A Novel Pharmacodynamic Assay to Measure Glutaminase Inhibition Following Oral Administration of CB-839 in Triple Negative Breast Cancer Biopsies

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Abstract # P1-08-07
Abstract

Triple negative breast cancer (TNBC) cell lines are highly dependent on glutamine (Gln) for growth and survival. A critical step in Gln utilization is its conversion to glutamate (Glu) by the mitochondrial enzyme glutaminase (GLS). CB-839 is a potent inhibitor of GLS that has anti-proliferative activity in TNBC cell lines and antitumor activity in TNBC xenograft models (Ref 1). Across a panel of breast cancer cell lines derived from both receptor positive and TNBC tumors, sensitivity to CB-839 was associated with (i) elevated GLS expression, (ii) elevated GLS activity, and (iii) the TNBC subtype. Importantly, many of the determinants of CB-839 sensitivity in cell lines are also present in primary tumor samples, including high mRNA and protein expression of GLS and a high Glu to Gln ratio in TNBC tumors as compared to receptor positive tumors. These observations motivate the Phase 1 clinical study of CB-839 in TNBC patients. To aid in the selection of a recommended Phase 2 dose, we sought to develop a pharmacodynamic (PD) assay to directly measure the GLS activity in breast tumor lysates in order to determine the extent of GLS inhibition in tumor biopsies from CB-839 treated patients. To develop a robust PD assay, we first identified conditions that maintain the GLS:CB-839 inhibitory complex during preparation of lysates from CB-839 treated samples. High concentrations of KCl (150 mM) and low concentrations of K-phosphate (15 mM) in the lysis buffer, as well as maintaining the lysate at a low temperature stabilized the inhibited complex. Following gel filtration of the lysate to remove unbound CB-839 and exchange the buffer, GLS activity was immediately measured with a coupled enzyme assay. The GLS activity measured at this step reflects the residual activity present in a sample that was exposed to CB-839. To quantify the amount of total GLS present in the sample, we incubated the same lysate for 3 hours at room temperature under conditions of low KCl and high phosphate to allow the GLS:CB-839 complex to fully dissociate prior to measuring activity. This assay format allows quantitation of the % GLS inhibition from a single tumor lysate sample and eliminates the requirement for multiple biopsies as well as any assay variability due to tumor heterogeneity. We utilized this tumor PD assay to determine the plasma drug levels required for maximal tumor GLS inhibition in a preclinical TNBC model. Mice bearing HCC1806 TNBC tumors were first treated with a range of CB-839 doses. Four hours after oral administration, a 10 mg/kg dose of CB-839 resulted in >90% inhibition of tumor GLS. CB-839 plasma concentrations of 100 nM corresponded to 50% inhibition of tumor GLS, while maximal inhibition occurred at plasma concentrations ≥300 nM. In xenograft studies, maximal anti-tumor efficacy was achieved with BID dosing at 200 mg/kg, a dose and schedule that resulted in trough plasma levels of CB-839 of ≥300 nM and sustained GLS inhibition in tumors. As part of an ongoing Phase 1 trial, this assay will be utilized to monitor tumor PD responses in TNBC patients undergoing treatment.
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San Antonio Breast Cancer Symposium -- December 10-13, 2014

**Glutamine is a Key Nutrient for TNBC Cells**

Compared to receptor positive cells, TNBC cell lines exhibit greater glutaminase expression, higher Gln consumption, lower intracellular Gln concentration, and greater dependence on extracellular Gln for growth, consistent with the notion that TNBC tumors are highly dependent on glutaminolysis (Ref 1). These tumor cells depend on the catabolism of glutamine to produce metabolic intermediates that fuel biosynthetic and bioenergetic demands (Ref 2, 3, 4). Glutaminase initiates this process by converting glutamine to glutamate which is subsequently used in multiple reactions that support tumor cell growth and survival. 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 in models of triple negative breast cancer. These findings provide a strong preclinical rationale for the further development of glutaminase inhibitors in triple negative breast cancer.
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Anti-Tumor Activity of Glutaminase Inhibition with CB-839

**In vitro efficacy**

A

Hs587T (triple-negative) vs T47D (ER+)

![Graph showing cell viability vs CB-839 concentration](image)

**In vivo efficacy**

C

Basal-like Breast Cancer JIMT-1 cells

![Graph showing tumor volume vs study day](image)

