Anti-Tumor Activity of Novel, Potent and Orally-Bioavailable Glutaminase Inhibitors

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Abstract

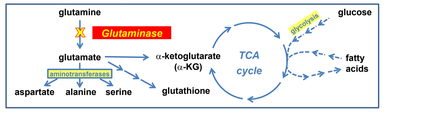
Glutamine is required for the growth of a broad range of tumor cells. An important step in the metabolism of glutamine is its conversion to glutamate, which is catalyzed by the mitochondrial enzyme glutaminase. GLS, the form of the enzyme expressed in most cells, is up-regulated in a sub-set of glutamine-requiring tumor cells. Suppression of GLS by genetic knockdown or with small molecule inhibitors slows the growth of these tumor cells. To further evaluate GLS as an oncology therapeutic target, we have developed a series of novel and potent GLS inhibitors that are orally bioavailable, permitting both *in vitro* and *in vivo* analysis of GLS inhibition.

In a reconstituted system with purified enzyme, the inhibitor CB-498 inhibited GLS with an IC_{50} of <15 nM. Kinetic analyses demonstrated that the mechanism of inhibition is allosteric, time dependent and slowly reversible. In a panel of over 30 tumor cell lines, CB-498 had broad anti-proliferative activity across multiple tumor cell types with IC_{50}'s ranging from 5 nM to 300 nM. While the cellular anti-proliferative response was cytostatic in the majority of cell lines, a few cell lines underwent apoptosis. Cellular levels of glutamate decreased and glutamine levels increased in a dose-dependent manner following CB-498 treatment, consistent with GLS inhibition. Flux studies with uniformly $^{15}N_2$ ^{13}C-labeled glutamine demonstrated that CB-498 blocks the incorporation of glutamine-derived carbon or nitrogen into TCA cycle intermediates, pyruvate, lactate, fatty acids and amino acids coupled to glutamate through transamination. Furthermore, a membrane permeable form of α-ketoglutarate reversed the anti-proliferative effects of CB-498 in most cell lines, suggesting that the anti-tumor activity of CB-498 primarily arises from decreased glutamine flux through the TCA cycle.

CB-839 is an orally bioavailable analog of CB-498 with comparable biochemical and cellular potency but with improved solubility and pharmacokinetic properties. In an H2122 lung adenocarcinoma xenograft model, CB-839 showed single-agent anti-tumor activity. This anti-tumor activity was associated with increased tumor glutamine levels and decreased levels of glutamate and aspartate. Importantly, these glutamate and aspartate decreases were not seen in kidney, liver or plasma, indicating a tumor specific response to GLS inhibition. Oral administration of CB-839 resulted in significant plasma exposure across multiple species and has been well tolerated in *in vivo* studies in rodents on either daily or twice-daily dosing schedules. These results motivate a further investigation of these potent and selective GLS inhibitors for clinical utility in oncology.

Introduction

Many tumors rely on the catabolism of glutamine to produce metabolic intermediates that fuel bioenergetic and biosynthetic demands (Ref 1, 2). Glutaminase initiates this process by converting glutamine to glutamate which is subsequently used in multiple reactions that support tumor cell growth and survival, including the generation of energy (TCA cycle), synthesis of amino acids and production of glutathione.

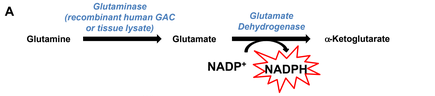


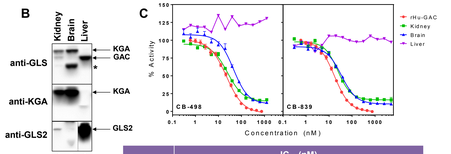
Two human genes encode glutaminase proteins, GLS (broadly expressed) and GLS2 (primarily expressed in liver). The GLS gene produces two alternatively spliced variants, KGA and GAC, that function as homo-tetrameric enzymes. Inhibition of these forms of glutaminase with siRNA knockdown or small molecules results in anti-proliferative effects in tumor cells both *in vitro* and *in vivo* (Ref 2, 3, 4, 5). Furthermore, the KGA and GAC splice variants show altered expression patterns in tumors (Ref 5) and their expression is associated with the aggressiveness of the cancer (Ref 6).

