CB-839, a Selective Glutaminase Inhibitor, has Anti-Tumor Activity in Renal Cell Carcinoma and Synergizes with Everolimus and Receptor Tyrosine Kinase Inhibitors

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Abstract

Many tumor cells utilize the amino acid glutamine to meet the elevated bioenergetic and biosynthetic demands of rapid cell growth. The enzyme glutaminase converts glutamine to glutamate, which is used to fuel the TCA cycle, synthesize amino acids, and balance cellular oxidative stress. CB-839 is a novel inhibitor of glutaminase that is currently in Phase 1 clinical trials for the treatment of cancer. To investigate the role of glutaminase in renal cancer, we tested CB-839 on a panel of 29 kidney tumor-derived cell lines. CB-839 caused tumor cell death in 18 out of 23 renal cell carcinoma (RCC) and 0 out of 6 non-RCC cell lines. Cell lines that were sensitive to CB-839 exhibited more pronounced decreases in aspartate and malate levels compared to resistant cell lines, indicating that sensitivity to glutaminase inhibition may be dependent on the intensity of TCA flux. Expression of the enzyme pyruvate carboxylase (PC), which converts pyruvate to oxaloacetate thereby fueling the TCA cycle, reduces the need for glutamine in cells and may be a biomarker of resistance to CB-839. Consistent with the strong dependency for glutamine metabolism, RCC cells and primary human RCC tumors expressed low levels of PC suggesting that RCC may lack this mechanism of resistance to CB-839. In addition, CB-839-sensitive RCC cell lines showed stronger pharmacodynamic decreases in mTOR signaling (decreased p-S6 and p-4EBP1 proteins), suggesting that nutrient deprivation by CB-839 treatment is sensed by the mTOR pathway. These observations motivated us to evaluate whether inhibitors of receptor tyrosine kinase (RTK) signaling or mTOR would combine with CB-839 to increase cytotoxicity in RCC cell lines and xenograft mouse models. We found that CB-839 synergized with the RTK inhibitors pazopanib, sunitinib, and cabozantib, and the mTOR inhibitor everolimus in proliferation assays with RCC cell lines. Mechanistic studies in Caki-1 cells revealed that the CB-839/cabozantinib combination reduced signaling through AKT and ERK, and reduced glycolytic and TCA cycle activity more than either single agent treatment. In the Caki-1 xenograft model, the combination of CB-839 and cabozantinib enhanced tumor growth inhibition compared to either monotherapy. Likewise, the combination of CB-839 and everolimus inhibited both glucose and glutamine consumption, leading to decreased glycolytic and TCA cycle function and enhanced anti-proliferative activity. The CB-839/everolimus combination showed enhanced anti-tumor activity in the Caki-1 xenograft model, and tumor metabolomics showed evidence of enhanced oxidative stress in tumors treated with this combination. CB-839 is currently being tested in phase 1 clinical trials of patients with solid and hematologic tumors, and is showing promising clinical activity in combination with everolimus in RCC patients.

CB-839 is a Potent and Selective GLS Inhibitor

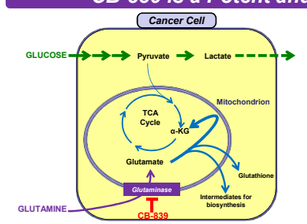


Figure 1. CB-839 inhibits tumor cell metabolism. Tumors rely on the catabolism of glucose and glutamine to produce metabolic intermediates that fuel bioenergetic and biosynthetic demands. Glutaminase initiates this process by converting glutamine to glutamate that is subsequently used in multiple reactions that support tumor cell growth. CB-839 is an orally-bioavailable glutaminase inhibitor that decreases levels of glutamate and other downstream metabolites thereby producing an anti-tumor effect in several in vitro and in vivo preclinical models. Phase 1 clinical trials in RCC patients treated with CB-839 are ongoing.

