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# Inhibition of mitochondrial translation suppresses glioblastoma stem cell growth

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## **SUMMARY**

Glioblastoma stem cells (GSCs) resist current glioblastoma (GBM) therapies. GSCs highly rely on oxidative phosphorylation (OXPHOS), which functioning requires mitochondrial translation. Here, we explore the therapeutic potential of targeting mitochondrial translation and report the results of a high-content screening with putative blockers of mitochondrial ribosomes. We identify the bacterial antibiotic quinupristin/dalfopristin (Q/D) as an effective suppressor of GSC growth. Q/D also decreases the clonogenicity of GSCs *in vitro*, consequently dysregulating cell cycle and inducing apoptosis. Cryo-EM reveals that Q/D binds to the large mitoribosomal subunit, thus inhibiting mitochondrial protein synthesis and functionally dysregulating OXPHOS complexes. These data suggest that targeting mitochondrial translation could be therapeutically explored to suppress GSC growth in GBM and that Q/D could potentially be repurposed for cancer treatment.

## **KEYWORDS**

Glioblastoma, glioblastoma stem cells, OXPHOS, mitoribosome, mitochondrial translation, high content screening, Cryo-EM, quinupristin/dalfopristin, drug repurposing.

## INTRODUCTION

Glioblastoma (GBM) is the most common malignant primary brain tumor in adults. Despite extensive treatment involving surgery, radiotherapy and chemotherapy, the survival of GBM patients remains extremely poor. The search for new effective therapies has proven challenging throughout the last decades, with temozolomide (TMZ) - the only available drug for newly diagnosed GBM - increasing the median patient survival from 12.1 to 14.6 months (Stupp et al., 2005). This is partially due to the presence of glioblastoma stem cells (GSCs), which are responsible for resistance to standard treatments as well as disease recurrence (Auffinger et al., 2014; Chen et al., 2012). Therefore, further research is needed to identify novel effective therapeutics targeting GSCs.

For decades, tumor metabolism was believed to heavily rely on aerobic glycolysis, a phenomenon known as the "Warburg effect" (Warburg et al., 1927), thought to be caused by a decreased or damaged oxidative phosphorylation (OXPHOS) (Warburg, 1956). However, in the past years several studies have demonstrated that mitochondria and OXPHOS play an essential role in tumorigenesis and tumor progression (Porporato et al., 2018). Rho<sup>0</sup> cells, which are devoid of any mitochondrial DNA, undergo a dramatic reduction in tumorigenic potential and tend to acquire the mitochondrial DNA from neighboring normal cells to rescue tumorigenesis (Cavalli et al., 1997; Tan et al., 2015). Furthermore, OXPHOS remains the major source of ATP in many cancer types, even in the presence of enhanced rates of glycolysis (Zu and Guppy, 2004). A growing body of literature reports that OXPHOS plays an important role in addressing energetic demands of cancer stem cells (CSCs) (Škrtić et al., 2011; Viale et al., 2014; Vlashi et al., 2014), including GSCs (Janiszewska et al., 2012; Vlashi et al., 2011). Moreover, chemotherapeutic genotoxic drugs induce a shift in cancer cell metabolism towards upregulated OXPHOS and mitochondrial biogenesis (Haq et al., 2013; Roesch et al., 2013).

The assembly of OXPHOS complexes in the inner mitochondrial membrane is largely orchestrated by mitoribosomes that synthesize thirteen transmembrane proteins. The mitochondrial translation machinery is upregulated in a subset of human tumors (Kim et al., 2017; Sotgia et al., 2012), and breast CSCs show metabolic reliance on mitoribosome synthesized proteins (Vlashi et al., 2014). Since human mitoribosomes, being descendants of bacterial ribosomes, differ from their cytosolic counterparts (Amunts et al., 2015; Petrov et al., 2019), they could be in principle selectively targeted to inhibit energy production. Indeed, mitoribosome sensitivity to antibiotics directed to bacterial ribosomes is a common side effect for these molecules (Zhang et al., 2005) and has been preliminarily exploited to target leukemia cells (Škrtić et al., 2011).

Given the emerging evidence on the GSC addiction to OXPHOS, we systematically investigated whether the inhibition of mitochondrial translation could be exploited to treat GBM by suppressing GSC growth. We performed a high-content screening using a focused, custom-made library of compounds known to affect the bacterial ribosome translation, hypothesizing that they could also act on mitoribosomes given the structural similarity of the ribosomal core. This screening identified an FDA-approved combination of two molecules, quinupristin/dalfopristin (30:70 w/w) (Q/D), as the most effective compound on GSCs. We present mechanistic evidence that Q/D acts by inhibiting mitochondrial translation, leading to dysregulation of OXPHOS and suppression of GSCs growth.

## RESULTS

### A screening of bacterial ribosome targeting antibiotics identifies Q/D as the most effective compound on GSCs

To identify potent compounds effective on GSCs, we performed a focused high-content screening using a custom-made library of potential mitochondrial translation inhibitors (**Figure 1A**). We mined the pharmacology of antibiotics targeting bacterial translation and compiled a custom-made library containing 71 compounds (**Table S1**). The majority of these compounds belong to eight molecular classes (tetracyclines, streptogramins, aminoglycosides, lincosamides, macrolides, amphenicols, oxazolidinones and pleuromutilins), and most of them are FDA-approved for the treatment of bacterial infections in humans. We grew two GSC lines, COMI and VIPI, as adherent cultures on laminin using a method documented to preserve their stemness properties (Pollard et al., 2009). We then showed that both lines indeed depend on OXPHOS by assessing their viability upon treatment with known inhibitors of the respiratory chain complexes or glycolysis (**Figure S1A**). The OXPHOS inhibitors rotenone, antimycin A and oligomycin (which inhibit complex I, complex III, and complex V, respectively) significantly decreased the viability of both lines. In contrast, the glycolysis inhibitors sodium dichloroacetate (DCA) and 2-deoxy-D-glucose (2-DG) did not have a significant effect, in line with a previous report on GSCs (Janiszewska et al., 2012).

We treated COMI and VIPI cells with the aforementioned custom-made library of potential mitochondrial translation inhibitors for 24 h at 100  $\mu$ M and stained them with two dyes: DRAQ5, a DNA dye which allowed us to quantify the number of cells displaying morphological features related to cytotoxicity, and MitoTracker Orange (MTO) dye, which accumulates in healthy mitochondria allowing to observe features related to mitotoxicity. The staining was followed by data acquisition using a high-content imaging system and data mining. As positive controls, we used three well known OXPHOS disruptors at doses and times inducing clear phenotypes in GSCs. Long term treatment (100  $\mu$ M for 24 h) with rotenone, oligomycin A or FCCP were paradigms of late cellular effects of OXPHOS inhibition, whereas an acute treatment with FCCP (100  $\mu$ M for 30 min) accounted for immediate mitochondrial toxicity without effects on cell viability (**Figure S1B**). We phenotypically characterized the cells by extracting six non-redundant morphological features (**Figure S1C** and **Table S2**), which were informative of either the cellular or mitochondrial toxicity, as specified. Next, the informative content of each feature was assessed by testing whether the positive controls behaved as expected (**Figure S2A**). The late cellular effects were described by the cell number, the nucleus area and the nucleus roundness, while the mitochondrial toxicity was described by the area of disrupted mitochondria, the number of MTO-positive spots detected in the cytoplasmic region of cells (mitochondrial spot number), and the area covered by the mitochondrial spots (mitochondrial spot area). We evaluated the effects of each library compound according to these six features (**Figure S2B**). A feature was defined as affected by a given compound when it deviated from the median value of all compounds by a distance larger than 1.5 times the median absolute deviation. In order to identify the most effective classes of drugs, we first calculated a compound score for each compound based on the number of features affected, and then the scores of all compounds in a given class were averaged to obtain the class score (**Figure 1B** and **Table S3**). On the basis of these results, we selected three classes

with the highest class score for further evaluation: streptogramins, pleuromutilins, and tetracyclines.

Streptogramins are a class of antibiotics consisting of a mixture of two structurally different compounds: group A (also called M) streptogramins, which are polyunsaturated macrolactones, and group B (also called S) streptogramins, which are cyclic hexadepsipeptides (Mast and Wohlleben, 2014). Streptogramins A and B are known to act synergistically against bacteria (Di Giambattista et al., 1989), and are used in combination in a fixed 70:30 (w/w) ratio. We, therefore, assayed streptogramins as combinations of group A and B compounds, whereas tetracyclines and pleuromutilins were tested as single agents. We validated a number of compounds from the three hit classes by constructing dose-response curves and calculating the growth inhibition 50 (GI<sub>50</sub>) values. For this purpose, we treated COMI and VIPI cells with a range of drug concentrations for 48 h and assessed viability following staining with the Hoechst 33342 and propidium iodide (PI) DNA dyes (**Figures 1C** and **1D**). This analysis pointed to streptogramins used as a combination of group A and B compounds as the most cytotoxic class on GSCs, and in particular to the combination of quinupristin/virginiamycin M1 (Q/VM1) and quinupristin/dalfopristin (Q/D) as the most potent. Dalfopristin is a semi-synthetic derivative of virginiamycin M1 (**Figure 1E**) and it rapidly hydrolyzes to virginiamycin M1 at pH 7.4 and 37°C (Noeske et al., 2014), which could account for the similar activity of Q/D and Q/VM1. Notably, the Q/D (30:70 w/w) combination is an FDA-approved antibiotic for the treatment of skin infections (Delgado et al., 2000) and is traded as Synercid®. Given the cytotoxicity induced by Q/D and the potential for its repurposing for cancer treatment, we selected this molecule combination for further investigations.

### **Q/D selectively inhibits the growth of GSCs at clinically relevant concentrations**

We assessed the activity of Q/D on a panel of GSCs composed of 21 lines derived from 18 patients with variable clinical features (**Table S4**, cell line characterization in Marziali et al., 2016). The GI<sub>50</sub> values spanned from 2.5 to 32.5 µM after 48 h of treatment, narrowing to a range varying from 1.7 to 12.2 µM after 72 h of treatment (**Figure 2A**). These values are in the range of the maximal blood concentration values achievable in patients treated with Q/D for bacterial infections, which varies between 14 and 32 µM (corresponding to 10.7 and 24.2 µg/mL) upon administration of 12.6 and 29.4 mg/kg, respectively (Bergeron and Montay, 1997). These results emphasize that the inhibitory effect of Q/D is time- and dose-dependent and is exerted at clinically relevant concentrations. We then attempted to correlate the Q/D sensitivity with clinical parameters of the patients from which these GSC lines were derived and with GBM markers, such as the EGFRvIII or PTEN status, but no statistically significant correlations emerged (**Table S5**). This negative result suggests that Q/D affects GSCs irrespective of the patient's clinical and basic molecular profile, although to unequivocally answer this question, a study on a larger panel of lines will have to be conducted.

To assess the selectivity of Q/D for GSCs, we evaluated its cytotoxicity in several normal diploid cells: astrocytes differentiated from three different human fetal neural stem cell lines (CB660, HNPC#13 and U3), a lung fibroblast cell line (MRC5) and a skin fibroblast cell line (Hs68), and compared it to the Q/D effects on a panel of 14 GSC lines. We treated the cells with a range of Q/D concentrations and assessed their viability (**Figure 2B**). The GI<sub>50</sub> values for the human fetal neural stem cell derived astrocytes and the fibroblast cells

ranged from  $68.3 \pm 15.5 \mu\text{M}$  to  $231.6 \pm 30.7 \mu\text{M}$ , which are substantially higher than the  $\text{GI}_{50}$  of the other 14 GSC lines tested.

We then examined the effect of Q/D treatment on the differentiated GSC progeny. To this aim, we exposed three GSC lines to a pro-differentiation environment by culturing them in media deprived of growth factors and supplemented with 10% FBS for 14 days, and then assessed their status by checking the expression of stemness (SOX2 and NESTIN) and differentiation (GFAP) markers by immunofluorescence (**Figure 2C**). COMI and GB7 cells differentiated to a greater extent than VIPI, as evidenced by a higher increase in the expression of GFAP and decrease of SOX2 and NESTIN at the end of the treatment (**Figure 2D**). The  $\text{GI}_{50}$  of the differentiated COMI and GB7 cells were higher than their stem counterpart, while the less differentiated VIPI cells showed a smaller increase in sensitivity to Q/D than their stem counterpart (**Figure 2D**). This suggests that well-differentiated GSCs are less sensitive to Q/D treatment.

Taken together, these results indicate that Q/D selectively inhibits GSCs growth at clinically achievable concentrations.

### **Q/D is active in hypoxic conditions and it is more effective than temozolomide.**

GSCs are known to reside in dedicated tumor niches, i.e. anatomical regions defined by a unique microenvironment, which preserves them in low oxygen and represses their differentiation. Despite their enrichment in these hypoxic niches (Li et al., 2009), as little as 1% oxygen is sufficient for effective OXPHOS in GSCs (Janiszewska et al., 2012). In this context, we evaluated the activity of Q/D on GSCs grown under normoxia and hypoxia (21% and 1% oxygen) by assessing cell viability after Hoechst 33342 and PI staining (**Figure 2E**). At 1%  $\text{O}_2$ , the viability of untreated cells was not affected and the cells still responded to Q/D treatment in a dose-dependent manner, supporting Q/D efficacy on GSCs in hypoxic conditions.

The only chemotherapeutic drug used in the clinic to treat newly diagnosed GBM is TMZ, a pro-drug that is spontaneously converted to the alkylating agent 5-(3-methyltriazen-1-yl)imidazole-4-carboxamide at physiological pH (Friedman et al., 2000). The most widely studied mechanism of resistance to TMZ in GBM is the expression of the  $\text{O}^6$ -methylguanine DNA-methyltransferase (MGMT), a DNA repair protein able to remove methyl adducts from  $\text{O}^6$ -methylguanine and repair DNA damage. Since GSCs tend to be resistant to TMZ (Chen et al., 2012), we compared the cytotoxicity of Q/D with that of TMZ in COMI and VIPI cells (**Figure 2F**). In COMI cells, where the MGMT gene is epigenetically silenced by promoter methylation, the  $\text{GI}_{50}$  value for Q/D was  $6.5 \pm 1.1 \mu\text{M}$  while that for TMZ was  $96.5 \pm 15.2 \mu\text{M}$ ; in VIPI cells, where, instead, the MGMT promoter is unmethylated, the  $\text{GI}_{50}$  value for Q/D was  $20.2 \pm 1.4 \mu\text{M}$  while that for TMZ was  $337.7 \pm 35.5 \mu\text{M}$ . These experiments demonstrate that Q/D is 15 times more effective than TMZ in terms of growth inhibition of GSCs, independently from MGMT promoter methylation status. Therefore, Q/D is equally able to affect GSCs in normoxia and hypoxia and is over an order of magnitude more potent than TMZ *in vitro*.

## **Q/D decreases clonogenicity, blocks cell cycle progression and promotes apoptosis**

We next investigated the extent of growth inhibition induced by Q/D in GSCs grown as gliomaspheres. Ten COMI cells were seeded per well in media with various Q/D concentrations and the area of gliomaspheres was measured over the course of nine days (**Figure 3A** and **3B**). We observed that the 1  $\mu\text{M}$  Q/D treatment only slightly inhibited gliosphere formation, but that the 5 and 10  $\mu\text{M}$  treatments, concentrations achievable in patients' blood, nearly completely abolished gliosphere formation.