We have developed a series of novel, potent, and selective glutaminase inhibitors that are orally bioavailable and show *in vitro* and *in vivo* anti-tumor activity. Our findings provide a strong preclinical rationale for the further development of glutaminase inhibitors in oncology.

Biochemical Potency and Selectivity

CB-498 and CB-839 are potent and selective glutaminase inhibitors



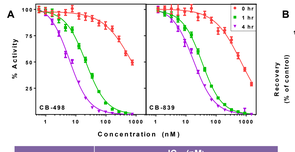


Inhibitor	IC_{50} (nM)			
	rHu-GAC	Kidney (KGA)	Brain (KGA)	Liver (GLS2)
CB-498	22	25	44	>5000
CB-839	34	24	34	>5000

Figure 1: (A) Schematic representation of the glutaminase-glutamate dehydrogenase (GDH) coupled reaction. Glutaminase converts glutamine to glutamate. GDH utilizes glutamate to convert $NADP^+$ to NADPH which can be measured fluorometrically [Ex:340 nm- Em:460 nm]. Glutaminase activity has been measured with a recombinant form of human glutaminase (rHu-GAC) and in lysates prepared from mouse tissues. **(B) Kidney and brain homogenates contain primarily KGA whereas liver homogenate contains primarily GLS2.** Lysates (20 µg) from mouse kidney, brain and liver were separated by SDS-PAGE, transferred to nitrocellulose and immunoblotted with either anti-GLS (which recognizes both splice variants of GLS, KGA and GAC), anti-KGA or anti-GLS2 antibodies. Arrows indicate bands corresponding to KGA, GAC and GLS2. Asterisk denotes anti-GLS reactive band in brain homogenate that likely corresponds to a KGA degradation product. **(C) CB-498 and CB-839 are potent inhibitors of both GAC and KGA splice variants of GLS.** Compounds were dose titrated and incubated with either purified rHu-GAC, mouse kidney lysate, mouse brain lysate or mouse liver lysate for 1 h prior to addition of glutamine. Upon addition of glutamine, NADPH production was monitored by fluorescence for 5 min and initial velocities were calculated and normalized to uninhibited control. Percent activity was plotted against compound concentration and resulting curves were fit using a four-parameter dose response to determine IC_{50} values.

Biochemical Potency and Selectivity

Glutaminase inhibition by CB-498 and CB-839 is time-dependent and slowly reversible



Inhibitor	IC_{50} (nM)		
	0 hr	1 hr	4 hr
CB-498	730	22	6
CB-839	670	34	18

Figure 2: (A) CB-498 and CB-839 show time-dependent inhibition of rHu-GAC. Compounds were dose titrated and preincubated with rHu-GAC for the indicated times prior to addition of glutamine. IC_{50} values were determined as described in (Figure 1C). **(B) Recovery of rHu-GAC activity following CB-498 or CB-839 inhibition is slow.** CB-498 or CB-839 were preincubated with rHu-GAC for 90 min prior to separation of free inhibitor from rHu-GAC:inhibitor complex by gel filtration immediately followed by dilution into GDH-containing buffer. Progress curves were generated at indicated times by addition of glutamine. Initial velocities for inhibitor-treated rHu-GAC were normalized to untreated rHu-GAC to calculate extent of recovery. The kinetics of recovery of rHu-GAC activity is plotted for each inhibitor. For CB-839, the half-life of recovery is calculated to be 45 min by fitting the recovery data to a one-phase association equation.

