regulates calcineurin function, thereby suggesting that calcineurin is a potential downstream target of TRPM7 signaling in U251 cell migration and invasion.

Keywords: Glioblastoma; Transient receptor potential melastatin 7 channels; Calcineurin

1. Introduction

The ubiquitously expressed transient receptor potential melastatin 7 (TRPM7) channel is involved in the cellular homeostasis of divalent ions[1-3]. Containing both an ion channel and a kinase domain[4-6], TRPM7 plays a critical role in cancer cell function, and inhibition of overexpressed TRPM7 has been identified as a potential pharmaceutical intervention[7-11].

Glioblastoma (GBM) is the most lethal malignant primary brain tumor in adults, with an estimated 5-year survival rate of 5.1%[12,13]. Current treatment approaches, including surgical resection, radiotherapy, and chemotherapy, are untargeted, resulting in suboptimal outcomes[14]. Thus, novel treatment options are urgently required. Over the past decade, there has been growing evidence demonstrating the critical role of TRPM7 in regulating...
GBM cell function. Several publications have shown that whereas TRPM7 inhibition in GBM reduced its cell function (i.e., migration, invasion, and proliferation)\cite{10-19}, TRPM7 potentiation conversely promoted GBM cell function\cite{20,21}. Furthermore, our previous studies have also revealed that changes in TRPM7 activity affected the phosphorylation of proteins in the PI3K/AKT and MEK/ERK pathways\cite{22,23}. Nonetheless, there needs to be a higher level of granularity in our understanding of the TRPM7 signaling pathway in GBM before TRPM7 can be pursued as a drug target for future clinical application.

Previous proteomic analysis data from our laboratory showed a change in the expression of the calcium-dependent Ser/Thr phosphatase, calcineurin, following pharmacological inhibition of TRPM7\cite{24,25}. Interestingly, inhibiting calcineurin has also been shown to reduce GBM cell function\cite{26,27}. However, it is unclear (1) if calcineurin and TRPM7 interact in GBM, and (2) whether calcineurin and TRPM7 act within the same pathway to regulate GBM cell function. In this study, we demonstrated the interaction between TRPM7 and calcineurin proteins using co-immunoprecipitation and pull-down assays. Furthermore, inhibiting calcineurin upregulated TRPM7 protein and mRNA expression levels in the human GBM cell line U251. Finally, following pharmacological potentiation of TRPM7 activity in U251, enhancement in cell migration and invasion was prevented by inhibition of calcineurin, thus suggesting that calcineurin is a downstream target of TRPM7 signaling.

2. Materials and methods

2.1. Cell culture

The human GBM cell line U251 was obtained from the American Type Culture Collection (Manassas, VA, USA) and maintained with Dulbecco’s Modified Eagle Medium (DMEM, Sigma-Aldrich) containing 10% heat-inactivated fetal bovine serum (FBS, Wisent, CA) and 100 U/mL penicillin-streptomycin (Gibco, CA). Stably transfected Flag-TRPM7 human embryonic kidney (HEK293) cells were maintained with Minimum Essential Medium (MEM, Sigma-Aldrich, USA) containing 10% heat-inactivated FBS and 100 U/mL penicillin-streptomycin. All cell cultures were maintained in an incubator at 37°C (5% CO₂, 95% humidified air).

2.2. Reagents

The stock solution of cyclosporine A (CsA, ab120114, Abcam, UK) was prepared in DMSO at 100 mM. The stock solution of Naftiben mesylate (Naftiben, 0892, Tocris, UK) was prepared in DMSO at 50 mM. Tetracycline (87128) and dimethyl sulfoxide (DMSO, 472301) were obtained from Sigma-Aldrich (USA).

