

Metformin Targets tmCLIC1 to Trigger Metabolic and Apoptotic Reprogramming in Glioblastoma

Rafael Mendoza^{1*}, Isabela Cruz¹, Carlos Lopez²

¹Department of Glioblastoma Research and Metabolic Reprogramming, Faculty of Medicine, National Autonomous University of Mexico, Mexico City, Mexico.

²Department of Apoptotic Signaling and Drug Repurposing, Faculty of Medicine, Monterrey Institute of Technology, Monterrey, Mexico.

*E-mail ✉ rafael.mendoza@unam.mx

Abstract

A major challenge in cancer therapy is overcoming the profound metabolic alterations that occur in malignant cells. These cells exhibit heightened energy and biosynthetic precursor requirements relative to their non-transformed counterparts. Tumor cells achieve metabolic flexibility by shifting between glycolysis and oxidative phosphorylation (OXPHOS), processes orchestrated by transmembrane proteins that detect changes in the external cellular environment. This investigation highlights the transmembrane variant of Chloride Intracellular Channel 1 (tmCLIC1) as an indicator of malignant phenotype and a viable target for intervention. Levels of tmCLIC1 are substantially upregulated across various solid malignancies, thereby promoting tumor growth and progression, whereas its expression remains negligible in normal cells. Our work establishes that tmCLIC1 serves as the direct molecular target of metformin, an agent known to suppress OXPHOS in neoplastic cells. Single-channel patch-clamp electrophysiology, combined with NMR spectroscopy, confirmed that tmCLIC1 is the main target of metformin action in glioblastoma stem-like cells. Experiments were conducted on multiple patient-derived glioblastoma lines representing heterogeneous genetic profiles. Genetic disruption of CLIC1 via CRISPR-Cas9 or introduction of an arginine 29 point mutation eliminated the drug's inhibitory influence on tumor cells. Subsequent analyses examined cellular proliferation, mitochondrial function, and tumorigenic potential through both in vitro assays and in vivo models, including zebrafish and mouse xenograft systems. Metformin exerts its inhibitory effect on tmCLIC1 by means of a precise physical interaction at arginine 29 within the channel's amino acid sequence. In addition, during periods of low glucose availability, metformin triggers programmed cell death in glioblastoma cells by suppressing the Cancerous Inhibitor of Protein Phosphatase 2A (CIP2A) and concurrently stimulating the PP2A B56δ regulatory subunit. The resulting pathway drives dephosphorylation of Glycogen Synthase Kinase 3 Beta (GSK3β), which in turn destabilizes the pro-survival factor MCL-1, ultimately promoting apoptosis. Blockade of tmCLIC1 function proves indispensable for metformin's anticancer properties, chiefly by governing the PP2A-GSK3β-MCL-1 signaling axis under nutrient-deprived conditions. Prolonged metformin exposure within tumor tissue effectively curbs in vivo expansion even at nanomolar levels. Although the potential benefit of metformin for brain tumor management remains controversial, the present results emphasize the critical need for adequate drug delivery to the tumor site. Notably, measurable suppression of tumor progression occurs in vivo at concentrations below 10 nanomolar. These observations indicate that achieving sustained therapeutic levels of metformin in the central nervous system could strengthen tmCLIC1 blockade, thereby laying the groundwork for refined therapeutic approaches that leverage metformin or structurally related molecules to augment treatment efficacy.

Keywords: Glioblastoma, Metformin, Oxidative phosphorylation, TmCLIC1, Cancer metabolism

Access this article online

<https://smerpub.com/>

Received: 27 January 2025; Accepted: 09 April 2025

Copyright CC BY-NC-SA 4.0

How to cite this article: Mendoza R, Cruz I, Lopez C. Metformin Targets tmCLIC1 to Trigger Metabolic and Apoptotic Reprogramming in Glioblastoma. Arch Int J Cancer Allied Sci. 2025;5(1):249-64. <https://doi.org/10.51847/1cOCZ992TC>

Introduction

Glioblastoma (GBM) is the most lethal form of primary brain cancer and continues to present substantial difficulties in clinical management [1]. Even with the implementation of conventional therapeutic protocols and emerging treatment approaches [2-6], long-term

patient survival remains markedly limited [7, 8]. The extensive cellular diversity within tumors and the highly invasive nature of GBM cells complicate complete surgical excision, commonly resulting in disease recurrence. Current understanding attributes tumor regrowth to a subset of progenitor or stem-like cells, known as glioblastoma stem cells (GSCs). These cells display exceptional oncogenic capabilities, notably their capacity for self-renewal and differentiation into various lineages, which help maintain tumor heterogeneity [9-14]. For this reason, GSCs represent a key focus for innovative therapeutic development.

In recent times, metformin has emerged as a compound with noteworthy anticancer potential. In patients undergoing treatment for type 2 diabetes, metformin use has been linked to lower rates of several solid malignancies and reduced cancer-associated deaths, including those from GBM. Furthermore, research specifically addressing GBM has revealed that metformin exerts its suppressive influence particularly on GSCs [15-19].

Despite these observations, the underlying molecular processes responsible for metformin's activity against cancer cells have yet to be completely clarified. Evidence points toward metformin's interference with the oxidative phosphorylation (OXPHOS) pathway [20-22]. The ability of GSCs to alternate between glycolysis and OXPHOS [23-25] may underlie the restricted clinical benefits observed in trials thus far [26].

Chloride Intracellular Channel 1 (CLIC1) functions as a versatile protein that is mainly cytoplasmic in unstimulated conditions. Under conditions of persistent cellular stress, it migrates to the cell surface, where it supports chloride ion movement across the membrane. Earlier investigations by our group have shown that the transmembrane isoform of CLIC1 (tmCLIC1) drives GBM progression in both cultured systems and living organisms [27, 28]. Its preferential presence and accumulation on the plasma membrane of GSCs highlight tmCLIC1 as an attractive candidate for targeted GBM therapy. Of particular interest, exposure to metformin or other tmCLIC1-blocking agents leads to equivalent decreases in GSC growth that are not further enhanced when the treatments are combined [29-31]. Employing a comprehensive strategy encompassing molecular, cellular, and animal model studies, the present research establishes that metformin modulates GSC proliferation, viability, and overall GBM expansion primarily by inhibiting tmCLIC1.

Materials and Methods

Reagents

IAA94 (Indanyloxyacetic acid 94; Sigma-Aldrich) was employed as a selective inhibitor of CLIC1. A stock solution of 50 mM was prepared in absolute ethanol and subsequently diluted to a final working concentration of 100 μ M. Metformin (1,1-dimethylbiguanide hydrochloride; Sigma-Aldrich) was examined as another CLIC1 inhibitor; it was formulated as a 1 M stock in ultrapure water and applied at concentrations of 5 or 10 mM. PD0332991 isethionate (Sigma-Aldrich), an inhibitor of CDK4/6, was used at 2.5 μ g/ml to induce G1-phase cell cycle synchronization. Niclosamide (Sigma-Aldrich), a mitochondrial uncoupling agent, was dissolved at 10 mM in DMSO and tested at 4 μ M. IACS-010759 (Sigma-Aldrich), an inhibitor of mitochondrial complex I, was added at 100 nM from a 10 mM stock solution in DMSO.

Cell culture

Primary glioblastoma stem cell (GSC) cultures, designated GBM1, GBM2, and GBM3, were isolated and propagated following well-established procedures. GBM1 cells were grown as floating neurospheres and passaged by mechanical dissociation twice each week. GBM2 and GBM3 cells adhered to standard cultureware and were maintained on Geltrex-coated surfaces as required.

Clic1^{-/-} mutant generation by CRISPR-Cas9 technology
Patient-derived GSCs underwent transduction with a transEDIT lentiviral delivery system that included both gRNA and Cas9 components (pCLIP-All-hCMV-ZsGreen V66), in accordance with the provider's recommendations (Transomic). Two separate plasmid constructs were applied: one containing a gRNA sequence directed at the *Clic1* coding region (TGAGTGGCCCTATACCTGGG) and a control gRNA targeting GFP (NC). These vectors expressed ZsGreen to facilitate selection of transduced cells.

Generation of GL261 R29A Knock-In cells using CRISPR-Cas9 technology

A donor double-stranded DNA (dsDNA) template was produced through PCR amplification of a mutated cDNA fragment in which the arginine codon at position 29 (AGA) was changed to an alanine codon (GCC), resulting in the R29A substitution. Amplification was

performed using the forward primer GAGCAGCCTGGTAAGGATGG and the reverse primer GTGGATGCACGTGCACTGCA, generating a 225 bp product. A quantity of 1 µg of this donor DNA was introduced into GL261 cells by electroporation with the Neon Transfection System (ThermoFisher). The procedure also included a guide RNA targeting the sequence immediately upstream of the arginine codon (gRNA sequence: GCAGTGATGGTGCCAAGATT, Integrated DNA Technology) together with the pre-formed Cas9 ribonucleoprotein complex (Integrated DNA Technology). Assembly of the Cas9-gRNA ribonucleoprotein occurred in vitro per the manufacturer's instructions. Verification of the intended genomic modification was achieved by DNA sequencing.

