**SUPPLEMENTARY MATERIAL TO**

# CHLORIDE INTRACELLULAR CHANNEL 1 ACTIVITY IS NOT REQUIRED FOR GLIOBLASTOMA DEVELOPMENT BUT ITS INHIBITION DICTATES GLIOMA STEM CELL RESPONSIVITY TO NOVEL BIGUANIDE DERIVATIVES

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1) **Supplementary Figures S1 – S14**

2) **Supplementary Tables S1 – S7**

# SUPPLEMENTARY FIGURES

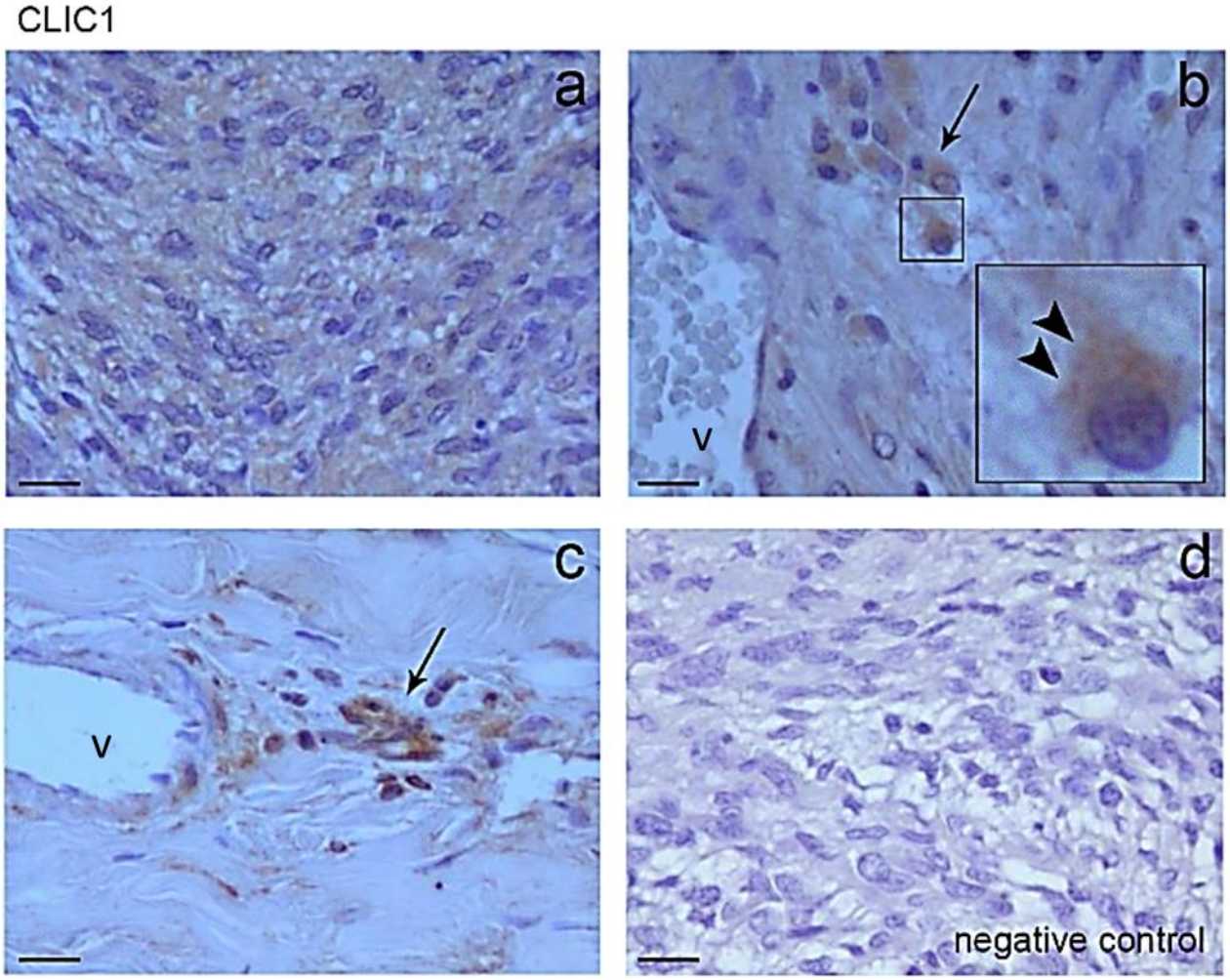



**FIGURE S1**

**Distribution of CLIC1 in GBM**

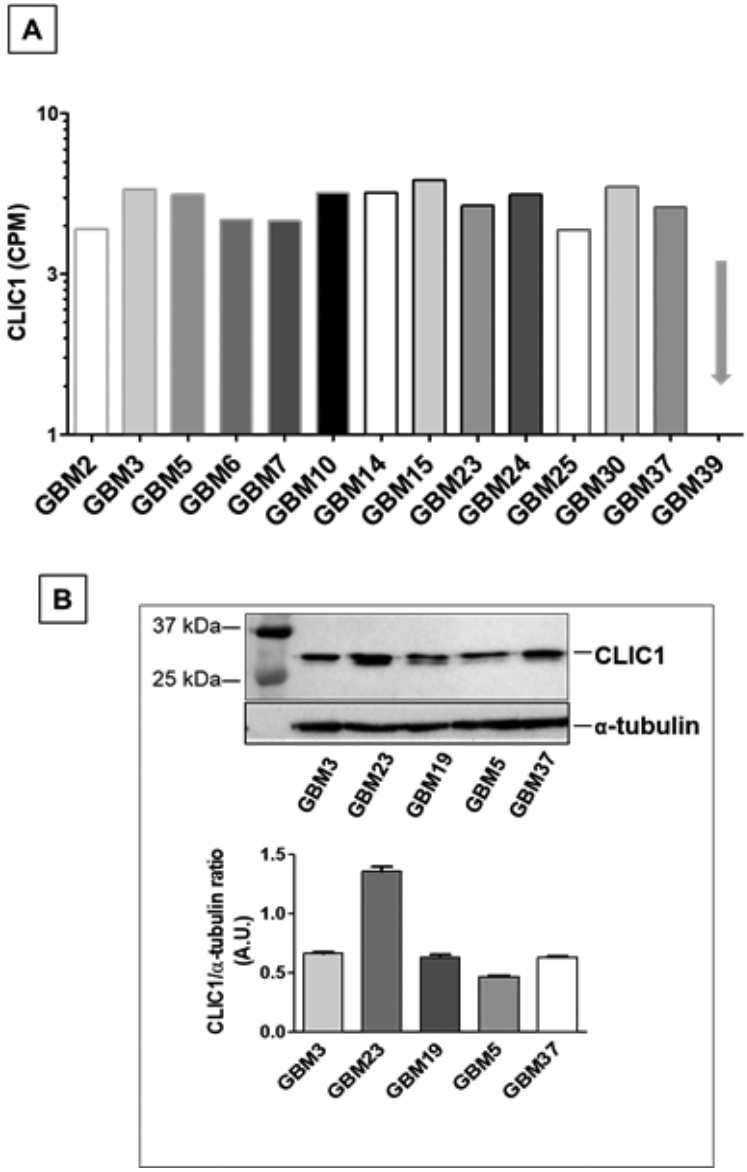
(a) GBM solid tumor cells CLIC1 staining is diffuse and uniform.

(b-c) CLIC1 intensely positive cells are visible near vessels (arrows), and the staining is dot-like and localized especially on plasma membrane (arrows-heads).

(d) Negative controls.

V: vessel.