2.3. Co-immunoprecipitation (co-IP)

Overexpression of recombinant mouse Flag-TRPM7 protein in HEK cells was induced by tetracycline (1 µM/mL in MilliQ water). After 18 – 24 h, the cells were lysed using co-IP lysis buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% NP-40, 0.25% sodium deoxycholate, protease inhibitors: 1 µg/mL pepstatin A, 5 µg/mL leupeptin, 2 µg/mL aprotinin, 1 mM sodium orthovanadate, 0.1 mM PMSF, 10 mM NaF). After centrifuging at 13,000 rpm, the protein concentration of the supernatant was determined using Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, USA). Anti-Flag antibody (F3165, Sigma-Aldrich, USA) was added to the protein lysate and incubated on a rotator overnight at 4°C. Protein A/G agarose beads (sc2003, Santa Cruz, USA) were then added and incubated for 1 – 3 h. The beads were washed three times with lysis buffer. An antibody-free negative control was run alongside the experimental samples. All samples were subsequently analyzed using Western blot.

2.4. His-tagged protein synthesis and purification

Human calmodulin (CALM1) cDNA was amplified using PCR with the following primers: ATGGCTGATCAGCTGACCGAA (forward), TTGGTGCAGTCATCATCTGTAGC (reverse). The product was inserted into a pET28-BIOH vector using the In-Fusion HD EcoDry Cloning Kit (Takara, Japan) and transformed into Escherichia coli DH5α cells by heat shock. DNA was then isolated using the QIAprep Spin Miniprep Kit (Qiagen, Germany) and sequenced. Plasmids were transformed into E. coli BL21 by heat shock and 1 mM IPTG (BioShop, CA) was used to induce protein overexpression. The bacterial broth was centrifuged at 6,000 rpm at 4°C and the pellet was sonicated in equilibrium buffer (20 mM Tris, 150 mM NaCl, pH 8.0, 2 mM 2-mercaptoethanol). The supernatant after centrifugation at 12,000 rpm was incubated with Ni-NTA agarose beads (Qiagen, Germany) and washed three times with lysis buffer. An antibody-free negative control was run alongside the experimental samples. All samples were subsequently analyzed using Western blot.
with a Superdex 16/60 75 columns (GE Healthcare, USA) with buffer (20 mM Tris, pH 8.0, 200 mM NaCl, 2 mM 2-mercaptoethanol). Samples containing proteins of desired molecular weight validated by SDS-PAGE were concentrated and quantified using NanoDrop (Thermo Fisher Scientific, USA). The quality of the final protein product was evaluated by mass spectrometry.

### 2.5. Pull-down assay

The pull-down assay was performed using HisPur cobalt beads according to the manufacturer's instructions (Thermo Fisher Scientific, USA). In brief, cells were lysed using pull-down lysis buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% NP-40, 0.25% sodium deoxycholate, and protease inhibitors), and the lysate was incubated with the 6×His-calmmodulin on a rotator at 4°C overnight. HisPur cobalt beads were incubated with the pull-down mixture, washed three times with lysis buffer, and eluted with pull-down lysis buffer containing 150 mM imidazole. All samples were subsequently analyzed using Western blot.

### 2.6. Cell viability assay

U251 cells were seeded at 3×10^4 cells/well in 96-well plates and incubated in a medium containing 10 – 80 µM CsA. After 24 h, the Cell Counting Kit-8 (CCK-8, Dojindo, Japan) solution was added and incubated for 3 h. The absorbance at 450 nm was determined using a microplate reader (Synergy H1, Biotek, USA).

### 2.7. Quantitative reverse transcription PCR (qRT-PCR)

Total RNA was extracted using the PureLink RNA Mini Kit (Thermo Fisher Scientific, USA) and quantified using Nanodrop (Thermo Fisher Scientific, USA). High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA) was used to synthesize cDNA. cDNA was then amplified with TRPM7 and GAPDH primers using Platinum SYBR Green qPCR SuperMix-UDG with ROX (Thermo Fisher Scientific, USA) in the 7900HT Fast Real-Time PCR System (Applied Biosystems, USA). TRPM7 RT-qPCR primers: CCAGAAACCAAGCCTCTTCC (forward); GCCATGACCTGCCTCTCAT (reverse). GAPDH primers: ACTCCACTCAGGCCAAATTC (forward); CCAGTAGACTCCACGGACATACT (reverse). The quantity mean of TRPM7 in each sample was calculated by SDS Software v2.4 (Thermo Fisher Scientific, USA) and normalized to the reference gene, GAPDH.