Plasmids

CLIC1wt-pIRES2-EGFP and CLIC1R29A-pIRES2-EGFP constructs were utilized for in vitro complementation experiments. For animal studies, both wild-type and R29A versions of Clic1 were inserted into pCDH vectors to enable lentiviral delivery.

OCR/ECAR measurements

Oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) were assessed using a Seahorse XF96 Analyzer (Agilent), with five technical replicates per experimental condition. During measurements, cells were treated with 10 mM metformin, and parameters were recorded for up to 120 minutes.

Mitochondrial membrane potential

Cells were exposed to 5 mM metformin and subsequently labeled with JC1 dye. Fluorescence signals were acquired on a BD FACSCelesta™ flow cytometer.

Intracellular ROS and mitochondrial superoxide

Cells were incubated for 3 hours with or without 5 mM metformin, then stained using CellROX™ and MitoSOX™ probes. Flow cytometric analysis was performed on a BD FACSCanto™ II instrument, with data analysis in FlowJo v10.

Protein extraction and western blotting

Harvested cells were disrupted in preheated lysis buffer, then sonicated, passed through a syringe, boiled, and centrifuged. Protein concentrations were determined

using the BCA assay. SDS-PAGE resolved samples, transferred onto nitrocellulose membranes, and incubated with appropriate primary and secondary antibodies. Chemiluminescent signals were visualized with a ChemiDoc Touch® imaging system.

Growth curves

Defined numbers of cells (2×10^4 for GBM1; 7×10^3 for GBM2 and GBM3) were seeded in culture plates and monitored over successive time points. Cell viability was assessed by Trypan Blue dye exclusion using an automated cell counter. All conditions were evaluated in triplicate.

3D cultures

Spheroids derived from GBM1 cells were generated and, after 24–48 hours, transferred into medium supplemented with 5 mM metformin. Bright-field images were obtained at the initial time point and after 72 hours. Spheroid size was determined by measuring the cross-sectional area using ImageJ.

Fluorescence intensity assay

Suspension cells (1×10^6 cells per well) underwent three washes and were then incubated in blocking buffer for 30 minutes on ice. Alexa Fluor 555-conjugated primary antibody was added, and samples were kept on ice for an additional 2 hours. Following three further washes, cells were transferred to a black 96-well plate. Fluorescence readings were collected using an EnSight Multimode Plate Reader (PerkinElmer). The intensity values obtained from samples treated with tmCLIC1omab® (3.5 µg/ml) corresponded to surface tmCLIC1 expression levels and were normalized against matched blank controls.

Patch clamp experiments

A voltage-step protocol was applied to generate current-voltage relationships, consisting of 800 ms depolarizing pulses ranging from −60 mV to +60 mV in 20 mV increments. The holding potential was maintained at the cell's resting membrane potential. CLIC1-specific chloride currents were isolated by bath application of 100 µM IAA94 and subsequent digital subtraction of the remaining current from control traces.

Outside-out patch recordings were conducted to examine single tmCLIC1 channel activity using two complementary protocols. Initially, channel identity was confirmed with a voltage-step protocol from −40 mV to

+40 mV (20 mV increments). Subsequently, the membrane potential was held at 0 mV, and pharmacological blockers (metformin followed by IAA94) were introduced after a minimum 3-minute baseline recording period.

For time-dependent experiments, the holding potential was fixed at -50 mV, and a +100 mV test pulse was delivered every 5 seconds. Current amplitude was quantified at the conclusion of each 800 ms step. Once stable currents were achieved, metformin and/or IAA94 were perfused, with bath solution exchanges performed as required. Data analysis was performed using Clampfit 10.2 (Molecular Devices) and OriginPro 9.1. The composition of the experimental solutions was as follows:

Bath solution: 140 mM NaCl, 5 mM KCl, 10 mM Hepes, 5 mM glucose, 2 mM CaCl₂, 1 mM MgCl₂, pH 7.4.

Pipette solution (whole cell): 135 mM KCl, 5 mM NaCl, 10 mM Hepes, 1 mM MgCl₂, 2 mM CaCl₂, pH 7.4.

(outside-out): 120 mM K gluconate, 20 mM KCl, 5 mM TEACl, 10 mM Hepes, 0.1 mM CaCl₂, pH 7.

Cyclin E1 expression

To examine Cyclin E1 levels, cells were first arrested with PD0332991 for 24 hours, then released into culture medium containing either 5 mM metformin or none.

Recombinant protein purification

Rosetta (DE3) *E. coli* strains were transformed with plasmids encoding either wild-type CLIC1 or the R29A mutant inserted into the pET28a vector. Bacterial cultures were incubated with vigorous shaking at 270 rpm and, upon reaching an OD of 1, induced for protein production with 1 mM IPTG for 3 hours. Following induction, cells were pelleted and disrupted via sonication. The clarified soluble lysate was obtained after centrifugation at 16,000 × g for 30 minutes at 4 °C.

This supernatant was applied to a HisTrap Nickel affinity column for initial capture of the His-tagged protein. A subsequent polishing step was performed by size-exclusion chromatography using a Superdex 75 column, with the purified protein exchanged into final storage buffer composed of 150 mM NaCl and 10 mM HEPES at pH 7.4. The resulting protein preparations were concentrated to 1-5 mg/mL.

NMR spectra were collected at 25 °C on a Bruker 600 MHz instrument. Samples consisting of 50 μM metformin were measured in isolation and in the presence of 10 μM recombinant CLIC1 protein (wild-type or

R29A mutant) prepared in PBS buffer at pH 7.4 containing 10% D₂O. WaterLOGSY, T2-filtered, and one-dimensional ¹H NMR spectra were recorded.

In-Cell NMR

Both CLIC1^{-/-} knockout cells and those rescued with the transgene were suspended at 6.5 × 10⁶ cells/mL in PBS buffer prepared with D₂O and supplemented with 2 mM metformin. WaterLOGSY and T2-filter experiments were performed at 37 °C utilizing a CryoProbe.

Zebrafish orthotopic xenografts

Zebrafish embryos at 48 hours post-fertilization were anesthetized before intracranial injection of approximately 150–200 fluorescently labeled cancer cells. Following implantation, embryos were maintained in medium supplemented with or without 5 mM metformin. Tumor volume was assessed by fluorescence quantification using ImageJ software immediately after injection and again after 3 days. All experiments were concluded by 5 days post-fertilization, before the larvae became capable of independent feeding. As such, these studies were exempt from institutional or national animal ethics review, in compliance with Directive 2010/63/EU and applicable national regulations.

Mouse orthotopic GBM model

Either wild-type or R29A mutant GL261 cells (10⁵ cells per mouse) were implanted stereotactically into the caudate nucleus of female C57BL/6 N mice. Metformin was provided ad libitum in the drinking water at 10 mM, beginning 1 week before tumor cell inoculation and continuing until study termination. At the experimental endpoint, brains were rapidly dissected and snap-frozen for further processing. All surgical and animal-handling procedures were performed by a certified external provider (Charles River Laboratories, Germany, under project code P1146A). Mice were randomly assigned to four experimental cohorts: five animals bearing GL261 wild-type tumors, five with GL261 wild-type tumors receiving metformin, five with GL261 R29A tumors, and five with GL261 R29A tumors treated with metformin.

Metformin brain concentration

Quantification of metformin levels in brain tissue was performed by Gas Chromatography-Mass Spectrometry (GC-MS) employing derivatized calibration standards and a deuterated metformin internal standard, according to a previously established method [32]. In short, tissue

samples were freeze-dried, extracted using methanol, and further purified by solid-phase extraction. Chromatographic separation was performed on a 25 m × 0.2 mm × 0.3 µm cross-linked methyl silicone gum column (Agilent Technologies, US). The oven temperature program was initiated at 120 °C, increased to 160 °C at 10 °C/min, then to 175 °C at 15 °C/min, and finally to 220 °C at 20 °C/min. Detection was conducted in selected-ion monitoring (SIM) mode with a cool-on-column injector maintained at constant flow. The ions monitored were $m/z = 303$ (corresponding to derivatized metformin hydrochloride) and $m/z = 309$ (derivatized deuterated metformin internal standard). The sample injection volume was approximately 1 µL, and extracts were stored at −20 °C until analysis.

In silico docking of metformin to CLIC1

Molecular docking simulations were conducted using the Schrödinger Maestro software suite, release 2023-3 (Maestro, Schrödinger, LLC, New York, NY, 2023). The atomic coordinates of human CLIC1 in its soluble dimeric conformation were retrieved from the Protein Data Bank under accession code 1RK4 [33]. Before docking, the structure underwent preparation steps that included adding missing atoms and determining optimal protonation states for ionizable residues at pH 7 using the Protein Preparation Wizard [34]. Energy minimization was subsequently performed employing the OPLS force field [35] to eliminate steric clashes and optimize interactions.

The metformin ligand was constructed from its SMILES string (PubChem CID 4091). Ligand preparation, including geometry optimization and generation of protonation states at pH 7, was carried out using LigPrep [34] and Epik [36]. A cubic docking grid with an edge length of 22 Å was centered on chain A of the protein at coordinates (42.193, 9.568, 34.707). Docking calculations utilized the GlideXP mode [37], permitting full ligand flexibility. The seven highest-ranking binding poses of metformin were examined in detail for specific molecular contacts with the protein. Protein-ligand interaction maps were produced with the Ligand Interaction module within the Schrödinger Maestro package, applying a 3 Å distance threshold from ligand atoms. Structural visualizations were generated using the open-source PyMOL distribution (Schrödinger, LLC). The prepared protein system and selected metformin docking poses have been deposited in Zenodo (DOI: <https://doi.org/10.5281/zenodo.14710698>) [38].

