Metabolomic, Proteomic and Genomic Profiling Identifies Biomarkers of Sensitivity to Glutaminase Inhibitor CB-839 in Multiple Myeloma

Andrew L. MacKinnon, Mark Bennett, Matt Gross, Julie Janes, Weiqin Li, Mirna Rodriguez, Tracy Wang, Winter Zhang and Francesco Parlati
Calithera Biosciences, South San Francisco, CA

Abstract

Introduction
Glutaminase is a mitochondrial enzyme that converts glutamine to glutamate to support several metabolic processes including amino acid and nucleotide synthesis, maintenance of cellular redox homeostasis, and the replacement of TCA cycle intermediates. Selective glutaminase inhibitors BPTES and CB-839 have anti-proliferative activity in several pre-clinical cancer models including breast, pancreatic, lung, renal, brain, leukemia, and lymphoma. Across a panel of twenty-nine multiple myeloma cell lines, we found that glutaminase inhibition with CB-839 caused tumor cell death or growth inhibition in only a subset of cell lines. To identify biomarkers that predict sensitivity to CB-839 in multiple myeloma cells, we profiled cellular metabolites, mRNA transcripts, and signaling pathways in eight multiple myeloma cell (four CB-839-sensitive and four CB-839-resistant).

Results
Proteomic analysis showed that CB-839 treatment suppressed the activity of the amino-acid sensing kinase mTORC1 in CB-839-sensitive cells, leading to down regulation of protein synthesis and expression of metabolic genes. Analysis of steady-state levels of intracellular metabolites revealed that CB-839-sensitive cells had more profound decreases in nucleotide levels and less pronounced increases in essential amino acids upon CB-839 treatment compared to CB-839-resistant cells. This suggests that the metabolic response to glutaminase inhibition is fundamentally different in sensitive versus resistant multiple myeloma cell lines. Consistent with the *in vitro* data, in a xenograft model with the CB-839-sensitive cell line RPMI8226, CB-839 treatment produced a 71% reduction in tumor growth that was associated with reduced levels of intratumoral nucleotides and no changes in the levels of essential amino acids. We next explored protein biomarkers that predict resistance to CB-839 and found that pyruvate carboxylase (PC) expression strongly correlated with resistance. siRNA-mediated knockdown of PC reduced TCA cycle activity and sensitized cells to CB-839 treatment, suggesting that PC can rescue cells from glutaminase inhibition by supporting anaplerurotic utilization of glucose. This hypothesis was further substantiated by the observation that treatment of CB-839-resistant cells with the AKT inhibitor MK2206 led to a decrease in glucose utilization, and when combined with CB-839, produced a significant decrease in TCA cycle activity and a profound synergistic anti-proliferative response.

Conclusion
Multiple myeloma cells show varying anti-proliferative responses to glutaminase inhibition by CB-839. CB-839 treatment inhibits mTORC1 pathway signaling and causes decreases in nucleotides in sensitive multiple myeloma cells. Multiple myeloma cells that are resistant to glutaminase inhibition have high expression of PC, which may allow these cells to utilize glucose instead of glutamine to resupply TCA cycle intermediates. Knockdown of PC or treatment with an AKT inhibitor causes cells to utilize less glucose and sensitizes resistant cells to glutaminase inhibition with CB-839. CB-839 is currently being evaluated in Phase 1 clinical trials for the treatment of various solid and hematological cancers including multiple myeloma. We are exploring the utility of PC and mTORC1 pathway signaling biomarkers to identify multiple myeloma patients that may respond to CB-839 treatment.

CB-839 Targets the Glutamine Dependence of Cancer Cells

Many tumors rely on the catabolism of glucose and glutamine to produce metabolic intermediates that fuel bioenergetic and biosynthetic demands. Glutaminase initiates this process by converting glutamine to glutamate that is subsequently used in multiple reactions that support tumor cell growth and survival. CB-839 is an orally-bioavailable glutaminase inhibitor that decreases levels of glutamate and other downstream metabolites (e.g. aspartate, TCA cycle intermediates, GSH) thereby producing an anti-tumor effect in several in vitro and in vivo preclinical models (Gross et al. (2014) *Mol Cancer Ther* 13:890-901). CB-839 is currently being tested in Phase 1 clinical trials for the treatment of cancer.