### 2.8. Western blot

Cells were resuspended in RIPA buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, protease inhibitors) and the protein concentration in the centrifuged supernatant was measured with Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, USA). Samples were boiled and 40 µg protein was loaded into each well of the SDS-PAGE gel. The gel was transferred to a 0.2 µm PVDF membrane (Bio-Rad, USA) and blocked using 5% skim milk. Membranes were incubated in the appropriate primary antibodies overnight at 4°C: anti-TRPM7 (1:1000, ab85016, Abcam, UK); anti-calcinemurin A (1:500, G182-1847; BD Pharmingen, CA); anti-phospho-AKT (1:1000, 9271S, CST, USA); anti-AKT (1:1000, 2920S, CST, USA); anti-phospho-ERK (1:1000, 5726S, CST, USA); anti-ERK (1:1000, 4695S, CST, USA); anti-GAPDH (1:5000, 2118S, CST, USA); anti-6×His (1:1000, Y1010, UBPBio, USA). Following incubation with the respective mouse (1:7500, 7076S, CST, USA) or rabbit (1:10000, 7074S, CST, USA) secondary antibodies, protein signals were detected using X-ray film (Clonex, CA) after incubating blots in enhanced chemiluminescence reagents (Bio-Rad, USA). The intensity of each protein band was analyzed using ImageJ.

### 2.9. Wound healing migration assay

A vertical wound was created on the U251 cell layer in 12-well plates. Culture medium containing 1% FBS and drug treatments were added to corresponding wells. Four representative images at marked locations along the wound were captured per well using a phase-contrast Olympus microscope (×10 objective, CKX41) at 0 and 24 h after treatment. The images were analyzed using ImageJ and the following formula: Percentage of closure = Gap(T\textsubscript{24} – T\textsubscript{0})/ Gap\textsubscript{T\textsubscript{0}} ×100%.

### 2.10. Oris migration assay

The Oris migration assay was performed according to the manufacturer's instructions (Platypus Technologies, USA). In brief, U251 cells were seeded into the Oris 96-well plate at 2.5 × 10^4 cells/mL and a circular wound was created in each well after the removal of the stoppers. A culture medium containing 1% FBS and drug treatments were added to each well. An image was taken for each well using a phase-contrast Olympus microscope (×4 objective) at 0-, 12-, 24-, and 48-h post-treatment. The percentage of closure was analyzed in the same fashion as the wound healing assay.

### 2.11. Cell invasion assay

The Corning BioCoat Matrigel invasion assay was conducted according to the manufacturer's instructions (Corning, USA). In summary, U251 cells were seeded into the 24-well Matrigel chambers at 5 × 10^4 cells/mL in serum-free DMEM and placed into the companion plate containing a chemoattractant (DMEM with 10% FBS).
After 24 h, the invaded cells were fixed with methanol and stained using 1% Toluidine blue. Four fields of each chamber were imaged using a phase-contrast Olympus microscope (×10 objective) and the cells were quantified using ImageJ.

### 2.12. Statistics and data analysis
Data are presented as means ± SEM. Student’s t-test was used to assess the statistical significance of the difference in two experimental groups or one-way ANOVA for more than two groups. Statistical significance was defined as a probability level lower than 0.05 ($P < 0.05$). For each of the figures shown below, the following summarizes the number of times each experiment was repeated to obtain the total indicated sample size, respectively: Figure 1A (4 times), Figure 1B (1 time), Figure 2 (1 time), Figure 3A (3 times), Figure 3B and C (4 times), Figure 3D (3 times), Figure 4A and B (6 times), Figure 4C and D (1 time), Figure 4E (1 time), and Figure 5 (4 times; AKT and ERK experiments were run together).

