Biomarkers of Response to the Glutaminase Inhibitor CB-839 in Multiple Myeloma Cells

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Abstract #3429
Abstract

CB-839 is a potent, orally bioavailable small-molecule inhibitor of the tumor metabolism target glutaminase (GLS) that is currently in Phase 1 clinical trials for the treatment of solid and hematological malignancies. GLS is a mitochondrial enzyme that converts Gln to glutamate (Glu) to support several metabolic processes including amino acid synthesis, maintenance of cellular redox homeostasis, and the replacement of TCA cycle intermediates. CB-839 has in vitro antiproliferative activity across a sub-set of cell lines from diverse hematological tumor types including multiple myeloma (MM), acute lymphocytic leukemia, and non-Hodgkin’s lymphoma [Pariati et al. Blood 2013 122:4226]. To identify biomarkers that would predict sensitivity to CB-839 in MM, we profiled cellular metabolites, mRNA transcripts, and oncogenic signaling pathways in eight MM cell lines representing four CB-839-sensitive cell lines (RPMI8226, MM.1S, KMS-11, and IM-9) and four CB-839-resistant cell lines (AMO-1, L-383, KMS-28PE, and OPM-2).

CB-839 treatment for 4 hours significantly decreased the levels of amino acids (Glu, aspartate, proline) as well as TCA cycle intermediates (fumarate, malate, succinate) across all cell lines. However, prior to treatment, CB-839-sensitive cells had significantly lower baseline levels of pyruvate-, fumarate-, and succinyl-CoA-related amino acids compared to CB-839-resistant cells. In addition, both the adenylate and guanylate energy charges, a measure of cellular metabolic activity [Atkinson and Walton, J. Biol. Chem. 1967 242: 3239-41], were significantly lower in CB-839 sensitive cells. These observations suggest that cells with low levels of amino acids and/or low cellular energy charge are more susceptible to the pharmacological effects of CB-839.

Reverse phase protein array and immunoblot analysis were used to evaluate the impact of CB-839 on signaling pathways across the panel of cell lines. Consistent with the observed decreases in amino acid pools, CB-839 treatment for 4 hours led to an acute inhibition of the amino-acid sensing kinase mTORC1 across all cell lines, as evidenced by decreased phosphorylation of p70S6K and S6. CB-839 treatment also reduced the levels of c-Myc across all cell lines, consistent with a block in five-prime cap-dependent translation as a likely consequence of mTORC1 inhibition. However, untreated sensitive cells had lower baseline levels of both 4E-BP1 and phospho-4E-BP1 in comparison to resistant cells, and at 24 hours following treatment, the mTORC1-dependent activation of CAD, a key enzyme in pyrimidine biosynthesis [Ben-Sahra et al., Science 2013 16: 1323-8], was reduced in CB-839-sensitive but not in resistant cells. These results suggest that CB-839 inhibits mTORC1 signaling in MM cells and that sensitive cells may lack compensatory mechanisms to overcome this inhibition.

Consistent with differential regulation of mTORC1 in CB-839-sensitive versus resistant cells, RNAseq analysis showed greater down-regulation of several mTORC1-regulated transcripts encoding glycolytic enzymes (such as ALDOA and TP1, Düvel et al., 2010 Mol. Cell 39: 171-83) in sensitive cells versus resistant cells following CB-839 treatment. RNAseq analysis also revealed elevated levels of pyruvate carboxylase (PC) in CB-839 resistant cells as compared to sensitive cells. Immunoblot analysis of twenty four MM cell lines supported this observation; PC protein levels were inversely correlated with response to CB-839 (r = 0.64, p = 0.001) and PC protein and mRNA transcript levels were highly correlated (r = 0.94, p = 0.0004). PC is an anaplerotic enzyme that converts pyruvate to oxaloacetate. Therefore, sufficiently high levels of glycolytic enzymes and PC could lessen the dependence of a MM cell line on Gln metabolism for replenishment of TCA intermediates.

The studies described herein elucidate differences in metabolic and signaling pathways among MM cell lines. These differences in metabolites, mTORC1 signaling, glycolytic enzymes and PC may account for differential sensitivity to CB-839. We are exploring the potential utility of PC expression as a biomarker in our ongoing clinical studies of CB-839.
Abstract #3429: MacKinnon et al.