Statistical analysis

Graphical representations and statistical analyses were performed using GraphPad Prism 7 (GraphPad Software, Inc., San Diego, California). Means, standard deviations, and standard errors of the mean were computed within the same software package. Comparisons between two groups were analyzed using unpaired t-tests. For comparisons involving more than two groups within a single condition, a one-way ANOVA was used. When multiple experimental factors were present, two-way ANOVA was employed. Each experimental condition was assessed in at least three independent biological replicates. A p-value threshold of < 0.05 was defined as statistically significant.

Results and Discussion

Metformin affects GSC proliferation and viability via tmCLIC1

To investigate the functional relationship between metformin and tmCLIC1, three patient-derived GSC-enriched cultures (GBM1–3) and one murine glioma cell line (GL261) were engineered to lack CLIC1 expression using CRISPR-Cas9 gene editing. Following verification of complete CLIC1 knockout, these lines were reconstituted with either wild-type (WT) CLIC1 or the arginine 29-to-alanine mutant (R29A), the latter predicted to disrupt a potential metformin-binding site based on structural data [29]. Successful gene deletion and functional rescue were validated by assessing total CLIC1 protein levels, tmCLIC1 membrane translocation, and ion channel activity across negative control (NC), knockout, and rescued populations.

Proliferation dynamics were then monitored over 96 hours in both two-dimensional and three-dimensional culture systems in the presence or absence of 5 mM metformin (**Figure 1**). Metformin treatment markedly suppressed the growth of NC GBM1 cells (**Figure 1a**). Untreated *Clic1*^{−/−} cells exhibited proliferation rates similar to those of metformin-treated NC cells, yet the drug produced no additional inhibitory effect on the knockout cells. Re-expression of either WT or R29A CLIC1 restored baseline proliferative capacity. Importantly, WT-rescued cells recovered sensitivity to metformin comparable to NC cells (metformin-treated NC vs. metformin-treated *Clic1*^{−/−} + CLIC1 WT, n.s., $p = 0.5683$), whereas R29A-rescued cells remained insensitive to the drug (metformin-treated NC vs. metformin-treated *Clic1*^{−/−} + CLIC1 R29A, $p = 0.0164$).

Parallel results were obtained in three-dimensional spheroid cultures.

To further confirm the importance of tmCLIC1 function in GSC expansion, cells were treated with tmCLIC1omab®, a monoclonal antibody targeting the transmembrane form of the channel. This antibody produced similar growth inhibition in both WT- and R29A-rescued cells, suggesting that the antibody and metformin interact with tmCLIC1 through distinct binding sites. Prior studies have shown that metformin synergizes with glucose restriction to promote cell death in various solid tumors by activating the CIP2A-GSK3 β -MCL1 pathway, leading to CIP2A downregulation [38]. Accordingly, the sensitivity of GBM1-3 cells to combined metformin treatment and low glucose (2.5 mM) was examined by quantifying proliferation and apoptosis. Consistent with findings in other tumor types, NC glioblastoma cells displayed a glucose-dependent transition from a cytostatic to a cytotoxic response upon metformin exposure. In contrast, both *Clic1*^{-/-} and R29A-rescued cells proved completely resistant to this cytotoxic combination, demonstrating that tmCLIC1 is required for metformin-mediated cell killing under hypoglycemic conditions (**Figures 1b and 1c**). Comparable patterns were observed in GBM2, GBM3, and GL261 lines. Treatment with tmCLIC1omab® recapitulated the cytotoxic effects of metformin plus low glucose in both WT- and R29A-rescued populations (**Figures 1d and 1e**).

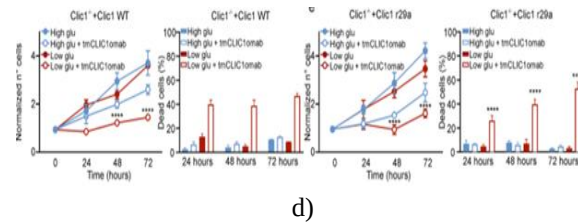
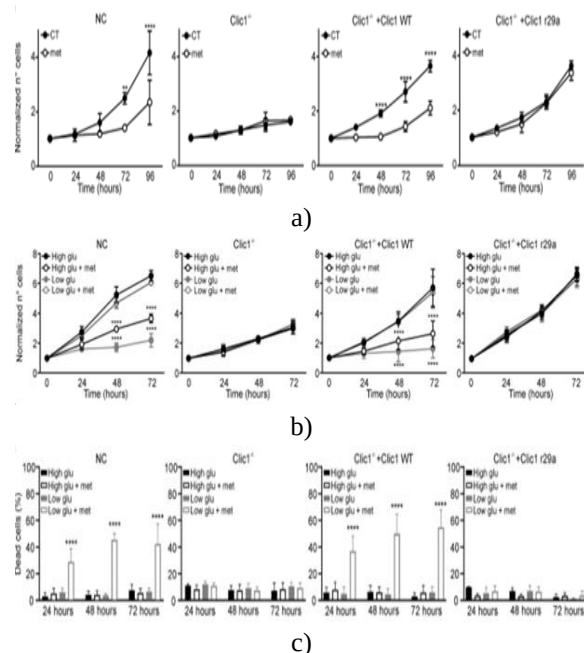


Figure 1. Metformin restricts cellular expansion exclusively in cells expressing wild-type CLIC1

(a) Proliferation kinetics of GBM1 cells tracked across 96 hours under untreated conditions (black circles) or with continuous exposure to 5 mM metformin (open circles) in NC, *Clic1*^{-/-}, *Clic1*^{-/-} + *Clic1* WT, and *Clic1*^{-/-} + *Clic1* R29A populations. (NC: CT, 24–96 hours, n=4; Met, 24–96 hours, n=7. At 72 hours, p=0.0011; at 96 hours, p<0.0001. *Clic1*^{-/-} CT, 24–72 hours, n=4; 96 hours, n=2; Met, 24–72 hours, n=7; 96 hours, n=3. *Clic1*^{-/-} + *Clic1* WT: CT, 24, 48, 96 hours, n=8; 72 hours, n=7; Met, 24 hours, n=5; 48–96 hours, n=6; 48–96 hours, p<0.0001. *Clic1*^{-/-} + *Clic1* R29A: CT, 24 hours and 96 hours, n=8; 48 hours and 72 hours, n=5; Met, 24 hours, n=5; 48–96 hours, n=6. Means $\pm$ SD, two-way ANOVA with Sidak's multiple comparisons test.) (b) GBM1 proliferation curves measured over 72 hours in high-glucose (black) versus low-glucose (gray) environments, either with or without 5 mM metformin supplementation, across NC, *Clic1*^{-/-}, *Clic1*^{-/-} + *Clic1* WT, and *Clic1*^{-/-} + *Clic1* R29A groups. (NC, low Glu vs high Glu: n=4; 48–72 hours, p<0.0001. *Clic1*^{-/-}: n=4. *Clic1*^{-/-} + *Clic1* WT: n=7, 48–72 hours, p<0.0001. *Clic1*^{-/-} + *Clic1* R29A: n=6. Means $\pm$ SD, two-way ANOVA with Tukey's multiple comparisons test.) (c) Quantification of cell death rates for the experimental conditions depicted in panel (a). (NC: n=4, 24–72 hours, p<0.0001. *Clic1*^{-/-} + *Clic1* WT: n=7, 24–72 hours, p<0.0001. Mean $\pm$ SD, two-way ANOVA with Tukey's multiple comparisons test.) (d) (left) Growth kinetics of GBM3 cells over 72 hours under high-glucose (blue) or low-glucose (dark red) conditions, treated with or without 3.5 μ g/ml tmCLIC1omab in the *Clic1*^{-/-} + *Clic1* WT background (n=3, 24 hours, p=0.0043; 48 hours, p=0.0021; 72 hours, p<0.0001; mean $\pm$ SD, two-way ANOVA with Tukey's multiple comparisons test). (right) Percentage of dead cells corresponding to the conditions illustrated in the left panel (n=3; 24–72 hours, p<0.0001; mean $\pm$ SD, one-way ANOVA with Tukey's multiple comparisons test), and (e) (left) Proliferation curves of GBM3 cells bearing the *Clic1*^{-/-} + *Clic1*

R29A genotype under identical conditions to those in panel (d) ($n=3$, 48–72 hours, $p<0.0001$; mean $\pm$ SD, two-way ANOVA with Tukey's multiple comparisons test). (right) Fraction of dead cells for the conditions shown in the left panel ($n=3$; 24–72 hours, $p<0.0001$; mean $\pm$ SD, one-way ANOVA with Tukey's multiple comparisons test).