### 3. Results

#### 3.1. Flag-TRPM7 immunoprecipitates calcineurin A-subunit from both HEK293 and U251 cell lysates
To investigate the interaction between TRPM7 and calcineurin, the tetracycline-inducible expression system (Figure S1) was used to overexpress Flag-tagged mouse TRPM7 protein in HEK293 cells. Anti-Flag antibody was used to immunoprecipitate Flag-TRPM7 and its associated protein complexes from HEK293 cells, as well as U251 cells. TRPM7 (212 kDa) and calcineurin A-subunit (61 kDa) were detected using Western blot (Figure 1). The regulatory calcineurin B-subunit (19 kDa), known to bind to calcineurin A, was also present in the precipitated mixture (Figure S2). This suggested that calcineurin binds TRPM7, either directly or indirectly, thus indicating that both proteins potentially participate in the same signaling pathway to regulate GBM cell function.

#### 3.2. Calmodulin does not function as a mediator in the TRPM7-calcineurin interaction
We hypothesized that there may be a protein mediator that binds both TRPM7 and calcineurin in this protein complex. One such candidate protein is calmodulin, which classically binds calcineurin[25] and also has the potential to bind to TRPM7 due to the presence of conserved calmodulin-interacting TRPM3 regions on TRPM7 (Figure S3). In U251 cells, 6×His-tagged human calmodulin (His-calmodulin) full-length protein was used for a pull-down assay (Figure 2). Both calcineurin A and His-calmodulin were present in the elution product of the pull-down experiment and absent from the negative control sample, which is consistent with previous reports[25]. However, TRPM7 was not pulled down. Thus, this suggested that calmodulin was unlikely to be involved in the interaction between TRPM7 and calcineurin in U251 cells.

#### 3.3. Inhibition of calcineurin increases the expression of TRPM7 protein
To investigate whether calcineurin is an upstream regulator of TRPM7 function in GBM, we inhibited calcineurin and examined the expression level of TRPM7 in U251 cells. When evaluating the cytotoxicity of the calcineurin inhibitor cyclosporine A (CsA), we found a concentration-
dependent decrease in U251 cell viability, consistent with a previous report\textsuperscript{[23]} (Figure 3A). Furthermore, we observed that treatment with 10 µM CsA had no significant effect on the viability of U251 cells, and thus this concentration was used for subsequent experiments.

Our data showed that CsA treatment significantly increased ($p < 0.05$) TRPM7 protein levels (Figure 3B and C). Consistently, CsA treatment resulted in a significant increase ($p < 0.001$) in mRNA levels of TRPM7 compared to control (Figure 3D). These findings indicated that calcineurin inhibition can increase protein and mRNA levels of TRPM7 in U251 cells.

### 3.4. Treatment with TRPM7 activator does not reverse the effects of calcineurin inhibition on U251 cell migration and invasion

Next, we wanted to examine whether TRPM7 acted upstream of calcineurin in regulating GBM cell function. After treating U251 cells for 24 h with 10 µM CsA, 25 µM naltriben, or co-treatment, the rate of cell migration was assessed using wound healing assays. We found that 10 µM CsA significantly lowered the rate of U251 cell migration whereas 25 µM naltriben significantly increased the rate of migration ($p < 0.05$) (Figure 4A–D), consistent with previous findings\textsuperscript{[20]}. However, naltriben was unable to reverse the inhibitory effect of CsA. Specifically, the rate of wound closure in the co-treatment group was significantly lower than in the naltriben-only group ($p < 0.0001$) and not significantly different compared to the CsA-only group. Since CsA appeared to occlude naltriben’s effect of enhancing U251 cell migration, this suggested that calcineurin acts downstream of TRPM7 to promote GBM cell migration.

In addition to migration, CsA treatment has also been previously shown to inhibit the rate of GBM cell invasion\textsuperscript{[24]}. Conversely, treatment with naltriben has been reported to increase invasion\textsuperscript{[20]}. We confirmed these findings by using the Corning BioCoat Matrigel Invasion Chamber. Specifically, we showed that the rate of U251 cell invasion was significantly increased following treatment with naltriben for 24 h ($p < 0.001$), whereas a decreasing trend for the invasion was observed in the CsA-treated group ($p = 0.131$). Consistent with migration assay results, co-treatment with CsA and naltriben resulted in an invasion rate that was not significantly different from that of the CsA-only group, while being significantly lower ($p < 0.0001$) than the naltriben-only group (Figure 4E). Thus, calcineurin may act downstream of the TRPM7 pathway that promotes GBM cell function.