Scale bar = 40 μm




| TUMOR CODE | NCBI Geo Data set code |
|---|---|
| GBM 2 | GSC#Ge002 |
| GBM 3 | GSC#Ge003 |
| GBM 5 | GSC#Ge005 |
| GBM 6 | GSC#Ge006 |
| GBM 7 | GSC#Ge007 |
| GBM 10 | GSC#Ge010 |
| GBM 14 | GSC#Ge014 |
| GBM 15 | GSC#Ge015 |
| GBM 23 | GSC#Ge023 |
| GBM 24 | GSC#Ge024 |
| GBM 25 | GSC#Ge025 |
| GBM 30 | GSC#Ge030 |
| GBM 37 | GSC#Ge037 |
| GBM 38 | GSC#Ge038 |

**FIGURE S2**

A) CLIC1 expression in GSCs isolated from 14 human GBMs, evaluated by RNA-seq, and expressed as counts per million reads mapped (CPM). Only GBM 39 (arrow) displayed low CLIC1 mRNA content. The Table reports the corresponding codes of the GSC cultures used in this study and the RNA-seq data deposited at NCBI Geo data set.

B) **Upper panel**: CLIC1 expression evaluated by WB, in total cell lysates from selected GSC cultures. Membranes were re-probed with α-tubulin antibody after stripping and used as a reference for protein loading.

**Lower panel:** Histograms report CLIC1/α-tubulin ratio of densitometric values and expressed in arbitrary units (A.U.) as mean ± S.D.

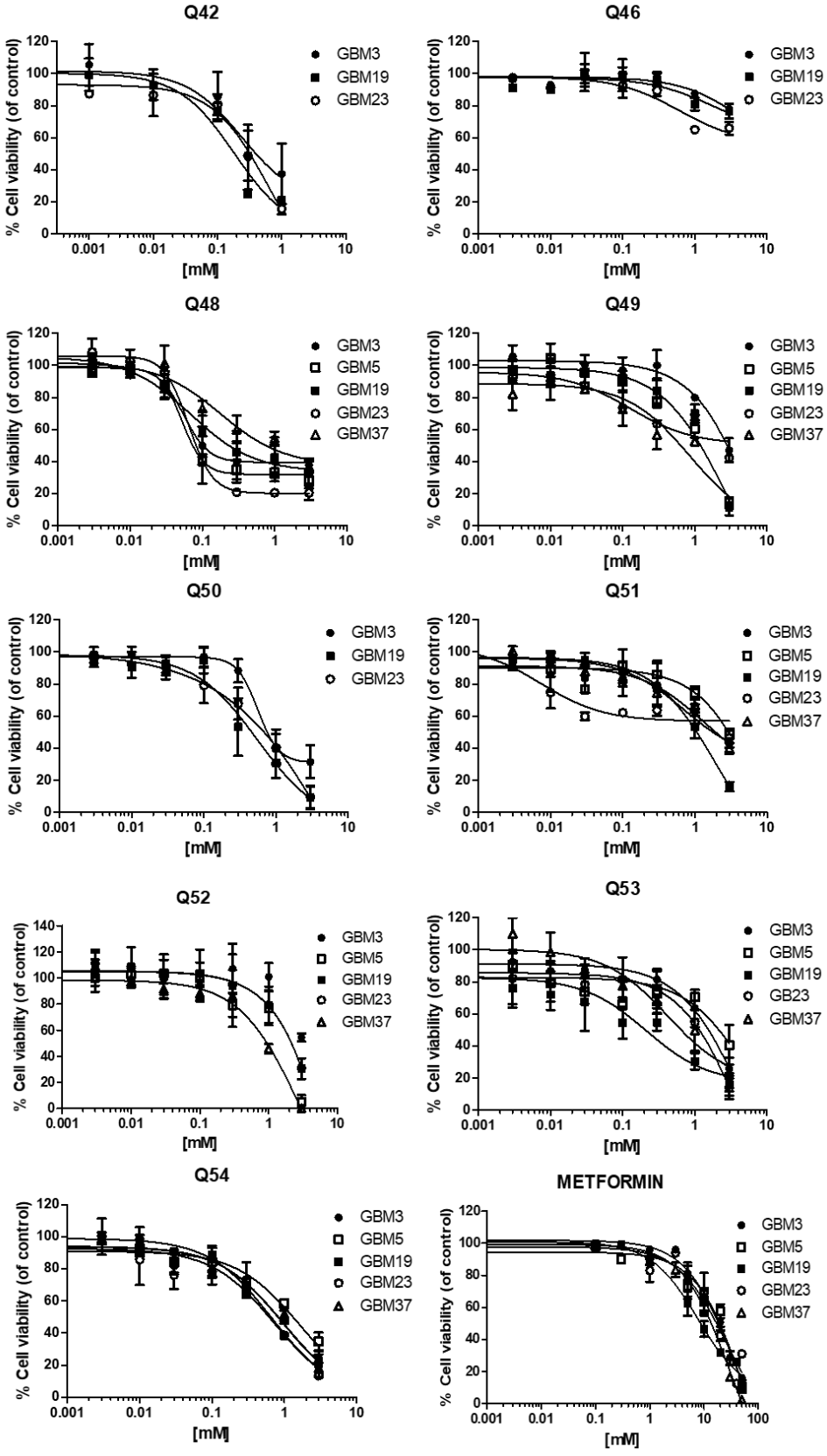



**FIGURE S3**

Dose-response curves of novel biguanide derivatives and metformin on individual GSC cultures. The average response is reported in the Figure 1A of the manuscript.