Given the inhibitory growth effect of Q/D on GSCs, we wondered whether this drug could impact GSC self-renewal and clonogenic maintenance. To assess the effects of Q/D on GSC clonogenic potential, we measured the gliosphere forming ability of COMI cells after Q/D treatment. COMI cells were grown in suspension with several concentrations of Q/D for 72 h, then the gliospheres were dissociated and seeded at a density of 10 or 100 cells per well in the absence of the drug. After 10 days the clonogenic potential was evaluated by counting the number of gliospheres reformed (**Figure 3C** and **3D**). Q/D decreased the gliosphere forming ability in a dose-dependent manner, confirming a substantial impact on GSC maintenance.

We then investigated the functional effects induced on the GSC cell cycle by performing EdU-PI staining upon treatment with Q/D, and by measuring the percentage of cells in each phase (**Figure 3E** and **3F**). Increasing concentrations of Q/D led to a marked dose-dependent decrease in the number of cells in the S-phase, indicating inhibition of proliferation. In addition, we observed a significant increase in the number of cells in the G0/G1 phase at 5  $\mu\text{M}$  Q/D treatment, indicating an accumulation of cells in this phase, followed by an increase in the number of cells in the G2/M starting at 5  $\mu\text{M}$  and culminating at 10  $\mu\text{M}$  Q/D treatments (**Figure 3F**).

As Q/D disrupts GSC maintenance and dysregulates the cell cycle, we next investigated whether Q/D induces apoptosis. We performed a flow cytometric analysis using Annexin V and PI staining and estimated the percentage of apoptotic cells by calculating the percentage of Annexin V positive cells (**Figure 3G** and **3H**). The treatment with 6.5 and 10  $\mu\text{M}$  Q/D led to an increase in the percentage of apoptotic cells, even though the effect was significant only at 10  $\mu\text{M}$ .

Since autophagy can be induced upon drug treatment in GSCs (Angeletti et al., 2016), we investigated its induction upon Q/D treatment. We treated cells with Q/D alone or in combination with autophagy inhibitors to investigate the autophagic flux, and performed immunofluorescence and immunoblotting experiments using an anti-LC3 antibody, which suggested that Q/D in fact causes an increase in the autophagic flux (**Figure S3A-D**). We next investigated whether this increase could have a pro-death or pro-survival function by simultaneously treating GSCs with Q/D and the autophagy inhibitor chloroquine. A small but significant decrease in the viability was observed upon the combination treatment (**Figure S3E**), indicating that autophagy is a pro-survival mechanism. These data are in line with those reported for other molecules such as the pan-histone deacetylase inhibitor givinostat (Angeletti et al., 2016).

Overall, this phenotypic analysis of GSCs treated with Q/D reveals a loss of clonogenic potential due to cell cycle arrest accompanied by apoptosis.

## Q/D inhibits mitochondrial translation and induces OXPHOS dysregulation

To investigate the molecular mechanism of action of Q/D, we determined the cryo-EM structure of the human mitoribosome from HEK293 cells treated with Q/D (**Figure S4** and **Table S6**). To ensure that the cryo-EM experiment reflects physiological conditions, Q/D was not added to any of the purification steps upon cell lysis (see Methods). The overall resolution of the reconstruction was 3.9 Å, and the local resolution of ~3.4 Å allowed identification of both molecules in the large ribosomal subunit - dalfopristin in the cryo-EM density at the peptidyl-transferase center (PTC) and quinupristin at the entrance to the exit tunnel for nascent polypeptides (**Figure 4A**). The density for dalfopristin was of comparable quality to the previously reported X-ray crystal structures of the bacterial counterparts (Harms et al., 2004; Noeske et al., 2014), which was sufficient to detect that the compound was hydrolyzed with the loss of the diethylaminoethylsulfonyl group to form virginiamycin M1 (**Figure 4B**), an effect reported also in the pharmacology studies on Q/D (Delgado et al., 2000). By comparing the Q/D bound structure to the previously published unbound state of the human mitoribosome (Amunts et al., 2015), we identified no protein conformational changes, and only a number of rRNA rearrangements induced by Q/D binding. The most prominent is the 90 degrees shift of A2725 (A2062 in *E. coli*) (**Figure 4C**). Other residues affected are U2993 (U2506 in *E. coli*) and U3072 (U2585 in *E. coli*), while the latter was not well resolved in the unbound structure (EMD2876), probably due to inherent flexibility. A comparison with the *E. coli* and *D. radiodurans* ribosome structures with Q/D (Harms et al., 2004; Noeske et al., 2014) shows that the bacterial counterpart of A2725 (A2062 in *E. coli*) adopts a similar shift, whereas the equivalent of U3072 (U2585 in *E. coli*) is displaced by nearly 180 degrees, which was not observed in the mitoribosome due to the absence of the diethylaminoethylsulfonyl group of dalfopristin. In addition, we observed a shift in the mitoribosomal U2993 (U2506 in *E. coli*) that was not observed in the bacterial counterpart. Since Q/D was added directly to the cells, the data suggest that Q/D is able to pass the mitochondrial membranes and bind to the mitoribosome.

We next evaluated the effects induced by Q/D on mitochondrial translation and OXPHOS functionality. To determine whether Q/D specifically affects mitoribosomal function, we assayed for *de novo* synthesized proteins by mitochondrial or cytosolic ribosomes. To this end, we conducted metabolic labeling with <sup>35</sup>S-methionine on COMI cells treated with Q/D for 24 h (**Figure 5A**). Q/D was very effective in inhibiting mitochondrial translation and nearly completely abolished it at 0.5 μM. Importantly, no effects on cytosolic translation were noted at this or even higher Q/D concentrations (up to 2.6 μM tested). We further confirmed the effects on proteins synthesized in the mitochondria and cytosol by performing immunoblotting and immunofluorescence analysis on COMI and VIPI cells treated with Q/D for 48 h (**Figure 5B** and **Figure S5A** and **S5B**). Cytochrome c oxidase subunit I (COX1) is synthesized in the mitochondria, whereas cytochrome c oxidase subunit IV (COX4), succinate dehydrogenase complex flavoprotein subunit A (SDHA) and β-tubulin are translated in the cytosol. The expression of COX1 was markedly decreased upon treatment with Q/D, whereas the expression of COX4, SDHA and β-tubulin remained unchanged. The decrease in COX1 protein levels upon Q/D treatment was confirmed also at 1% O<sub>2</sub> (**Figure S5C**), suggesting that at 1% O<sub>2</sub> Q/D behaves in the same manner as at 21%. We then assessed the effects of Q/D on mRNAs encoding COX1 and COX4 but we did not observe any significant changes (**Figure 5C**). Therefore, Q/D acts specifically on mitochondrial translation.

Since the thirteen proteins synthesized by mitoribosomes are an essential part of the OXPHOS complexes, we tested the effects of Q/D on the functionality of these complexes by performing blue native polyacrylamide gel electrophoresis (BN-PAGE) followed by in-gel activity assays on COM1 and VIPI cells. The activity of complex I, IV and V, which are composed of proteins both mitochondrial and cytosolic in origin, was decreased upon Q/D treatment, whereas the activity of complex II, which is composed entirely of nuclearly encoded proteins, was unaffected (**Figure 5D**). In parallel, we assessed the amount of these complexes by BN-PAGE followed by immunoblotting, and we consistently found that the levels of complex I, IV and V, but not those of complex II, were decreased (**Figure S5D**).

To verify whether the altered stoichiometry of the OXPHOS complexes led to a decreased mitochondrial respiratory capacity upon treatment with Q/D, we tested mitochondrial respiration using high-resolution respirometry. For both COM1 and VIPI cells the functionality of complex I and complex II was decreased upon 1  $\mu\text{M}$  Q/D treatment, even if this did not have an apparent impact on the basal respiration capacity (R). When we measured the maximal oxygen consumption (ETS) through injection of the FCCP uncoupler, we found that it was decreased, indicating a substantial loss of reserve respiratory capacity (**Figure 5E**).

Since functional OXPHOS is necessary to maintain the mitochondrial membrane potential (MMP), we investigated whether Q/D induced a consequent loss in the MMP. We used JC-1 dye to stain cells treated with Q/D at various concentrations and analyzed them by flow cytometry. In this experimental setting mitochondrial depolarization is indicated by a shift from red to green fluorescence. We determined the percentage of cells with normal MMP (having high red fluorescence and low green fluorescence), as well as the percentage of cells with lower MMP (having low red fluorescence and high green fluorescence), and subsequently quantified these changes (**Figure 5F**). We observed little changes upon 2.5  $\mu\text{M}$  Q/D treatment, while from 5  $\mu\text{M}$  onwards the percentage of cells with disrupted MMP increased in a dose-dependent manner, suggesting that indeed Q/D affects the MMP.

Another process by which cells can fulfill their ATP requirements is glycolysis, and switching to glycolysis is a potential mechanism of bioenergetic flexibility for OXPHOS targeting drugs in cancer cells. To verify whether upon treatment with Q/D GSCs switch to glycolysis, we measured the levels of L-lactate, a product of the glycolytic pathway. We observed a small but significant increase in L-lactate production starting from 1  $\mu\text{M}$  Q/D treatment for both COM1 and VIPI cells (**Figure 5G**), indicating a tendency to glycolytic switch.

Finally, we checked the correlation of the relative quantity of mitochondrial mass with the Q/D treatment response in GSC lines. We assessed eight GSC lines by treating them with different concentrations of Q/D for 48 h (**Figure S5E**). The data show that the viability after Q/D treatment is negatively correlated to the mitochondrial mass at 2.5  $\mu\text{M}$  ( $r=-0.882$ ,  $p<0.001$ ), 5  $\mu\text{M}$  ( $r=-0.758$ ,  $p<0.05$ ) and 10  $\mu\text{M}$  treatment ( $r=-0.682$ ,  $p=0.06$ ), indicating that GSCs with more mitochondria are more susceptible to Q/D-induced death.

Overall, these results clearly demonstrate that Q/D acts by selectively interfering with mitochondrial translation and consequently dysregulating OXPHOS in GSCs.

## **Genetic inhibition of mitochondrial translation suppresses GSC growth, recapitulating Q/D effects.**

To further assess if mitochondrial translation is the target of Q/D activity on GSCs, we investigated whether genetic strategies would lead to a similar effect as Q/D treatment. To address this question, we used CRISPR/Cas9 to knockout genes involved in mitochondrial translation. We first generated Cas9 expressing COMI and VIPI cells, and then transduced them with lentiviral vectors bearing sgRNAs specific for TUFM or MRPS18A and expressing GFP. A non-targeting sequence (sgNT) was used as a negative control. TUFM is a mitochondrial translation elongation factor, which shRNA-mediated knockdown was shown to inhibit mitochondrial translation in acute myeloid leukemia cells (Škrtić et al., 2011). MRPS18A is a mitoribosome small subunit protein that has a zinc-binding function and which was shown to be upregulated in breast cancer (Sørensen et al., 2017).

We evaluated the effects of two sgRNAs targeting each gene on cell growth using a competition assay (**Figure 6A**). Both guides showed a similar inhibitory effect on cell growth and we chose one guide for further validation (sgTUFM\_1 and sgMRPS18A\_1). We verified the efficiency of the Cas9 cutting by sequencing the target site (**Figure 6B**) and by performing immunoblotting (**Figure 6C**). To confirm that TUFM and MRPS18A knockout resulted in mitochondrial translation inhibition, we measured the expression of COX1 and COX4 proteins. The expression of COX1 was markedly decreased, whereas the expression of COX4, and  $\beta$ -tubulin remained unchanged (**Figure 6D**).

Next, we evaluated the effects of TUFM and MRPS18A knockout on GSCs grown as gliomaspheres by measuring the area of the sphere formed over the course of fifteen days. We observed that genetic inhibition of mitochondrial translation nearly completely abolished gliosphere formation (**Figure 6E** and **Figure 6F**). The same effect was observed also on COMI and VIPI cells grown as adherent cultures (**Figure 6G**). To assess the impact of genetic inhibition of mitochondrial translation on GSC self-renewal and clonogenic maintenance, we measured the gliosphere forming ability of TUFM and MRPS18A knockout COMI and VIPI cells. COMI and VIPI were seeded at two different cell densities and the clonogenic potential was evaluated by counting the number of gliospheres formed after 10-15 days (**Figure 6H**). TUFM and MRPS18A knockout substantially decreased the gliosphere forming ability, confirming a strong impact on GSC maintenance.

These results show that genetic inhibition of mitochondrial translation almost completely suppresses GSC proliferation, strongly impacting GSC self-renewal and clonogenic potential maintenance, recapitulating the effects observed upon Q/D treatment.

## DISCUSSION

OXPHOS targeting leads to destabilization of energy homeostasis in CSCs, providing a possible target in oncology (Weinberg and Chandel, 2015). At least fourteen inhibitors of one of the OXPHOS complexes have been evaluated *in vivo* or in the clinic, while at least six are in preclinical testing (Ashton et al., 2018). The anti-diabetic drug metformin was shown to reversibly inhibit complex I (Wheaton et al., 2014), resulting in cytotoxic effects in different CSC types, including GSCs (Würth et al., 2013). Metformin is currently in more than three hundred ongoing clinical trials in combination with standard treatments (Weinberg and Chandel, 2015). In GBM high OXPHOS levels measured by complex IV activity are an independent negative prognostic factor (Griguer et al., 2013), suggesting that molecules able to affect OXPHOS activity might represent an interesting new therapeutic strategy also for this tumor type. Two different small molecules have been demonstrated to efficiently target complex IV function in GSCs both *in vitro* and *in vivo* (Oliva et al., 2016, 2017). Recently, IACS-010759 and Gboxin, two inhibitors of complex I and complex V, respectively, have been shown to inhibit proliferation of GSCs *in vitro* and *in vivo* (Molina et al., 2018; Shi et al., 2019).

Here, we adopted a strategy of OXPHOS targeting by selectively suppressing its assembly. In detail, we hypothesized that mitochondrial translation inhibition would hamper the formation and functionality of the complexes I, III, IV and V, leading to pronounced detrimental effects on cell viability. By performing a high-content imaging-driven screening on a library encompassing all known bacterial ribosome-targeting antibiotic classes, we identified streptogramins quinupristin/dalfopristin (Q/D) as the most potent combination leading to significant growth inhibition of GSCs. We showed by cryo-EM that Q/D added to actively dividing cells binds to the mitoribosomal large subunit stalling polypeptide synthesis. This leads to inhibition of mitochondrial translation, which in turn dysregulates OXPHOS leading to loss of clonogenic potential, cell cycle arrest and death by apoptosis. Our data is consistent with the effect of other ribosome targeting antibiotics, which have been shown to decrease tumor-sphere formation in cell lines from eight different tumor types (Lamb et al., 2015). Among them, tigecycline, a tetracycline antibiotic, has been reported to be cytotoxic for acute myeloid leukemia stem cells (Kuntz et al., 2017; Ravà et al., 2018; Škrtić et al., 2011). Therefore, our results add to the increasing evidence that targeting OXPHOS, and in particular mitochondrial translation, could be clinically exploitable in cancer therapy.