In Vitro Anti-Proliferative Activity

Glutaminase inhibitor has anti-proliferative activity in a wide-range of tumor cell types

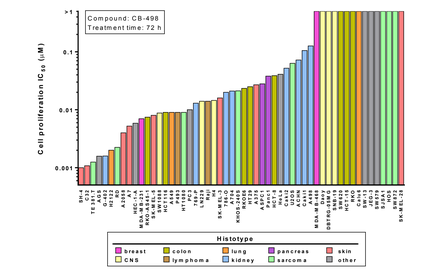


Figure 3: CB-498 has broad anti-proliferation activity across a panel of tumor cell lines representing a range of histotypes. Cells were treated with a range of CB-498 concentrations for 72 h in RMPI-1640 media containing 10% fetal bovine serum and 2 mM glutamine. The effect on cell proliferation was measured using CellTiterGlo reagent (Promega) or by cell counting. IC_{50} values were calculated and graphed in ascending order of sensitivity.

Sensitivity to glutaminase inhibition correlates with sensitivity to glutamine withdrawal

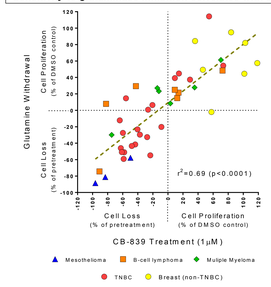


Figure 4: The effects of glutamine withdrawal and treatment with CB-839 on cell proliferation or cell loss are correlated across a panel of breast cancer, B-cell lymphoma, multiple myeloma and mesothelioma cell lines. Cells were plated in RMPI-1640 media containing 10% FBS and 2 mM glutamine. After 24 h the media was replaced with either glutamine-free media (glutamine withdrawal) or media containing 1µM CB-839. Cell proliferation and cell loss was measured after 72 h using the CellTiterGlo reagent by comparison with the CellTiterGlo signal measured at the time of glutamine withdrawal or CB-839 addition (t=0). For cell lines where the signal was greater than at t=0, proliferation was calculated as a percentage of growth relative to cells treated with DMSO in complete media (with glutamine). For cell lines where the signal was less than at t=0, cell loss was calculated as the percentage reduction relative to the t=0 signal. Each datapoint on the bivariant plots depicts an individual cell line (response to glutamine withdrawal on the y-axis and response to CB-839 on the x-axis). TNBC=triple-negative breast cancer.

In Vitro Anti-Proliferative Activity

The anti-proliferative effect of glutaminase inhibition is correlated with glutamine/glutamate changes and is reversed by the downstream product α-KG

Cell line: MDA-MB-231 Compound: CB-839

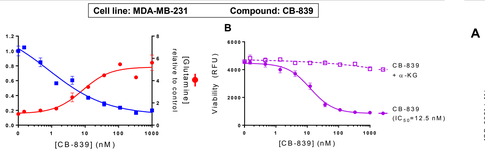


Figure 5: (A) CB-839 treatment results in an increase in cellular glutamine (substrate) and a decrease in cellular glutamate (product). MDA-MB-231 breast adenocarcinoma cells were treated with CB-839 for 24 h. Cells were harvested and the amounts of glutamine and glutamate were measured by LC/MS/MS. The measured concentrations were normalized to cell number determined on a parallel plate and plotted as a fraction relative to the DMSO control. **(B) The anti-proliferative activity of CB-839 is correlated with changes in glutamine/glutamate levels (see panel A) and is reversed by addition of the downstream product α-ketoglutarate (α-KG).** MDA-MB-231 cells were treated with a range of CB-839 concentrations for 72 h in the presence or absence of 1 mM di-methyl α-KG, a membrane permeable form α-KG. The effect on cell proliferation was measured using CellTiterGlo.

