Metabolic changes upon GLS inhibition by CB-839 in glioma cell lines

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Introduction

Many tumors use Gln for both energy generation and as a biosynthetic precursor. Glutaminases (GAs) catalyze the first step of glutaminolysis by converting glutamine (Gln) into glutamate and ammonia in the mitochondria. In humans, two genes encode for glutaminases: *GLS* and *GLS2*. *GLS* is widely considered as a tumor promoting gene, and encodes for two GA isoenzymes named KGA and GAC, that are usually referred to as GLS isoenzymes. *GLS2* encodes for LGA and GAB isoforms and appears to have more complicated roles, including tumor-suppressive functions in some contexts. In glioma, *GLS2* is commonly silenced and *GLS* is usually overexpressed. We examined the metabolic consequences of inhibiting GLS activity in glioma cells by using the clinically relevant inhibitor CB-839. We treated three glioblastoma (GBM) cell lines with CB-839 and performed untargeted metabolomics and isotope tracing experiments using U-^{13}C-labeled Gln and ^{15}N-labeled Gln in the amido group to ascertain the metabolic fates of Gln carbon and nitrogen.

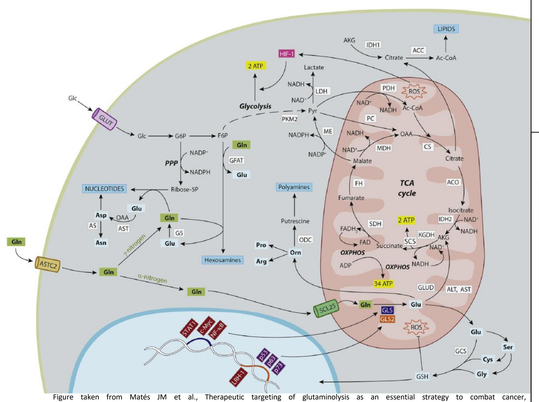


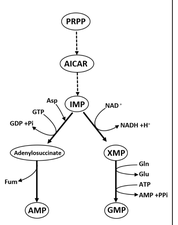
Figure taken from Matés JM et al., Therapeutic targeting of glutaminolysis as an essential strategy to combat cancer, Sem. in Cell & Dev. Biol. 98, 2020, 34-43

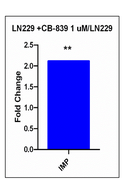
Methods

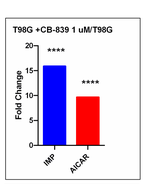
LN229, T98G and U87MG human GBM cell lines were cultured in standard conditions. In all cases, cells were treated with 1 μM CB-839 or vehicle (DMSO) for 24h. For untargeted metabolomics, after treatment cell dishes were washed with ice-cold normal saline solution. Then 1 mL 80% methanol was added per dish. Dishes were scraped on ice, transferred to microfuge tubes and subjected to three freeze-thaw cycles. Extracts were centrifuged for 15 min. at 17,000xg and supernatant was transferred to new tubes and dried overnight in a Speed-Vac concentrator. Metabolite pellet was resuspended and analyzed on an Agilent Quadrupole Time of Flight LCMS. For U-^{13}C- Gln tracing experiments, after 24h treatment, cells were washed with PBS and traced for 6h with media containing treatment and labeled Gln. After tracing, cells were washed with ice-cold saline, processed as described before, derivatized with tert-Butyldimethylsilyl ether (TBDMS) and analyzed on an Agilent GC-MS. For ^{15}N-Gln tracing, cells were traced for 6h, washed with saline and 0.5 mL 80% acetonitrile was added per dish. Samples were processed as described before and analyzed by LC-MS. Isotopic tracing data were expressed as fractions of the total metabolite. For untargeted metabolomics, metabolite abundances were normalized to the total ion count of each sample and data were expressed as a fold-change against the control sample. For tracing experiments, three independent experiments were made in triplicate; untargeted metabolomics was made twice, in triplicate. Heatmaps were generated by using the web tool Metaboanalyst; dot and bar graphs and statistical analysis (Student's T test for experimental against control samples) were made using GraphPad Prism software.

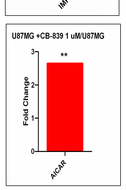
Nucleotide *de novo* biosynthesis

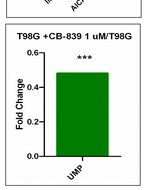
Metabolomics data showed that CB-839 treatment caused an accumulation in the levels of some intermediate metabolites in the *de novo* purine biosynthesis pathways, such as IMP in LN229 and T98G and AICAR in T98G and U87MG. Also, decreased levels of pyrimidine *de novo* biosynthesis pathways intermediate UMP were found in T98G treated with CB-839. Moreover, tracing experiments using Gln labeled with 15N in the amido in T98G treated with CB-839, showed decreased labeling in some purines and pyrimidine metabolites, and also in Asparagine.