Q/D was approved by the FDA for the treatment of persistent bacterial infections and could be repurposed for other uses. The suitability of Q/D repurposing in GBM is supported by the fact that the range of GI<sub>50</sub> values obtained *in vitro* matches blood concentration values achievable in patients treated with Q/D for bacterial infections (Bergeron and Montay, 1997), however, further *in vivo* studies will be necessary to determine whether such concentrations are sufficient to exert desired effects on tumors. We further explored the possibility of repurposing Q/D for GBM by demonstrating that Q/D preferentially targets GSCs rather than astrocytes or primary fibroblasts, suggesting a suitable therapeutic window. *In vitro* GSCs are more sensitive to Q/D compared with their differentiated progeny, revealing a preferential GSC targeting for Q/D, however, further experiments will have to be conducted to understand the molecular basis of the preferential targeting of GSC. In addition, a pre-clinical mouse model will have to be established to demonstrate Q/D effectiveness *in vivo*. GBM tumors are largely hypoxic, and GSCs were identified in both GBM perivascular areas and hypoxic regions (Calabrese et al., 2007; Li et al., 2009). Importantly for GBM therapy, Q/D

can exert its cytotoxic effects and inhibit mitochondrial translation under hypoxic conditions. Moreover, since drugs targeting the OXPHOS complexes alleviate or even eradicate tumor hypoxia (Ashton et al., 2018), Q/D could exert an important, indirect antitumor effect in GBMs by promoting oxygenation and downregulating neoangiogenesis. Further experiments are needed to investigate this possibility.

Similarly to any newly proposed anticancer drug, a major issue for favoring the transfer of Q/D to the clinical setting is the possibility to stratify patients. We proved that Q/D decreased cell viability of GSCs grown both as adherent cultures and as gliomaspheres. We observed the effect on a large panel of GSCs, and did not find any correlation between the sensitivity and the molecular features of the cells, or the variable clinical features of the patients from whom these lines were established. This suggests that Q/D could be extensively used on GBM patients, even though this data needs to be verified on a larger panel of lines to account for tumor variability. Some CSCs, including GSCs (Vlashi et al., 2011), present a certain degree of metabolic flexibility after inhibition of either glycolysis or OXPHOS, upregulating one of the two metabolic pathways in order to compensate for the inhibition of the other. GSCs from GBM patients bearing a homozygous deletion of the key glycolytic enzyme enolase 1 (ENO1) (3.3% of the cases) present a reduced capacity for compensatory glycolysis and thus an increased sensitivity to the complex I inhibitor IACS-010759 (Molina et al., 2018). Other molecular alterations can produce a similar effect, for example, the FGFR3-TACC3 gene fusion (3% of GBM cases) confers special sensitivity to OXPHOS inhibitors by activating mitochondrial metabolism (Frattoni et al., 2018). Very recently, a mitochondrial GBM subtype was identified using a computational approach for unbiased characterization of core biological features from single cells and bulk tumor transcriptome analysis (Garofano et al., 2021). This GBM subtype was shown to rely exclusively on OXPHOS for energy production and to exhibit a marked vulnerability to OXPHOS inhibitors. Therefore, despite the fact that we did not observe a marked variability in the GI<sub>50</sub> spectrum in GSCs from 18 different patients, a search for more responsive patients to Q/D based on genome features is feasible, exploiting single genotypes as ENO1 loss-of-function or the FGFR3-TACC3 translocation, or more complex molecular signatures able to predict either high reliance on OXPHOS or glycolysis impairment.

Overall, we describe the identification and detailed characterization of Q/D action. Q/D selectively inhibits mitochondrial translation and consequently dysregulates OXPHOS. Notably, Q/D is an FDA-approved drug and our study sets the stage for a potential repurposing effort of Q/D for GBM treatment.

## **ACKNOWLEDGEMENTS**

COMI and VIPI cells were a kind gift from Antonio Daga (Azienda Ospedaliera Universitaria San Martino di Genova, Italy), and 030616 from Rossella Galli (H. S. Raffaele, Milan, Italy). Human GSC lines GB6, GB7, GB8, G144, G166 and the human fetal neural stem cell line CB660 and U3 were kind gifts from Luciano Conti (Department CIBIO, University of Trento). We thank the HTS, the CASF, the CT and the Imaging Core Facilities of CIBIO for their assistance with experimentation. We thank Camilla Maffezzini at the Karolinska Institute for the help with BN-PAGE and in-gel activity assay. The cryo-EM data was collected at the Swedish National Facility funded by the Knut and Alice Wallenberg, Family Erling Persson and Kempe Foundations. We thank M. Carroni, J.M. de la Rosa Trevin and S. Fleischmann for the smooth running of the data collection and processing. This work was supported by

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## AUTHOR CONTRIBUTIONS

Conceptualization, A.M. and A.Q.; Methodology, D.S., A.M.; Formal analysis, A.R.; Investigation, D.S., M.N., S.A., G.R., V. A., M.G., A.S., C.A., F.B, E.F.R., M.B., Q.D., S.G., S.P., R.P., A.A. and A.M.; Writing - Original Draft, A.M. Writing – Review & Editing, A.M., D.S. and A.Q.; Funding Acquisition, A.M., A.Q. and A.A.; Resources, A.Q., L.R-V., R.P., J.R., A.A., S.R. and I.M.; Supervision, A.M. and A.Q.

## DECLARATION OF INTERESTS

D.S., I.M., A.M. and A.Q. have applied for protection of intellectual property related to the results in the manuscript and to Q/D analogues in cancer therapy (Italian patent number 102020000012601). The authors have no additional conflict of interests.

## MAIN FIGURE TITLES AND LEGENDS

**Figure 1. Q/D is the most effective prospective mitochondrial translation inhibitor in GSCs.** (A) An outline of the screening workflow. Each treatment was performed in a technical triplicate. (B) Identification of hit classes based on class score calculation. (C) Representative dose-response curves for all tested compounds belonging to the three hit classes validated on COMI and VIPI cells, n=4 technical replicates, mean  $\pm$  SD. (D) The GI<sub>50</sub> values of the tested compounds. (E) The chemical structures of the lead compound quinupristin/dalfopristin (30:70 w/w) and virginiamycin M1, the product of dalfopristin hydrolysis. See also [Figures S1](#), and [S2](#) and [Tables S1](#), [S2](#) and [S3](#).

**Figure 2. Q/D selectively inhibits the growth of GSCs at clinically relevant concentrations, it is effective in hypoxic conditions and it is more potent than TMZ.** (A) GI<sub>50</sub> values of a panel of 21 GSC lines derived from 18 tumor samples at 48 and 72 h post-Q/D treatment, n=4 technical replicates. (B) Q/D GI<sub>50</sub> values for 14 GSC lines compared to Q/D GI<sub>50</sub> values for astrocytes derived from human fetal neural stem cells (CB660, HNPC#13 and U3), for human lung fibroblasts (MRC5), and for human skin fibroblast (Hs68), n=3 biological replicates, mean  $\pm$  SD. (C) Representative immunofluorescence images for SOX2, NESTIN and GFAP staining on COMI, GB7 and VIPI cells grown in stemness (Stem) and differentiation (Diff) conditions. Scale bar 100  $\mu$ m. (D) Quantification of fluorescence intensity of SOX2, NESTIN and GFAP immunostaining (*left*) and Q/D GI<sub>50</sub> values for COMI, GB7 and VIPI cells grown in stemness and differentiation conditions (*right*). For immunostaining quantification n=6000 objects for stem cells and n=1000 objects for differentiated

cells, mean,  $\pm$  SEM. Differentiated cell values were normalized to those of stem cells (dashed line). The GI<sub>50</sub> values were calculated using n=4-7 biological replicates, mean  $\pm$  SD. (E) Viability of COMI and VIPI cells grown in normal and hypoxic conditions upon different doses of Q/D, n=4 technical replicates, mean  $\pm$  SD. Representative results of n=3 biological replicates. (F) Representative dose-response curves to Q/D and temozolomide (TMZ) for COMI and VIPI cells, n=3 biological replicates, mean  $\pm$  SD. See also [Tables S4](#) and [S5](#).

**Figure 3. Q/D decreases clonogenicity, dysregulates cell cycle and promotes apoptosis.** (A) Effects of Q/D treatment on COMI cells grown in suspension. Example images from days 0, 4 and 9, scale bar 100  $\mu$ m. (B) Sphere area measured over the course of the nine-day experiment. The data were normalized to day 0. n=30 technical replicates, mean  $\pm$  SEM. One representative experiment is shown, n=3 biological replicates. (C) Representative images of the gliomasphere formation assay, scale bar 1000  $\mu$ m. (D) Quantification of the number of spheres greater than 100  $\mu$ m, n=20 technical replicates, mean  $\pm$  SEM. \*\*\*p < 0.001, unpaired two-tailed t-test. One representative result is shown, n=3 biological replicates. (E) Representative FACS analysis of the effects of Q/D on the cell cycle in COMI cells assayed using EdU incorporation and PI staining. (F) Quantification of the percentage of cells in each cell cycle phase, n=3 biological replicates, mean  $\pm$  SD. \*p < 0.05 \*\*p < 0.01 \*\*\*p < 0.001, unpaired two-tailed t-test. (G) Representative FACS analysis of apoptosis upon treatment with Q/D in COMI cells as evaluated by Annexin V and PI staining. (H) Quantification of the percentage of Annexin V positive cells, n=6 biological replicates, mean  $\pm$ SD. \*\*p < 0.01, unpaired two-tailed t-test. See also [Figure S3](#).

**Figure 4. Cryo-EM of the mitoribosome from Q/D treated cells.** (A) A model of the mitoribosomal large subunit with quinupristin and virginiamycin M1 (the hydrolysis product of dalfopristin). The two compounds are found in the entrance to the exit tunnel and the peptidyl transferase center (PTC). (B) View of the cryo-EM density at overall resolution of 3.9 Å around quinupristin (purple) and virginiamycin M1 (green). The two compounds make interactions with the surrounding rRNA. (C) Comparison between the human mitoribosomal RNA when bound to quinupristin and virginiamycin M1 (black) and when unbound (PDB ID: 3J9M, light gray). Red arrows indicate the rRNA movement to accommodate the molecules. See also [Figure S4](#) and [Table S6](#).

**Figure 5. Q/D inhibits mitochondrial translation and negatively affects OXPHOS functionality.** (A) <sup>35</sup>S metabolic labeling assay on mitochondrial (*left*) and cytosolic (*right*) translation on COMI cells after 24 h treatment with Q/D. One representative result is shown, n=3 biological replicates. (B) Effects of increasing concentrations of Q/D on COX1, COX4, SDHA and  $\beta$ -tubulin proteins in COMI and VIPI cells after 48 h treatment as assayed by immunoblotting. One representative result is shown, n=2 biological replicates. (C) Effects of Q/D on COX1 and COX4 mRNA levels on COMI and VIPI cells after 48 h treatment assessed by RT-qPCR. Data presented as fold change over control. n=4-5 biological replicates, mean  $\pm$  SD. Unpaired two-tailed t-test. (D) Effects of Q/D on the functionality of OXPHOS complexes as assessed using BN-PAGE and in-gel activity assay on COMI and VIPI cells after 48, 72 and 96 h of drug treatment. Coomassie staining serves as the loading control. Representative results of n=2 biological replicates. (E) Oxygen consumption of COMI and VIPI cells upon treatment with Q/D for 48 h as measured using Oxygraph-2k. Cells were evaluated for routine (R), complex I (CI), complex I and II (CI&II), uncoupled (ETS) and complex II (CII) respiration. Representative results of n=3 biological replicates. (F) Quantification of the changes in the mitochondrial membrane potential (MMP) as assessed by JC-1 staining in COMI cells. FCCP treatment was used as a positive control, n=4 biological replicates, mean  $\pm$  SD. \*p < 0.05, unpaired two-tailed t-test as compared to non-treated. (G) Effects of increasing concentrations of Q/D on L-lactate production in COMI and VIPI cells after 48 h of Q/D treatment. The L-lactate levels were normalized to the number of cells, n=3 technical replicates, mean  $\pm$  SD. \*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001, unpaired two-tailed t-test. Representative results of n=3 biological replicates. See also [Figure S5](#).

**Figure 6. Genetic inhibition of mitochondrial translation suppresses GSC growth, recapitulating Q/D effects.** (A) Competition assay in VIPI cells transduced with viruses expressing sgRNAs for target genes. Data normalized to day 1. n=4 biological replicates, mean  $\pm$  SD. (B) Percentage of indels analyzed by decomposition (TIDE) analysis in Cas9 expressing COMI and VIPI cells following lentiviral transduction of the sgRNAs selected in (A) (sgTUFM\_1 and sgMRPS18A\_1). n=3-4 biological replicates, mean  $\pm$  SD. (C) Effects of TUFM and MRPS18A knockout on TUFM, MRPS18A and  $\beta$ -tubulin proteins in COMI and VIPI cells as assayed by immunoblotting. One representative result is shown, n=2 biological replicates. (D) Effects of TUFM and MRPS18A knockout on COX1, COX4, and  $\beta$ -tubulin proteins in COMI and VIPI cells as assayed by immunoblotting. One representative result is shown, n=2 biological replicates. (E) Effects of TUFM and MRPS18A knockout on COMI and VIPI cells grown in suspension. Example images from days 0, 4, 10 and 15, scale bar 200  $\mu$ m. (F) Sphere area measured over the course of the fifteen-day experiment. The data were normalized to day 0. n=15 technical replicates, mean  $\pm$  SEM. One representative experiment is shown, n=3 biological replicates. (G) Effects of TUFM and MRPS18A knockout on COMI and VIPI proliferation when grown as adherent cultures. The data were normalized to day 0. n=3 biological replicates, mean  $\pm$  SD. (H) Quantification of the number of spheres greater than 100  $\mu$ m for COMI and VIPI cells, n=20 technical replicates, mean  $\pm$  SEM. \*\*\*\*p < 0.0001, unpaired two-tailed t-test. One representative result is shown, n=3 biological replicates.

## STAR METHODS

### RESOURCE AVAILABILITY

#### Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Alessandro Quattrone (alessandro.quattrone@unitn.it).

#### Materials availability

Cell lines generated in this study can be requested from the lead contact.

#### Data and code availability

3D cryo-EM density maps and the coordinates of the atomic model have been deposited in the Worldwide Protein Data Bank under the accession numbers EMD-4434 and 6I9R, respectively.

### EXPERIMENTAL MODEL AND SUBJECT DETAILS

#### Cell Lines

Human glioblastoma stem cell lines COMI and VIPI were kind gifts from Antonio Daga (Azienda Ospedaliera Universitaria San Martino di Genova, Italy), and 030616 from Rossella Galli (H. S. Raffaele, Milan, Italy). COMI, VIPI and 030616 were cultured in DMEM/F-12 and Neurobasal media (Thermo Fisher Scientific, 1:1 ratio), supplemented with GlutaMAX (2 mM; Thermo Fisher Scientific), B27 supplement (1%; Thermo Fisher Scientific), Penicillin G (100 U/mL; Sigma Aldrich), recombinant human fibroblast growth factor-2 (bFGF) (10 ng/mL; R&D Systems), recombinant human epidermal growth factor (EGF) (20 ng/mL; R&D Systems) and heparin (2 µg/mL; Sigma Aldrich) at 37°C, 5% CO<sub>2</sub>. Cells were grown either as spheres in suspension or as adherent cultures on laminin-coated flasks where they maintain intact self-renewal capacity (Pollard et al., 2009).

Human glioblastoma stem cell lines GB6, GB7, GB8, G144, G166 and the human fetal neural stem cell lines CB660 and U3 were kind gifts from Luciano Conti (Department CIBIO, University of Trento, Italy) and were cultured as adherent cultures on laminin-coated flasks in Euromed-N media (Euroclone), supplemented with GlutaMax (2mM), B27 supplement (2%), N2 (1%; Thermo Fisher Scientific), Penicillin G (100 U/mL), recombinant human fibroblast growth factor-2 (bFGF) (20 ng/mL), and recombinant human epidermal growth factor (EGF) (20 ng/mL) at 37°C 5% CO<sub>2</sub>. Culture flasks were coated with laminin (10 µg/mL final, Thermo Fisher Scientific, cat. 23017015) and incubated for 3 h at 37°C or overnight at 4°C prior to use.