Table 1: IC_{50} determination for CB-839

Enzyme	Splice Variant	Tissue Distribution	IC_{50} (nM)
GLS	GAC	Tumor	24
	KGA	Broad	29
GLS2	-	Liver	>5000

CB-839 Potently Inhibits Glutaminase in RCC Cell Lines

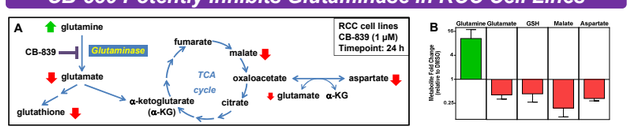


Figure 2. Steady state metabolites decrease in response to CB-839. (A) Schematic representation of glutamine metabolism showing experimentally observed changes to levels of glutamine-derived metabolites following treatment with CB-839. **(B)** CB-839 promotes a consistent metabolic response that includes a suppression of glutamate and downstream metabolites (amino acids, TCA cycle intermediates).

RCC Cells are Sensitive to CB-839

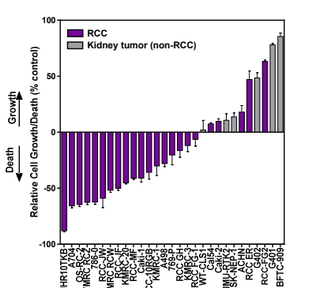


Figure 3 CB-839 has potent anti-proliferative activity in RCC cells that correlates with glutamine dependency. (A) Relative cell growth or cell death across a panel of kidney tumor-derived cell lines following a 72 h treatment with CB-839 (1 μM)

CB-839 Treatment Suppresses the mTOR Pathway

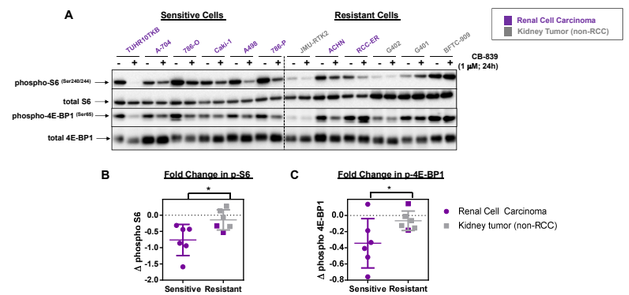


Figure 4. CB-839 inhibits the mTORC1 pathway in sensitive RCC cells. (A) Western blots of the mTORC1 pathway signaling following 24 h treatment with CB-839 (1 μM) or DMSO. **(B, C)** Fold change (log 2 treated/control) in phospho-S6 and phospho-4E-BP1 were determined using densitometry by comparing protein levels in CB-839-treated to DMSO treated cells. Each point represents a single CB-839 resistant or sensitive cell line. * (p = 0.05 – 0.01)

CB-839 Synergizes with Signal Transduction Inhibitors

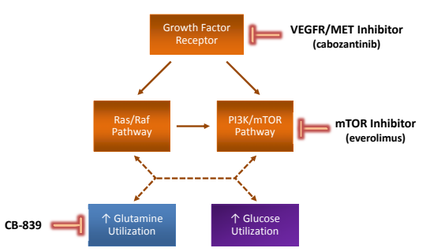


Figure 5. Combination strategy between glutaminase inhibitor CB-839 and signal transduction inhibitors everolimus and cabozantinib. Signal transduction inhibition has been shown to reduce glucose and glutamine utilization, and in combination with CB-839, can synergize to kill cancer cells.