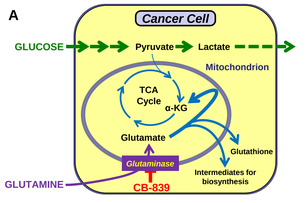


Figure 1. The glutaminase Inhibitor CB-839 has potent anti-tumor activity across multiple tumor types. (A) Schematic diagram of glutamine metabolism by tumor cells. **(B)** Correlation between glutamine dependence (y=axis) and CB-839 sensitivity (x-axis) across a panel of tumor cell lines as assessed by relative cell growth or death (Parlati et al. (2014) *Cancer Res* 74(19):Abstract #1416).

Discovery of Biomarkers That Predict CB-839 Sensitivity

Objective: To identify metabolites, proteins, and gene expression patterns that distinguish CB-839-sensitive from CB-839-resistant multiple myeloma cells before and/or after treatment with CB-839

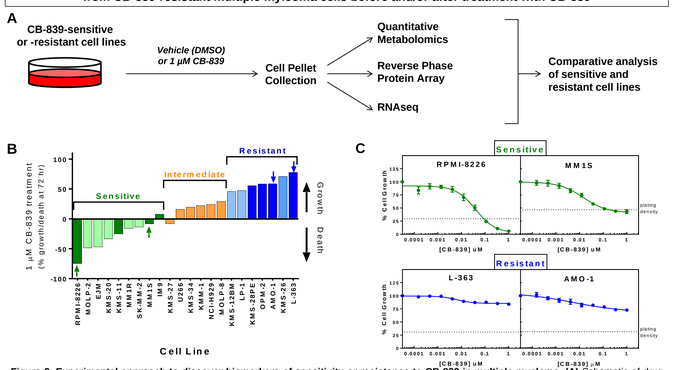
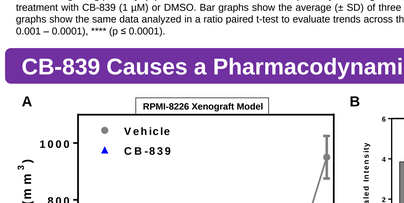
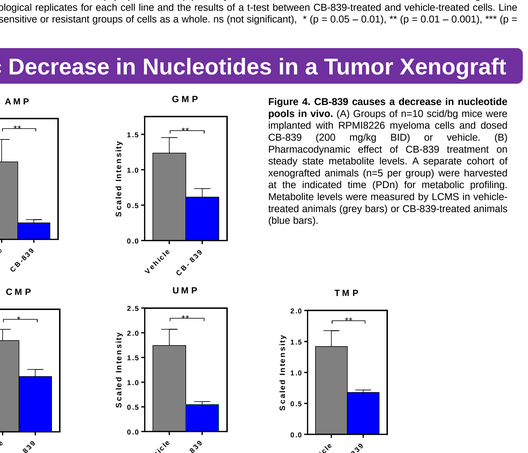


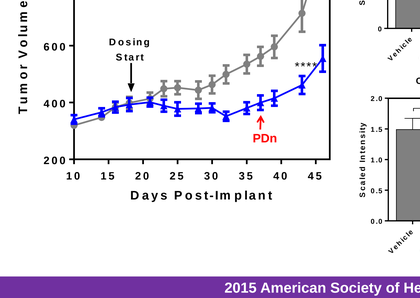
Figure 2. Experimental approach to discover biomarkers of sensitivity or resistance to CB-839 in multiple myeloma. (A) Schematic of drug treatment regimens and experimental platforms employed in the study. **(B)** Relative cell growth or cell death across a panel of multiple myeloma cell lines following treatment with CB-839 (sensitive and resistant cell lines used for biomarker discovery are depicted by the dark green and blue bars, respectively). **(C)** Representative CB-839 dose response curves for two sensitive and two resistant myeloma cell lines (see arrows in panel B).