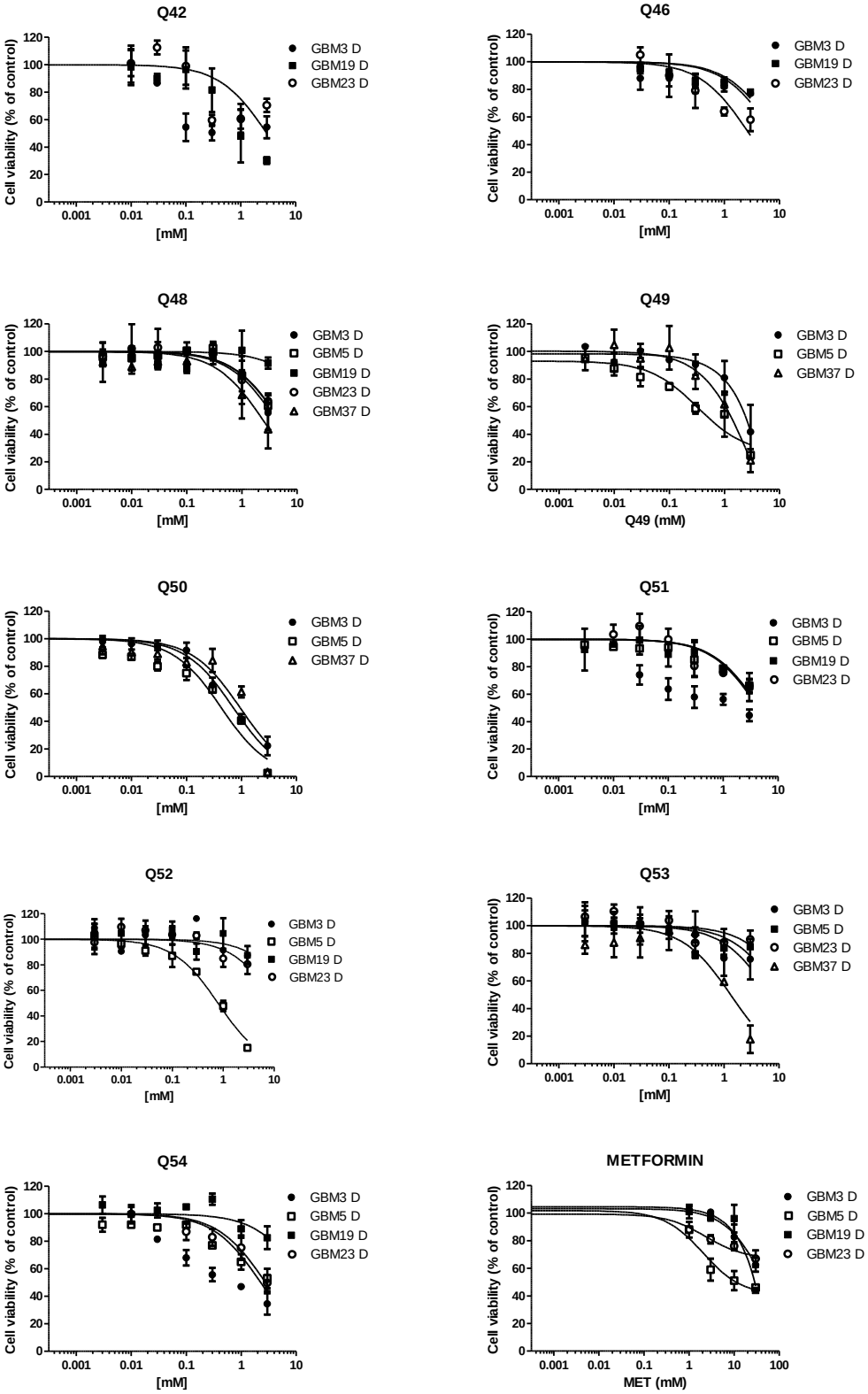



**FIGURE S4**

Dose-response curves of novel biguanide derivatives and metformin on individual non-stem differentiated GBM cell cultures (GBM D). The average response is reported in the Figure 1B of the manuscript.

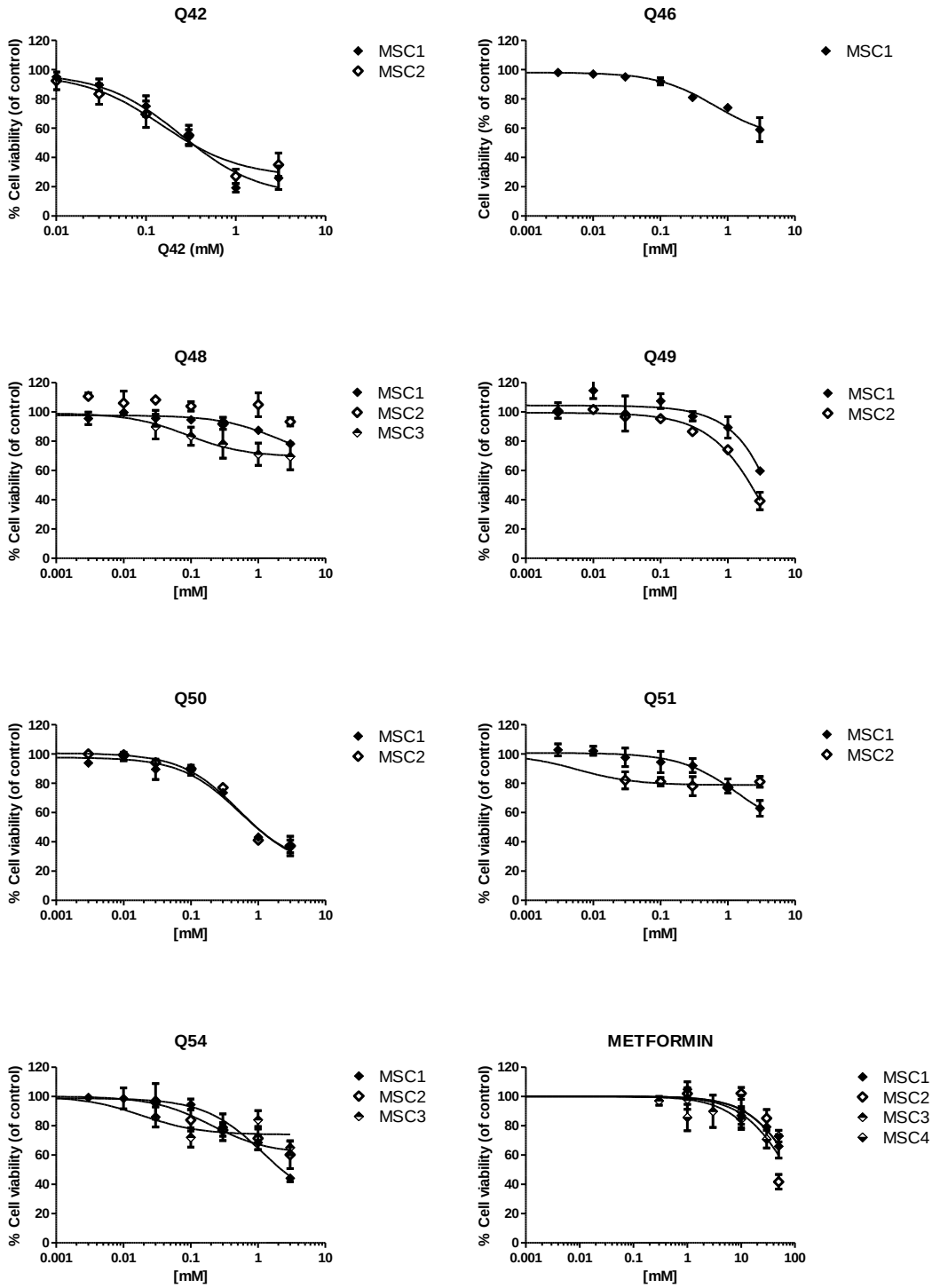



**FIGURE S5**

Dose-response curves of metformin and novel biguanide derivatives on individual ucMSC cultures. The average response is reported in the Figure 1C of the manuscript.