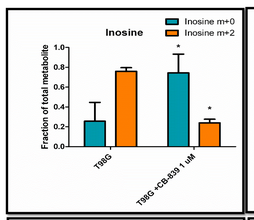


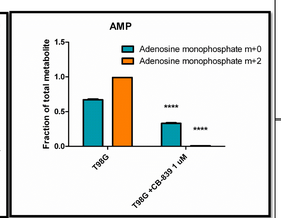


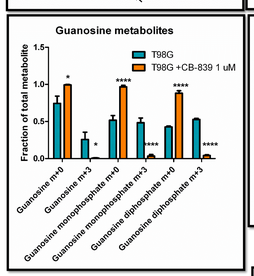


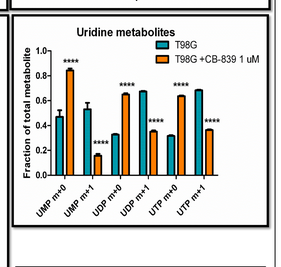


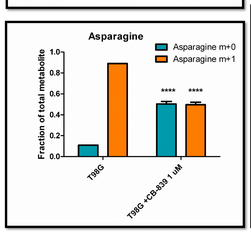
15N-Gln (Amido) tracing in T98G

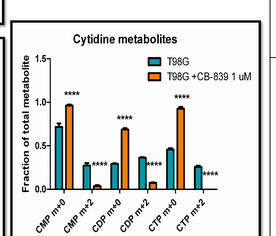








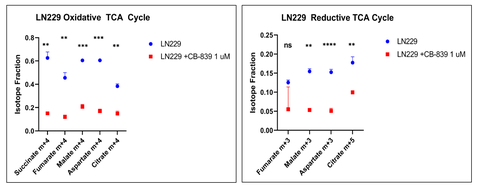




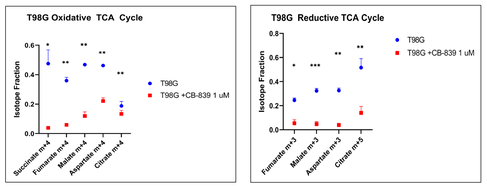
Tricarboxilic acid cycle related metabolites

Untargeted metabolomics results showed that CB-839 treatment significantly changed levels of many metabolites from different pathways. Regarding tricarboxilyc acid cycle (TCAC), there was a depletion of intermediates and related metabolites in the three human glioblastoma cell lines assayed, which showed an accumulation of glutamine and lower levels of downstream metabolites towards TCAC anaplerosis, including aspartate. This result was also confirmed by a lower oxidative labeling from U-^{13}C- Gln (m+4) in TCAC cycle intermediates succinate, fumarate, malate, citrate and also aspartate. U-^{13}C- Gln tracing also revealed reductive carboxylation-related labeling (m+3, m+5) from glutamine in fumarate, malate, aspartate and citrate in these cell lines, and this pathways was also significantly suppressed by CB-839.