We next investigated whether the combined presence of metformin and glucose restriction in GBM1 cells engages the CIP2A-GSK3 β -MCL1 signaling cascade, thereby promoting cell death [38]. In line with expectations, exposure to metformin under low-glucose conditions triggered dephosphorylation of GSK3 β accompanied by downregulation of CIP2A and consequent destabilization of MCL1, correlating with elevated cell death rates (**Figure 2**). Strikingly, neither Clic1 $^{-/-}$ knockout cells nor those rescued with the R29A variant exhibited metformin-induced reductions in CIP2A expression or alterations in the phosphorylation status of GSK3 β (Ser9) and MCL1 levels (**Figure 2a**). In contrast, treatment with tmCLIC1omab® disrupted the CIP2A-GSK3 β -MCL1 pathway in R29A-rescued cells, further underscoring the central involvement of tmCLIC1 in this response.

To confirm the specificity of the arginine 29 residue, Clic1 $^{-/-}$ cells were reconstituted with a lysine 37 to alanine substitution (K37A), representing the sole additional positively charged amino acid within the transmembrane domain of tmCLIC1 besides Arg29. These K37A-rescued cells retained full sensitivity to metformin, thereby confirming that Arg29 is the critical determinant for metformin interaction and subsequent biological activity.

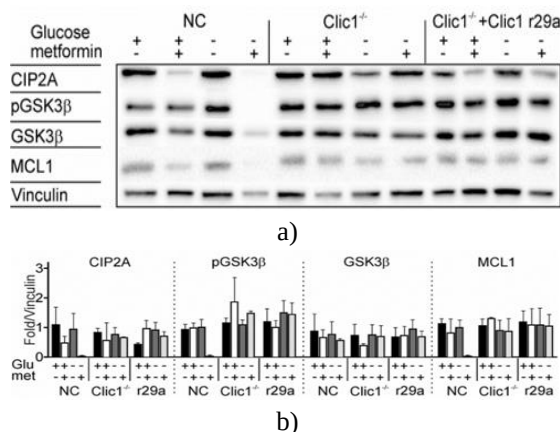


Figure 2. tmCLIC1 blockade during glucose deprivation modulates the CIP2A-GSK3 β -MCL1 signaling pathway

(a) Representative immunoblots depicting protein levels of the CIP2A-GSK3 β -MCL1 axis in NC, Clic1 $^{-/-}$, and Clic1 $^{-/-}$ + Clic1 R29A cells following 72 hours of culture in the presence or absence of metformin under high- or low-glucose conditions and (b) Densitometric quantification of Western blot signals for CIP2A, phosphorylated GSK3 β (pGSK3 β), total GSK3 β , and MCL-1 in cells maintained in high glucose (black filled bars) or low glucose (dark gray filled bars). Metformin (5 mM) was added under high-glucose (white bars) or low-glucose (light gray bars) conditions. (NC: CIP2A high glucose vs. low glucose met $p=0.0023$; low glucose vs. low glucose met $p=0.0083$. pGSK3 β : high glucose vs. low glucose met $p=0.0193$; high glucose met vs. low glucose met $p=0.0124$; low glucose vs. low glucose met $p=0.0103$. MCL-1: high glucose vs. low glucose met $p=0.0053$; low glucose vs. low glucose met $p=0.015$. Mean $\pm$ SD, two-way ANOVA with Tukey's multiple comparisons test. $n = 3$).

Metformin promotes G1-phase accumulation in GSCs

Earlier findings established that tmCLIC1 function is tightly linked to dynamic signals regulating its membrane trafficking and plays an indispensable role at the G1/S checkpoint [28]. In light of this, the current experiments assessed how tmCLIC1 status and metformin exposure influence G1-to-S phase progression in GBM1 cells with four distinct genetic configurations. Cyclin E1 protein levels rise sharply at the G1/S transition and decline rapidly once cells enter S phase. To follow cell cycle dynamics, Cyclin E1 abundance was measured at successive time points after release from early G1 synchronization. While the overall pattern of Cyclin E1 fluctuation was largely preserved across the lines, Clic1 $^{-/-}$ cells exhibited a noticeable delay in cell cycle advancement, aligning with their slower growth rate (**Figure 1**).

Metformin delayed the maximal accumulation of Cyclin E1 in both NC cells and wild-type CLIC1-rescued populations, as predicted. However, the R29A-rescued cells showed no shift in Cyclin E1 timing upon drug treatment, consistent with their lack of response to metformin. Parallel experiments demonstrated that tmCLIC1omab® produced equivalent delays in NC cells and retained activity against R29A-rescued cells. Similar responses were documented in GBM2 and GBM3 cultures, supporting the notion that this regulatory axis operates consistently across independent patient-derived models.

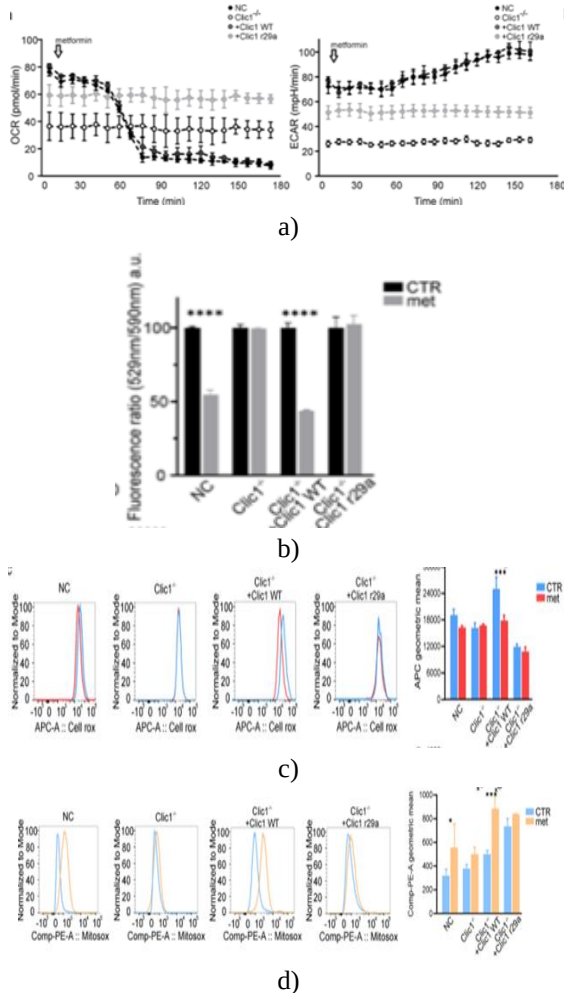


Figure 3. The metabolic actions of metformin depend entirely on CLIC1

(a) Real-time traces of oxygen consumption rate (left) and extracellular acidification rate (right) in GBM1 cells, serving as readouts for oxidative phosphorylation and glycolysis, respectively, following acute addition of 10 mM metformin. Profiles correspond to NC (black circles), Clic1^{-/-} (open circles), Clic1^{-/-} + Clic1 WT (black dotted circles), and Clic1^{-/-} + Clic1 R29A (gray dotted circles) groups, with readings taken at 6-minute intervals, (b) Mitochondrial membrane potential determined by JC-1 staining in GBM1 cells from NC, Clic1^{-/-}, Clic1^{-/-} + Clic1 WT, and Clic1^{-/-} + Clic1 R29A lines, either untreated (black bars) or exposed to 5 mM metformin (gray bars). N=2; p<0.0001; results shown as mean $\pm$ SD, evaluated by two-way ANOVA using Sidak's multiple comparisons test. (c) (left) Exemplary flow cytometry overlay of CellROXTM Deep Red signal indicating cellular oxidative stress in GBM3 cells after

3-hour incubation in the absence (blue) or presence (red) of 5 mM metformin. (right) Summary of mean CellROXTM Deep Red fluorescence intensity. N=3, Clic1^{-/-} + Clic1 WT: CT vs Met; p=0.0002; data presented as mean $\pm$ SD, analyzed via two-way ANOVA with Tukey's multiple comparisons test, and (d) (left) Representative flow cytometry histograms depicting MitoSOXTM Red signal for mitochondrial superoxide levels in GBM3 cells following 3-hour incubation without (blue) or with (yellow) 5 mM metformin. (right) Average MitoSOXTM Red fluorescence intensity. N=4, NC: CT vs Met, p=0.0361. Clic1^{-/-} + Clic1 WT: CT vs Met, p=0.0005. Data expressed as mean $\pm$ SD, analyzed by two-way ANOVA with Tukey's multiple comparisons test.

Metabolism modulation by metformin depends on CLIC1

Metformin is widely acknowledged as a potent suppressor of oxidative phosphorylation (OXPHOS) across diverse cancer cell types. In the present study, we assessed alterations in GBM cell metabolic profiles—specifically mitochondrial respiration and glycolytic activity—following acute exposure to a high dose of metformin (10 mM) in NC, Clic1^{-/-}, WT-rescued, and R29A-rescued populations. Both NC and WT-rescued cells displayed comparable baseline mitochondrial respiration rates. Upon metformin administration, these cells showed an immediate drop in oxygen consumption accompanied by a progressive rise in extracellular acidification, consistent with OXPHOS inhibition. In marked contrast, Clic1^{-/-} cells exhibited substantially lower basal metabolic activity and remained unresponsive to metformin. R29A-rescued cells maintained basal metabolism levels similar to their WT-rescued counterparts yet failed to exhibit any metabolic shift upon drug treatment (**Figure 3a**) (for GBM1). Parallel metabolic suppression was observed following tmCLIC10mab® treatment, suggesting that interference with tmCLIC1 rapidly compromises mitochondrial respiratory function.