Human glioblastoma stem cell lines GSC23p, GSC163, GSC67, GSC62, GSC23C, GSC76, GSC30PT, GSC28, GSC148, GSC83, GSC1, GSC30p, GSC74, GSC120, GSC61, GSC70, GSC83\_2, GSC112, GSC151, GSC147 and GSC68 were isolated from patients' surgical samples at the Institute of Neurosurgery, Catholic University of Rome as previously described (Marziali et al., 2016). Human fetal neural stem cell line HNPC#13 was obtained from the brain of a 12-week-old embryo legally aborted, according to the ethical guidelines of the European Network for Transplantation (NECTAR), as previously described (Ricci-Vitiani

et al., 2004). These lines were grown in suspension in DMEMF12 serum-free medium (Thermo Fisher Scientific) containing 2 mM L-glutamine, 0.6% glucose, 9.6 mg/mL putrescine, 6.3 ng/mL progesterone, 5.2 ng/mL sodium selenite, 0.025 mg/mL insulin, 0.1 mg/mL transferrin sodium salt (Sigma, St Louis, MO), EGF (20 ng/mL), bFGF (10 ng/mL) and heparin (2 µg/mL; Sigma Aldrich) at 37°C, 5% CO<sub>2</sub>.

Human lung fibroblasts, MRC5, were cultured in EMEM media (Thermo Fisher Scientific), supplemented with 10% FBS, GlutaMAX (2 mM; Thermo Fisher Scientific) and Penicillin G (100 U/mL; Sigma Aldrich) at 37°C, 5% CO<sub>2</sub>.

Human skin fibroblast, Hs68, were cultured in DMEM media (Thermo Fisher Scientific), supplemented with 10% FBS, GlutaMAX (2 mM; Thermo Fisher Scientific) and Penicillin G (100 U/mL; Sigma Aldrich) at 37°C, 5% CO<sub>2</sub>.

For GSCs differentiation, cells were grown on laminin-coated plates in the above media without growth factors and with the addition of 10% FBS (Thermo Fisher Scientific) for 14 days. For astrocyte differentiation, CB660, U3 and HNPC#13 cells were grown on laminin-coated plates in the above media without growth factors and with the addition of 5% FBS for 3 weeks.

For hypoxia and anoxia experiments, cells after treatment were grown in a hypoxic chamber (Invivo<sub>2</sub> 200, Baker Ruskinn, Hypoxic, 1% O<sub>2</sub>).

## METHODS DETAILS

### Cas9 cell line generation

**sgRNA cloning.** pLKO.1-puro U6 sgRNA BfuAI large stuffer plasmid where the puromycin gene was removed and replaced with a puro-GFP fusion protein as previously described by Phelan et al., 2018 was used (gift from Dr. Louis Staudt, National Cancer Institute, USA). The resulting plasmid was digested with BfuAI (NEB cat. R0701S). Complementary sgRNA sequences flanked by ACCG on the 5' end, and CTTT on the 3' of the reverse strand were annealed, diluted and ligated into the cut vector with T4 ligase (NEB cat. M0202M) according to the manufacturer's instructions. All transformations were performed in *Stb13* bacteria (Thermo Fisher Scientific cat. C737303) and sequencing was performed to verify the correctness of the inserts. The sgRNA sequences are listed in the Key Resources Table.

**Lentiviral vectors production.** Lentiviral particles were produced in 293FT cells (Thermo Fisher Scientific cat. R70007) by transfecting Cas9 vector or sgRNA vectors with packaging vectors psPAX2 (Addgene cat. 12260) and pMD2.G (Addgene cat. 12259) in a 4:3:1 ratio in serum-free Opti-MEM. Trans-IT 293T (Mirus cat. MIR2705) was added and incubated for 15 min before adding the mixture dropwise to cells. Supernatants were harvested 48 and 72 h later, filtered through a 45µm filter and then incubated with Lenti-X concentrator (TakaraBio cat. 631232). Virus was concentrated according to the manufacturer's instructions, aliquoted and stored frozen at -80°C.

**Cas9 cell line generation.** COMI-Cas9 and VIPI-Cas9 cells were generated by transducing COMI and VIPI cells twice with lenti-SpCas9 blast (Addgene cat. 104997) and selected with 100 µg/ml blasticidin. The generated cell lines were tested for functional Cas9 cutting after transduction with sgRNA targeting the essential ribosomal subunit RPL6 and expressing a GFP reporter. The loss of GFP was assessed by FACS for 15-17 days. Since the efficiency of COMI-Cas9 cell pool cutting was not sufficient, this line was dilution cloned. Single-cell

clones were picked and tested for functional Cas9 cutting and the best cutting clone was selected for further experiments.

**Generation of knockout cell lines.** COMI and VIPI Cas9-expressing cells were transduced with sgRNA lentiviral vectors targeting sgNT (negative control), sgTUFM and sgMRPS18A and expressing a GFP reporter and selected with 1 µg/ml puromycin for COMI and 1.25 µg/ml puromycin for VIPI for 72h. The transduction and selection were performed in biological triplicates. These cell lines were then used to perform the 3D viability, proliferation and gliomasphere formation assays.

### Screening and analysis

A custom-made library was created using compounds available from the Selleck Translation Inhibitors and MS Discovery libraries, and additional compounds acquired from other companies (**Table S1**). All compounds were solubilized in dimethyl sulfoxide (DMSO, Sigma Aldrich) and stored frozen prior to use. COMI and VIPI cells were plated in 96-well microtiter plates in 150 µL of media at plating densities of 6,000 and 4,500 cells/well, respectively, and incubated for 24 h prior to drug treatment. The drugs were added to a final concentration of 100 µM and the plates were incubated for 24 h at 37°C. Each treatment was performed in a technical triplicate. The positive control wells were treated with FCCP, rotenone and oligomycin A at 100 µM for 24 h or with FCCP (Cayman Chemical, cat.15218) at 100 µM for 30 min at 37°C. Next, cells were stained with 200 nM MitoTracker Orange (Thermo Fisher Scientific, cat. M7510) and incubated for 30 min at 37°C. Subsequently, cells were fixed by direct addition of paraformaldehyde solution to a final concentration of 4% v/v and incubation for 15 min at room temperature, followed by a wash with 200 µL of PBS. The cells were then stained with 5 µM DRAQ5 (Cell Signaling, cat. 4084) for 20 min at room temperature protected from light. Drug dilution and addition were performed using Tecan Evo 200 liquid handler, whereas fixing, PBS washing, and dyes dispensation were performed using BioTek EL406 washer dispenser. The plates were read using the Operetta-High Content Imaging System (Perkin Elmer). Compound precipitation was visually assessed, and interfering compounds were excluded from the analysis.

In order to perform phenotypic profiling of the compound-induced toxicity effects, the image data were visualized and analyzed using the Harmony Software. The data were plate-wise normalized by computing the compound percentage of the vehicle control treatment (DMSO). Six informative features describing cellular compactness and intracellular fluorescence distribution were extracted to describe cellular or mitochondrial toxicity phenotypes (**Table S2**). The informative content of a feature was assessed by testing whether the feature values for the positive controls were significantly different from the vehicle treatment (t-test, FDR < 0.05). For each library compound, we assessed the size of the effect induced on each feature. A feature for a given compound was defined as a hit when the feature deviated from the median value of all compounds by a distance larger than 1.5 times the median absolute deviation. Individual compounds were scored by the number of affected features. When a feature was affected by a compound, it accounted for one over the number of informative features N (in this case  $\frac{1}{6}$ , since N = 6). Therefore, the maximal attainable score when all the features are affected for a given compound is one (or 100%). We next assigned a percentage score to each class of compounds by calculating the average of the scores attained by all the compounds included in each class.

## Viability assays

For the evaluation of the effect of OXPHOS and glycolysis inhibitors on GSCs viability, cells were plated in a 96 laminin-coated well plate (2,500 cells/well of COMI and VIPI) and treated the following day with rotenone (1  $\mu$ M, Sigma Aldrich, cat. R8875), Oligomycin (1  $\mu$ M, Sigma Aldrich, cat. O4876), Antimycin A (1  $\mu$ M, Sigma Aldrich, cat. A8674), 2-deoxyglucose (2-DG, 250  $\mu$ M, Sigma Aldrich, D8375) and dichloroacetic acid (DCA, 5 mM, Sigma Aldrich, cat. 347795) for 72 h. Each treatment was performed in technical triplicate.

For the evaluation of the effect of streptogramins, tetracyclines and pleuromutilins on GSCs viability, cells were seeded into 96-well laminin-coated microtiter plates in 150  $\mu$ L of media at plating densities ranging from 3,500-6,000 cells/well. The plates were incubated for 24 h prior to drug treatment. Serial drug dilutions were prepared in PBS to provide a total of seven drug concentrations plus control. 10  $\mu$ L of these dilutions were added to each well, and the plates were incubated for additional 48 h. Each treatment was performed in technical quadruplicate. The following compounds were tested: Dalfopristin (Santa Cruz, cat. sc-362728), Quinupristin (Bioaustralis, cat. BIA-Q1354), Quinupristin-dalfopristin mesylate complex (Santa Cruz, cat. sc-391726), Virginiamycin M1 (Sigma Aldrich, cat. V2753), Virginiamycin B (Santa Cruz, cat. sc-362038), Virginiamycin S1 (Apollo Scientific, cat. BIV1004), Griseoviridin (Apollo Scientific, cat. BIBR1137), Viridogrisein (Apollo Scientific, cat. BIBR1132), Tetracycline (Sigma Aldrich, cat. 87128-25G), Methacycline HCl (Sigma Aldrich, cat.37906-100MG-R), Oxytetracycline (Sigma Aldrich, cat. PHR1537-1G), Doxycycline HCl (Sigma Aldrich, cat. D3447-500MG), Demeclocycline HCl hydrate (Sigma Aldrich, cat. 46161-100MG), Meclocycline Sulfosalicylate (Sigma Aldrich, cat. M1388-500 MG), Minocycline HCl (Santa Cruz, cat. sc-203339), Pipacycline (Santa Cruz, cat. sc-391690), Tigecycline (Santa Cruz, cat. sc-394197), Valnemulin (MicroSource Discovery library) and Tiamulin (Sigma Aldrich, cat., 34044).

After the drug treatments, the cells were stained with Hoechst 33342 (1 $\mu$ g/mL; Thermo Fisher Scientific, cat. H1399) and Propidium Iodide (PI, 1 $\mu$ g/mL; Sigma Aldrich, cat. P4170) and incubated for 20 min shaking in the dark. The plates were then read using Operetta-High Content Imaging System (Perkin Elmer) and analyzed using the Harmony Software. The number of viable cells was calculated by subtracting PI positive cells from the total number of cells estimated by Hoechst 33342 staining and normalized to the non-treated control.

For cytotoxicity analysis on GSCs grown as gliomaspheres, the cells were mechanically dissociated and plated at a density of 20,000 cells/mL in a 96-well plate, in triplicate. Quinupristin/dalfopristin (Q/D) was added 24 h after cell plating. ATP levels were measured using the CellTiter-Glo® Luminescent Cell Viability Assay (Promega, cat. G7570) as per the manufacturer's instructions after 48 h and 72 h of treatment. Percentage viability was calculated upon normalization on the non-treated control.

Dose-response curves were plotted, and growth inhibition 50 (GI<sub>50</sub>) values calculated using the GraphPad Prism software.

## 3D Viability assay

Ten COMI cells/well were plated in Ultra-Low attachment round bottom 96 well plates (Costar) and treated with desired Q/D concentrations. The cells were centrifuged at 300 g for 30 sec, followed by the first acquisition using Operetta-High Content Imaging System (Perkin Elmer). The images were subsequently acquired over the course of 9-10 days. The area of the spheres formed was assessed using the Harmony Software.

Ten COMI-Cas9 cells/well and twenty-five VIPI-Cas9 cells/well transduced with the respective sgRNA lentiviral vectors were plated in Ultra-Low attachment round bottom 96 well plates (Costar). The experiment was then performed as described above. The images were acquired over the course of 15 days. The area of the spheres formed was assessed using the Harmony Software.

### **Gliomasphere formation assay**

COMI cells grown in suspension were plated at a density of 50,000 cells/mL and treated with Q/D for 72 h. The spheres were then dissociated, cells counted and plated at a density of 10 or 100 cells/well in a 96 well plate without the drug. After 10 days, the spheres formed were stained with 1  $\mu$ M Calcein AM (Thermo Fisher Scientific, cat. C3100MP), incubated for 20 min at 37°C, after which the spheres were imaged using Operetta-High Content Imaging System (Perkin Elmer) and analyzed using the Harmony Software. Only spheres greater than 100  $\mu$ m were quantified. The experiment was performed in a biological triplicate, with 20 technical replicates each.

COMI-Cas9 cells and VIPI-Cas9 cells transduced with the respective sgRNA lentiviral vectors were plated at two different cell densities (10 and 100 cells/well for COMI, and 100 and 250 cells/well for VIPI). After 10 days, the spheres formed were stained and analyzed as described above.

### **Cell cycle assay**

Cells were plated 24 h prior to treatment (120,000 cells/well of COMI cells in a 6 well plate) and incubated with Q/D for additional 48h. Cell cycle analysis was conducted using FACS (BD FACSCanto II) after staining with the Click-IT EdU Flow Cytometry Assay kit (Thermo Fisher Scientific, cat.C10634) according to the manufacturer's instructions. The experiment was performed in a biological triplicate. Data were processed by the BD FACS DIVA V8.0.1™ software.

### **Apoptosis assays**

Apoptosis was assessed using FITC Annexin V Apoptosis Detection Kit I (BD Pharmingen, BD Biosciences, cat. 556547). Cells were plated 24 h prior to treatment (270,000 cells/6cm<sup>2</sup> plate of COMI cells) and incubated with Q/D for further 48h. 200,000 cells were stained according to the manufacturer's instructions and analyzed using FACS (BD FACSCanto II). Data were processed by BD FACS DIVA V8.0.1™ software.

### **Autophagy assays**

To assess the autophagic flux, cells were plated 24 h prior to treatment and incubated with 6.5  $\mu$ M Q/D for further 48 h. In addition, for immunoblotting the cells were treated with 60  $\mu$ M chloroquine for 24 h (Sigma Aldrich, cat. C6628), 6.5 nM bafilomycin for 3 h (Sigma Aldrich, cat. B1793) or 5 mM NH<sub>4</sub>Cl for 3 h (Sigma Aldrich, cat. A9434). Immunoblotting was performed as described in the Immunoblotting section. LC3B-II was quantified by densitometric analysis (Image Lab 2.0.1 software, Biorad) and normalized to  $\beta$ -tubulin as a loading control. For immunofluorescence analysis, the cells were treated with 60  $\mu$ M chloroquine, 6.5 nM bafilomycin or 10 mM NH<sub>4</sub>Cl for 24 h. Immunofluorescence for LC3 staining was carried out according to the procedure described in the Immunofluorescence section. Cell morphology was determined by staining with CellMask Deep Red Stain (Thermo Fisher Scientific, cat. H32721).

In order to evaluate the role of autophagy in Q/D cytotoxic activity, COM1 cells were seeded into 96-well microtiter plates in 150  $\mu$ L of media at plating densities of 4,000 cells/well. The plates were incubated for 24 h prior to drug treatment. Cells were pretreated with chloroquine (5, 10 and 20  $\mu$ M) for 3 h and then treated with 6.5  $\mu$ M of Q/D for further 48 h. The cells were stained with Hoechst 33342 (1  $\mu$ g/mL; Thermo Fisher Scientific, cat. H1399) and Propidium Iodide (PI, 1  $\mu$ g/mL; Sigma Aldrich, cat. P4170) and incubated for 20 min shaking in the dark. The plates were then read using Operetta-High Content Imaging System (Perkin Elmer) and analyzed using the Harmony Software. The number of viable cells was calculated by subtracting PI positive cells from the total number of cells estimated by Hoechst 33342 staining and normalized to the control. Each treatment was performed in technical quadruplicate and in biological triplicate.

