Antitumor Activity of Glutaminase Inhibitor CB-839 in Hematological Tumors

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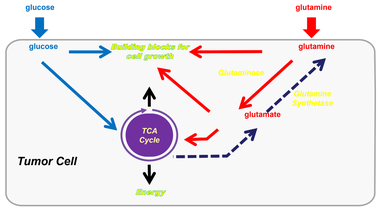
Abstract- Updated

Many tumor cells are dependent on glutamine (Gln) for growth and survival. Gln is the most abundant amino acid in plasma and tumor cells can utilize Gln for energy production and generation of building blocks for the synthesis of macromolecules. A key step in Gln utilization is its conversion to glutamate (Glu) by the mitochondrial enzyme glutaminase (GLS). Suppressing GLS activity via either siRNA knockdown or small molecule inhibitor treatment has anti-proliferative activity in several tumor types. mRNA levels for splice variant GAC are elevated in several hematological malignancies, particularly those derived from the B-cell lineage including multiple myeloma (MM), acute lymphocytic leukemia (ALL), chronic lymphocytic leukemia (CLL) and several types of non-Hodgkin's lymphoma (NHL). High GAC expression is observed in triple negative breast cancer (TNBC), a tumor type that we and others have shown to be highly dependent on exogenous Gln for cell proliferation and sensitive to GLS inhibitor treatment. CB-839 is a novel, potent, orally bioavailable GLS inhibitor that inhibits recombinant human GLS with an IC_{50}~30 nM. Given the similarity of the GAC expression pattern observed in TNBC with some hematologic malignancies, we evaluated the anti-tumor activity of CB-839 in cell culture and xenograft models of hematological malignancies predicted to be dependent on Gln for growth. Across a panel of 44 cell lines derived from MM, NHL and ALL, Gln deprivation resulted in a minimum of 70% reduction in growth for 41 cell lines and induced cell death in 11 cell lines. Across the same panel of cell lines, the majority were sensitive to CB-839 treatment with 36 cell lines exhibiting an anti-proliferative IC_{50} of < 100nM (range 2-98 nM). Importantly, there was a strong correlation between sensitivity to Gln withdrawal and CB-839 sensitivity (r=0.56, p<0.001), suggesting that the dominant pathway by which Gln supports tumor cell growth and survival is through its conversation to Glu by GLS. The in vivo antitumor activity of CB-839 was also evaluated in a xenograft model using the multiple myeloma RPMI-8226 model. Oral, twice daily dosing of CB-839 resulted in a significant decrease in tumor volume (71% tumor growth inhibition). As observed in vitro, CB-839 dosing caused an increase in tumor Gln levels and a decrease in the level of tumor Glu. In a dose dependent fashion, there was a correlation between tumor Gln accumulation, tumor GLS inhibition and antitumor activity. Efficacious doses of CB-839 were well tolerated with no effect on hematological cell counts or body weight. These data suggest that GLS inhibition with CB-839 may provide therapeutic benefit in hematological malignances and motivate the evaluation of this compound in clinical trials that include patients with B-cell malignancies.

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 two 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.

Glutaminase Expression Analysis

Higher Glutaminase Expression in B-cell-derived Malignancies as Compared to Normal B-cells

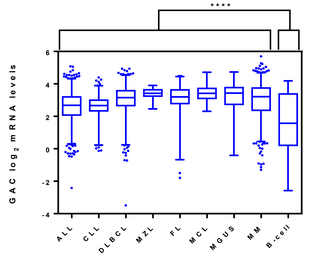
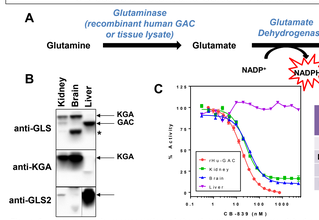


Figure 1: High mRNA expression of GAC in B-cell-derived malignancies as compared to normal B-cells. Box and whisker plot representing the distribution of GAC mRNA levels in acute lymphocytic leukemia (ALL; n=1989), chronic lymphocytic leukemia (CLL; n=605), diffuse large B-cell lymphoma (DLBCL, n=1118), marginal zone lymphoma (MZL, n=39), follicular lymphoma (FL, n=243), mantle cell lymphoma (MCL, n=86), (MGUS, n=57), multiple myeloma (MM=1869) and normal B-cells (n=56). Whiskers capture data that fall within the 1-99 percentile and statistics were performed using One-Way ANOVA to generate a P value < 0.001 (****). Normal B-cells include the following cell types: CD10-positive CD19-positive hematogone; centroblast; germinal center B-lymphocyte; memory B-lymphocyte; naive pregerminal center B-lymphocyte; plasma cell; small cleaved follicle center cell. GAC mRNA levels were obtained from Compendia Bioscience™ Translational Bioinformatics Services (Life Technologies, Ann Arbor, MI).