To substantiate that the metformin-tmCLIC1 interaction initiates a coordinated stress response linking channel activity, mitochondrial function, and cell proliferation in GBM, mitochondrial membrane potential was quantified using the JC-1 probe 30 minutes after metformin exposure. NC and WT-rescued cells rapidly displayed reduced membrane potential, whereas both Clic1^{-/-} and R29A-rescued populations were insensitive to the treatment (GBM1): (**Figure 3b**).

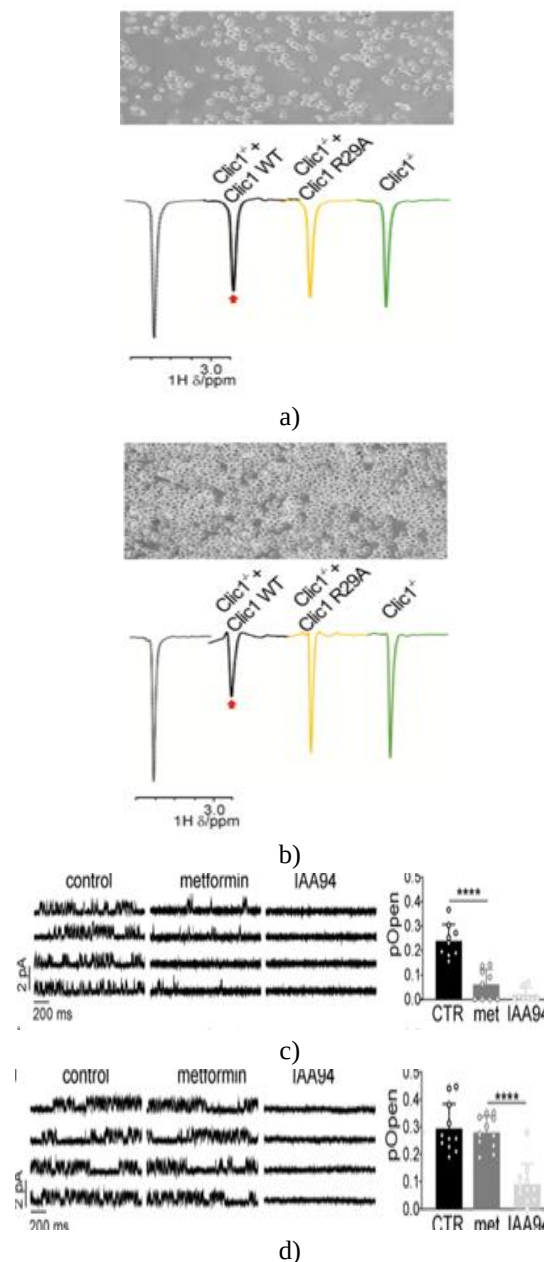
The potential impact of metformin on oxidative stress was further explored by quantifying intracellular reactive oxygen species (ROS) and mitochondrial superoxide (O_2^-) levels across the four genetic variants in GBM1–3 cultures, with and without metformin exposure (**Figures 3c and 3d**). Short-term metformin treatment led to decreased ROS generation in NC and WT-rescued cells. Still, it produced no change in *Clic1*^{-/-} or R29A-rescued lines (**Figure 3c**). Likewise, metformin failed to modify superoxide production in knockout and R29A-rescued cells. However, in cells expressing functional tmCLIC1, metformin provoked a significant elevation in mitochondrial superoxide, suggestive of acute mitochondrial dysfunction (**Figure 3d**). Supporting these observations, GBM1 *Clic1*^{-/-} and R29A-rescued cells demonstrated pronounced sensitivity to the mitochondrial complex I inhibitors IACS and niclosamide. Collectively, these findings indicate that tmCLIC1 located at the plasma membrane serves as the primary mediator of metformin's upstream inhibitory action on OXPHOS.

Evidence of direct binding between metformin and CLIC1 protein

Direct physical interaction between metformin and tmCLIC1 was verified through WaterLOGSY (Water-Ligand Observation with Gradient Spectroscopy) [39, 40] and ¹H T2-filter (transverse relaxation filter) [41] NMR spectroscopy. Experiments were carried out with 2 mM metformin, either alone or in combination with *Clic1*^{-/-}, *Clic1*^{-/-} + *Clic1* WT, or *Clic1*^{-/-} + *Clic1* R29A cells (**Figure 4a**). A pronounced binding signal appeared when metformin was incubated with WT-rescued cells, whereas interaction remained weak with *Clic1*^{-/-} cells. The minor residual binding in knockout cells most likely arises from association with secondary cellular targets. Notably, the R29A mutation led to a sharp reduction in binding, implying that this residue plays a pivotal role in metformin recognition by CLIC1. Increasing cell density in the NMR samples further accentuated the specific interaction with WT-rescued cells above background levels seen in knockout cells (**Figure 4b**).

To exclude indirect cellular effects, parallel NMR analyses were performed on purified recombinant proteins (see Methods). Both WaterLOGSY and ¹H T2-filter spectra confirmed robust binding of metformin to wild-type CLIC1 at 25 °C, with no detectable interaction occurring with the R29A mutant. These observations demonstrate at the molecular level that replacement of

the polar, positively charged arginine at position 29 with a non-polar alanine substantially abolishes metformin's capacity to engage CLIC1.



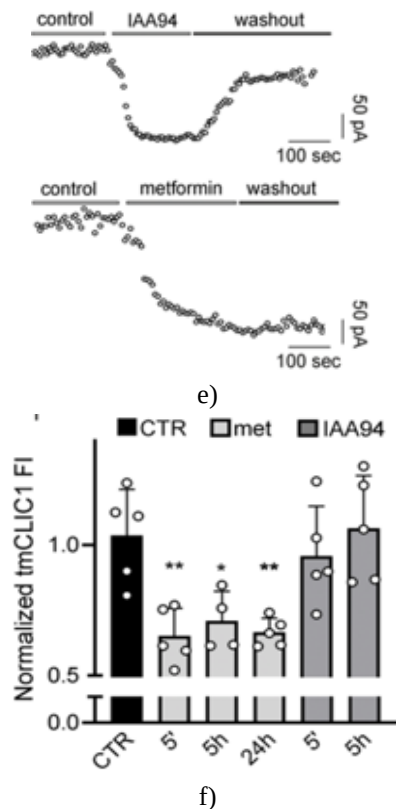


Figure 4. Metformin directly interacts with tmCLIC1

(a) WaterLOGSY NMR spectra obtained for 2 mM metformin recorded without cells (dashed line) or in the presence of Clic1^{-/-} (green), Clic1^{-/-} + Clic1 WT (black), and Clic1^{-/-} + Clic1 R29A (yellow) cells. Arrows denote prominent signal alterations indicative of strong metformin binding selectively in Clic1^{-/-} + Clic1 WT cells compared with the other genotypes, (b) Repetition of the experiment shown in (a) using twice the cell concentration, (c) Exemplary single-channel outside-out patch recordings from GBM3 Clic1^{-/-} + Clic1 WT cells during application of vehicle (CTR) or test agents (left panel). Summary graph of tmCLIC1 single-channel open probability (right panel). CTR and met n=9; IAA94, n=8; p<0.0001; mean ± SD, one-way ANOVA with Tukey's multiple comparisons test. (d) Parallel recordings performed in GBM3 Clic1^{-/-} + Clic1 R29A cells (left panel). Corresponding quantification of tmCLIC1 single-channel open probability (right panel). n=11; p<0.0001; mean ± SD, one-way ANOVA with Tukey's multiple comparisons test. (e) Representative whole-cell current time-course in WT cells. Pulses of 800 ms at +60 mV were delivered every 5 seconds from the resting

potential. After current stabilization, either 100 μM IAA94 (top) or 10 mM metformin (bottom) was perfused, followed by washout, and (f) Relative surface tmCLIC1 fluorescence intensity was measured in WT cells following incubation with metformin (gray bars) or IAA94 (dark gray bars) for the specified periods. Mean ± SD, one-way ANOVA with Tukey's multiple comparisons test (CTR n=5, met 5' n=5, met 5 h n=4, met 24 h n=5, IAA94 5' n=5, IAA94 5 h n=5. CTR vs met 5' p=0.0060, CTR vs met 5 h p=0.0378; CTR vs met 24 h p=0.0086; CTR vs IAA94 5' n.s. p=0.9614; CTR vs IAA94 5 h n.s. p=0.9997).

Patch-clamp electrophysiology was utilized to directly evaluate the functional consequences of metformin on tmCLIC1 channel activity over time. The selective CLIC1 blocker IAA94 was used for comparison. Application of metformin suppressed macroscopic chloride currents in NC and WT-rescued cells. Consistent with earlier data, R29A-rescued cells showed no reduction in tmCLIC1 current upon metformin perfusion.

Single-channel outside-out recordings provided detailed molecular-level confirmation. In WT-rescued cells, metformin potently inhibited tmCLIC1 unitary currents, rendering subsequent IAA94 addition ineffective (**Figure 4c**). In R29A-rescued cells, metformin exerted no inhibitory action, while IAA94 produced complete channel blockade (**Figure 4d**). Together, these results demonstrate that metformin physically engages tmCLIC1 within glioblastoma stem cells.