### **Cryo-EM data collection**

Human mitoribosomes and grids were prepared as described previously (Brown et al., 2014) with modifications involving scaling down the prep in order to minimize the time from the antibiotic treatment to cryo-EM (Aibara et al., 2018). Briefly, 2 liters HEK293S TetR GnTi-cells lacking N-acetyl-glucosaminyltransferase I were grown to a final concentration of  $4.2 \times 10^6$  cell/mL. Prior to cell collection and mitoribosome isolation, the cells were treated with 5  $\mu$ M Q/D for 24 h. Three  $\mu$ L of purified mitochondrial ribosomes at a concentration of 3.6 Abs at 280 nm were incubated for 30 sec on glow-discharged holey carbon grids (Cu Quantifoil R2/2), onto which a home-made continuous carbon film had previously been deposited, prior to vitrification in a Vitrobot MKIV (3 second blot, 4°C, 100 % humidity). Images were collected on a 300 kV FEI Titan Krios electron microscope, a slit width of 20 eV on a GIF-Quantum energy filter. A Gatan K2-Summit detector was used in counting mode at a magnification of 130,000 (yielding a pixel size of 1.05 Å), and a dose rate of  $\sim 5.2$  electrons per pixel per second. Exposures of 8 sec (yielding a total dose of  $38 \text{ e} \cdot \text{Å}^{-2}$ ) were dose-fractionated into 20 movie frames that were stacked into a single MRC stack using newstack. 4225 images were collected using EPU automatic data collection with defocus values ranging from 0.4 to 4.5  $\mu$ m.

### **Cryo-EM image processing, model building and refinement**

Collected micrographs were corrected for local-frame movement as well as dose filtered using UcsfDfCorr. The contrast transfer function parameters were estimated using GCTF-0.5, for all other image processing steps RELION-2.1.b1. 578,573 particles were picked using reference based picking, where bad particles were removed by particle sorting (546,701 particles retained) and subjected to reference-free 2D class averaging, resulting in 387,428 particles for 3D classification. 4 classes containing 138,030 particles were pooled and subjected to 3D auto-refinement, yielding a map of the human mitochondrial ribosome. However, since there was some heterogeneity visible in the reconstruction, the particles were subjected to further fine angular classification and 100,000 particles were selected for another 3D auto-refinement step. Since Q/D is known to interact with the bacterial large subunit and to improve the quality of the map, masked focused-refinement was used to focus on the mitoribosomal large subunit. The resolution of the final reconstruction was 3.9 Å.

Reported resolutions are based on gold-standard refinement applying the 0.143-criterion on the FSC between reconstructed half-maps. The FSC was corrected for the effects of a soft mask on the using high-resolution noise substitution. All 3D refinements used a 70 Å low-

pass filtered initial model, the first of which was a previous low resolution reconstruction of the human mitochondrial ribosome (EMDB-2876 (Amunts et al., 2015)). Before visualization, all density maps were corrected for the modulation transfer function of the detector, and then sharpened by applying a negative B-factor that was estimated using automated procedures. The quality of the map allowed unambiguous model building with Coot. Stereochemical refinement was performed using phenix.real\_space\_refine as implemented in the PHENIX suite. The final model was validated using MolProbity. Refinement statistics are given in **Table S6**.

### **Protein synthesis assay**

Cells were plated 24 h prior to treatment (180,000 cells/well of COMI cells in a 6 well plate) and incubated with Q/D for further 24 h prior to <sup>35</sup>S labelling. To assay for mitochondrial protein synthesis, growth medium was removed and cells were washed twice with methionine/cysteine-free DMEM medium, followed by an incubation in methionine/cysteine-free DMEM medium containing 96 µg/mL Cysteine, 1% B27 supplement, 1% GlutaMax; 1% Sodium Pyruvate, 10 ng/mL bFGF, 20 ng/mL EGF, 2 µg/mL heparin and 80 µg/mL emetine (Sigma Aldrich, cat. E2375) for 15 min at 37°C. Subsequently, [<sup>35</sup>S]-methionine (Perkin Elmer, cat. NEG709A005MC) was added to a final concentration of 166.6 µCi/mL and the labeling was performed for 20 min at 37°C. The cells were then detached and pelleted at 4,000 rpm for 5 min. The pellet was washed three times with 1 mL of PBS. Cell pellets were resuspended in protein lysis buffer containing protease inhibitors and 1.25 U/µL benzonase. Protein concentrations were measured with Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific, cat. 23227) and equal amount of protein samples were separated on SDS-PAGE gels (NuPAGE™ 12% Bis-Tris Protein Gels, Thermo Fisher Scientific, cat. NP0343BOX). The labelled proteins were visualized and quantified using a PhosphorImager system and ImageQuant software (Molecular Dynamics, GE Healthcare). To assay for cytosolic translation, the above procedure was used without the addition of emetine.

### **Immunoblotting**

Total cell lysates were prepared from cells. Briefly, cells were washed with PBS and resuspended in lysis buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.25% NP-40, 0.1% Triton X-100, 0.1% SDS and supplemented with protease inhibitors). Protein concentrations were measured with Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific, cat. 23227). Equal amounts of protein were separated on SDS-PAGE and transferred to nitrocellulose or PVDF (for anti-LC3 antibody only) membrane. Membranes were probed with anti-MTCO1 (COX1, Abcam, cat. ab14705), anti-COX4 (Cell Signaling, cat. 4850), anti-SDHA (Cell Signaling, cat. 5839), anti-β-tubulin (Santa Cruz, cat. sc-53140), anti-TUFM (Thermo Fisher Scientific, cat. MA5-31363), anti-MRPS18A (Thermo Fisher Scientific, cat. PA5-57274), anti-LC3 (Cell Signaling, cat. 3868S), and secondary HRP-conjugated antibodies (Santa Cruz Biotechnology). Detection was performed using Amersham ECL Prime or Select Western Blotting Detection Reagent (GE Healthcare Life Sciences) and ChemiDoc Imaging System (Bio-Rad). Data were analyzed using ImageLab software.

### **Immunofluorescence**

The cells were fixed either with paraformaldehyde solution (4% v/v final, 15 min incubation at room temperature) or with 100% ice-cold methanol (5 min incubation at room temperature,

only for LC3B IF), followed by two washes with PBS. The cells were then permeabilized with 0.3% Triton X-100 - 3% BSA in PBS for 45 min at room temperature. The primary and secondary antibodies were diluted in 3% BSA-PBS solution. The incubation with the primary antibody was carried out at 4°C overnight, followed by a wash with 3% BSA-PBS solution and incubation with the secondary antibody for 1 h at room temperature. Cell morphology was determined by staining with HCS CellMask™ Deep Red Stain (Thermo Fisher Scientific, cat. H32721, 1:2000, 20 min, room temperature). The plates were then read either using Operetta-High Content Imaging System (Perkin Elmer) and analyzed by the Harmony Software or using the Leica TCS SP5 confocal microscope and processed by imaging softwares ImageJ (version v1.51w) and Photoshop. For the latter, z-stack images were acquired and LC3 puncta quantification was performed on image stacks of region of interests containing single cells, using the “3D Maxima finder” plugin of ImageJ. Both size and intensity threshold constraints were applied to the quantification.

### **Confocal imaging**

Images were acquired on a Leica TCS SP5 confocal microscope with a 63x oil immersion objective, 2x zoom, 1024x1024 resolution, 200Hz speed, lasers Argon 488 nm and Diode laser 633 nm, step 0.89 µm. Images were further analyzed and processed using imaging softwares ImageJ (Fiji) and Photoshop.

### **RNA extraction, reverse transcription and qPCR**

Total RNA was extracted using QIAzol reagent (QIAGEN) according to the manufacturer’s instructions. Reverse transcription was performed using iScript Reverse Transcription Supermix (BioRad, cat. 170-8891) on C1000 Thermal Cycler (BioRad) and quantitative real-time PCR was performed using 2x qPCR SyGreen Mix Separate ROX (PCR Biosystems, cat. PB20.14-05) following the manufacturer’s instructions on CFX384 Real-Time System (BioRad). All assays were performed in triplicate in 4-5 independent experiments. Data was analyzed using the CFX Manager software (BioRad). Relative expression values of each target gene were normalized to GAPDH and 18S RNA level. The following primers were used at 500 nM concentration:

COX1: 5'- CTATACCTATTATTCGGCGCATGA-3' and 5'-CAGCTCGGCTCGAATAAGGA - 3',

COX4: 5'-GCCATGTTCTTCATCGGTTTC-3' and 5'-GGCCGTACACATAGTGCTTCTG-3',

18S: 5'-GGACATCTAAGGGCATCAC-3' and 5'-AGGAATTGACGGAAGGGCAC-3',

GAPDH: 5'-CAACGAATTTGGCTACAGCA-3' and 5'-AGGGGTCTACATGGCAACTG-3'.

### **BN-PAGE and in gel complex activity assay**

Cells were plated in two T75 flasks (700,000 cells/flask) and treated with Q/D after 24 h for additional 48 h, 72 h and 96 h. Mitochondria were isolated in the following manner: cells were detached, pelleted and resuspended in 750 µL of MIB+BSA buffer (0.32 M sucrose, 1 mM EGTA pH 8, 20 mM Tris-HCl, pH 7.2, 0.1% fatty acid-free BSA). The cells were homogenized using Potter-Elvehjem homogeniser and centrifuged at 1,000g for 5 min at 4°C. The supernatant was collected and the pellet resuspended in MIB+BSA, rehomogenised and centrifuged again. The supernatant was collected and pooled with the first one, then centrifuged at 12,000g for 10 min at 4°C to pellet the mitochondria. The pellet of mitochondria was subsequently washed once with MIB+BSA, once with MIB, resuspended in 100 µL of ACNA buffer (1.5 M aminocaproic acid (Sigma Aldrich, cat.

A2504), 50 mM BisTris, pH 7.00) and quantified using Qubit™ Protein Assay Kit (Thermo Fisher Scientific, cat. Q33212). Digitonin (Sigma Aldrich, cat. D5628-1G) was added to a final concentration of 1% w/v, the samples were vortexed and incubated on ice for 20 min, followed by a centrifugation at 14,000g for 30 min at 4°C. The supernatant was mixed with the loading buffer, and 50 µg of protein was separated on Blue Native PAGE gels (NativePAGE™ 3-12% BisTris Protein Gels, Thermo Fisher Scientific, cat. BN1001BOX). The gels were incubated overnight at room temperature with the respective complex substrates: Complex I: 2 mM Tris HCl, pH 7.4; 0,1 mg/mL NADH (Sigma Aldrich, cat. N8129-50MG); 2.5 mg/mL iodinitrotetrazolium chloride (Sigma Aldrich, cat. I8377-1G); Complex II: 4.5 mM EDTA, 0.2 mM phenazine methosulfate (Sigma Aldrich, cat. P9625-500MG), 84 mM succinic acid (Sigma Aldrich, cat. S2378-100G) and 0.5 mg/mL iodinitrotetrazolium chloride (Sigma Aldrich, cat. I8377-1G); Complex IV: 0.5 mg/mL 3,3'-diamidobenzidine tetrahydrochloride (DAB) (Sigma Aldrich, cat. D5637-1G), 50 mM phosphate buffer pH 7.4; 1 mg/mL cytochrome *c* (Sigma Aldrich, cat. C2506-1G), 0.2 M sucrose, 20 µg/mL (1 nM) catalase (Sigma Aldrich, cat. C9322-1G), Complex V: 3.76 mg/mL glycine, 5 mM MgCl<sub>2</sub>, Triton X-100, 0.5 mg/mL lead nitrate (Sigma Aldrich, cat. 203580), ATP (Sigma Aldrich, cat. A7699-5G), pH 8.4.

### **Immunoblotting after BN-PAGE**

Proteins separated on Blue Native PAGE gels (NativePAGE™ 3-12% BisTris Protein Gels, Thermo Fisher Scientific, cat. BN1001BOX) were transferred to PVDF membrane (25V, 4°C, overnight, wet-transfer, transfer buffer with 20% MeOH and 2 mL of 10% SDS in 1L). Membranes were stripped to remove the blue-staining (Restore™ PLUS Western Blot Stripping Buffer, Thermo Fisher Scientific, cat. 46430, 3 min) and probed with anti-OxPhos complex I (39kDa subunit; clone 20C11, Thermo Fisher Scientific, cat. A21344), anti-SDHA complex II (2E3GC12FB2AE2, Abcam, cat. ab14715), anti-OxPhos complex III (core 1 subunit; clone 16D10, Thermo Fisher Scientific, cat. A21362), anti-OxPhos complex IV (subunit 1; clone 1D6E1A8, Invitrogen cat. 459600), anti-ATP5A complex V (15H4C4, Abcam, cat. ab14748) and Amersham ECL secondary HRP-conjugated antibodies (GE Healthcare). Proteins detection was performed using Clarity™ Western ECL Substrate Detection Reagent (Biorad, cat. 1705061) and the film exposure time was adjusted according to signal intensity.

### **Respiration assay**

High-resolution respirometry was performed using a 2 mL chamber OROBOROS Oxygraph-2k (Oroboros Instruments) at 37°C. Respiration rates were calculated as the negative time derivative of oxygen concentration measured in the closed respirometer and expressed per number of viable cells and corrected by residual oxygen consumption (ROX, after antimycin A addition). The amplified signal was recorded on a computer with online display of the calibrated oxygen concentration and oxygen flux (DatLab software for data acquisition and analysis; Oroboros Instruments). Cells were plated 24 h prior to treatment and incubated with Q/D for further 48 h. The cells were then detached and 1,000,000 cells were injected into each chamber. Oxygen consumption was evaluated for cellular ROUTINE respiration, and then cells were permeabilized with digitonin 4.1 µM (Fluka, cat. 37008) in MiR05 medium (10 mM KH<sub>2</sub>PO<sub>4</sub>, 60 mM lactobionic acid (Sigma Aldrich, cat. 153516), 20 mM HEPES, 3 mM MgCl<sub>2</sub>, 0.5 mM EGTA, 20 mM taurine (Sigma Aldrich, cat. T0625), 110 mM D-sucrose, and 1 mg/mL BSA fatty acid free). Complex I activity was measured after malate

(2 mM, Sigma Aldrich, cat. M1000), glutamate (10 mM, Sigma Aldrich, cat. G1626) and ADP (5 mM, Calbiochem, cat. 117105) injection, and complex I&II activity after additional succinate (10 mM, Sigma Aldrich, cat. S2378) injection. The ETS capacity (maximum uncoupled respiration) was induced by stepwise titration of carbonyl cyanide p-(trifluoromethoxy) phenylhydrazone (FCCP, Sigma Aldrich, cat. C2920) (typically 3–4 steps, 1 ul each of 1 mM FCCP). Complex II activity was measured after the addition of rotenone (0.5 uM, Sigma Aldrich, cat. R8875). Residual respiration (ROX) was measured after inhibition with antimycin A (2.5 uM, Sigma Aldrich, cat. A8674).