Biochemical Potency and Selectivity

CB-839 is a Potent and Selective Glutaminase Inhibitor

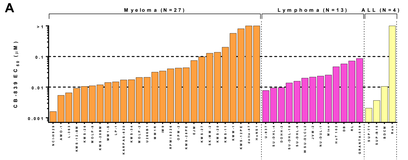


Enzyme	IC_{50} (nM)
rHu-GAC	34
Kidney (KGA)	24
Brain (KGA)	34
Liver (GLS2)	>5000

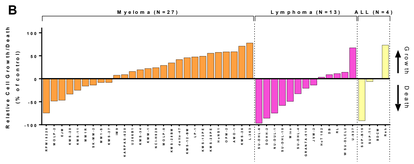
Figure 2: (A) Schematic representation of the glutaminase-glutamate dehydrogenase (GDH) coupled reaction. Glutaminase converts glutamine to glutamate. GDH utilizes glutamate to convert NAD^+ to NADPH which can be measured fluorometrically. **(B) Kidney and brain homogenates contain primarily KGA whereas liver homogenate contains primarily GLS2.** Lysates (20 μg) from mouse kidney, brain and liver were 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-839 is a potent inhibitors of both GAC and KGA splice variants of GLS.** Compounds were dose titrated and incubated with either purified rHu-GAC, kidney, brain lysate or liver lysates for 1 h prior to addition of glutamine. Upon addition of glutamine, NADPH production was monitored 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.

In Vitro Anti-Proliferative Activity

Glutaminase Inhibition Blocks Growth in the Majority of Hematological Tumor Cells



Maximum Glutaminase Inhibition Induces Death in Several Hematological Tumor Cells



Glutamine Withdrawal Induces Death in the Several Hematological Tumor Cells

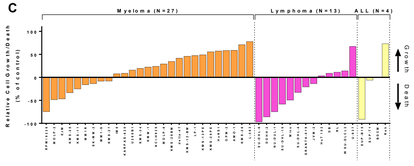
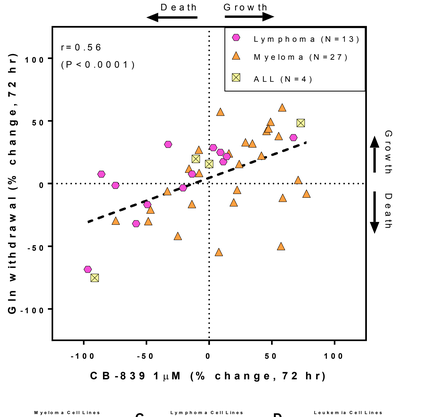


Figure 3: CB-839 has broad anti-proliferation activity across a panel of hematological tumor cell lines representing multiple myeloma, lymphoma and acute lymphocytic leukemia (ALL). (**A**) IC_{50} values were calculated and graphed in ascending order of sensitivity. Cell proliferation and cell loss was measured after 72 h using the CellTiterGlo reagent by comparison with the CellTiterGlo signal measured at the time of (**B**) 1μM CB-839 addition (t=0) or (**C**) glutamine withdrawal (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

In Vitro Anti-Proliferative Activity

CB-839 Sensitivity is Correlated with Glutamine-dependence in Heme Tumor Cells



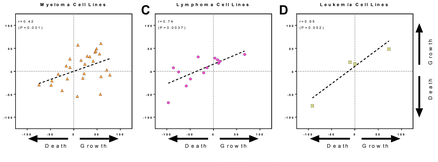


Figure 4: Anti-proliferative activity CB-839 correlates with glutamine withdrawal sensitivity in multiple myeloma, lymphoma and acute lymphocytic leukemia (ALL). (**A-D**) Each data point on the bivariate plots depicts an individual cell line (response to glutamine withdrawal on the y-axis and response to CB-839 on the x-axis). Correlation between CB-839 sensitivity and glutamine withdrawal in (**A**) broad panel of hematological malignancies , (**B**) multiple myeloma, (**C**) Lymphoma and (**D**) acute lymphocytic leukemia cells.

Effect of CB-839 on cellular metabolites

The anti-proliferative effect of CB-839 is correlated with glutamine/glutamate changes

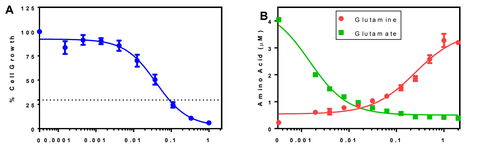


Figure 5: The anti-proliferative activity of CB-839 is correlated with changes in glutamine/glutamate levels. RPMI-8226 multiple myeloma cells were treated with a range of CB-839 concentrations for 72 h. (**A**) The effect of CB-839 on cell proliferation was measured using CellTiterGlo and the EC_{50} was determined to be 40 nM. (**B**) CB-839 treatment results in an increase in cellular glutamine (substrate) and a decrease in cellular glutamate (product). RPMI-8226 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 the CellTiterGlo signal determined on a parallel plate and plotted as a fraction relative to the DMSO control. The EC_{50} for glutamate depletion was calculated to be 1.5 nM.

Pharmacodynamic Response and In Vivo Efficacy

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

Figure 6: CB-839 produces a strong pharmacodynamic response in RPMI 8226 multiple myeloma tumor xenografts 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 RPMI 8226 tumor xenografts. 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 and 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 small subset 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 (**)].