CB-839 Synergizes with Cabozantinib in RCC cells

Synergistic anti-proliferative activity | **Combination inhibits signal transduction** | **Combination inhibits TCA cycle activity**

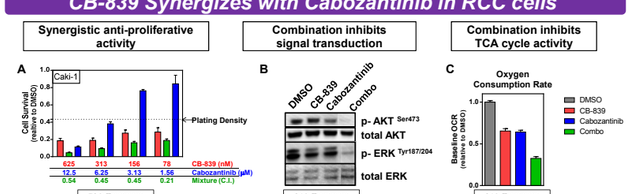


Figure 6. CB-839 synergizes with cabozantinib to decrease proliferation, signal transduction and TCA cycle activity in RCC cell line Caki-1. (A) Viability of Caki-1 cells treated with CB-839, cabozantinib or a combination of both inhibitors for 72 h. Combination Index (C.I.) was calculated using the Calcusyn Software (BioSoft). **(B)** Measurement of signal transduction in cells treated with DMSO, CB-839 (1 μM), cabozantinib (6 μM) or a combination of both drugs for 24 . **(C)** Determination of oxygen consumption rate using the Seahorse Metabolic Analyzer in cells treated for 24 hours as outlined in *(B)*.

Enhanced In Vivo Efficacy of CB-839 plus Cabozantinib

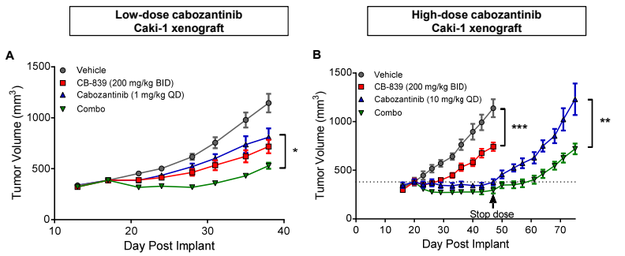


Figure 7. CB-839 shows significant single-agent activity and combines with cabozantinib to produce strong anti-tumor activity. (A) Mice were implanted subcutaneously with Caki-1 RCC cells. When tumor volumes reached ~400mm³, mice were randomized into the following four treatment groups: (i) vehicle, (ii) CB-839 at 200 mg/kg and dosed orally BID, (iii) cabozantinib at 1 mg/kg dosed orally QD, and (iv) CB-839 and cabozantinib. **(B)** Same as in (A) except: (i) cabozantinib was dosed at 10 mg/kg QD orally and (ii) dosing was stopped in the cabozantinib and combination groups after day 47 . * (p=.05-.01), ** (p=.01-.001), *** (p=.001-.0001).

CB-839 Synergizes with Everolimus in RCC cells

Synergistic anti-proliferative activity | **Combination inhibits mTORC1 signal transduction** | **Combination inhibits glucose and glutamine consumption**

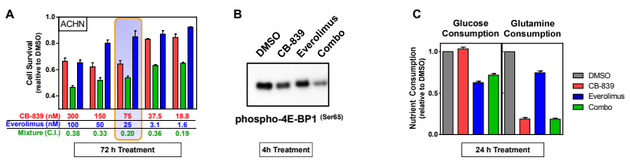
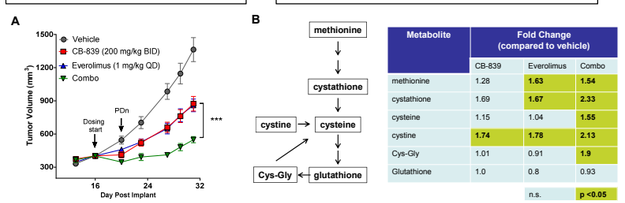


Figure 8. CB-839 synergizes with everolimus to decrease proliferation, mTORC1 signaling and metabolism in RCC cell line ACHN. (A) Viability of ACHN cells treated with CB-839, everolimus or a combination of both inhibitors for 72 h. Combination Index (C.I.) was calculated using the Calcusyn Software (BioSoft). **(B)** Measurement of mTORC1 signaling in cells treated with DMSO, CB-839 (75 nM), everlolimus (25 nM) or a combination of both drugs for 4 h and cell lysates were probed with anti-phospho-4E-BP1 antibody. **(C)** Measurements of glucose or glutamine consumption using the YSI analyzer in cells treated for 24 h as outlined in *(B)*.