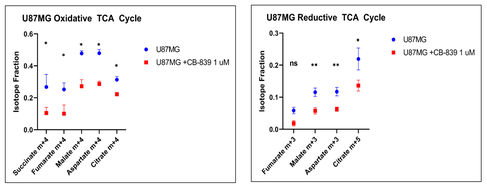
U13C-Gln tracing in LN229



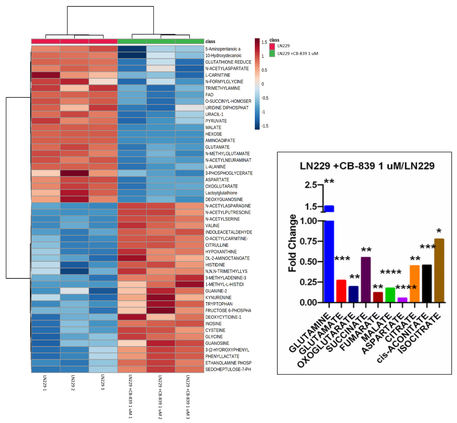
U13C-Gln tracing in T98G



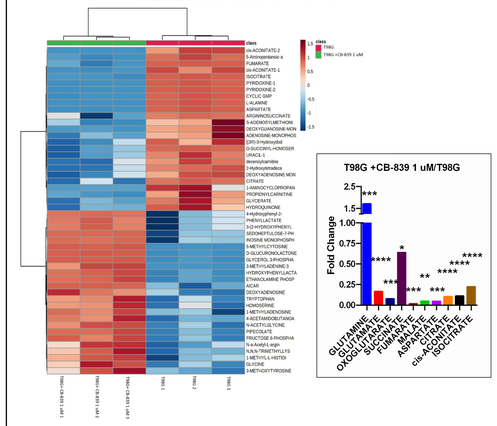
U13C-Gln tracing in U87MG



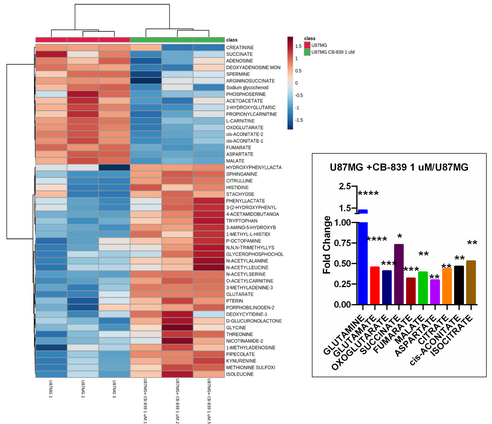
Metabolomics in LN229



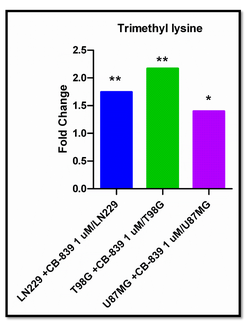
Metabolomics in T98G

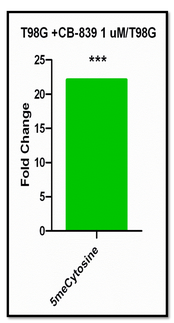


Metabolomics in U87MG

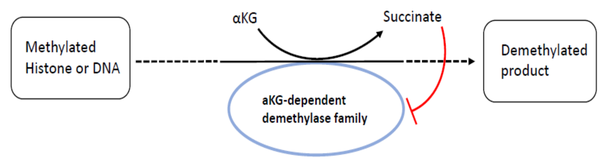


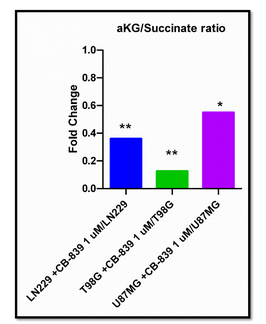
Changes in methylation patterns





Levels of methylated metabolite Trimethyl lysine were significantly increased in all three cell lines analyzed when treated with CB-839; on the other hand, T98G treated with CB-839 showed a 22-fold increase in 5-methyl-Cytosine levels. We wondered if higher levels of methylated metabolites could be attributable to a potential lower activity of Alpha-ketoglutarate-dependent demethylase family, which are implicated in a multi-step demethylation process of methylated substrates such as DNA or proteins, including histones. AKG-dependent demethylases use aKG as a substrate and generate succinate as a product, which in high levels can inhibit activity. Accordingly, we calculated the aKG/Succinate ratio and a significant lower ratio was found for all three cell lines.





Discussion

Although metabolomics showed lower levels of TCAC metabolites in LN229, T98G and U87MG treated with CB-839, there were prominent quantitative differences among the cell lines. T98G showed the largest decrease in TCAC metabolite levels, followed by LN229. U87MG showed smaller differences. This likely reflects differential dependencies of glioblastoma cells on GLS isoenzymes for maintaining levels of TCAC metabolites, which was also noted by a lower labeling from glutamine carbon in TCAC related metabolites. Interestingly, we found reductive-carboxylation associated labeling in all three cell lines, so it may be of interest to further study the importance of reductive-carboxylation pathways from glutamine-derived alpha-ketoglutarate in glioma. CB-839 also decreased nucleotide biosynthesis, causing accumulation of purine *de novo* biosynthesis intermediates IMP and/or AICAR and depletion of UMP; using glutamine labeled with 15N in the amido group, which functions as a nitrogen donor group for different pathways, we found a significant lower labeling in many nucleotides and in asparagine in T98G, all of this is most likely explained due to aspartate depletion. CB-839 also appeared to increase methylation in nucleotides and proteins, noted by increased levels of 5-methyl-cytosine in T98G, and trimethyllysine in all three cell lines. These metabolites likely arise from degradation of DNA and proteins, including histones. We speculate that these effects result from a reduced activity of α-KG-dependent demethylases, which use α-KG as a substrate and can be inhibited by succinate or fumarate. Accordingly, the α-KG/succinate ratio was calculated and we found that was significantly decreased in all three CB-839 treated cell lines.

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