### **Mitochondrial membrane potential assessment**

Cells were plated 24 h prior to treatment (120,000 cells/well of COMI cells in a 6 well plate) and incubated with Q/D for further 48h. Positive control was treated with 100 uM FCCP for 10 min. Cells were detached and 500,000 cells were resuspended in fresh media containing 5 uM JC-1 (Abcam, cat. ab113850), and incubated at 37°C for 30 min. Cells were then centrifuged at 400 g for 5 min and resuspended in 0.5 mL PBS and analyzed using FACS (BD FACSCanto II). Data were processed by BD FACS Diva V8.0.1™ software.

### **Lactate Assay**

Cells were plated in a 96 well plate (4,000 cells/well of COMI and 3,500 cells/well of VIPI) and treated the following day with Q/D for 48h. Media was collected and the lactate production was measured using Glycolysis Cell-Based Assay Kit (Cayman Chemical, cat. 600450) according to the manufacturer's instructions. The cells were then fixed with 4% v/v paraformaldehyde for 15 min at room temperature and stained with Hoechst 33342 (1 uM). The nuclei were quantified using Operetta-High Content Imaging System (Perkin Elmer) and analyzed by the Harmony Software. The lactate production was normalized to the number of cells.

### **Mitochondrial mass**

Mitochondrial mass was assayed by staining the mitochondria with MitoTracker Orange (Thermo Fisher Scientific) or anti-COX4 antibody (Abcam) as described in the immunofluorescence section. The number of mitochondria was estimated by counting the number of MitoTracker Orange or COX4 positive spots per area of cytoplasm using Operetta-High Content Imaging System (Perkin Elmer) and analyzed by the Harmony Software.

### **Competition assay**

VIPI-Cas9 cells were transduced with sgRNA lentiviral vectors targeting TUFM and MRPS18A and expressing a GFP reporter. sgNT was used as a negative control. Three days after transduction, GFP levels were measured by flow cytometry on a BD LSR II using Diva version 9.0 and analyzed with FlowJo version 10.7.1. GFP levels were followed every 3-4 days for 21 days and normalized to the day 1 measurement.

### **Analysis of Cas9-induced indels**

Genomic DNA was extracted using QuickExtract™ DNA Extraction Solution and the target site was PCR-amplified with Taq DNA polymerase (Meridian Bioscience).

The following primers were used at a concentration of 400 nM:

TUFM for: GTCTAAGCTCTGCCTCTAGC

TUFM rev: CATCTCAATGCCTAGGACGG  
MRPS18A for: GTGGAGGAAGTGATCTCAGC  
MRPS18A rev: GGTAATAGACCTGAGTGGCG

PCR products were purified and sequenced. To assess the Cas9-induced indels with each sgRNA, tracking of indels by decomposition (TIDE) analysis was performed as reported in Brinkman et al., 2014.

## **2D viability assay**

COMI-Cas9 and VIPI-Cas9 cells transduced with the respective sgRNA lentiviral vectors were plated in 96 well plates at a cell density of 1500 cells/well. Every 24 h, five wells per condition were stained with Hoechst 33342 (1 $\mu$ g/mL; Thermo Fisher Scientific, cat. H1399) and Propidium Iodide (PI, 1 $\mu$ g/mL; Sigma Aldrich, cat. P4170) and incubated for 20 min shaking in the dark. The plates were then read using Operetta-High Content Imaging System (Perkin Elmer) and analyzed using the Harmony Software. The number of viable cells was calculated by subtracting PI positive cells from the total number of cells estimated by Hoechst 33342 staining and normalized to day 0.

## **QUANTIFICATION AND STATISTICAL ANALYSIS**

Results were reported as mean  $\pm$  SD (standard deviation) or mean  $\pm$  SEM (standard error of the mean), as indicated in the figure legend. Details of each analysis are in figure legends. Statistical significance is determined by unpaired two-tailed t-test (\* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , \*\*\*\* $p < 0.0001$ ; ns  $p \geq 0.05$ ). n represents number of biological or technical replicates, as indicated in figure legends. All the experiments with representative images (including immunoblotting and immunofluorescence) were repeated at least twice and representative images are shown. The association of GI<sub>50</sub> values with continuous clinical features was computed by Pearson's correlation coefficient and with categorical values by ANOVA test.