Biomarkers of Response to the Glutaminase Inhibitor CB-839 in Multiple Myeloma Cells

American Society of Hematology Annual Meeting -- December 6-9, 2014 -- San Francisco, CA

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 (Metallo and Vander Heiden (2013) Mol Cell 49: 388-98). 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).
Abstract #3429: MacKinnon et al.
Biomarkers of Response to the Glutaminase Inhibitor CB-839 in Multiple Myeloma Cells
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**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

![Diagram](image)

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).
Abstract #3429: MacKinnon et al.  
Biomarkers of Response to the Glutaminase Inhibitor CB-839 in Multiple Myeloma Cells  
American Society of Hematology Annual Meeting -- December 6-9, 2014 -- San Francisco, CA

Figure 3. Steady state metabolites at baseline and in response to CB-839 measured by CE/MS (Human Metabolome Technologies).  
(A) CB-839 promotes a consistent metabolic response that includes a suppression of glutamate and downstream metabolites (amino acids, TCA cycle intermediates) and an increase in many essential amino acids. These changes were noted in both sensitive and resistant cell lines to a similar extent.  
(B) Baseline energy charge (adenylate and guanylate) and the levels of several amino acids are lower in sensitive cell lines in comparison to resistant lines. Metabolite levels were quantitated in extracts from cells (biological triplicates) treated with DMSO or 1 µM CB-839 for 4 h * (p = 0.05 – 0.01), ** (p = 0.01 – 0.001), *** (p = 0.001 – 0.0001), **** (p ≤ 0.0001).
Baseline Metabolite Levels Correlate with Drug Sensitivity

Lower baseline metabolite levels distinguish sensitive from resistant cell lines

Abstract #3429: MacKinnon et al.
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American Society of Hematology Annual Meeting -- December 6-9, 2014 -- San Francisco, CA

**Baseline Metabolite Levels Correlate with Drug Sensitivity**

Low expression of amino acid transporters may account for reduced amino acid pools in sensitive cells

![Graph](image-url)

*Figure 4. Transcripts encoding multiple amino acid transporters trend lower in sensitive myeloma cell lines in comparison to resistant lines. Baseline transcript levels were measured by RNAseq.*
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**Biomarkers of Response to the Glutaminase Inhibitor CB-839 in Multiple Myeloma Cells**  
American Society of Hematology Annual Meeting -- December 6-9, 2014 -- San Francisco, CA

**Inhibition of mTORC1 is Sustained in Sensitive Cells**

CB-839 inhibits signaling downstream of mTORC1 within four hours in both sensitive and resistant cells.

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**Figure 5.** Reverse phase protein array (RPPA) analysis reveals differential inhibition of mTORC1 signaling by CB-839 in sensitive versus resistant multiple myeloma cells.  
(A) Heat map from RPPA analysis (MD Anderson Cancer Center) of myeloma cells treated with DMSO or CB-839 (1 μM) for 4 h. Lysates were probed with 217 validated antibodies representing most major intracellular signaling pathways. Data were quantified by densitometry, normalized to total protein and median centered.  
(B) Proteins whose levels changed most significantly after 4 h treatment with CB-839.  
(C) Comparison of mTORC1 pathway components between sensitive and resistant cells after 4 h treatment with CB-839. Only samples after drug treatment are shown.  
(D) Proteins whose levels changed most significantly after 24 h treatment with CB-839.  
(E) Comparison of mTORC1 pathway components between sensitive and resistant cells after 24 h of treatment with CB-839. Only samples after drug treatment are shown.  
(F) Western blots showing differential responses in CAD phosphorylation and levels of SREBP after 24h of CB-839 treatment.  
(G) Pathway diagram highlighting the most significant changes in protein phosphorylation observed across RPPA data sets.  
(H) Work flow and methodology to detect nascent protein synthesis in myeloma cells using Click-IT technology (Life Technologies).  
(I) Nascent protein synthesis in sensitive or resistant cell lines after treatment with DMSO or CB-839 for 24 h.
Inhibition of mTORC1 is Sustained in Sensitive Cells

mTORC1 pathway inhibition following CB-839 treatment is sustained in sensitive cells.
Abstract #3429: MacKinnon et al.
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**Inhibition of mTORC1 is Sustained in Sensitive Cells**

**Effect of CB-839 on mTORC1 signaling pathway**

CB-839 suppresses nascent protein synthesis to a greater extent in sensitive cells.