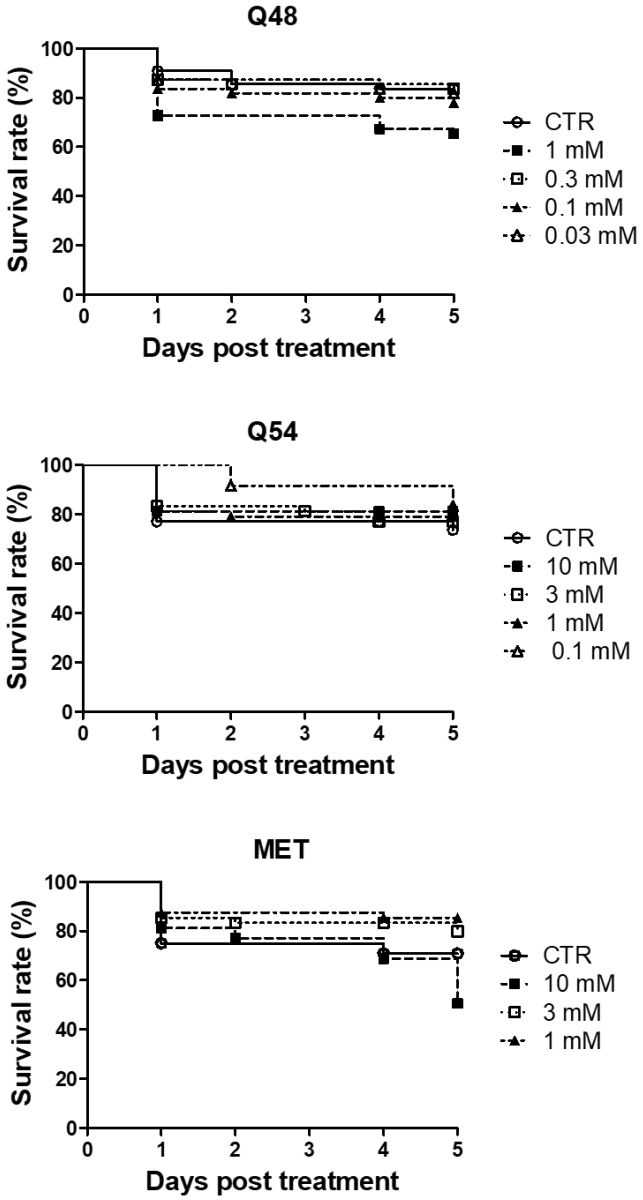



**FIGURE S6**

Kaplan-Mayer curves of Q48, Q54, and metformin (MET) depicting the effect of supramaximal concentration of these compounds on zebrafish embryos survival. Limited toxicity, not different from controls, was observed for all the compounds up to 5 days of treatment. Experiments were repeated twice, n = 20 per experimental group. Q48: log rank test for trend *p*=0.62; Q54: log rank test for trend *p*=0.64; metformin: log rank test for trend *p*=0.38.

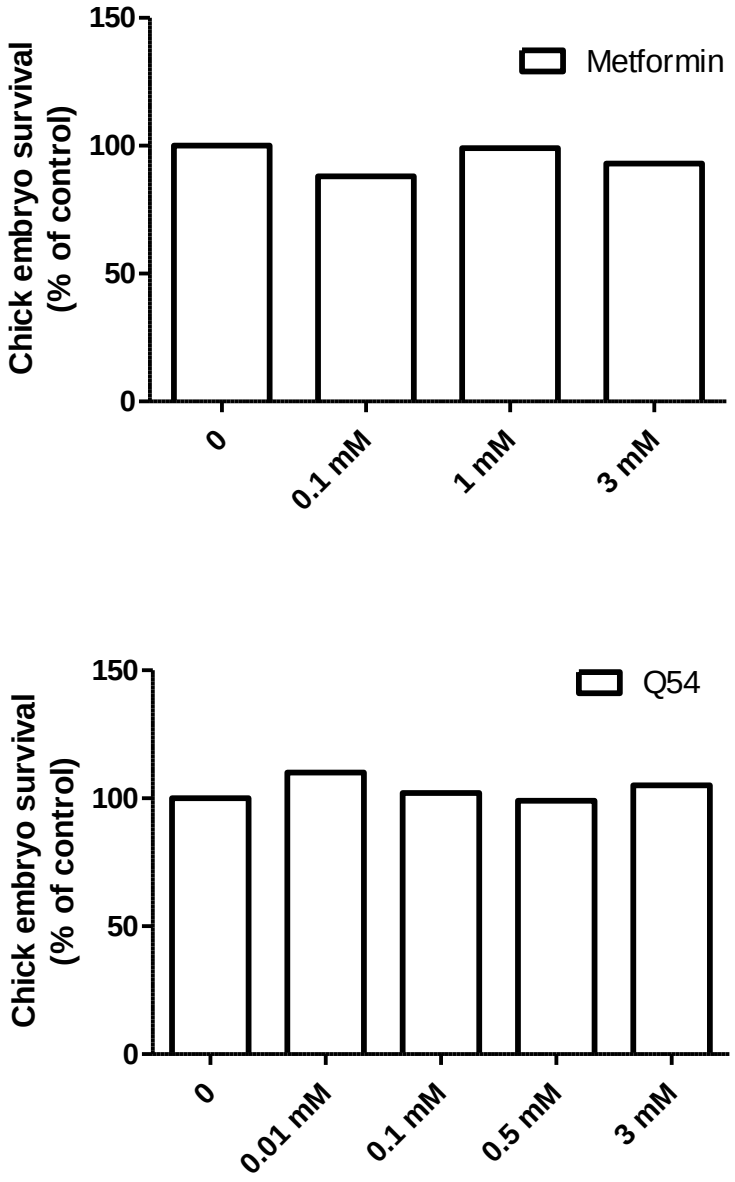
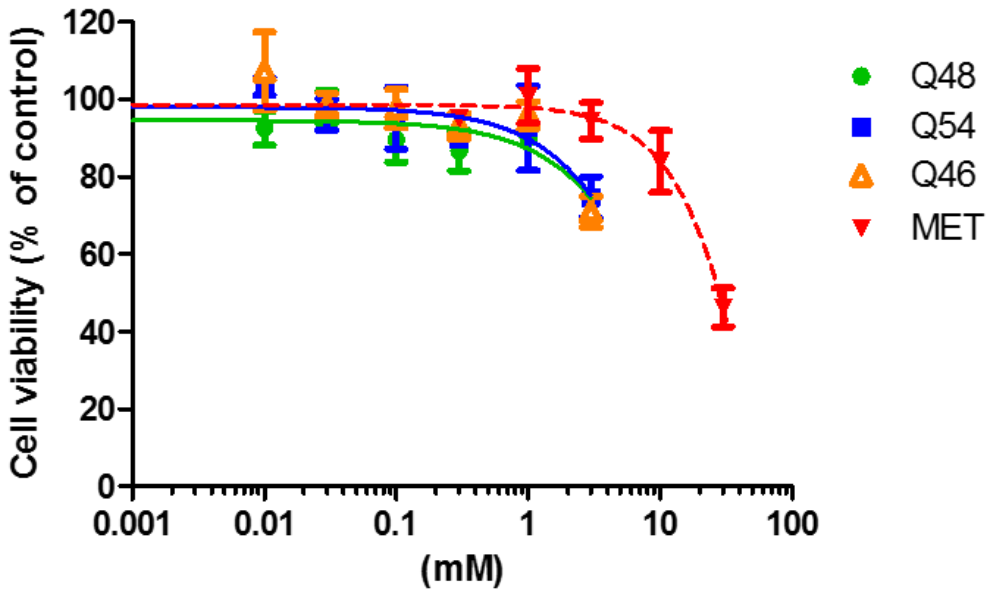



**FIGURE S7**

Effect of different doses of metformin and Q54 on chick embryo survival after 10 days of incubation. Experiments were performed by Inovotion (La Tronche, France). No toxicity was observed for these compounds up to 3 mM. Experiments were repeated twice, n = 18 per experimental group.

**A**




**B**

| Compound# | $IC_{50}$ (mM) Rat astrocytes | $IC_{50}$ (mM) Human GSCs | Selectivity index $\left( SI=\frac{IC_{50}\ no\ cancer\ cells}{IC_{50}\ cancer\ cells} \right)$ |
|---|---|---|---|
| Q48 | 20.19 | 0.082 | 246,21 |
| Q54 | 22.35 | 0.43 | 51.97 |
| Q46 | n.r.° | n.r.° | - |
| MET | 9.8 | 106 | 10,81 |

°n.r.= not reached

**FIGURE S8**

A. Dose-response curves of Q46, Q48, Q54 and metformin on rat astrocyte cultures. Limited toxicity is observed for all the novel compounds. Only metformin reduced astrocyte viability (-50%) at the higher concentration tested (30 mM). Data are expressed as average of experiments preformed in quadruplicate and repeated twice.

B. Table reports $IC_{50}$ values in non-malignant rat astrocytes and GSCs and the calculated selectivity indices for each compound. According to the "selectivity criteria" all biguanides are considered selective compounds against GSCs (selectivity index >10) (see reference 62).