Inhibition of glutaminolysis by CB-839 affects levels of several metabolites in RPMI-8226 tumor xenografts following BID oral administration of CB-839 for 20 days

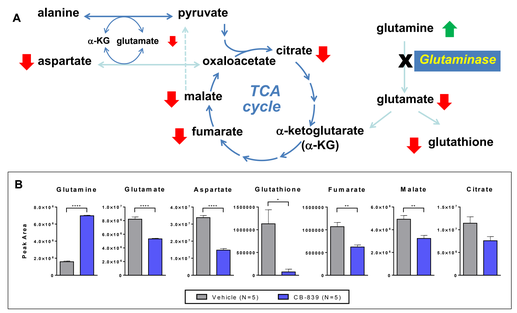
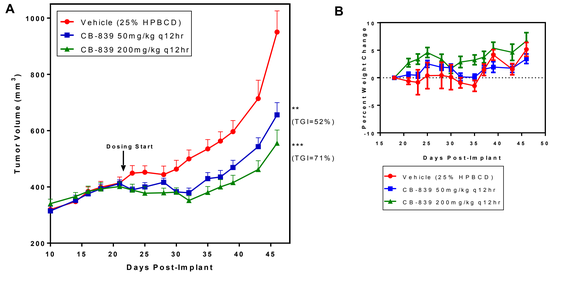


Figure 7: CB-839 treatment of RPMI 8226 tumor xenografts causes increases in glutamine, decreases in glutamate and downstream metabolites. Groups of n=5 scid/bg mice were implanted with 3 x 10^7 RPMI 8226 myeloma cells mixed 1:1 with matrigel SC in flank. BID dosing with CB-839 (200 mg/kg PO) or vehicle was started when tumors showed consistent growth for three consecutive measurements. Following 20 consecutive days of dosing, mice were sacrificed 4h after the last dose and tumors were harvested and analyzed for metabolite levels by Metabolon Inc. of Durham, NC. (**A**) Schematic representation of downstream product of glutamine upon production of glutamate by glutaminase. Downstream metabolites with significant decreases following CB-839 administration are noted with a downward red arrow. (**B**) CB-839 causes increases in glutamine and decreases in glutamate, aspartate, glutathione, fumarate, malate and citrate in RPMI 8226 tumors. For each metabolite, average peak areas and standard deviations measured in vehicle-treated and CB-839-treated tumors are plotted. Significant changes in metabolites are noted by:* (p < 0.05); ** (p <0.01); *** (p < 0.001); and **** (p < 0.0001).

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



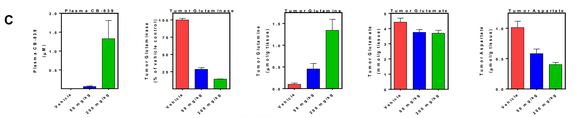


Figure 8: (A) Dose dependent anti-tumor efficacy is observed in a RPMI 8226 multiple myeloma xenograft model. Groups of n=10 scid/bg mice were implanted with 3 x 10^7 RPMI 8226 myeloma cells mixed 1:1 with matrigel SC in flank. BID dosing with CB-839 (50 or 200 mg/kg PO) or vehicle was started when tumors showed consistent growth for three consecutive measurements. ** P value < 0.01, *** P value < 0.001 (ANOVA). **(B)** Efficacious doses of CB-839 have 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). **(C)** CB-839 treatment results in dose dependent pharmacodynamic effects. Plasma and tumor samples were collected on Day 46 approximately 12 h from the last dose. Plasma samples were analyzed by LC/MS/MS for CB-839 levels (1st panel). Tumors were homogenized and assayed for glutaminase activity (2nd panel) and glutamine (3rd panel), glutamate (4th panel) and aspartate (5th panel) concentrations by LC/MS/MS .

Conclusions

- **Glutaminase splice variant GAC is overexpressed in several B-cell malignancies as compared to normal B-cells**
- **CB-839 is a novel, potent and selective small molecule glutaminase inhibitor**
- **CB-839 has *in vitro* antiproliferative activity on a wide range of hematological tumor cells including multiple myeloma, non-hogkins lymphoma and acute lymphocytic leukemia cells**
- **CB-839 has on-target *in vitro* anti-proliferative activity as demonstrated by:**
  - **Concordance with glutamine withdrawal sensitivity in multiple myeloma, non-hodgkin's lymphoma and acute lymphocytic leukemia cells**
  - **Correlation with changes in steady state metabolite levels (↑ glutamine and ↓ glutamate)**
- **CB-839 is orally bioavailable in mice and induces pharmacodynamic changes in the tumor (↑ glutamine, ↓ glutamate, and ↓ aspartate) with minor effects in other tissues**
- **Repeated administration of CB-839 in mice induces changes metabolite levels in tumors (↑ glutamine, ↓ glutamate, ↓ aspartate, ↓ glutathione, ↓ fumarate, ↓ malate and ↓ citrate)**
- **CB-839 has significant single agent anti-tumor efficacy in a RPMI-8226 multiple myeloma xenograft model and efficacy is correlated with pharmacodynamic response**
- **The development of CB-839 in hematological malignancies is supported by the preclinical data in this study**

References

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