### 3.5. CsA-induced changes in PI3K/AKT and MAPK/ERK signaling pathways are not affected by naltriben

Previous studies have demonstrated that both pharmacological inhibition and genetic knockdown of TRPM7 resulted in decreased phosphorylation of AKT and ERK\textsuperscript{[16,17]}. In contrast, calcineurin inhibition by CsA has been reported to increase p-AKT\textsuperscript{[24]}. In the present study, we showed that 24-h CsA treatment elevated p-AKT levels significantly, whereas naltriben treatment had no significant effect (Figure 5A and B). Levels of p-AKT levels were significantly increased in the naltriben and CsA co-treatment group compared to the naltriben-only group, but they were not significantly different from the CsA-only group.

Interestingly, although statistical significance was not observed, treatment of U251 cells with CsA appeared to trend towards a decrease in ERK phosphorylation. Similarly, the naltriben and CsA co-treatment group also showed a decreasing trend in p-ERK levels compared to the control group (Figure 5D and E). These findings provide further evidence that calcineurin acts as a downstream target in the TRPM7-mediated signaling that is involved in regulating GBM cell function.
4. Discussion

The current study investigated the relationship between TRPM7 and calcineurin, and the role of this interaction in GBM cell migration and invasion. We found that: (1) There is a possible interaction between TRPM7 and the calcineurin A-subunit proteins; (2) calcineurin inhibition appears to affect TRPM7 protein and mRNA levels; and (3) calcineurin potentially acts as a downstream target of TRPM7 and is likely involved in one of the pathways by which TRPM7 promotes GBM cell function.

We showed that the calcineurin A-subunit was immunoprecipitated with Flag-tagged mouse TRPM7 protein in both HEK293 and U251 cells (Figure 1A and B), thus suggesting a potential interaction between TRPM7 and calcineurin A. It has been previously reported that the calcineurin phosphatase region can directly bind to phosphorylation sites on the C-terminus of TRPM7\[^{27}\]. This may modulate TRPM7 kinase activity and its subsequent signaling pathways\[^{28}\]. In contrast, the function of N-terminal phosphorylation sites on TRPM7 remains largely undefined\[^{29}\], and none of the known consensus sequences of calcineurin (i.e., PXIXIT\[^{30}\], LXVP\[^{31}\], and LQLP\[^{32}\]) are found at the TRPM7 N-terminus.

If the interaction between TRPM7 and calcineurin is indirect, a mediator protein would be required to facilitate the binding. Calmodulin (CaM), a calcium-binding protein,
may mediate this binding since CaM has been shown to interact with both TRPM7 and calcineurin. Consistent with a previous finding, we showed that calcineurin A was precipitated alongside His-CaM. However, TRPM7 was not found in the pull-down protein mixture in the absence of Ca\(^{2+}\) (Figure 2). This suggested that either Ca\(^{2+}\) is crucial for this interaction, or calmodulin may not be required for the TRPM7/calcineurin interaction. Nonetheless, there are limitations to our current experimental approach. Future co-IP studies may consider using anti-TRPM7 antibodies to precipitate endogenous human TRPM7 protein and reverse IP experiments to precipitate endogenous or overexpressed CaM. Importantly, reciprocal co-immunoprecipitation assay should be employed to validate the interaction between Flag-TRPM7 and the calcineurin A-subunit. Furthermore, investigators can employ approaches such as PLA assay or confocal microscopy to examine the subcellular dispositions of TRPM7 and calcineurin. Finally, potential mediators of this interaction can be investigated using in silico protein docking simulations or affinity purification-mass spectrometry approaches, potentially testing different concentrations of Ca\(^{2+}\) to investigate the Ca\(^{2+}\) dependency of this interaction.