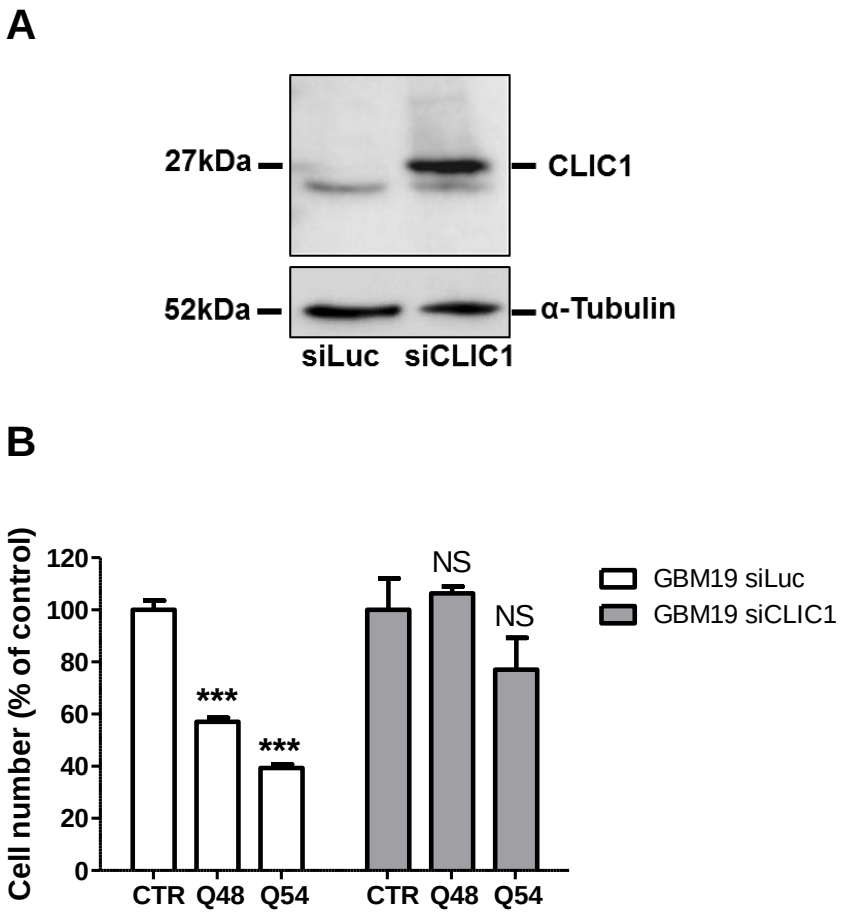



**FIGURE S9**

A. CLIC1 expression in GBM19 GSCs carrying siRNA for both Luciferase (siLuc, silencing control) and CLIC1 (siCLIC1).

B. Cell proliferation of GBM19 siLuc and GBM19 siCLIC1, treated with Q48 and Q54 (100μM) for 48h and evaluated by counting live cells. Data represent the mean ± S.E.M..

$^{***}p < 0.001$ *vs.* respective control (CTR).

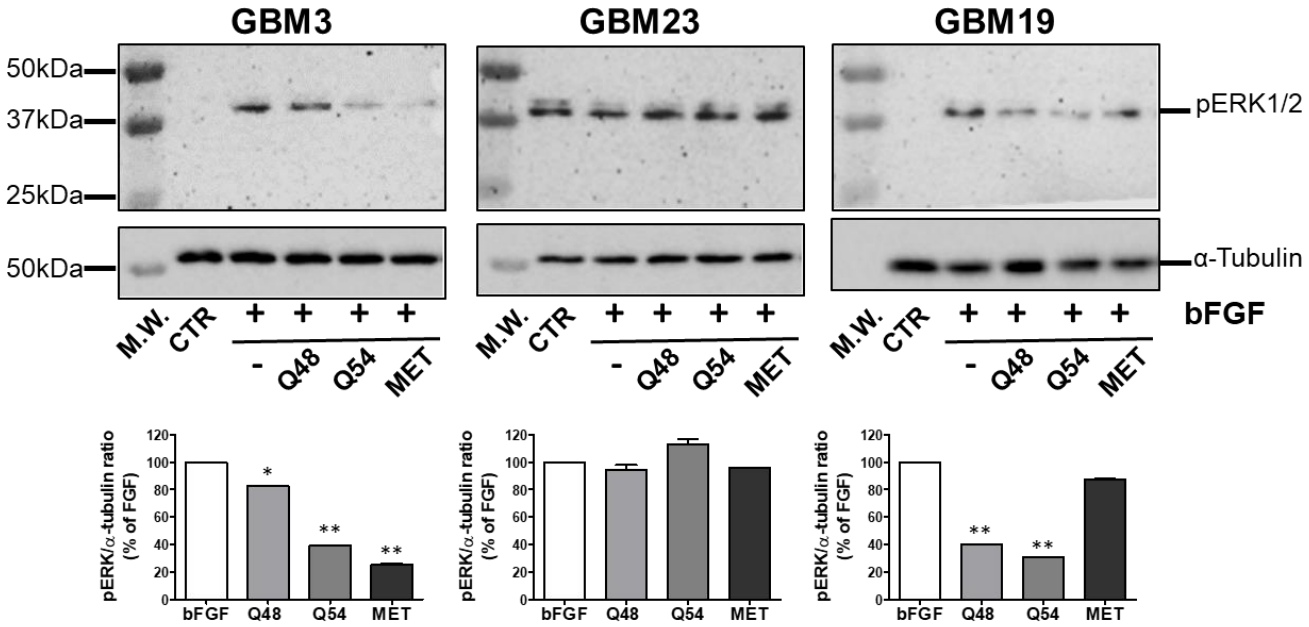
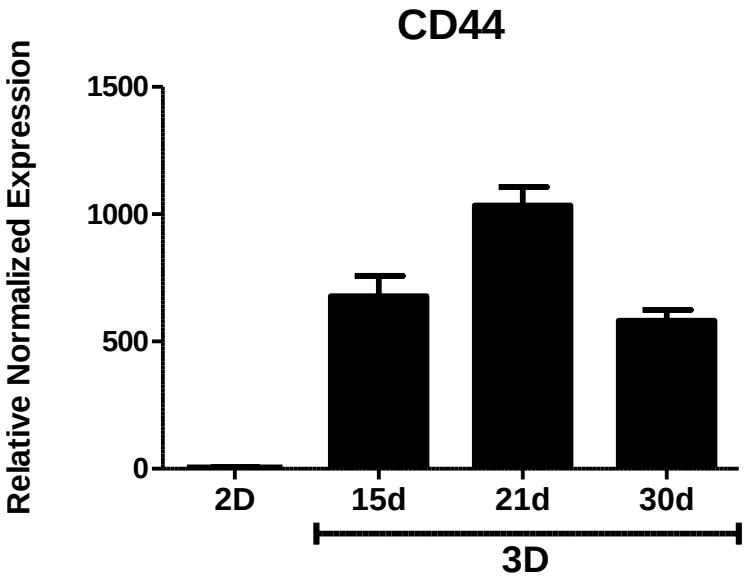



**FIGURE S10**

Upper panels: Western blot depicting phospho-ERK1/2 (pERK) levels in GSC cultures in control conditions (CTR), after 5 min stimulation with bFGF (40ng/ml) and after treatment of bFGF-stimulated cells with with Q48 (100µM), Q54 and Q46 (300µM), and metformin (10mM) for 24h. α-tubulin was used as loading reference. M.W.= molecular weight markers.

Lower panels: densitometric analysis of pERK normalized for α-tubulin levels, * = p< 0.05; ** =p<0.01.

