Many tumor cells utilize glutamine to meet the elevated biochemical and synthetic 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. Since glutaminase is highly expressed in RCC tumors, we tested the anti-tumor effect of the glutaminase inhibitor CB-839 in preclinical models. CB-839 had a cytotoxic effect in 18 out of 23 tested RCC cell lines. Kidney tumor cells that were CB-839-sensitive exhibited more pronounced decreases in aspartate, malate levels, and reduced glutamine consumption compared to resistant cells, indicating that sensitivity to glutaminase inhibition may be dependent on the intensity of TCA cycle flux. CB-839-sensitive cells showed greater pharmacodynamic decreases in mTOR signaling, suggesting that nutrient deprivation by CB-839 treatment is sensed by the mTOR pathway. We tested synergy between CB-839 and the signal transduction inhibitors cabozantinib and everolimus and found that CB-839 synergized with either agent. The CB-839/cabozantinib combination showed enhanced reduction in signaling via AKT and ERK, and TCA cycle activity as compared to a single agent treatment. In the Caki-1 xenograft model, the CB-839/cabozantinib combination enhanced tumor growth inhibition compared to either monotherapy. Similarly, the combination of CB-839/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 greater anti-tumor activity and tumor metabolism changes evidenced by oxidative stress in tumors treated with this combination. The combination of CB-839 and everolimus is currently being tested in patients with advanced RCC and is showing encouraging clinical activity.

**Table 1: IC50 determination for CB-839**

- **RCC cell lines** (IC50 values in μM)
  - OS-RC 2: 0.17
  - RCC-2: 0.21
  - RCC-JW: 0.24
  - RCC-JF: 0.28
  - RCC-MF: 0.32
  - Caki-1: 0.35
  - DMSO: 0.5

**Figure 1. CB-839 inhibits tumor cell metabolism.** Tumors rely on the catalysis of glucose and glutamine to produce metabolic intermediates that fuel tRNA synthesis and bioenergetics. 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.

**Figure 2. Steady state metabolite changes 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 treatment shows a consistent response pattern that includes a suppression of glutamine and downstream metabolites (amino acids, TCA cycle intermediates).

**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). (B) Correlation between CB-839 sensitivity (μM) and glutamine withdrawal. Each data point on the bi-plot depicts an individual cell line (response to glutamine withdrawal on the y-axis and response to CB-839 on the x-axis).

**Figure 4. 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.

**Figure 5. CB-839 inhibits the mTORC1 pathway in sensitive RCC cell lines.** (A) Western blots of the mTORC1 pathway signaling following 24 h treatment with CB-839 (1 μM) or DMSO. (B, C) Fold change in phospho-S6 and phospho-4E-BP1 were determined using densitometry by comparing protein levels in CB-839-treated to DMSO-treated cells. (D) Determination of oxygen consumption rate using the Seahorse Metabolic Analyzer in cells treated for 24 h as outlined in (B).

**Figure 6. CB-839 synergizes with everolimus to decrease proliferation, signal transduction and TCA cycle activity following 24 h treatment with CB-839 (1 µM) or DMSO.** (A) Viability of Caki-1 cells treated with CB-839, everolimus or a combination of both drugs. (B) Western blots of the PI3K/mTOR pathway signaling following 24 h treatment with CB-839 (1 µM) or DMSO.

**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 xenografts. (B-C) Bioluminescence imaging showing best change in tumor burden. (D) Determination of oxygen consumption rate using the Seahorse Metabolic Analyzer in cells treated for 24 h as outlined in (B).

**Figure 8. CB-839 synergizes with everolimus in RCC cell line Caki-1.** (A) Viability of Caki-1 cells treated with CB-839, everolimus or a combination of both inhibitors for 72 h. Combination Index (CI) was calculated using the CalcuSyn Software (Biosoft). (B) Measurement of signal transduction in cells treated with DMSO, CB-839 (1 μM), cabozantinib (1 μM) or a combination of both drugs for 24 h and cell lysates were probed with anti-phospho-AKT and anti-phospho-ERK antibodies. (C) Determination of oxygen consumption rate using the Seahorse Metabolic Analyzer in cells treated for 24 h as outlined in (B).