CB-839 Treatment Suppresses mTORC1 Signaling in Sensitive Cells

Reverse phase protein array (RPPA) and immuno-blots demonstrate that CB-839 inhibits signaling downstream of mTORC1 in sensitive cells







CB-839 treatment causes significant drops in nucleotide pools in sensitive MM cells

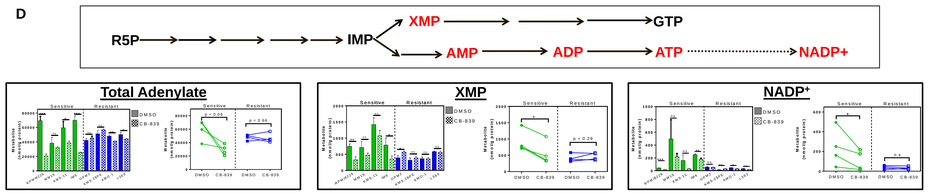
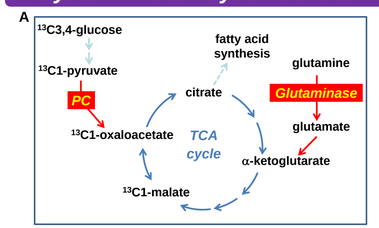


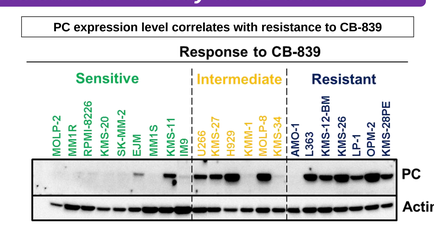
Figure 3. CB-839 inhibits the mTORC1 pathway in sensitive MM cells. (A) Reverse phase protein arrays (RPPA) analysis of mTORC1 pathway following CB-839 (1 μM) treatment. (B) Schematic of the mTORC1 signaling pathway. (C) Western blots of the mTORC1 pathway following 24 hour treatment with CB-839 (1 μM) or DMSO. (D) Steady-state concentrations of intracellular metabolites following 24 hour treatment with CB-839 (1 μM) or DMSO. Bar graphs show the average (± SD) of three biological replicates for each cell line and the results of a t-test between CB-839-treated and vehicle-treated cells. Line graphs show the same data analyzed in a ratio paired t-test to evaluate trends across the sensitive or resistant groups of cells as a whole. ns (not significant), * ($p = 0.05 – 0.01$), ** ($p = 0.01 – 0.001$), *** ($p = 0.001 – 0.0001$), **** ($p \leq 0.0001$).

CB-839 Causes a Pharmacodynamic Decrease in Nucleotides in a Tumor Xenograft

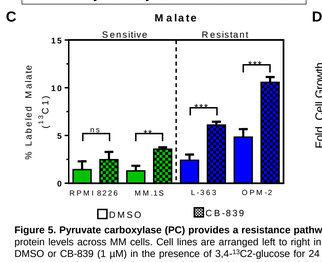
Figure 4. CB-839 causes a decrease in nucleotide pools in vivo. (A) Groups of n=10 scid/bg mice were implanted with RPMI8226 myeloma cells and dosed CB-839 (200 mg/kg BID) or vehicle. (B) Pharmacodynamic effect of CB-839 treatment on steady state metabolite levels. A separate cohort of xenografted animals (n=5 per group) were harvested at the indicated time (PDn) for metabolic profiling. Metabolite levels were measured by LCMS in vehicle-treated animals (grey bars) or CB-839-treated animals (blue bars).