**Measurement of nascent protein synthesis by pulse labeling with azido-homoalanine (AHA):**

- Vehicle or 1 μM CB-839 for 24 hr
- AHA pulse label for 3 hr

**Cell Pellet Collection**

- Extract protein, click chemistry with biotin-alkyne
- Western Blot (Streptavidin-HRP)

**Nascent Protein Synthesis**

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<tr>
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<th>Sensitive</th>
<th>Resistant</th>
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<tr>
<td>RPMI-8226</td>
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<td>OPM-2</td>
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**Strep-HRP**

**Actin**

24 hour treatment
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American Society of Hematology Annual Meeting -- December 6-9, 2014 -- San Francisco, CA

**CB-839 Reduces Expression of Glycolytic Enzymes in Sensitive Cells**

**A**  
Four Sensitive Cell Lines  
Vehicle or CB-839 treatment for 24 h  
RNASEq  
Identify genes that change after drug treatment for every cell line in the sensitive or resistant group  
44 genes  
22 genes  
26 genes  

**B**  
Flow diagram of data analysis. The union of the top 500 genes showing the largest fold change in expression after treatment for each cell line of the sensitive and resistant groups yielded 66 and 48 genes, respectively. **(B)** Changes in genes encoding glycolytic enzymes upon treatment with CB-839. **(C)** Schematic of the glycolysis pathway. Genes whose levels decreased in every sensitive cell line are highlighted in green. Genes whose fold-change after treatment are significantly greater for sensitive versus resistance cells are outlined.

**C**  
Glycolysis  
Glucose $\rightarrow$ G-6P $\rightarrow$ F-6P $\rightarrow$ F-1,6P $\rightarrow$ G3P $\rightarrow$ 1,3-PG $\rightarrow$ 3-PG $\rightarrow$ 2-PG $\rightarrow$ PEP $\rightarrow$ Pyruvate $\rightarrow$ Lactate  

Figure 6. RNAseq analysis of mRNA from multiple myeloma cell lines treated with DMSO or 1 μM CB-839 for 24 h. (A) Flow diagram of data analysis. The union of the top 500 genes showing the largest fold change in expression after treatment for each cell line of the sensitive and resistant groups yielded 66 and 48 genes, respectively. (B) Changes in genes encoding glycolytic enzymes upon treatment with CB-839. (C) Schematic of the glycolysis pathway. Genes whose levels decreased in every sensitive cell line are highlighted in green. Genes whose fold-change after treatment are significantly greater for sensitive versus resistance cells are outlined.
Low Pyruvate Carboxylase Levels Correlate with Sensitivity to CB-839

Figure 7. Pyruvate carboxylase (PC) levels in multiple myeloma cells correlate with resistance to CB-839. (A) Analysis of PC mRNA expression levels in sensitive and resistant multiple myeloma cell lines from the RNAseq and CCLE (Barretina et al. (2012) Nature 483:603-7) datasets. (B) Western blot analysis of PC in multiple myeloma cells. (C) Correlation between PC protein levels measured in (B) and response to CB-839 as measured by relative cell growth or death. (D) Western blots of sensitive and resistant cell lines treated with DMSO or CB-839 (1 μM) for 24 h PDH (pyruvate dehydrogenase), PDP1 (pyruvate dehydrogenase phosphatase 1). (E) Metabolism diagram showing the location of PC, PDH, and PDP1 within the major anaplerotic pathways of mammalian cells.
Summary and Conclusions

- CB-839, a selective and potent inhibitor of glutaminase, blocks proliferation and induces apoptosis in a sub-set of multiple myeloma cell lines.

- To discover biomarkers that could identify CB-839 sensitive myeloma cells, a comprehensive metabolic, proteomic, and expression analysis was performed.

- CB-839 caused prolonged suppression of the mTORC1 pathway in sensitive cells, likely linked to the observed suppression of glycolytic gene expression and new protein synthesis in these cells.

- The baseline levels of several amino acids, amino acid transporters, and the adenylate and guanylate energy charge were lower in sensitive cell lines, suggesting that the nutrient state and energy storage level in myeloma cells is an important factor in determining response to CB-839.

- A low baseline expression of pyruvate carboxylase, an enzyme that allows pyruvate to anaplerotically supply the TCA cycle, was also correlated with sensitivity to CB-839.

- We are exploring pyruvate carboxylase, the mTORC1 pathway, and steady state metabolite levels as possible biomarkers in clinical studies of CB-839.