Further experiments addressed the reversibility of inhibition. Following stabilization of whole-cell currents in WT cells, perfusion with either IAA94 or metformin was followed by drug washout. Although both compounds reduced current amplitude, IAA94 inhibition was largely reversible upon removal. In contrast, metformin's suppression persisted (**Figure 4e**). Structural information [42] localizes the IAA94 binding site to extracellular cysteine 24, while metformin is predicted to interact within the transmembrane region. The persistent inhibition by metformin may therefore result from reduced surface expression of tmCLIC1 following direct ligand binding. Surface tmCLIC1 abundance was quantified via fluorescence intensity assay after brief or prolonged exposure to either compound, followed by washout and measurement 72 hours later. Metformin caused a rapid and sustained decrease in membrane tmCLIC1 levels detectable after only 5 minutes, whereas IAA94 treatment left surface

expression unchanged at all examined time points (Figure 4f).

Metformin affects tumor expansion in zebrafish embryos and mouse glioblastoma models

To investigate whether metformin retains its efficacy in whole-organism contexts, zebrafish embryos were subjected to orthotopic implantation of GBM1 cells at 48 hours after fertilization. Tumor volume was evaluated 72 hours later, with or without 5 mM metformin added to the surrounding water. Aligning with the *in vitro* findings (Figure 1), metformin markedly suppressed tumor progression in xenografts established from NC and WT-rescued cells (Figures 5a and 5b), whereas it showed no activity against tumors formed by *Clic1*^{-/-} cells or those harboring the R29A mutation (Figures 5a and 5b).

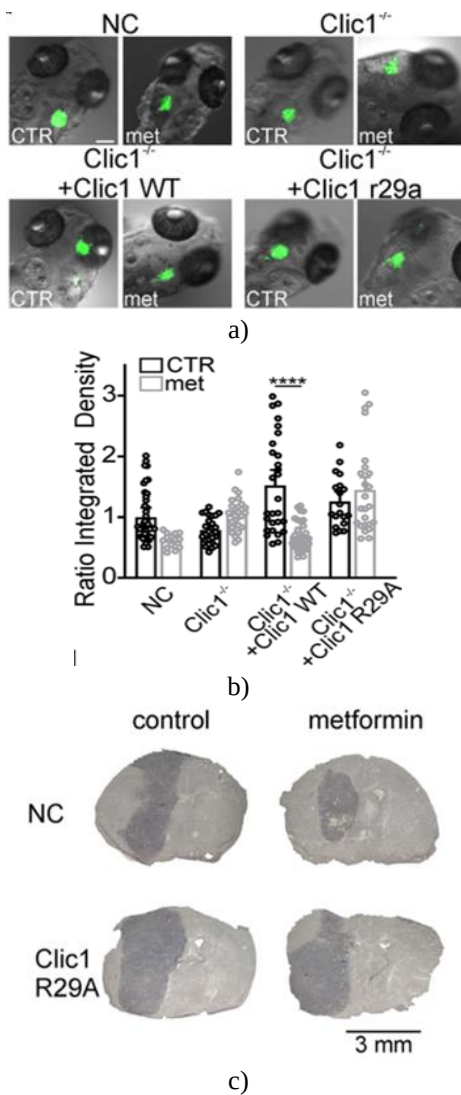


Figure 5. Metformin restricts the growth of GBM xenografts in zebrafish embryos and immunocompetent mice

(a) Typical fluorescence micrographs illustrating tumor development at 72 hours post-injection in the brain of zebrafish embryos injected with NC, *Clic1*^{-/-}, *Clic1*^{-/-} + *Clic1* WT, *Clic1*^{-/-} + *Clic1* R29A, or GBM1 cells and cultured with or without 5 mM metformin in the medium. Scale bar: 100 μm. (b) Quantitative analysis of integrated green fluorescence density within the tumor mass without (black) or with (gray) metformin treatment. Individual points correspond to tumor size measurements from single embryo brains at 4 hours post-injection and 3 days post-injection. NC: CTR, n=44, Met, n=17; n.s. p=0.1036. *Clic1*^{-/-} + *Clic1* WT: CTR, n=28, Met, n=31; p<0.0001. Mean ± SD, two-way ANOVA with Tukey's multiple comparisons test, (c) Representative hematoxylin-stained brain sections from mice intracranially injected with GL261 NC cells (upper panels) or GL261 R29A knock-in cells (lower panels), either left untreated (left) or administered 10 mM metformin via drinking water (right), and (d) Quantified tumor area normalized from hematoxylin-stained sections in untreated mice (black bars) versus those receiving 10 mM metformin (white bars). Control, n=5; metformin, n=5; *** p=0.0003, one-way ANOVA with Tukey's multiple comparisons test.

To extend these observations to a mammalian system, orthotopic syngeneic models were generated in mice by implanting GL261 wild-type or R29A knock-in cells [43]. Metformin was provided continuously in the drinking water at a concentration of 10 mM for a maximum of 50 days, yielding measurable drug levels in brain parenchyma [32] (average 7.25 nM ± 0.48, n = 12), (Table 1). Mice were euthanized on day 52 and brains collected (WT cells n = 6, *Clic1* R29A n = 6). Histopathology demonstrated that metformin treatment reduced tumor burden and invasion in animals bearing

wild-type tumors relative to untreated controls (**Figures 5c and 5d**). Conversely, tumor sizes remained equivalent

between treated and untreated groups in mice injected with R29A-mutant cells (**Figures 5c and 5d**).

Table 1. Measurement of metformin concentration in the mouse brain

SAMPLE NAME	SAMPLE WEIGHT (mg)	WATER CONTENT (ml)	METFORMIN (nM)
M78	155	131.75	5.39
M82	77.5	65.875	5.39
M86	106	90.1	5.14
M90	117	99.45	7.73
P02	141	119.85	8.54
P04	180	153	9.62
M79	69.4	58.99	5.16
M83	66.7	56.695	8.72
M87	84	71.4	6.88
M91	125	106.25	6.67
P01	164	139.4	8.64
P03	145	123.25	9.12
AVERAGES	119.22±11.2	101.33±9.5	7.25±0.48

Metformin binds the CLIC1 oxidized dimer in an amino acid pocket coordinated by R29

Molecular docking simulations performed with the soluble dimeric structure of CLIC1 identified a well-defined binding cavity for metformin. They underscored the importance of particular amino acids in ligand coordination and maintenance of the pocket's conformation (**Figure 6**). Evaluation of the seven generated poses, which yielded docking scores from -2.141 to -1.191 , revealed that metformin consistently occupied the same general area of the binding site in six instances, albeit with modest differences in spatial arrangement while retaining core interactions (**Figure 6**). Within these six poses, residue E81 stood out as the dominant contact point, establishing hydrogen bonds or ionic interactions with metformin and thus contributing substantially to stable ligand positioning. Pose seven diverged notably, relocating metformin to a peripheral pocket region where D76 mainly mediated interactions. The side chain of R29 establishes a relatively weak hydrogen bond with the central nitrogen of metformin solely in the sixth pose. This nitrogen atom remains unprotonated under physiological conditions and serves as a hydrogen-bond acceptor, pairing with donor groups such as the positively charged guanidinium moiety of arginine. In addition to this potential direct contact, R29 plays a structural role by participating in a network of hydrogen bonds that involves D76 via both side-chain and main-chain atoms, as well as the side chain of E81, thereby helping to maintain the overall geometry of the

binding cavity. Hydrophobic contributions from L175 and A176 further stabilize the ligand within the predominantly polar environment. The backbone of L175 forms hydrogen bonds with metformin in four of the poses. Residue N104 provides supplementary polar contacts in certain configurations, while E82 engages in salt bridges and hydrogen bonding with metformin in the two highest-scoring poses (**Figure 6**).

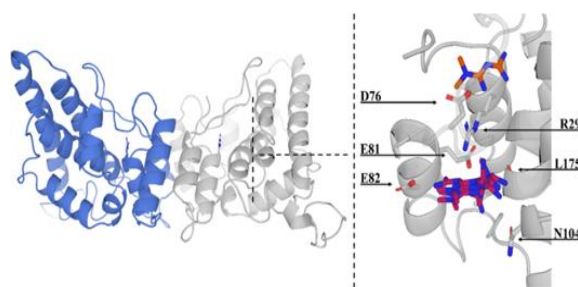


Figure 6. In silico model of CLIC1-metformin interaction.

Crystal structure of the human soluble dimeric oxidized form of CLIC1 (left) (PDB ID 1RK4) represented in cartoon format, with chain A depicted in light gray and chain B in light blue. Arginine 29 is illustrated as sticks, with nitrogen (N) and carbon (C) atoms colored to match their parent chain (light gray for chain A and light blue for chain B). Detailed view of the candidate metformin-binding pocket on CLIC1 (right), showing all 7 docking poses. Poses 1–6 are superimposed and rendered as sticks with carbon atoms in purple and nitrogen atoms in

blue. Pose 7, located outside the primary binding region, is shown with carbon atoms in orange. Critical residues involved in interactions across the poses (R29, D76, E81, E82, N104, and L175) are shown as sticks, with carbon atoms in light gray, oxygen atoms in red, and nitrogen atoms in blue. Hydrogen atoms are excluded for visual clarity.