Impact on Cellular Metabolite Levels and Metabolic Flux

Glutaminase inhibition decreases levels of metabolites downstream of glutamate

Cell line: HCT-116 Compound: CB-498

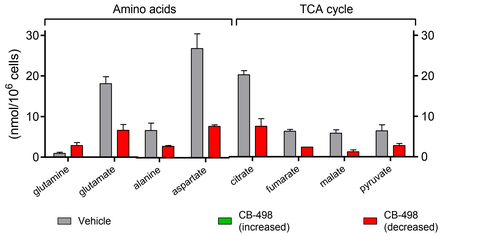


Figure 6: Glutaminase inhibition decreases the levels of metabolites linked to glutamate, including amino acids generated via transaminases and TCA cycle intermediates downstream of α-KG. HCT116 colon carcinoma cells grown in RMPI-1640 with 10% FBS and 2 mM glutamine were treated with 0.5 µM CB-498 or vehicle control (DMSO). After 24 h, cells were harvested and cell number was determined for each condition. The absolute levels of individual metabolites in cellular extracts were determined by GC/MS and amount of each metabolite per 10^6 cells was calculated.

Glutaminase inhibition decreases glutamine flux and has minimal impact on glucose flux

Cell line: HCT-116 Compound: CB-498

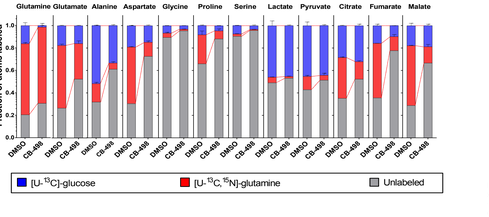


Figure 7: Glutaminase inhibition reduces the incorporation of atoms from isotopically-labeled glutamine into multiple cellular metabolites with minimal impact on metabolic flux originating from isotopically-labeled glucose. HCT116 colon carcinoma cells were incubated in RMPI-1640 with 10% FBS containing either 2 mM $^{13}C_5$,$^{15}N_2$-Glutamine and 2 g/L unlabeled glucose or 2 g/L $^{13}C_6$-Glucose with unlabeled 2 mM glutamine for 24 h in the presence or absence of 0.5 µM CB-498. After 24 h, cells were harvested and cell number was determined for each condition. The total amount of metabolite in the cell population was determined by GC/MS and normalized to cell number in order to calculate the amount of metabolite per cell. For each condition, the fraction of atoms labeled by glucose or glutamine was determined for each metabolite by summing the total number of labeled atoms and normalizing it to the total amount of metabolite.

Oral Bioavailability, Pharmacodynamic Response and In Vivo Efficacy

Oral dosing of CB-839 in rats results in prolonged plasma exposure that is well-correlated with a dose-dependent increase in plasma glutamine (a pharmacodynamic marker)

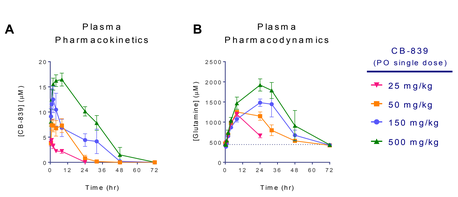
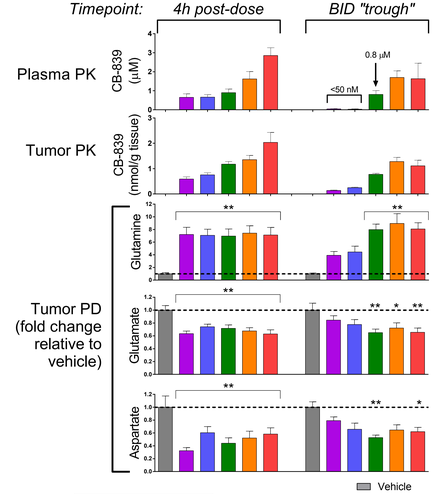


Figure 8: (A) CB-839 is orally bioavailable in rats (>50 %F) and generates dose-dependent and sustained exposure after a single dose. (B) A single oral dose of CB-839 elicits a sustained and dose-dependent pharmacodynamic response (increase in plasma glutamine) that is correlated with drug exposure. CB-839 was administered as a single oral gavage to female Sprague Dawley rats (n=3 per group) and plasma samples were collected at various times post-dose. Samples were analyzed by LC/MS/MS for the concentration of CB-839 (A) and glutamine (B). Plasma concentration vs. time profiles are plotted.