## **KEY RESOURCES TABLE**

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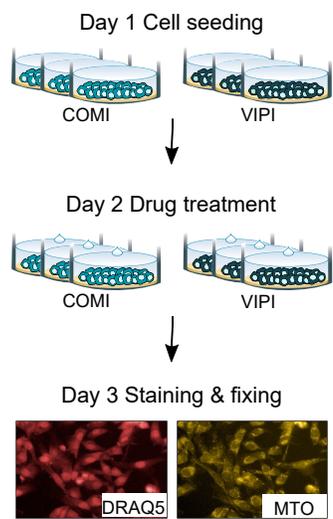
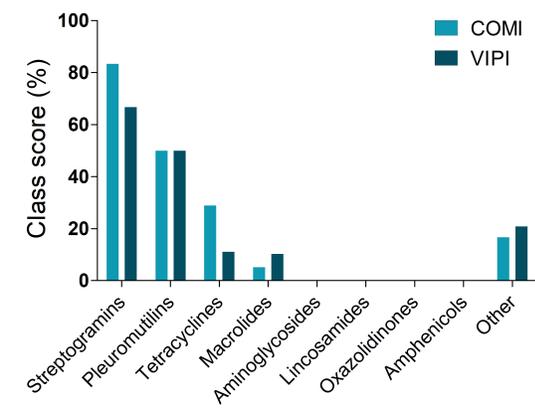
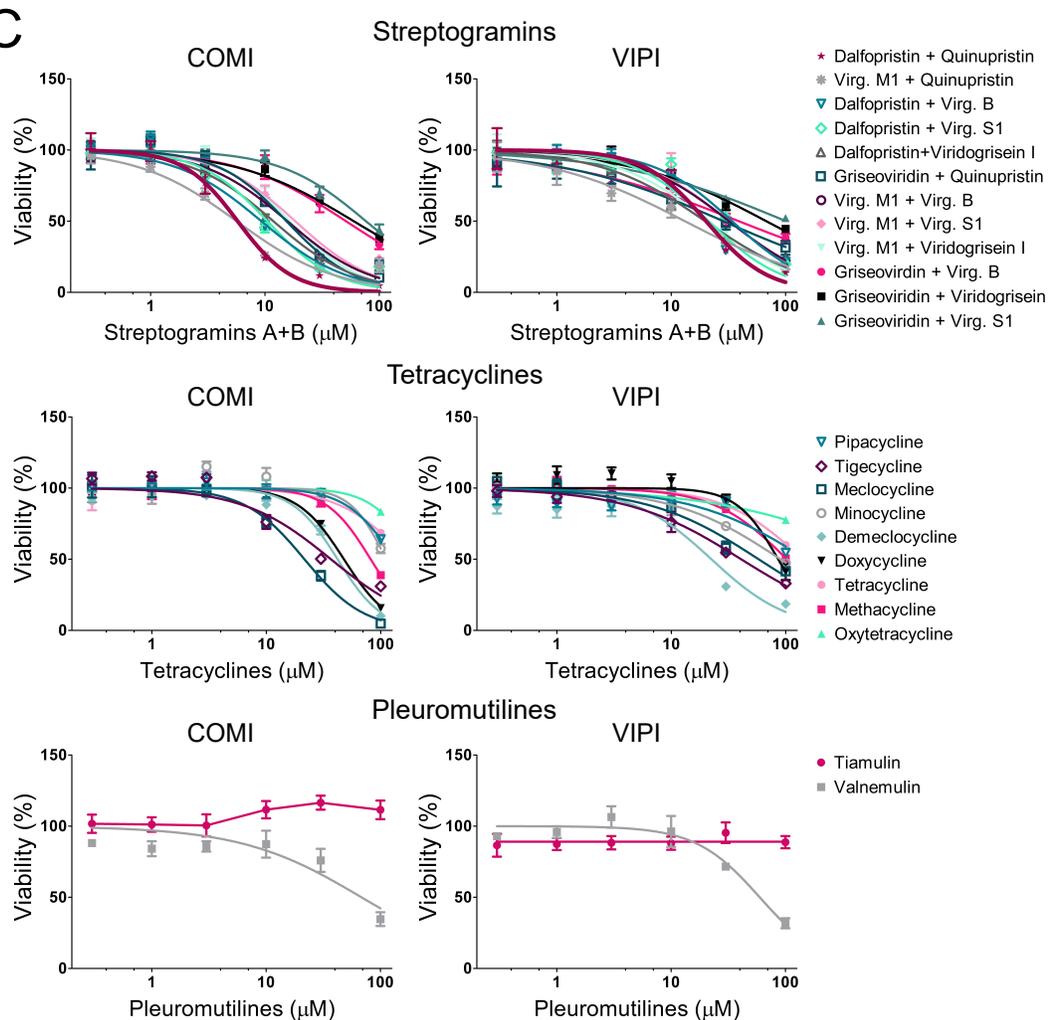
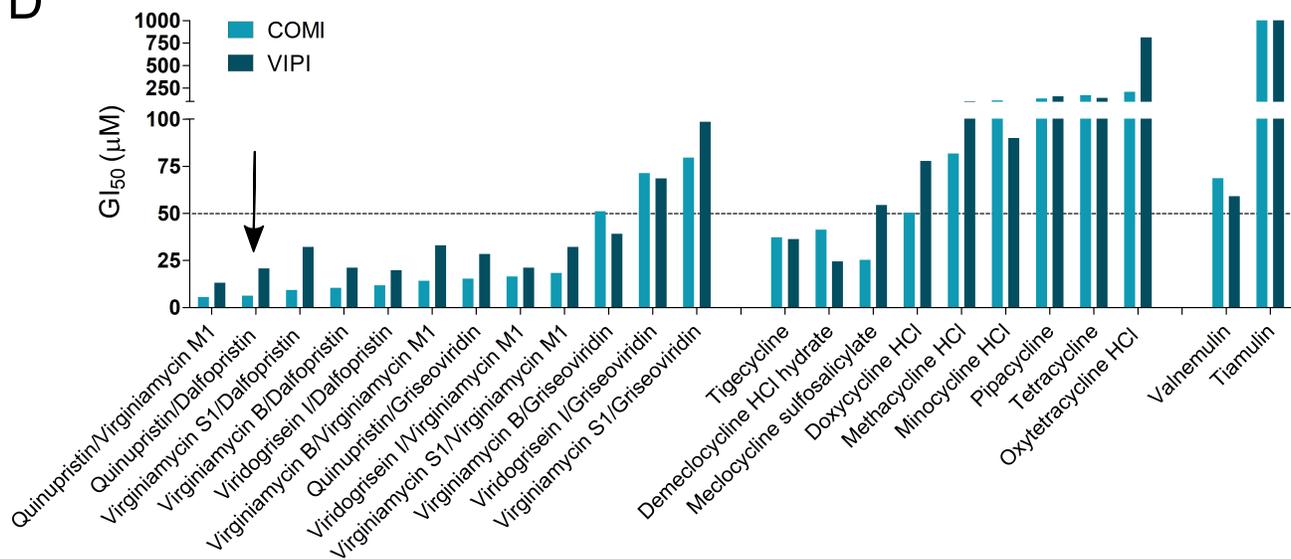
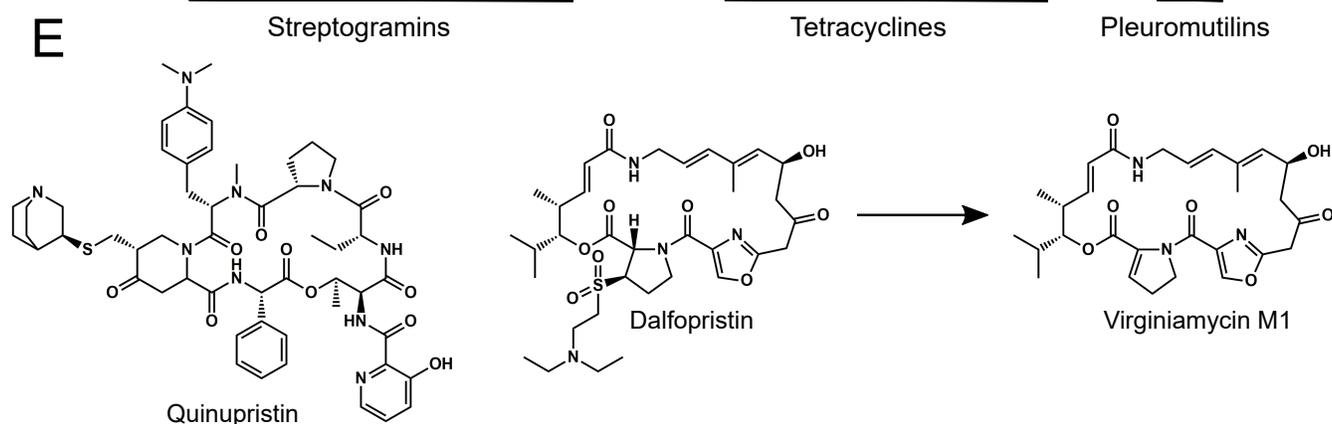
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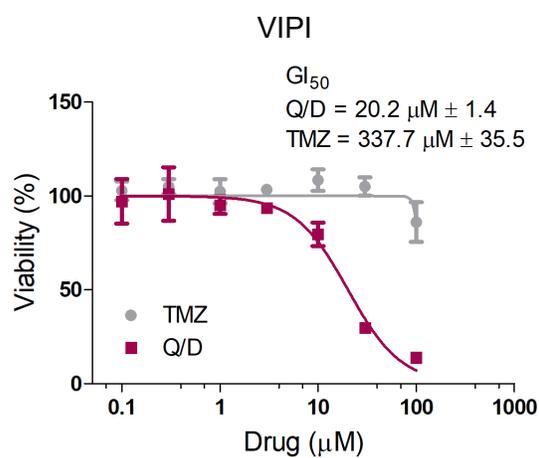
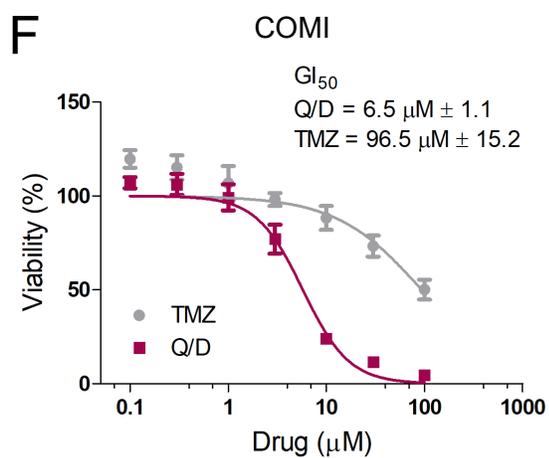
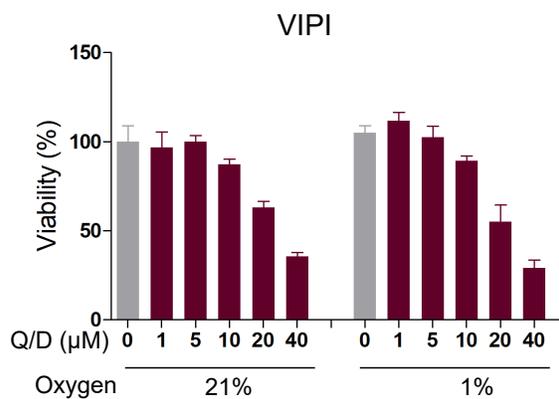
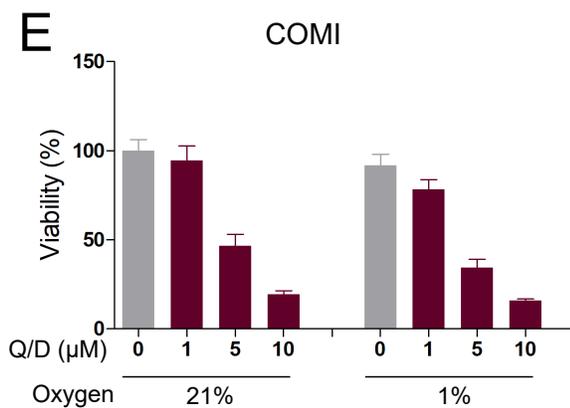
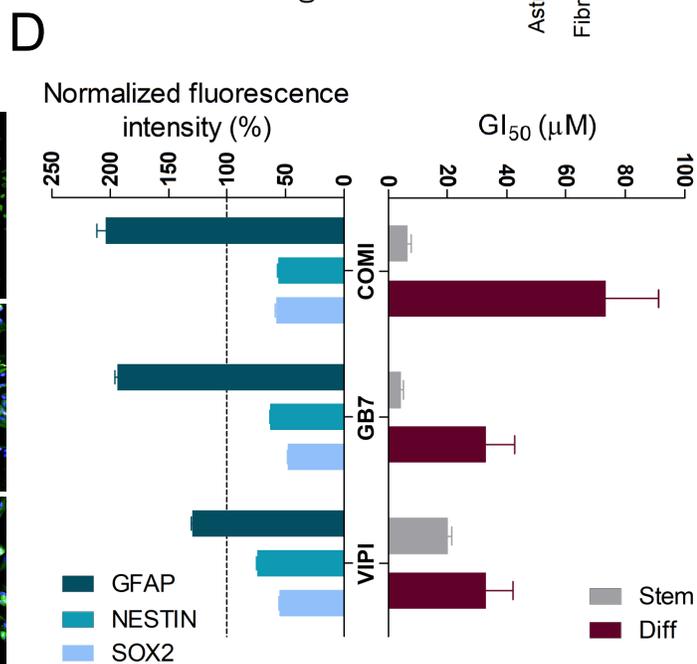
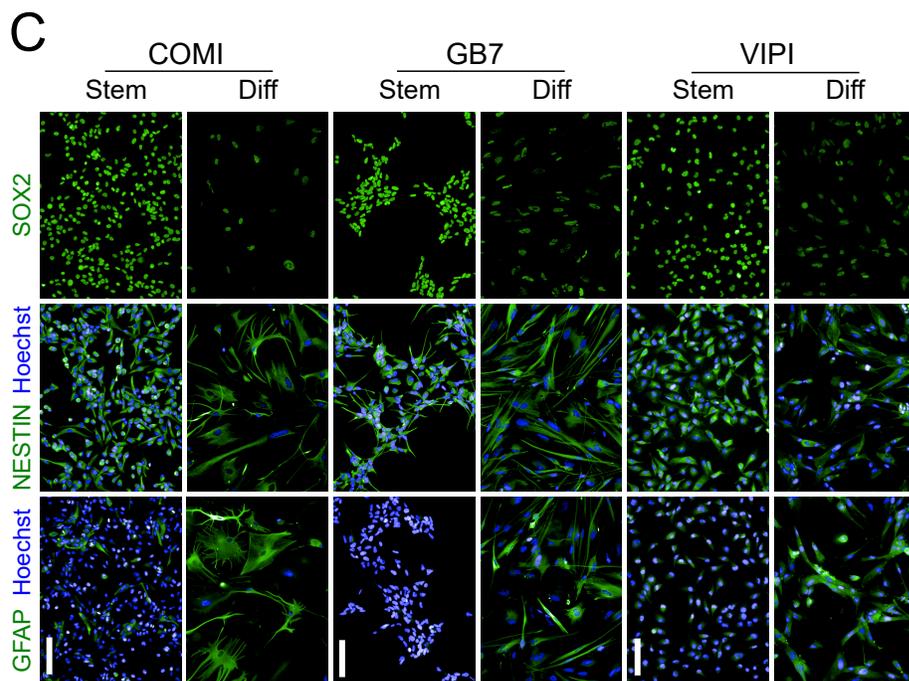
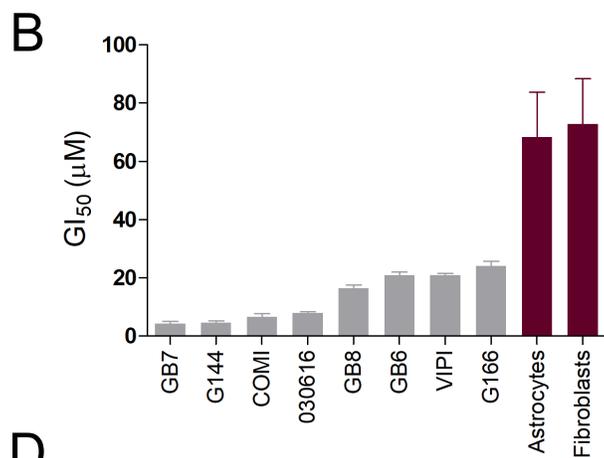
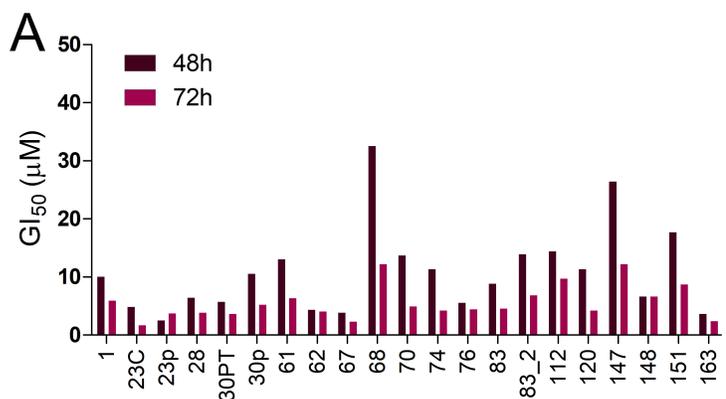
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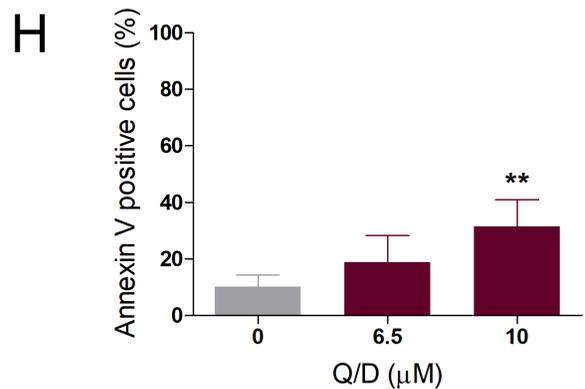
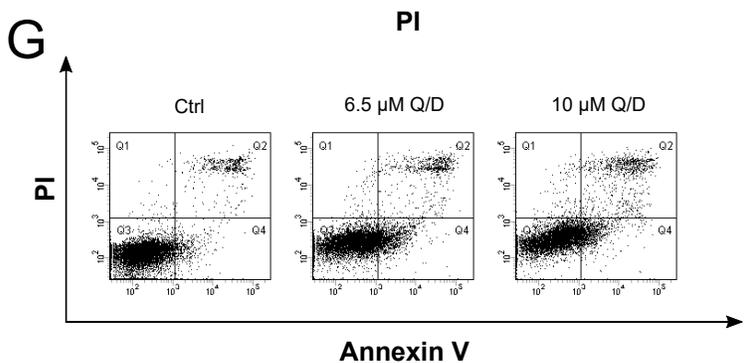
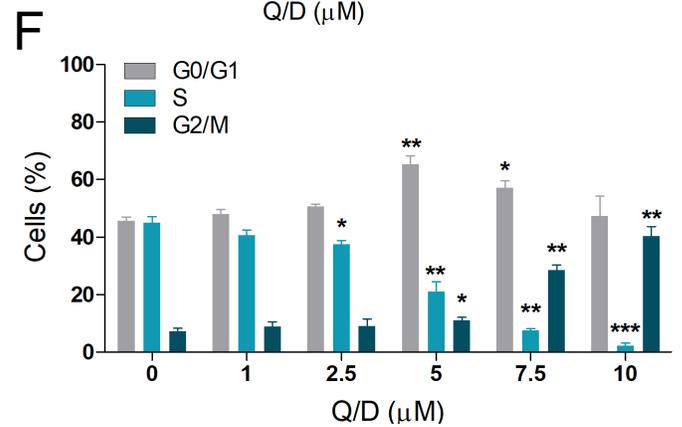
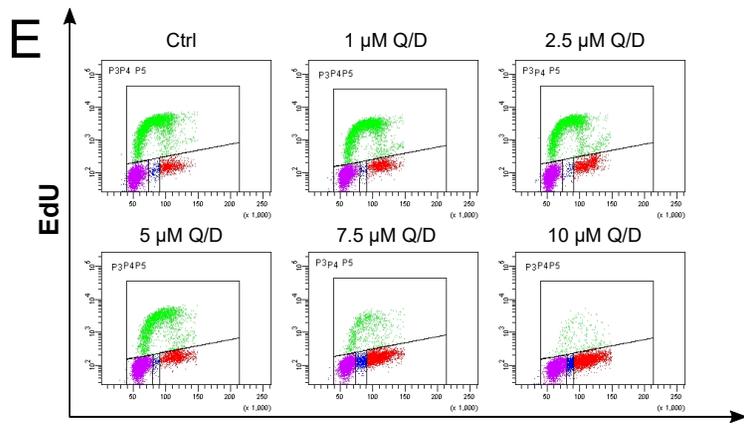
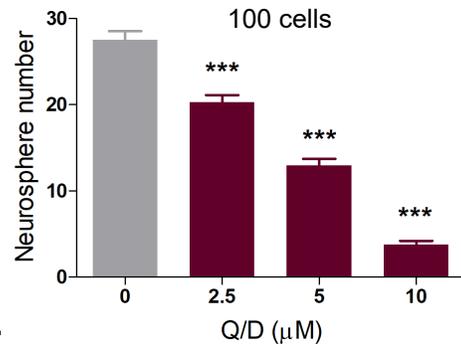
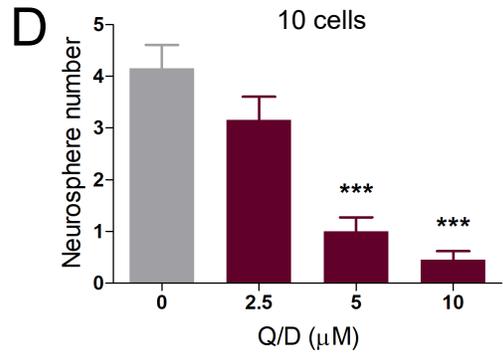
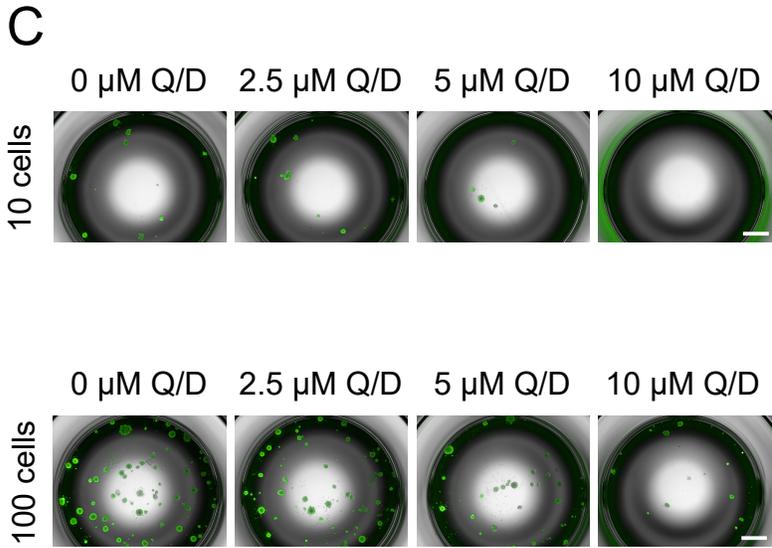
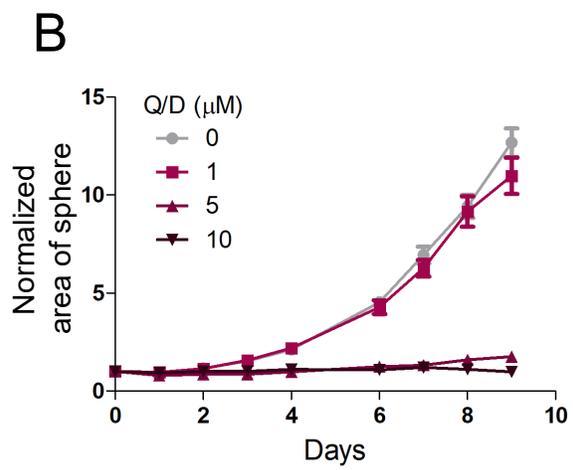
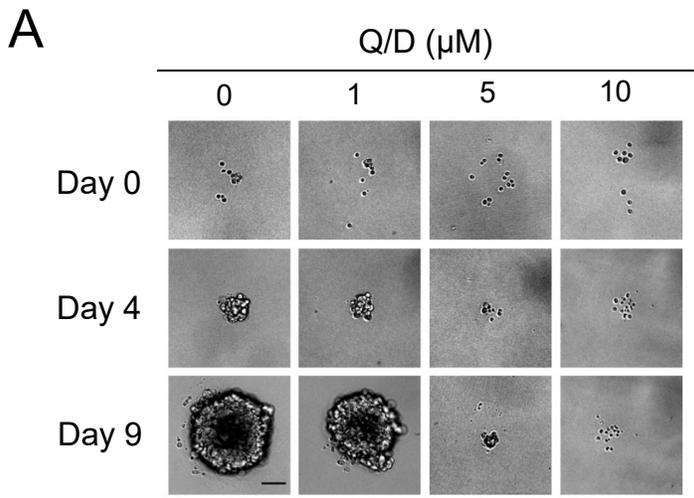
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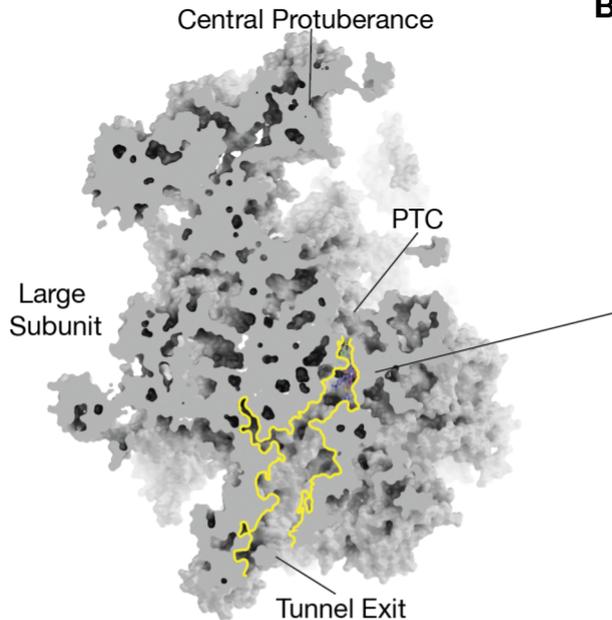
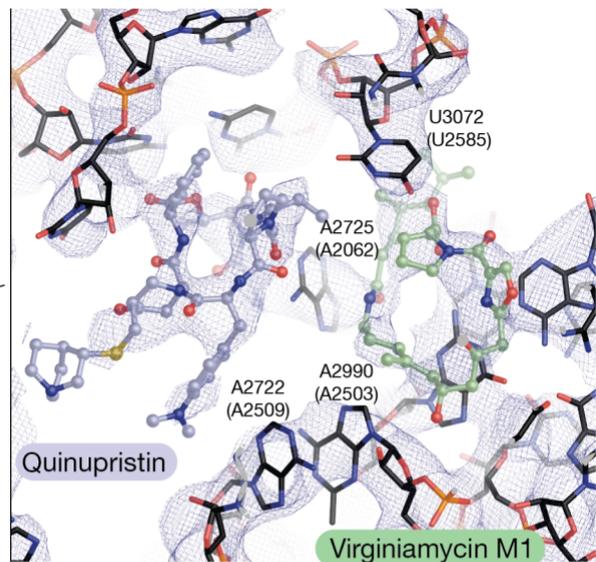
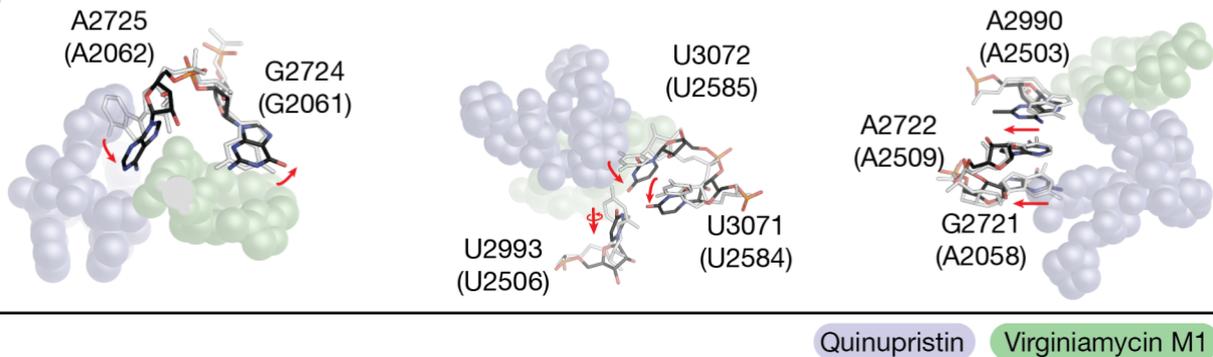
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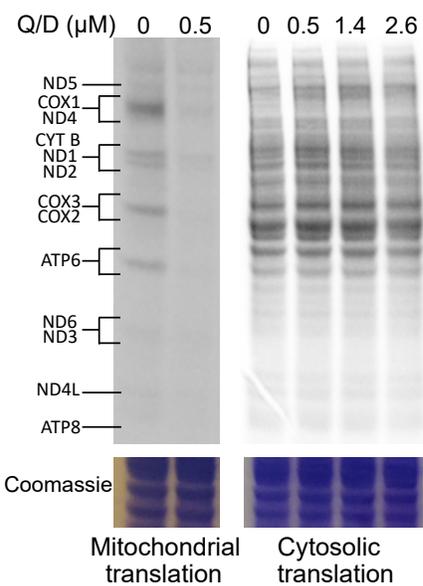
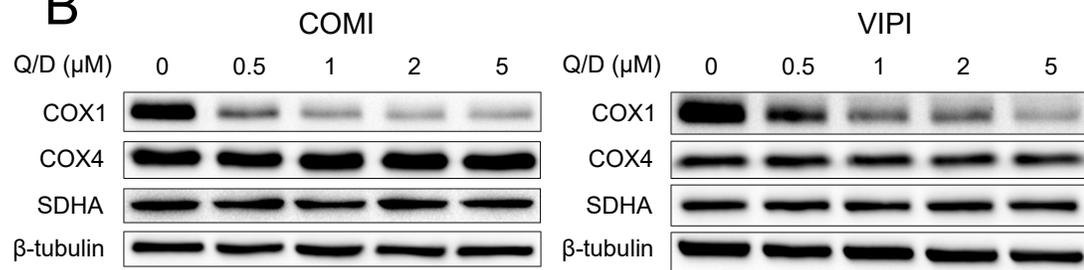
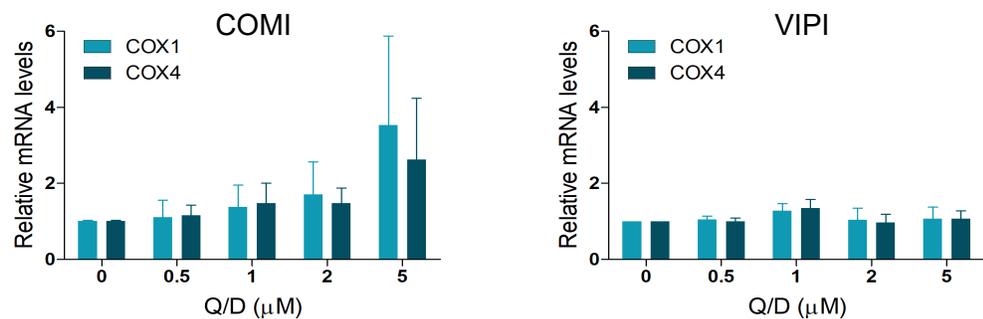
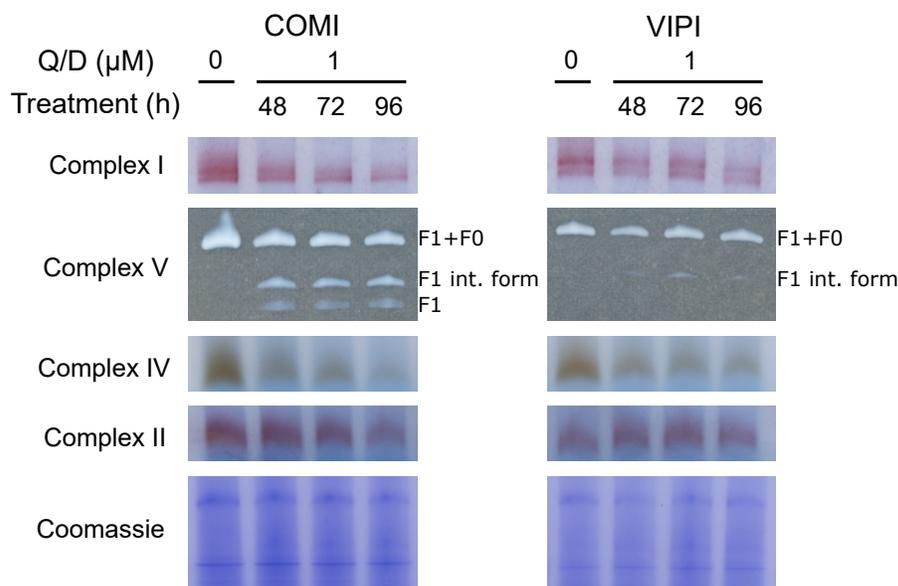
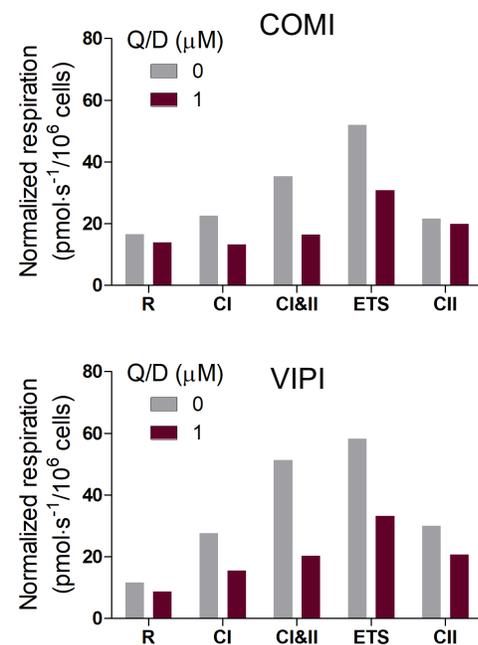
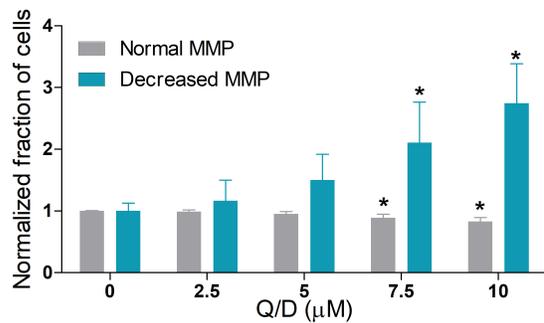
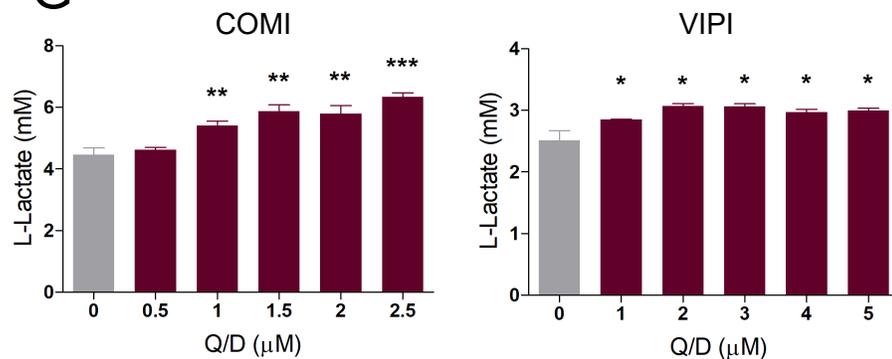
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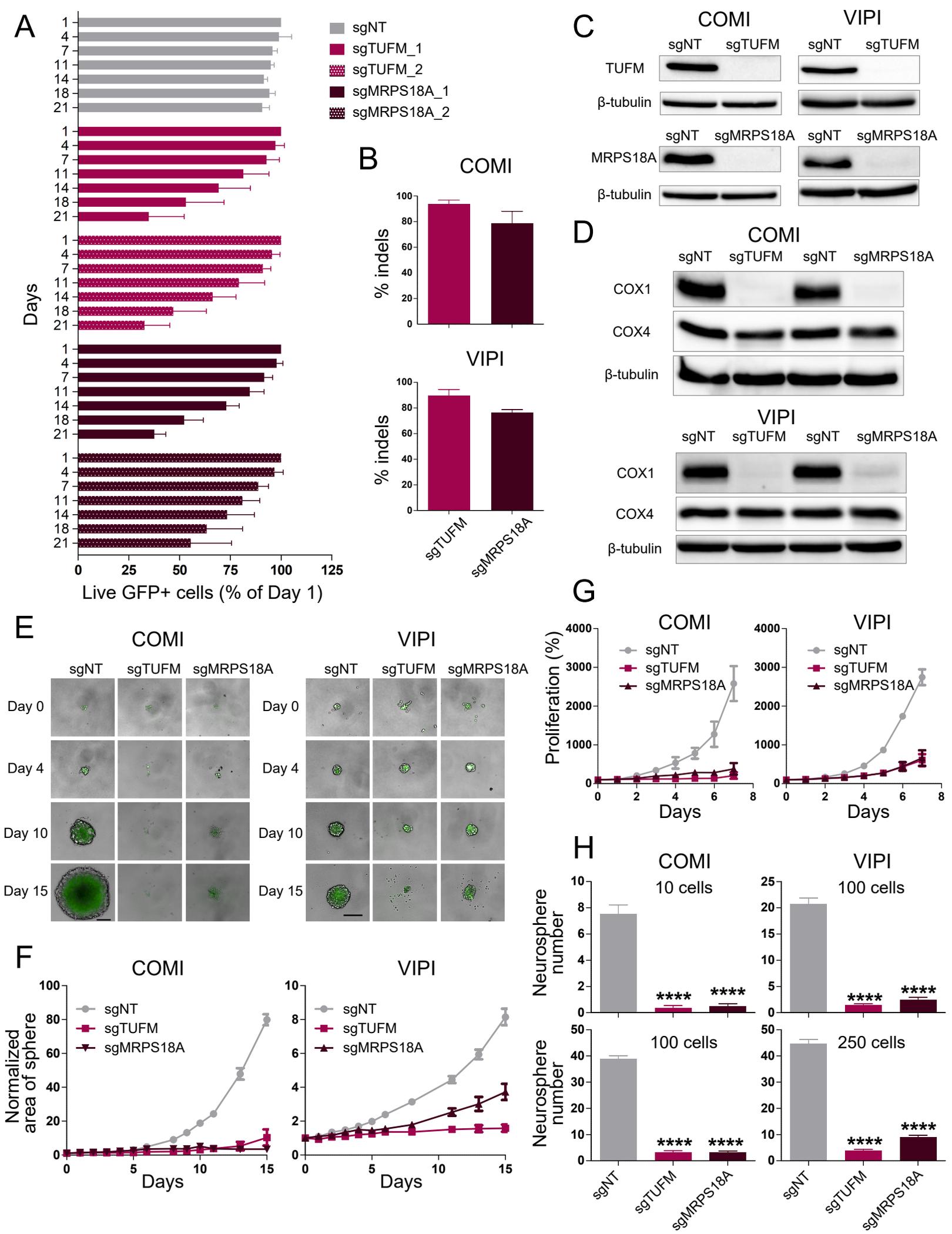
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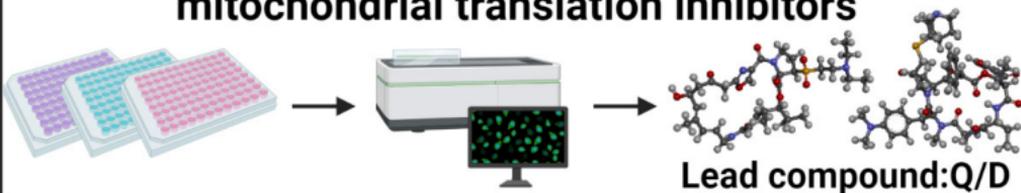