B

1 μM CB-839

![Graph showing cell proliferation and cell loss](image)

D

TNBC PDX: CTG-0052 (Champions Oncology)

![Graph showing tumor volume vs study day](image)

Figure 1: Anti-tumor activity CB-839 in triple negative breast cancer models. (A) The effect of CB-839 following 72 h treatment on cell proliferation was measured and the EC_{50} was determined to be 13 nM (Hs578T) and > 1 μM (T47D). (B) Cell proliferation or loss measured in breast cell lines after treatment with 1 μM CB-839 for 72 h. The basal-like ER-/HER2+ cell lines JIMT-1 and HCC1954 are annotated with asterisks (*). (C) Anti-tumor efficacy is observed in a JIMT-1 basal-like breast cancer model. (D) Anti-tumor efficacy is observed in a triple negative breast cancer primary tumourgraft model.
Elevated Glutaminase Levels Correlate with CB-839 Sensitivity

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Figure 2: Glutaminase activity correlates with CB-839 sensitivity in breast cancer cell lines (A) Schematic representation of the glutaminase-glutamate dehydrogenase (GDH) coupled reaction. (B) TNBC cell lines have elevated glutaminase protein and activity levels. Upper Panel, SDS-PAGE and immunoblot analyses of a breast cancer cell line panel using antibodies that recognize glutaminase (GAC and KGA), and actin. Lower Panel, TNBC cell lines have elevated glutaminase activity levels. Phosphate-activated glutaminase activity was measured in cell line homogenates and normalized to total protein amount used in the assay. (C) Correlation of CB-839 sensitivity with glutaminase activity. Correlation between cell proliferation or loss measured after CB-839 treatment plotted on the x-axis and glutaminase activity (log2 transformed values) plotted on the y-axis.
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Elevated Glutaminase Expression in Primary TNBC Biopsies

Primary TNBC tumors show increased expression of GAC mRNA, increased glutaminase activity and increased glutaminase protein expression as compared to primary ER+ breast tumors.

Figure 3. TNBC primary tumors have elevated glutaminase mRNA, activity and protein. (A) GAC mRNA levels are elevated in TNBC. Expression levels for glutaminase (GAC) in normal breast tissue, TNBC tumors, and receptor-positive tumors from the TCGA breast invasive carcinoma dataset (Ref 5). (B) Glutaminase activity was determined from fresh frozen biopsies from triple-negative and ER+ breast cancer patients. Statistical comparisons were performed by (A) one-way ANOVA or (B) unpaired t-test to generate P values: *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001. (C) GLS protein levels are increased in primary TNBC samples. Human formalin-fixed paraffin-embedded tumor samples from TNBC patients (upper rows) and ER+ patients (lower rows) were stained with H&E or anti-GLS antibody. Samples marked with an asterisk were used for the Western blot in Figure 4A.
Elevated Glutaminase Activity in Primary TNBC Biopsies Compared to Normal Tissue

Figure 4. TNBC primary tumors have elevated glutaminase activity compared to normal tissue. (A) Glutaminase activity and expression is elevated in TNBC compared to normal tissues. *Upper panel:* Mean and error values are plotted for normal tissue samples derived from an individual donor whereas TNBC and ER+ breast samples represent the average from 17 and 10 separate individual samples, respectively. *Lower panel:* SDS-PAGE and immunoblot analyses of normal tissue and tumor samples using an antibody that recognizes glutaminase (GAC and KGA). (B) Glutamate to glutamine ratios are elevated in primary TNBC tumors compared to normal breast tissue. Metabolite levels in patient samples from a published report (Ref 6).
Development of Pharmacodynamic Assay to Monitor Glutaminase Inhibition by CB-839 in Tumor Biopsies

Objective: Measure glutaminase inhibition in tissues from CB-839-treated patients

Challenges:
- CB-839 interaction with glutaminase is reversible
- Under standard tissue lysis conditions, CB-839 dissociates from glutaminase

Reversibility experiment to identify conditions that stabilize the CB839:Glutaminase complex

Figure 5. Identification of conditions that stabilize the CB-839:Glutaminase complex. (A) Schematic of experimental procedure. (B) Recovery of glutaminase activity under distinct conditions. Glutaminase activities in CB-839-treated samples were normalized to DMSO-treated samples for each time-point. Percent recovered glutaminase activity was plotted versus time for each condition. (C) Table of conditions used to isolate CB-839/glutaminase complex. Condition that represents total glutaminase activity and inhibited glutaminase activity are highlighted.
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Glutaminase Inhibition in Primary TNBC Tumor Lysates