Pyruvate Carboxylase Provides a Resistance Pathway to CB-839 in MM





PC is enzymatically active in resistant cells



PC knockdown sensitizes resistant cells to CB-839

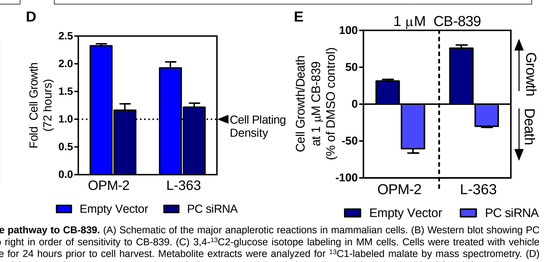
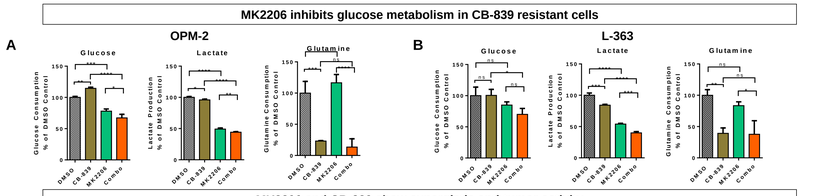


Figure 5. Pyruvate carboxylase (PC) provides a resistance pathway to CB-839. (A) Schematic of the major anaplerotic reactions in mammalian cells. (B) Western blot showing PC protein levels across MM cells. Cell lines are arranged left to right in order of sensitivity to CB-839. (C) 3,4-^{13}C2-glucose isotope labeling in MM cells. Cells were treated with vehicle DMSO or CB-839 (1 μM) in the presence of 3,4-^{13}C2-glucose for 24 hours prior to cell harvest. Metabolite extracts were analyzed for ^{13}C1-labeled malate by mass spectrometry. (D) Fold growth of CB-839-resistant cells infected with lentiviral particles harboring an empty vector or a PC siRNA vector. (E) Relative cell growth/cell death of lentivirus-infected or empty vector-infected MM cells after 72 hour treatment with CB-839 (1 μM).

MK2206 Blocks Glucose Metabolism and Synergizes with CB-839

MK2206 inhibits glucose metabolism in CB-839 resistant cells



MK2206 and CB-839 show synergistic anti-tumor activity

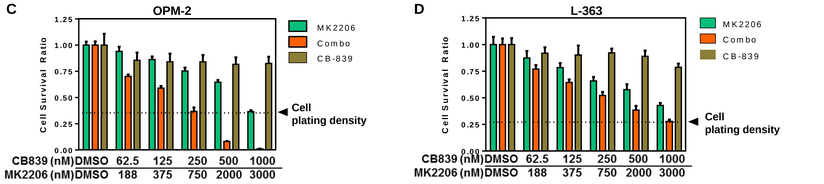


Figure 6. MK2206 combines with CB-839 to increase cell death in CB-839 resistant cells. (A, B) Measurements of glucose or glutamine consumption or lactate production in MM cells treated for 24 hours with DMSO, CB-839 (1 μM), MK2206 (1.5 μM) or the two drug combination. Cells of all treatment groups maintained >90% viability at this time point. (C, D) Viability assays of CB-839-resistant MM cells treated with CB-839, MK2206 or a combination of both inhibitors.

Summary and Conclusions

- **CB-839, a selective and potent inhibitor of glutaminase, blocks proliferation and induces apoptosis in a sub-set of multiple myeloma cells**
- **To discover biomarkers for CB-839 sensitivity in multiple myeloma cells, a comprehensive metabolic and proteomic analysis was performed**
- **CB-839 suppressed the mTORC1 pathway in multiple myeloma sensitive cells which lead to lower nucleotide levels**
- **Low pyruvate carboxylase expression was correlated with sensitivity to CB-839**
- **Knockdown of PC sensitized resistant cells to CB-839**
- **AKT inhibition decreased glucose metabolism and sensitized resistant cells to CB-839**
- **Pyruvate carboxylase expression and the mTORC1 pathway modulation are being explored as biomarkers in clinical studies of CB-839**

2015 American Society of Hematology Annual Meeting -- December 5-8, 2015 – Orlando, FL