**A****B****C**

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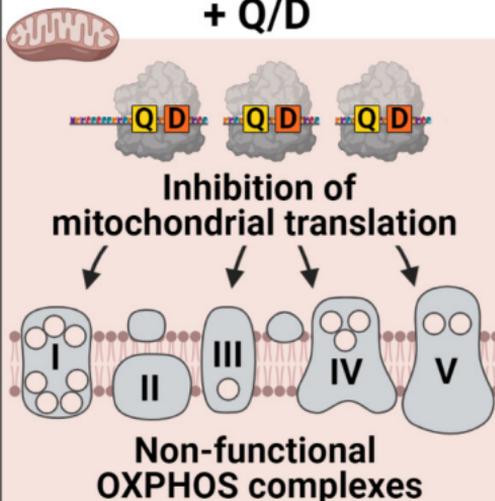
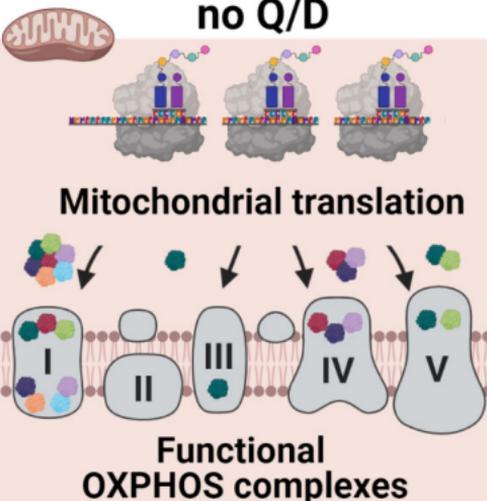
# Screening of antibiotics as mitochondrial translation inhibitors



## Effects of Q/D on glioblastoma stem cells (GSCs)

no Q/D

+ Q/D



GSC growth

Suppression of GSC growth