A




B

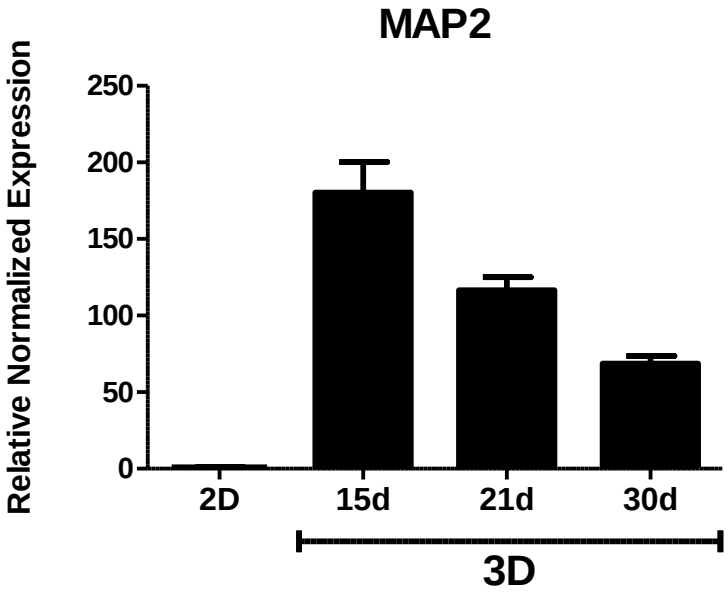



**FIGURE S11**

Comparison of CD44 (A) and MAP2 (B) mRNA expression in GBM3 GSCs cultivated as 2D monolayer or grown as 3D organoids, for 15, 21, and 30 days. Data are obtained by quantitative RT-PCR experiments.

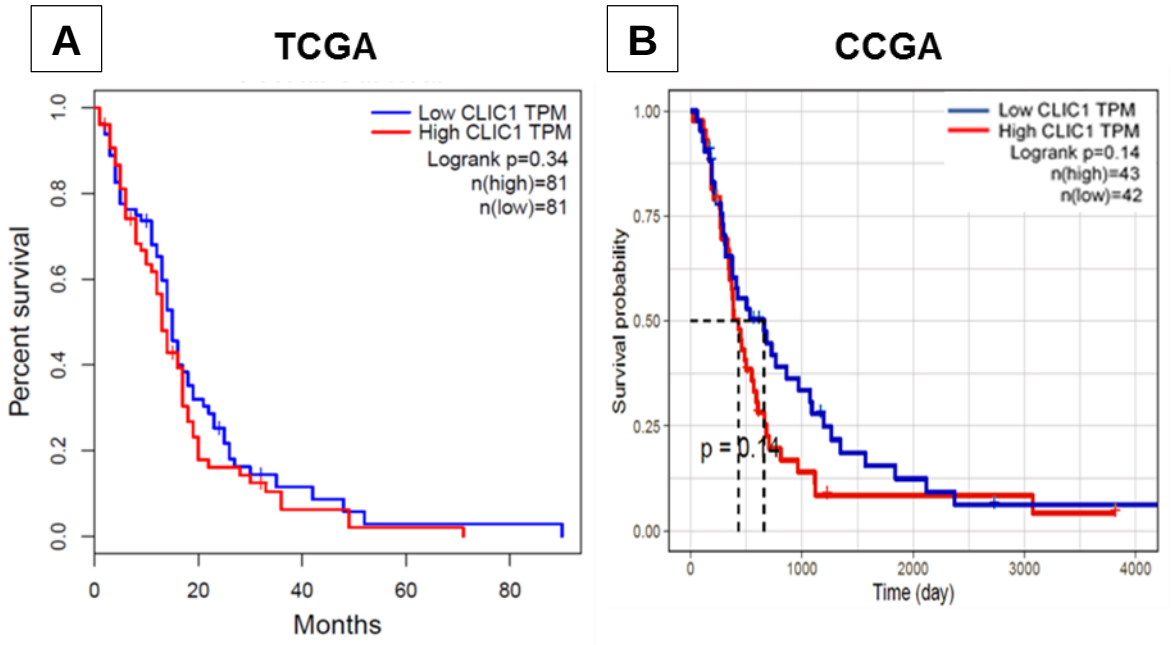



**FIGURE S12**

Kaplan-Meier analysis of the relationship between overall survival and CLIC1 expression. The statistical difference between the curves is measured by log-rank test.

The prognostic effect of CLIC1 mRNA level in GBM according to The Cancer Genome Atlas (TGCA) databasea nalyzed using the GEPIA (Gene Expression Profiling Interactive Analysis database) software (**A**) and Chinese Glioma Genome Atlas (CGCA) **(B**). TPM: transcripts of per million; n(high): samples with expression level higher than the median of TPM; n(low): samples with expression level lower than the median of TPM.