Tumor pharmacodynamic response is maximal at 25 mg/kg CB-839 in mice



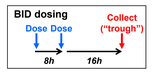


Figure 9: CB-839 elicits a sustained pharmacodynamic response in HCC-1806 (triple negative breast cancer) tumor xenografts. Female scid/bg mice were implanted with HCC1806 xenografts in the mammary fat pad. When tumors reached ~500mm^3, groups of n=5 mice were treated with vehicle or increasing dose levels of CB-839. Four hours after a single dose or 24 h after a series of 2 doses (dose at 0 and 8 h, harvest at 24 h (BID "trough")), tumors were collected and glutamine, glutamate and aspartate were measured by LC/MS/MS. The level of metabolites in tumors from CB-839 treated animals were normalized to the corresponding vehicle control values and are presented as fold change relative to vehicle. Plasma and tumor samples were also analyzed by LC/MS for CB-839 levels. CB-839 treatment results in increased tumor glutamine concentrations and reduced glutamate and aspartate concentrations confirming glutaminase inhibition in the tumor. The magnitude of the pharmacodynamic response (glutamine increase and glutamate/aspartate decrease) was comparable across all dose levels 4 h after CB-839 dosing despite a greater than 4-fold increase in CB-839 exposures across the range of doses, suggesting maximal pharmacodynamic effect at 25 mg/kg. When plasma CB-839 concentrations are maintained above 600 nM, the magnitude of pharmacodynamic response is also maintained through 24 h (16 h after a series of two doses). When plasma CB-839 levels dropped below 100 nM, the pharmacodynamic response was reduced.

CB-839 has wide tissue distribution (except brain) after PO dosing and elicits a pharmacodynamic response that is more pronounced in tumor than in normal tissues

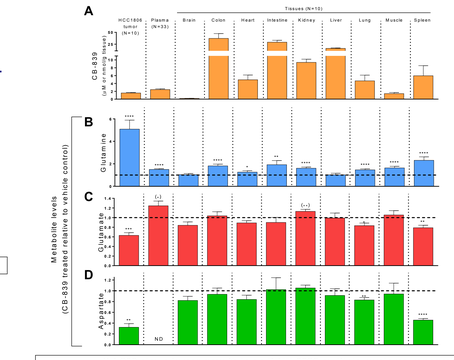


Figure 10: CB-839 produces a strong pharmacodynamic response in xenografted HCC-1806 triple-negative breast cancer tumors that includes an increase in glutamine and decreases in glutamate and aspartate. (A) CB-839 exposure is high in all tissues evaluated, except brain. CB-839 was administered as a single oral gavage at 200 mg/kg to scid/bg mice bearing HCC-1806 tumors xenografted in the mammary fat pad. Four hours post-dose, plasma, tumor and tissues samples were collected and flash frozen in liquid nitrogen. Drug levels were measured by LC.MS. **(B) Glutamine increases are greatest in tumor but are significant in all other tested tissues except liver and brain.** Glutamine levels in tumor, normal tissues and plasma from CB-839-treated animals were quantified by LC/MS/MS and compared to corresponding samples from vehicle-treated animals. Relative increase/decrease in glutamine levels in tumor/tissues/plasma from CB-839-treated versus vehicle-treated animals are plotted. Significant increases in glutamine are noted by: * (p < 0.05); ** (p <0.01); *** (p< 0.001); and **** (p < 0.0001). Dotted line denotes no change in glutamine levels. **(C,D) Glutamate and aspartate decreases are significant in tumor and a sub-set of tissues**. Glutamate and aspartate levels in tumor, normal tissues, and plasma from CB-839-treated animals were quantified by LC/MS/MS and compared to corresponding samples from vehicle-treated animals. Relative increase/decrease in **(C)** glutamate and **(D)** aspartate levels in tumor tissues/plasma from CB-839-treated versus vehicle-treated animals are plotted. Following CB-839 treatment, glutamate and aspartate levels decrease significantly in tumor, lung and spleen [see (B) above for significance key]. Following CB-839 treatment, small increases in glutamate levels in plasma and kidney are observed [indicated by (-) and (+)].