Glioblastoma ranks among the most aggressive solid malignancies, exhibiting elevated rates of recurrence even after maximal surgical resection and intensive pharmacological intervention. Contemporary therapeutic strategies increasingly emphasize eliminating glioblastoma stem cells (GSCs), which are considered primary drivers of tumor maintenance and disease relapse. Malignant cells, particularly GSCs, exhibit elevated energy and biosynthetic precursor requirements compared with normal cells. This metabolic dependency enables flexible switching between glycolysis and oxidative phosphorylation (OXPHOS). Consequently, considerable research efforts have focused on disrupting tumor bioenergetics [44, 45], with ion channel dysregulation emerging as a critical factor. Previous investigations from our laboratory have positioned tmCLIC1 as a candidate therapeutic target [27, 28]. The transmembrane isoform of CLIC1 (tmCLIC1) undergoes pronounced upregulation at the plasma membrane in numerous solid tumors (47–53), where it actively supports tumor expansion and advancement, while remaining minimally expressed in healthy tissues.

The present study establishes that inhibition of tmCLIC1 in GSCs extends beyond merely contributing to the growth-suppressive activity of metformin [29]—instead, tmCLIC1 functions as a primary receptor for this widely used antidiabetic agent. The R29A mutant of tmCLIC1 retains biophysical properties closely resembling those of the wild-type channel. Although metformin may engage additional cellular targets in glioblastoma, cells harboring the R29A point mutation exhibit complete loss of functional response to the drug. Consequently, introduction of metformin into cancer cells carrying the arginine 29 substitution abolishes its antiproliferative capacity.

Metformin suppresses mitochondrial respiration and thereby restrains tumor cell proliferation and invasiveness exclusively upon binding to tmCLIC1. When combined with hypoglycemic conditions, this interaction further perturbs the PP2A-GSK3 β -MCL-1 signaling axis, culminating in cancer cell apoptosis. These actions, however, require millimolar

concentrations in vitro, with electrophysiological data indicating an affinity of approximately 4 mM for the metformin-tmCLIC1 interaction. Notably, in vivo administration of metformin through drinking water produced substantial retardation of glioblastoma progression despite achieving only nanomolar drug concentrations in brain tissue (**Table 1**) [46]. This pronounced difference between effective in vivo and in vitro concentrations merits additional exploration.

We propose that tmCLIC1 does not operate as a conventional ion channel. Its membrane-embedded structure remains unresolved. Existing evidence suggests that the transmembrane form exists as homo-oligomeric complexes comprising two, three, or possibly four dimers. Its amino acid sequence and dimeric architecture diverge considerably from those of classical ion channels [36, 47]. Insertion into the membrane requires reorganization of the hydrophilic monomeric form into a dimeric assembly [48]. In particular, the putative transmembrane segment (amino acids 24–46) bears no resemblance to known channel architectures. We hypothesize that tmCLIC1 assembles into a multimeric complex that facilitates chloride conductance through a pathway largely independent of voltage and time. This notion is supported by the flickering behavior observed in single-channel recordings (**Figures 4c and 4d**). Within this framework, metformin does not function as a conventional pore blocker. Rather, it accumulates irreversibly within the tmCLIC1 complex, thereby attenuating chloride currents. Sustained delivery of metformin in drinking water has been shown to maintain stable brain levels over extended periods. Such continuous exposure was not achieved when the drug was administered by oral gavage, correlating with diminished antiproliferative efficacy. We posit that prolonged tissue accumulation enables effective tmCLIC1 inhibition at far lower concentrations than those needed for acute in vitro exposure. Preliminary data indicate that the concentration required for tmCLIC1 inhibition is inversely proportional to duration of exposure.

The interaction between tmCLIC1 and metformin critically depends on the positively charged arginine residue at position 29. Nevertheless, an unresolved question remains: how does a positively charged molecule such as metformin bind to an arginine residue and subsequently influence mitochondrial activity? Computational docking analyses revealed that, despite electrostatic repulsion, metformin localizes near R29,

underscoring the functional importance of this residue. Examination of the local environment surrounding R29 uncovered a pocket enriched in negatively or partially negatively charged amino acids that likely promotes metformin accommodation (**Figure 6**). Pharmacological agents such as IAA94 and the monoclonal antibody tmCLIC1omab® replicate metformin's functional outcomes despite interacting at distinct sites. Both disrupt tmCLIC1 channel activity even in the presence of the R29A mutation. Functional impairment of tmCLIC1 consequently perturbs GSC metabolism. Prior work has linked tmCLIC1 to maintenance of membrane potential and support of membrane-associated NADPH oxidase function [28]. We propose that tmCLIC1 contributes substantially to reactive oxygen species (ROS) generation, thereby facilitating metabolic reprogramming in GSCs. Moreover, tmCLIC1 operates upstream of the PP2A-GSK3 β -MCL-1 cascade, thereby promoting GSC survival. Sustained metformin exposure may therefore amplify both cytostatic and cytotoxic responses.

Conclusion

In summary, interference with tmCLIC1 function through small molecules, monoclonal antibodies, or metformin compromises glioblastoma cell viability by substantially diminishing cellular energy production. Metformin offers distinct advantages as a repurposed therapeutic agent, with an established safety profile, minimal adverse effects, and widespread clinical approval. Nevertheless, outcomes from clinical trials evaluating metformin have been inconsistent [47-49]. The current investigation elucidates mechanistic details of metformin's interaction with the tmCLIC1 complex. A central contribution of this work lies in the optimization of drug delivery. Continuous administration via drinking water achieves steady-state metformin concentrations in the brain, enabling effective tmCLIC1 inhibition at nanomolar levels. Targeting tmCLIC1 emerges as a crucial strategy for attenuating tumor aggressiveness and preventing recurrence driven by cancer stem cells. Such broad suppression of tumor fitness may enhance the efficacy of conventional therapies and support combination regimens aimed at limiting glioblastoma growth and dissemination.

Acknowledgments: None

Conflict of Interest: None

Financial Support: None

Ethics Statement: None

References

1. Louis DN, Perry A, Wesseling P, Brat DJ, Cree IA, Figarella-Branger D, et al. The 2021 WHO classification of tumors of the central nervous system: a summary. *Neuro Oncol.* 2021;23(8):1231-51.
2. Stupp R, Hegi ME, Mason WP, van den Bent MJ, Taphoorn MJ, Janzer RC, et al. Effects of radiotherapy with concomitant and adjuvant temozolomide versus radiotherapy alone on survival in glioblastoma in a randomised phase III study: 5-year analysis of the EORTC-NCIC trial. *Lancet Oncol.* 2009;10(5):459-66.
3. Van Meir EG, Hadjipanayis CG, Norden AD, Shu HK, Wen PY, Olson JJ. Exciting new advances in neuro-oncology: the avenue to a cure for malignant glioma. *CA Cancer J Clin.* 2010;60(3):166-93.
4. Davies AM, Weinberg U, Palti Y. Tumor treating fields: a new frontier in cancer therapy. *Ann N Y Acad Sci.* 2013;1291:86-95.
5. Paolillo M, Boselli C, Schinelli S. Glioblastoma under siege: an overview of current therapeutic strategies. *Brain Sci.* 2018;8(1):15.
6. Weller M, van den Bent M, Hopkins K, Tonn JC, Stupp R, Falini A, et al. EANO guideline for the diagnosis and treatment of anaplastic gliomas and glioblastoma. *Lancet Oncol.* 2014;15(9):e395-403.
7. Stewart LA. Chemotherapy in adult high-grade glioma: a systematic review and meta-analysis of individual patient data from 12 randomised trials. *Lancet.* 2002;359(9311):1011-8.
8. Bi WL, Beroukhi R. Beating the odds: extreme long-term survival with glioblastoma. *Neuro Oncol.* 2014;16(9):1159-60.
9. Wang X, Prager BC, Wu Q, Kim LJY, Gimple RC, Shi Y, et al. Reciprocal signaling between glioblastoma stem cells and differentiated tumor cells promotes malignant progression. *Cell Stem Cell.* 2018;22(4):514-28.e5.
10. Yi Y, Hsieh IY, Huang X, Li J, Zhao W. Glioblastoma stem-like cells: characteristics,