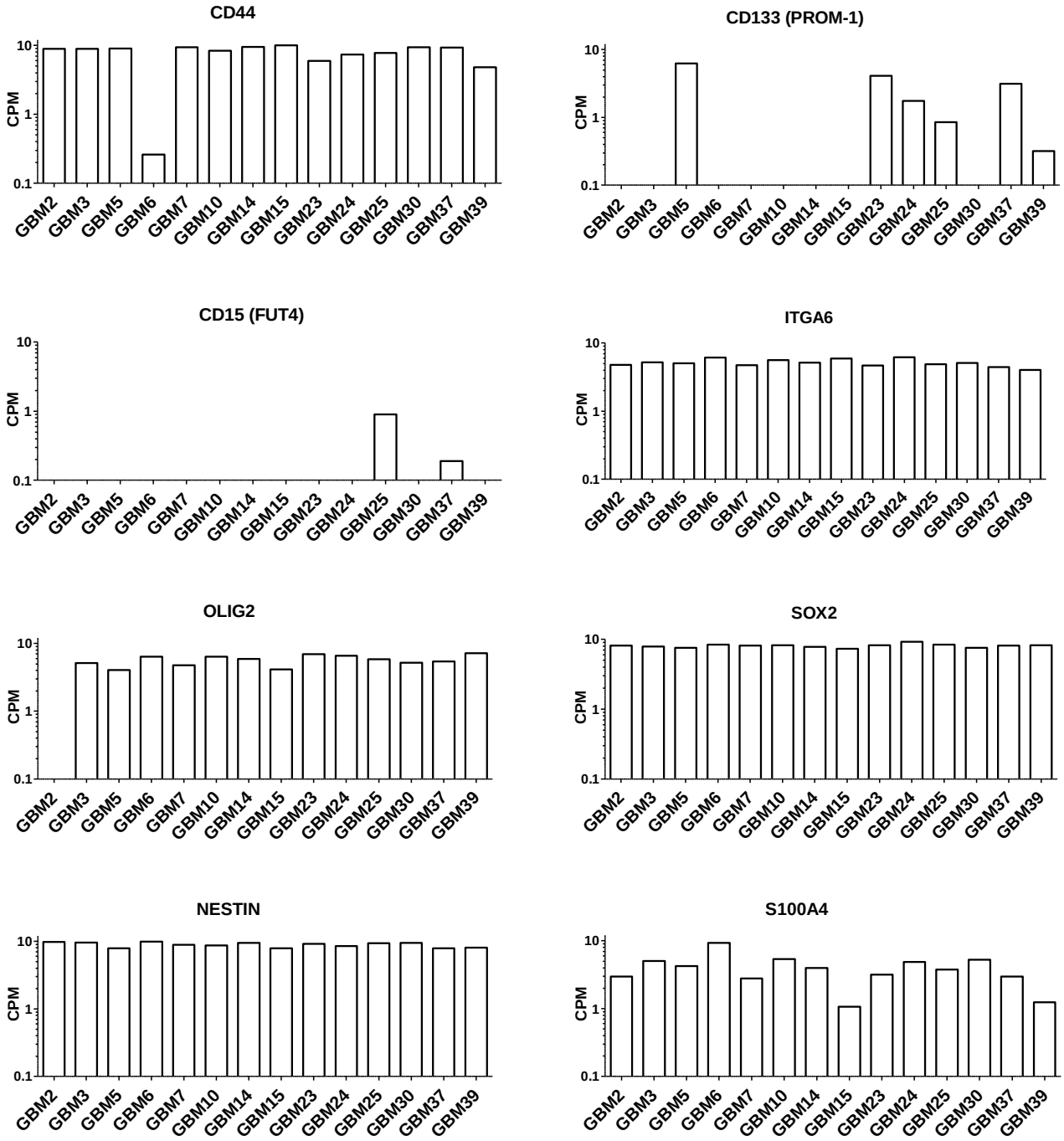



**FIGURE S13**

Expression stem cell-related markers (CD44, CD133, CD15, integrin-α6 ITGA6, Olig2, SOX2, S100A4, and nestin) in GSCs isolated from 14 human GBMs, evaluated by RNA-seq, and expressed as counts per million reads mapped (CPM). RNA-seq have been data deposited at NCBI Geo data set (see Figure S2 for the relative codes). Low CLIC1-expressing cells (GBM39) do not display differences as compared to high expressing GBMs.

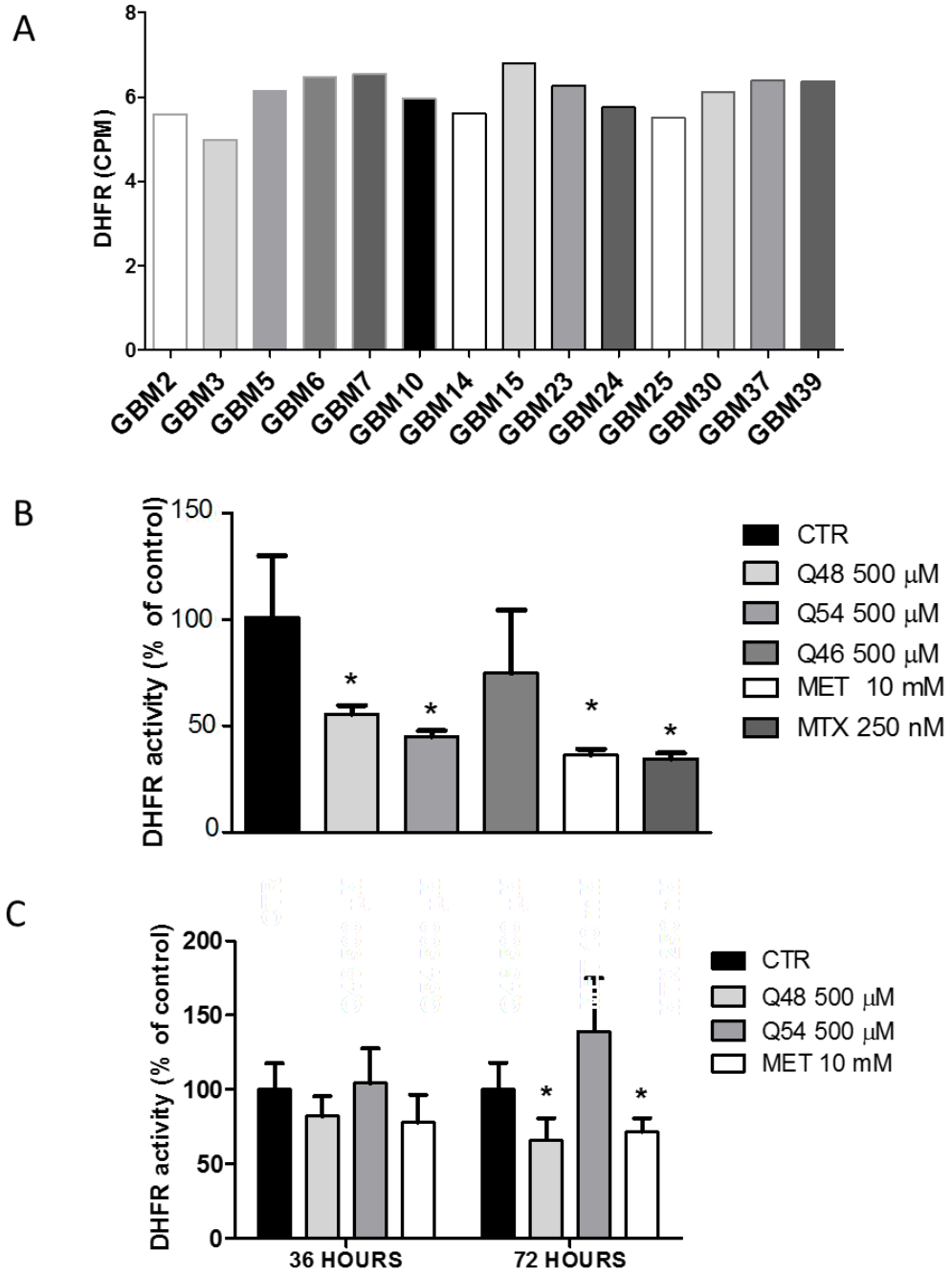



**FIGURE S14**

A) Dihydrofolate reductase (DHFR) expression in 14 human GSC cultures evaluated by RNA-seq, and expressed as counts per million reads mapped (CPM). Comparable expression was detected in cells derived from all the GBM analyzed. RNA-seq have been data deposited at NCBI Geo data set (see Figure S2 for the relative codes).

B) Effect of Q48, Q54, Q46 and metformin (MET) on DHFR activity, incubating the compounds with purified enzyme. Methotrexate (MTX) was used as positive control. Q48, Q54, and MET, but not Q46, inhibited DHFR activity with an efficacy comparable to MTX. * $p < 0.05$.

C) Effect of Q48, Q54, and metformin, on DHFR activity in living cells. Q48 and metformin, but not Q54, caused a moderate inhibition of enzyme activity in a time-dependent manner. * $p < 0.05$.

# SUPPLEMENTARY TABLES

**Table S1:**

**Patients' and tumors' characteristics**

| Code | Sex | Age (yrs) | WHO grade | Molecular subtype | IDH and 1p/19q co-deletional status | NOD/SCID mice survival time (days) |
|---|---|---|---|---|---|---|
| **GBM 3** | M | 48 | IV | Neural | | 120 |
| **GBM 23** | F | 70 | IV (primary multicentric) | Neural | | 100 |
| **GBM 19** | F | 41 | IV (secondary to oligondoglioma) | Mesenchymal | | 100 |
| **GBM 5** | M | 67 | IV (primary) | Neural | | 55 |
| **GBM 10** | F | 70 | IV | | | 180 |
| **GBM 37** | F | 73 | IV | | | 150 |
| **GBM 39** | M | 52 | IV | | | 100 |
| **GBM 44** | F | 69 | IV | | Wt / non-codeleted | n.d. |
| **GBM 50** | M | 71 | III | | Wt / non-codeleted | n.d. |

n. d. = not determined

## Table S2:

## Elemental analysis of biguanide derivatives

| | | Calculated % | | | Found % | | |
|---|---|---|---|---|---|---|---|
| **Compound #** | **Formula** | **C** | **H** | **N** | **C** | **H** | **N** |
| **Q48** | $C_9H_{13}N_5O$ HCl | 44.36 | 5.79 | 28.74 | 44.31 | 5.65 | 28.41 |
| **Q49** | $C_9H_{13}N_5$ HCl | 47.48 | 6.20 | 30.76 | 47.19 | 6.03 | 30.57 |
| **Q50** | $C_9H_{10}F_3N_5$ HCl | 38.12 | 3.94 | 24.86 | 38.03 | 3.88 | 24.71 |
| **Q51** | $C_{11}H_{15}N_5$ HCl | 52.07 | 6.36 | 27.60 | 51.93 | 6.16 | 27.87 |
| **Q52** | $C_{11}H_{14}ClN_5$ HCl | 45.85 | 5.25 | 24.30 | 45.64 | 5.00 | 23.92 |
| **Q53** | $C_{11}H_{14}ClN_5$ HCl | 45.85 | 5.25 | 24.30 | 46.02, | 5.17 | 23.90 |
| **Q54** | $C_{12}H_{17}N_5O$ HCl | 50.79 | 6.39 | 24.68 | 50.36 | 6.23 | 24.45 |