Measurement of both inhibited and total glutaminase activity in CB-839-treated tumor homogenates

A

Homogenize primary TNBC tumors
↓
Treat TNBC homogenates with CB-839
↓
Isolate glutaminase:CB-839 by gel filtration
↓
Assay glutaminase activity under conditions that:

Preserve glutaminase:CB-839 (Inhibited Glutaminase)
Dissociate glutaminase:CB-839 (Total Glutaminase)

B

Tumor 1

IC50 = 15 nM

% Activity (DMSO control)

Tumor 2

IC50 = 6 nM

% Activity (DMSO control)

Tumor 3

IC50 = 35 nM

% Activity (DMSO control)

Tumor 4

IC50 = 6 nM

% Activity (DMSO control)

C

CB-839 IC50 in other assay formats (nM)

Recombinant GLS 24-29
Tumor cells in vitro 10-30
Whole blood (platelets) 29

Figure 6. Measuring glutaminase inhibition in TNBC tumor lysates

(A) Schematic of experimental procedure. (B) Left Panel, IC50 determination for CB-839 inhibition of TNBC glutaminase activity. % glutaminase activity was calculated by determining the ratio of inhibited glutaminase relative to total glutaminase activity. Right Panel, % recovered activity in each sample by comparing activity of dissociated glutaminase:CB-839 in treated lysates to activity in lysates prior to CB-839 treatment. (C) The IC50 for CB-839 inhibition of glutaminase in a biochemical (Ref 1), cell-based (Ref 1) and intact platelet assay (Ref 7) are noted.
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Glutaminase Inhibition in TNBC Xenograft Tumors

300 nM CB-839 plasma concentration results in near maximal glutaminase inhibition in tumors

Figure 7. Correlation between plasma exposure and tumor glutaminase inhibition. (A) Outline of the experimental schema. (B) CB-839 concentrations corresponding to greater or equal than 300 nM produced maximal inhibition of glutaminase (upper right panel), and maximal increases and decreases in glutamine and glutamate, respectively (lower panels).
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San Antonio Breast Cancer Symposium -- December 10-13, 2014

Figure 8. Glutaminase is inhibited in tumor biopsies from patients treated with CB-839 from early dose cohorts. (A) Drug levels were measured in clinical plasma samples collected on day 15 of dosing (steady state). (B) CB-839 was administered for 22 consecutive days prior to acquiring a tumor biopsy. Glutaminase inhibition was assessed as described in Figure 6. (C) SDS-PAGE and immunoblot analyses of tumor biopsy samples using antibodies that recognizes glutaminase (GAC and KGA) and actin.
Summary and Conclusions

• CB-839, a potent inhibitor of the mitochondrial enzyme glutaminase, blocks the ability of tumor cells to use glutamine as a fuel.

• TNBC cells express higher levels of glutaminase, are more dependent on glutamine for growth, and exhibit a greater sensitivity to the antiproliferative effects of glutaminase inhibition with CB-839 in comparison to ER+ cells.

• Glutaminase is expressed at higher levels in TNBC tumors than in ER+ breast tumors and most normal tissues (except brain).

• These observations motivated the Phase 1 clinical testing of CB-839 in TNBC patients and patients with other glutamine dependent solid and hematological tumors.

• To assess glutaminase inhibition in tumors from patients treated with CB-839, we developed a novel pharmacodynamic assay.

• The pharmacodynamic assay determines the extent of inhibition in a single post-dose tumor biopsy sample by:
  • measuring residual glutaminase activity utilizing condition that preserve the CB-839:glutaminase complex (inhibited state)
  • measuring total glutaminase activity utilizing conditions that dissociate CB-839:glutaminase complex (uninhibited state)

• The pharmacodynamic assay was used to demonstrate glutaminase inhibition by CB-839 in:
  • lysates prepared from four primary TNBC biopsies (IC50 = 16-35nM)
  • tumors from a TNBC xenograft model (near complete inhibition at ~300 nM plasma concentrations)

• 74%-84% glutaminase inhibition was observed in tumor biopsy samples from patients treated with CB-839 in an early dose cohort in a Phase I clinical study, confirming target engagement in tumor tissue.

References