CB-839 slows tumor growth in an RPMI-8226 multiple myeloma xenograft model at well-tolerated doses

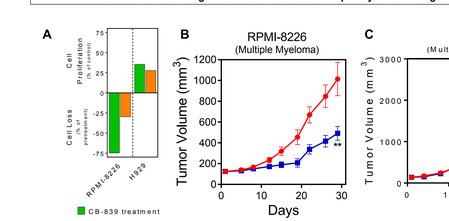


Figure 11: CB-839 has *in vitro* and *in vivo* anti-tumor activity in an RPMI-8266 but not a H929 multiple myeloma model. (A) RPMI-8266 multiple myeloma cell line but not H929 multiple myeloma cell line is sensitive to CB-839 treatment and glutamine withdrawal. RPMI-8226 and H929 cells were plated in RMPI-1640 media containing 10% FBS and 2 mM glutamine. After 24 h the media was replaced with either glutamine-free media (glutamine withdrawal) or media containing 1µM CB-839. Cell proliferation and cell loss was measured after 72 h using the CellTiterGlo reagent by comparison with the CellTiterGlo signal measured at the time of glutamine withdrawal or CB-839 addition (t=0). Calculations for cell proliferation and cell loss were performed as described in Figure 4. **(B) Anti-tumor efficacy is observed in an RPMI-8226 multiple myeloma model.** Groups of n=10 female CB.17 SCID mice were implanted with 1 x 10^7 RPMI-8226 myeloma cells mixed 1:1 with matrigel SC in flank. BID dosing with CB-839 (200 mg/kg) or vehicle was started when tumors reached ~150mm^3. Tumor growth inhibition of 59% was achieved (**, p < 0.01). **(C) Anti-tumor efficacy is not observed in a H929 multiple myeloma xenograft model.** Groups of n=8 female CB.17 SCID mice were implanted with 1 x 10^7 H929 myeloma cells mixed 1:1 with matrigel SC in flank. BID dosing with CB-839 (200 mg/kg) or vehicle was started when tumors reached ~150mm^3. **(D) CB-839 is well tolerated on a multi-day BID dosing schedule at 200 mg/kg.** An efficacious dose of CB-839 (200 mg/kg BID) has no impact on weight gain in mice from xenograft efficacy studies. Weight change is plotted relative to the body weight taken before the start of dosing (Day 0) (upper panel: RPMI-822 study; lower panel: H929 study).

Conclusions

- **CB-839 and CB-498 are novel, potent, small molecule glutaminase inhibitors**
- **CB-839 and CB-498 have on-target *in vitro* anti-proliferative activity as demonstrated by:**
  - **α-ketoglutarate rescue experiments**
  - **Correlation with changes in steady state metabolite levels (↑ glutamine and ↓ glutamate)**
  - **Impact on levels of TCA intermediates and on metabolic flux through pathways downstream of glutamine and glutamate**
  - **Concordance with glutamine withdrawal sensitivity**
- **CB-839 and CB-498 have *in vitro* antiproliferative activity on a wide range of tumor types**
- **CB-839 is orally bioavailable with prolonged exposure in rodents**
- **There is a dose dependent pharmacodynamic response in rodents treated with CB-839**
- **In tumor-bearing mice, CB-839 induces pharmacodynamic changes in the tumor (↑ glutamine, ↓ glutamate, and ↓ aspartate) with minor effects in other tissues**
- **CB-839 has significant single agent anti-tumor efficacy in a RPMI-8226 multiple myeloma xenograft model**
- **The development of CB-839 in oncology is supported by the preclinical data in this study**

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