*Q42 (1-(4-chlorophenyl) -biguanide hydrochloride) and Q46 (1-phenylbiguanide hydrochloride) are commercially available and have been obtained by Sigma-Aldrich (purity 97% and 98%, respectively).*

**Table S3:**

**Acidity constant (pKa) and distribution constant (LogD) at pH 7 of the novel biguanide derivatives and reference drugs (metformin and cycloguanil)**

| Compound | pKa* | LogD (pH 7.0)* |
|---|---|---|
| | | |
| ***Metformin*** | *12.27* | *-3.25* |
| **Q46** | 11.19 | -0.68 |
| **Q42** | 11.39 | 0.09 |
| **Q48** | 11.61 | -0.89 |
| **Q49** | 11.67 | -0.28 |
| **Q50** | 11.40 | 0.50 |
| | | |
| ***Cycloguanil*** | *9.27* | *-0.87* |
| **Q51** | 9.77 | -1.64 |
| **Q52** | 8.96 | -0.73 |
| **Q53** | 9.20 | -0.83 |
| **Q54** | 10.4 | -1.81 |

*predicted pKa and LogD values using the Advanced Chemistry Development (ACD/Labs) Software V11.02

**Table S4:**

**Statistical analysis of the effects of novel biguanide derivatives and metformin on GSCs**

| [mM] | Q42 | Q46 | Q48 | Q49 | Q50 | Q51 | Q52 | Q53 | Q54 | MET |
|---|---|---|---|---|---|---|---|---|---|---|
| **0.003** | n.s. | n.s. | n.s. | n.s. | n.s. | n.s. | n.s. | n.s. | n.s. | - |
| **0.01** | n.s. | n.s. | n.s. | n.s. | n.s. | n.s. | n.s. | n.s. | n.s. | - |
| **0.03** | ** | n.s. | *** | n.s. | n.s. | * | n.s. | * | * | - |
| **0.1** | *** | n.s. | *** | n.s. | n.s. | * | n.s. | ** | ** | n.s. |
| **0.3** | *** | n.s. | *** | ** | * | ** | n.s. | *** | *** | n.s. |
| **1** | - | ** | *** | *** | *** | *** | * | *** | *** | n.s. |
| **3** | - | *** | *** | *** | *** | *** | *** | *** | *** | ** |
| **10** | - | - | - | - | - | - | - | - | - | *** |
| **30** | - | - | - | - | - | - | - | - | - | *** |

*p≤0.05; **p≤0.01; ***p≤0.001; n. s. = not significant

(One-way ANOVA, Dunnett's post-test)

**Table S5:**

**Statistical analysis of the effects of novel biguanide derivatives and metformin on differentiated non-stem GBM cells**

| [mM] | Q42 | Q46 | Q48 | Q49 | Q50 | Q51 | Q52 | Q53 | Q54 | MET |
|---|---|---|---|---|---|---|---|---|---|---|
| **0.003** | - | - | n.s. | n.s. | n.s. | n.s. | n.s. | n.s. | n.s. | - |
| **0.01** | n.s. | - | n.s. | n.s. | n.s. | n.s. | n.s. | n.s. | n.s. | - |
| **0.03** | n.s. | n.s. | n.s. | n.s. | n.s. | n.s. | n.s. | n.s. | n.s. | - |
| **0.1** | n.s. | n.s. | n.s. | n.s. | n.s. | n.s. | n.s. | n.s. | n.s. | - |
| **0.3** | * | n.s. | n.s. | n.s. | ** | n.s. | * | n.s. | n.s. | - |
| **1** | * | * | n.s. | ** | *** | * | *** | n.s. | n.s. | n.s. |
| **3** | ** | ** | *** | *** | *** | *** | *** | * | * | n.s. |
| **10** | - | - | - | - | - | - | - | - | - | n.s. |
| **30** | - | - | - | - | - | - | - | - | - | * |

*p≤0.05; **p≤0.01; ***p≤0.001; n. s. = not significant

(One-way ANOVA, Dunnett's post-test)

**Table S6:**

**Statistical analysis of the effects of novel biguanide derivatives and metformin on ucMSCs**

| [mM] | Q42 | Q46 | Q48 | Q49 | Q50 | Q51 | Q54 | MET |
|---|---|---|---|---|---|---|---|---|
| **0.003** | * | n.s. | n.s. | n.s. | n.s. | n.s. | n.s. | - |
| **0.01** | * | n.s. | n.s. | n.s. | n.s. | n.s. | n.s. | - |
| **0.03** | *** | n.s. | n.s. | n.s. | *** | n.s. | n.s. | - |
| **0.1** | *** | n.s. | n.s. | n.s. | *** | n.s. | n.s. | - |
| **0.3** | *** | n.s. | n.s. | n.s. | *** | * | * | n.s. |
| **1** | *** | * | n.s. | ** | *** | ** | *** | n.s. |
| **3** | *** | ** | * | *** | *** | *** | *** | n.s. |
| **10** | - | - | - | - | - | - | - | n.s. |
| **30** | - | - | - | - | - | - | - | ** |

*p≤0.05; **p≤0.01; ***p≤0.001; n. s. = not significant

(One-way ANOVA, Dunnett's post-test)

**Table S7:**

**Statistical analysis of the effects of novel biguanides on low CLIC1-expressing GSCs (GBM39 and GBM44) and the average results from high CLIC1-expressing GSCs (GBM3, 5, 19, 23, and 37).**

| | GBM39 | | | GBM44 | | | High-expressing CLIC1 GSC | | |
|---|---|---|---|---|---|---|---|---|---|
| **[mM]** | **Q48** | **Q54** | **MET** | **Q48** | **Q54** | **MET** | **Q48** | **Q54** | **MET** |
| **0.03** | n.s. | n.s. | - | n.s. | n.s. | - | n.s. | * | - |
| **0.1** | n.s. | n.s. | - | n.s. | n.s. | n.s. | *** | ** | n.s. |
| **0.3** | n.s. | n.s. | n.s. | n.s. | n.s. | n.s. | *** | *** | n.s. |
| **1** | n.s. | n.s. | n.s. | n.s. | n.s. | n.s. | *** | *** | n.s. |
| **3** | n.s. | n.s. | n.s. | n.s. | * | n.s. | *** | *** | ** |
| **10** | - | - | n.s. | - | - | n.s. | - | - | *** |
| **30** | - | - | ** | - | - | ** | - | - | *** |

*p≤0.05; **p≤0.01; ***p≤0.001; n. s. = not significant

(One-way ANOVA, Dunnett's post-test)