Enhanced In Vivo Efficacy of CB-839 plus Everolimus

Anti-tumor efficacy in Caki-1 xenograft | **Evidence for oxidative stress in Caki-1 xenograft**



Metabolite	Fold Change (compared to vehicle)		
	CB-839	Everolimus	Combo
methionine	1.28	**1.63**	**1.54**
cystathione	1.69	**1.67**	**2.33**
cysteine	1.15	1.04	**1.55**
cystine	**1.74**	**1.78**	**2.13**
Cys-Gly	1.01	0.91	**1.9**
Glutathione	1.0	0.8	0.93
		n.s.	p <0.05

Figure 9. CB-839 combines with everolimus to produce strong anti-tumor activity. (A) Mice were implanted subcutaneously with Caki-1 RCC cells. When tumors reached ~400mm³ (day 16), mice were randomized into the following four treatment groups : (i) vehicle, (ii) CB-839 at 200 mg/kg and dosed orally BID, (iii) everolimus at 1 mg/kg dosed orally QD, and (iv) CB-839 and everolimus *** (p=.001-.0001). **(B)** Caki-1 xenografted tumors were harvested following 4 consecutive days of dosing (PDn, day 20), and metabolites were measured by LC/MS (Metabolon Inc.). Metabolites involved in glutathione synthesis were upregulated in treatment groups compared to vehicle control. Fold changes that are statistically significant than vehicle treated groups are highlighted in yellow.

Human ccRCC Tumors Express Glutaminase

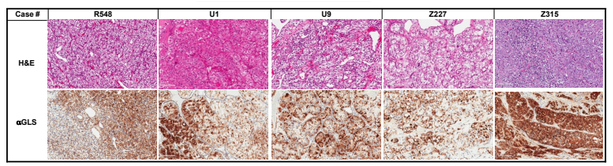


Figure 10. RCC tumors express high levels glutaminase (GLS). GLS protein levels are high in primary ccRCC samples. Human FFPE tumor samples from ccRCC patients were stained with H&E, anti-GLS antibody (Abcam) Samples were purchased from Indivumed (Hamburg, Germany).

Clinical Outcome for Patients Treated with CB-839 and Everolimus

- **15 evaluable patients: 12 ccRCC and 3 pRCC**
- **One PR in a ccRCC patient**
- **Stabilization or decreased tumor burden in the majority of patients**
- **Median PFS for CB-839 plus everolimus treated patients is currently 8.5 months**
- **Median PFS for everolimus treated RCC patients has been reported to be 3.9* to 4.4^ months**

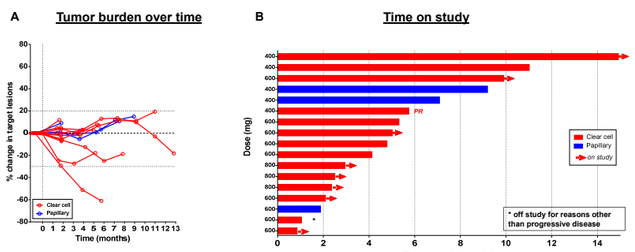


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Figure 11. Treatment outcomes for RCC patients treated with the combination of CB-839 and everolimus. (A) Spider plot showing tumor burden over time. **(B)** Swim plot showing time on study.
* 3.9 month PFS for everolimus treated patients in the cabozantinib vs. everolimus Phase 3 clinical trial (Choueiri et al. Lancet Oncol. (2016) 17:917-27)
^ 4.4 month PFS for everolimus treated patients in the nivolumab vs. everolimus Phase 3 clinical trial (Motzer et al. N Engl J Med. (2015) 373:1803-13)

Conclusions

- **CB-839 potently inhibits glutaminase, proliferation of RCC cell lines and tumor growth in a RCC xenograft mouse model**
- **CB-839 synergizes with everolimus and cabozantinib to decrease:**
  - **Proliferation, signal transduction and metabolism in RCC cells**
  - **Tumor growth in RCC xenograft mouse models**
- **Clinical trials of CB-839 in combination with everolimus or cabozantinib are ongoing**
- **CB-839 in combination with everolimus has efficacy in RCC patients**