The inhibition of either TRPM7 or calcineurin has previously been shown to decrease the rate of migration and invasion of GBM cells. The AKT and ERK pathways, which play important roles in GBM function, have been reported to act downstream of both TRPM7 and calcineurin. Thus, in U251 cells, we applied the TRPM7 agonist naltriben together with the calcineurin inhibitor CsA to determine whether TRPM7 activation can reverse the CsA-induced reduction in GBM cell function and signaling. Consistent with previous findings, results
from our migration assay showed that CsA significantly decreased U251 cell migration, and naltriben alone had the opposite effect (Figure 4A-D). However, CsA and naltriben co-treatment significantly reduced migration compared to the naltriben-only group, and not significantly different compared to the CsA-only group. Similar trends were found in the rate of cell invasion (Figure 4E), and phosphorylation levels of AKT and ERK (Figure 5). Taken together, CsA treatment appears to occlude the potentiating effect of naltriben on GBM cell function, thereby suggesting that calcineurin acts downstream of TRPM7. To strengthen this finding, future studies can simultaneously investigate if pharmacological TRPM7 inhibition, such as with the use of carvacrol or waixenicin A \(^{[15,16]} \), has similar effects on GBM cell function as pharmacological inhibition of calcineurin.

In addition, 24-h CsA treatment increased TRPM7 mRNA and protein levels in U251 cells (Figure 3B-D). One possible explanation is that reduction of calcineurin activity due to CsA-mediated inhibition may stimulate the cell to synthesize excess TRPM7 protein as compensation for the loss of downstream calcineurin-related signaling. Overall, our results suggested that TRPM7 may function as an upstream regulator of calcineurin activity in GBM.

Since the activation of the MEK/ERK pathway is involved in GBM invasiveness, migration, and death resistance \(^{[35,36]} \), the decreasing trend in p-ERK level following CsA treatment was expected (Figure 5E). However, in contrast with a previous report \(^{[20]} \), higher p-ERK levels following naltriben treatment were not observed. This may be due to the lower dosage (i.e., 25 µM instead of 50 µM) used in the present experiments to preserve cell viability in the naltriben and CsA co-treatment group. Furthermore, we employed a different GBM cell line from this previous study (i.e., U251 instead of U87), suggesting potential cell line-dependent effects \(^{[20]} \). Nevertheless, the co-treatment group showed a decreasing trend in p-ERK levels, similar to the CsA-only group, and trended towards reduction when compared to control or naltriben groups. These results were consistent with our migration and invasion assay findings. Interestingly, p-AKT levels in U251 cells were significantly elevated with CsA treatment, consistent with previous reports in other cell types, where it has been rationalized that the loss of dephosphorylating activity due to calcineurin inhibition would lead to higher p-AKT levels \(^{[24,30]} \). However, the literature shows that the activation of the PI3K/AKT pathway promotes GBM proliferation and tumor invasion, and inhibiting TRPM7 decreases the p-AKT level \(^{[16,37]} \). One possible explanation is that any potential GBM-enhancing effects due to CsA-mediated upregulation of p-AKT are outweighed by the collective inhibitory effects of CsA on other mechanisms involved in GBM cell function. To build on our present findings, the next steps should involve genetic manipulation of calcineurin levels by employing techniques such as RNA interference. In summary, calcineurin is a downstream player in the TRPM7-mediated signaling pathway that regulates GBM cell function.

5. Conclusion

The present findings provide evidence that the transient receptor potential melastatin 7 (TRPM7) channel interacts with calcineurin A-subunit proteins, either directly or indirectly. Aberrant TRPM7 activity can upregulate GBM cell function through various pathways, and calcineurin is potentially one of its downstream targets (Figure 6). In this study, we employed one GBM cell line (i.e., U251) to elucidate the proposed mechanism. It is important to note that the present findings with the U251 cell line may not necessarily reflect the nature of TRPM7-mediated signaling in glioma stem cells. Moving forward, future studies should consider confirming the interaction between TRPM7 and calcineurin using other GBM cell lines as well as glioma stem cells. By identifying potential drug targets within the TRPM7-mediated signaling pathway, the ultimate goal is to advance the development of novel chemotherapeutic agents for the treatment of GBM.

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Conflict of interest

The authors declare that they have no conflicts of interest with the contents of this article.

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Ethics approval and consent to participate

Not applicable.
Consent for publication
Not applicable.

Availability of data
The data that support the findings of this study are available from the corresponding author upon reasonable request.

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