- microenvironment, and therapy. *Front Pharmacol*. 2016;7:477.
11. Sharifzad F, Ghavami S, Verdi J, Mardpour S, Mollapour Sisakht M, Azizi Z, et al. Glioblastoma cancer stem cell biology: potential theranostic targets. *Drug Resist Updat*. 2019;42:35-45.
 12. Campos B, Olsen LR, Urup T, Poulsen HS. A comprehensive profile of recurrent glioblastoma. *Oncogene*. 2016;35(45):5819-25.
 13. Dirkse A, Golebiewska A, Buder T, Nazarov PV, Muller A, Poovathingal S, et al. Stem cell-associated heterogeneity in glioblastoma results from intrinsic tumor plasticity shaped by the microenvironment. *Nat Commun*. 2019;10(1):1787.
 14. Florio T, Barbieri F. The status of the art of human malignant glioma management: the promising role of targeting tumor-initiating cells. *Drug Discov Today*. 2012;17(19-20):1103-10.
 15. Saini N, Yang X. Metformin as an anti-cancer agent: actions and mechanisms targeting cancer stem cells. *Acta Biochim Biophys Sin*. 2018;50(2):133-43.
 16. Carmignani M, Volpe AR, Aldea M, Soritau O, Irimie A, Florian IS, et al. Glioblastoma stem cells: a new target for Metformin and arsenic trioxide. *J Biol Regul Homeost Agents*. 2014;28(1):1-15.
 17. Leidgens V, Proske J, Rauer L, Moeckel S, Renner K, Bogdahn U, et al. Stattic and Metformin inhibit brain tumor initiating cells by reducing STAT3-phosphorylation. *Oncotarget*. 2017;8(5):8250-63.
 18. Valtorta S, Lo Dico A, Raccagni I, Gaglio D, Belloli S, Politi LS, et al. Metformin and temozolomide, a synergic option to overcome resistance in glioblastoma multiforme models. *Oncotarget*. 2017;8(68):113090-104.
 19. Wurth R, Pattarozzi A, Gatti M, Bajetto A, Corsaro A, Parodi A, et al. Metformin selectively affects human glioblastoma tumor-initiating cell viability: a role for metformin-induced inhibition of Akt. *Cell Cycle*. 2013;12(1):145-56.
 20. El-Mir MY, Nogueira V, Fontaine E, Averet N, Rigoulet M, Leverve X. Dimethylbiguanide inhibits cell respiration via an indirect effect targeted on the respiratory chain complex I. *J Biol Chem*. 2000;275(1):223-8.
 21. Bridges HR, Jones AJ, Pollak MN, Hirst J. Effects of metformin and other biguanides on oxidative phosphorylation in mitochondria. *Biochem J*. 2014;462(3):475-87.
 22. Loubiere C, Clavel S, Gilleron J, Harisseh R, Fauconnier J, Ben-Sahra I, et al. The energy disruptor Metformin targets mitochondrial integrity via modification of calcium flux in cancer cells. *Sci Rep*. 2017;7(1):5040.
 23. Garnier D, Renoult O, Alves-Guerra MC, Paris F, Pecqueur C. Glioblastoma stem-like cells, metabolic strategy to kill a challenging target. *Front Oncol*. 2019;9:118.
 24. Shibao S, Minami N, Koike N, Fukui N, Yoshida K, Saya H, et al. Metabolic heterogeneity and plasticity of glioma stem cells in a mouse glioblastoma model. *Neuro Oncol*. 2018;20(3):343-54.
 25. Fidoamore A, Cristiano L, Laezza C, Galzio R, Benedetti E, Cinque B, et al. Energy metabolism in glioblastoma stem cells: PPARalpha a metabolic adaptor to intratumoral microenvironment. *Oncotarget*. 2017;8(65):108430-50.
 26. Chae YK, Arya A, Malecek MK, Shin DS, Carneiro B, Chandra S, et al. Repurposing metformin for cancer treatment: current clinical studies. *Oncotarget*. 2016;7(26):40767-80.
 27. Setti M, Savalli N, Osti D, Richichi C, Angelini M, Brescia P, et al. Functional role of CLIC1 ion channel in glioblastoma-derived stem/progenitor cells. *J Natl Cancer Inst*. 2013;105(21):1644-55.
 28. Peretti M, Raciti FM, Carlini V, Verduci I, Sertic S, Barozzi S, et al. Mutual influence of ROS, pH, and CLIC1 membrane protein in the regulation of G1-S phase progression in human glioblastoma stem cells. *Mol Cancer Ther*. 2018;17(11):2451-61.
 29. Gritti M, Wurth R, Angelini M, Barbieri F, Peretti M, Pizzi E, et al. Metformin repositioning as antitumoral agent: selective antiproliferative effects in human glioblastoma stem cells, via inhibition of CLIC1-mediated ion current. *Oncotarget*. 2014;5(22):11252-68.
 30. Barbieri F, Wurth R, Pattarozzi A, Verduci I, Mazzola C, Cattaneo MG, et al. Inhibition of chloride intracellular channel 1 (CLIC1) as biguanide class-effect to impair human glioblastoma stem cell viability. *Front Pharmacol*. 2018;9:899.
 31. Barbieri F, Bosio AG, Pattarozzi A, Tonelli M, Bajetto A, Verduci I, et al. Chloride intracellular channel 1 activity is not required for glioblastoma development but its inhibition dictates glioma stem cell responsivity to novel biguanide derivatives. *J Exp Clin Cancer Res*. 2022;41(1):53.

32. Ailuno G, Baldassari S, Balboni A, Drava G, Spalletti C, Tantillo E, et al. Development and validation of a GC-MS method for determination of Metformin in normal brain and in glioblastoma tissues. *J Pharm Biomed Anal.* 2023;234:115503.
33. Berman HM, Westbrook J, Feng Z, Gilliland G, Bhat TN, Weissig H, et al. The protein data bank. *Nucleic Acids Res.* 2000;28(1):235-42.
34. Sastry GM, Adzhigirey M, Day T, Annabhimoju R, Sherman W. Protein and ligand preparation: parameters, protocols, and influence on virtual screening enrichments. *J Comput Aided Mol Des.* 2013;27(3):221-34.
35. Roos K, Wu C, Damm W, Reboul M, Stevenson JM, Lu C, et al. OPLS3e: extending force field coverage for drug-like small molecules. *J Chem Theory Comput.* 2019;15(3):1863-74.
36. Johnston RC, Yao K, Kaplan Z, Chelliah M, Leswing K, Seekins S, et al. Epik: pK(a) and protonation state prediction through machine learning. *J Chem Theory Comput.* 2023;19(8):2380-8.
37. Friesner RA, Murphy RB, Repasky MP, Frye LL, Greenwood JR, Halgren TA, et al. Extra precision glide: docking and scoring incorporating a model of hydrophobic enclosure for protein-ligand complexes. *J Med Chem.* 2006;49(21):6177-96.
38. Elgendy M, Ciro M, Hosseini A, Weiszmann J, Mazzarella L, Ferrari E, et al. Combination of hypoglycemia and Metformin impairs tumor metabolic plasticity and growth by modulating the PP2A-GSK3 β -MCL-1 axis. *Cancer Cell.* 2019;35(5):798-815.e5.
39. Dalvit C, Pevarello P, Tato M, Veronesi M, Vulpetti A, Sundstrom M. Identification of compounds with binding affinity to proteins via magnetization transfer from bulk water. *J Biomol NMR.* 2000;18(1):65-8.
40. Dalvit C, Fogliatto G, Stewart A, Veronesi M, Stockman B. WaterLOGSY as a method for primary NMR screening: practical aspects and range of applicability. *J Biomol NMR.* 2001;21(4):349-59.
41. Hajduk PJ, Olejniczak ET, Fesik SW. One-dimensional relaxation- and diffusion-edited NMR methods for screening compounds that bind to macromolecules. *J Am Chem Soc.* 1997;119(50):12257-61.
42. Harrop SJ, DeMaere MZ, Fairlie WD, Reztsova T, Valenzuela SM, Mazzanti M, et al. Crystal structure of a soluble form of the intracellular chloride ion channel CLIC1 (NCC27) at 1.4-Å resolution. *J Biol Chem.* 2001;276(48):44993-5000.
43. Zagzag D, Esencay M, Mendez O, Yee H, Smirnova I, Huang Y, et al. Hypoxia- and vascular endothelial growth factor-induced stromal cell-derived factor-1 α /CXCR4 expression in glioblastomas: one plausible explanation of Scherer's structures. *Am J Pathol.* 2008;173(2):545-60.
44. Zhao J, Ma X, Gao P, Han X, Zhao P, Xie F, et al. Advancing glioblastoma treatment by targeting metabolism. *Neoplasia.* 2024;51:100985.
45. Zarzuela L, Duran RV, Tome M. Metabolism and signaling crosstalk in glioblastoma progression and therapy resistance. *Mol Oncol.* 2025;19(3):592-613.
46. Wilkinson MD, Dumontier M, Aalbersberg IJ, Appleton G, Axton M, Baak A, et al. The FAIR guiding principles for scientific data management and stewardship. *Sci Data.* 2016;3:160018.
47. Yoon WS, Chang JH, Kim JH, Kim YJ, Jung TY, Yoo H, et al. Efficacy and safety of metformin plus low-dose temozolomide in patients with recurrent or refractory glioblastoma: a randomized, prospective, multicenter, double-blind, controlled, phase 2 trial (KNOG-1501 study). *Discover Oncol.* 2023;14(1):90.
48. Fuentes-Fayos AC, Perez-Gomez MEGG, Montero-Hidalgo JM, Martin-Colom AJ, Doval-Rosa J. Metformin and simvastatin exert additive antitumour effects in glioblastoma via senescence-state: clinical and translational evidence. *EBioMedicine.* 2023;90:104484.
49. Basheer HA, Salman NM, Abdullah RM, Elsalem L, Afarinkia K. Metformin and glioma: targeting metabolic dysregulation for enhanced therapeutic outcomes. *Transl Oncol.* 2025;53:102323.