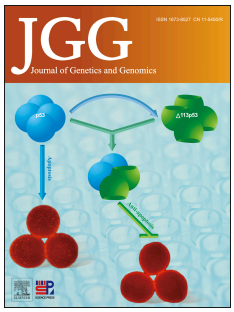

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# A facile and sensitive method of quantifying glutaminase binding to its inhibitor CB-839 in tissues

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## ABSTRACT

Many cancer types reprogram their metabolism to become addicted to glutamine. One of the critical enzymes in the utilization of glutamine in these cells is glutaminase. CB-839 (telaglenastat) is a drug that targets glutaminase that is currently being evaluated in many clinical trials for efficacy in various cancer types that are known to be driven by glutamine metabolism. Despite its use, there are limited assays available for testing the pharmacodynamic on-target effects of CB-839 on the limited, small volume patient samples that are obtained in early phase clinical trials. Thus, we developed an assay based on the cellular thermal shift assay (CETSA) technique using AlphaLISA technology to show that CB-839 specifically engages glutaminase in colon cancer cell lines in vitro and in minute quantities of mouse xenograft tumors. Notably, we show that this assay detects CB-839 binding to glutaminase in platelets of patients collected while receiving CB-839 on a clinical trial. This assay may be used to study the pharmacodynamic profile of CB-839 in very small tissue samples obtained from patients on a clinical trial and may be useful in future studies designed to screen other inhibitors of glutaminase.

## INTRODUCTION

Glutamine addiction is increasingly recognized as a driving metabolic alteration in some cancer types (Feng et al., 2016). This has prompted efforts to target the key steps in the glutamine metabolic pathway to exploit this glutamine addiction for therapeutic benefit for patients with cancer. One of these key steps is the conversion of extracellularly-sourced glutamine to glutamate by the enzyme glutaminase (GLS) (Cassago et al., 2012).

Specific inhibitors of GLS that can be safely used in humans have been long sought-after, but have been largely ineffective or toxic (Ahluwalia et al., 1990; Catane et al., 1979; Masisi et al., 2019; Ovejera et al., 1979; Raczka and Reynolds, 2019). More recently, an allosteric inhibitor of GLS, CB-839 (Gross et al., 2014), which was developed by Calithera Biosciences, Inc, has garnered significant interest in the clinical setting and is currently being evaluated in 20 clinical trials in humans with advanced malignancies (ClinicalTrials.gov, accessed February 2020). One of the common charges of early-phase therapeutic clinical trials is to ensure the investigational drug effect is on target or acting via its proposed mechanism. In the case of CB-839, assays do exist for measuring GLS activity in cells and tissues; however, these assays typically require large quantities of tissue, which is rarely feasible when working with limited patient samples enrolled in clinical trials. Additionally, these assays are technically challenging, as common cell lysis techniques dissociate CB-839 from GLS (Parlati, 2014). Thus, there is a pressing need to develop an assay to quantify CB-839's ability to target GLS in small quantities of patient tumor samples or in surrogate tissues such as platelets, which are more abundantly and safely available from patients compared to tumor biopsies. Interestingly, a previous study showed that

glutaminase inhibition by CB-839 in patients' platelets correlated with these in tumor tissues (Meric-Bernstam et al., 2016).

We recently discovered that activating mutations in *PIK3CA* render colorectal cancers addicted to glutamine (Hao et al., 2016; Zhao et al., 2019). Based on this discovery, we opened an investigator-initiated Phase I/II clinical trial using the glutaminase inhibitor CB-839 in combination with standard cytotoxic chemotherapy capecitabine in patients with metastatic *PIK3CA*-mutant colorectal cancer (NCT02861300). We sought to develop an assay that would serve as a biomarker using minute quantities of tissue or in surrogate pharmacodynamic samples such as platelets to show that CB-839 effectively targets GLS in our patients on the clinical trial, and that could be used to measure the drug's engagement with GLS in other clinical trials using CB-839.

Thus, we used the techniques initially described by Martinez Molina et al and Almqvist et al (Almqvist et al., 2016; Martinez Molina et al., 2013), which exploit the phenomenon of stabilization from thermal precipitation by drug binding of an enzyme. When heated, the drug-free proteins denature and precipitate, and are unable to be recognized by an antibody.

## RESULTS

### Development of Western blot-based assay

Our ultimate goal was to develop an assay that could be used to sensitively and quantitatively determine CB-839's engagement of GLS that could be used in preclinical models as well as in

minute amounts of human tissue samples that may be obtained from patients on clinical trials. First, we wanted to show that CB-839 engagement of GLS protects GLS from thermal denaturation. We treated HCT116 colon cancer cells with 10 μM CB-839, a concentration known to be cytotoxic to this cell line (data not shown), trypsinized cells, and heated them to varying temperatures for 3 minutes. We then performed Western blot of the heated and unheated samples and stained for GLS. As expected, we found that at lower temperatures (e.g., 42ºC), GLS structure remained intact to be recognized by the antibody, regardless of its engagement with CB-839 (Figure 1A). In contrast, at increasing temperatures, GLS structure became denatured in the absence of CB-839, but remained intact in the presence of CB-839, with the biggest effect seen between 52ºC and 56ºC. Based on these results, we selected 52ºC for future thermal shift assay experiments.

Next, we wanted to understand if the thermal protection of CB-839 to GLS was concentration-dependent. We incubated HCT116 cells with varying concentrations of CB-839 and tested them using our Western-blot-based CETSA (Figure 1B). We observed a concentration-dependent thermal protection of GLS, which suggests that this is a sensitive assay to detect concentration-dependent binding of the drug CB-839 to GLS. To better understand the rapidity with which CB-839 can engage GLS within cells, we incubated HCT116 cells with CB-839 for various durations and noted rapid engagement of GLS after only 5min exposure to the drug (Figure 1C), which means that CB-839 can rapidly be incorporated into cells to inhibit GLS.

We then determined if we could use our assay to detect CB-839 engagement in tumor tissue. We generated mouse xenograft tumors from HCT116 colon cancer cells and treated the mice with daily CB-839 or vehicle by oral gavage. After 5 days of treatment, we harvested the tumors

and used approximately half of the xenograft tumors (~200 mg) to perform the assay. We saw that CB-839 treatment in mice protects tumoral GLS from thermal aggregation (Figure 1D), which means that CB-839 is delivered to tumor cells *in vivo* to engage GLS.

Development of AlphaLISA-based assay

These Western blot-based thermal shift assays showed that CB-839 specifically targets GLS in cells and in xenograft tumors. We next sought to develop a more robust assay that can very sensitively, rapidly, and reliably quantify the extent of drug engagement to GLS in minute tissue samples. We thus employed the AlphaLISA-based CETSA technique (Almqvist et al., 2016), which has the distinct advantage of being able to use very small amounts of biological samples in 384-well plates. Samples are processed similarly as in the Western blot-based assay, then very small (3 µL in our experiments) volumes of heated lysates are added to 384-well plate with protein-specific antibodies and alpha donor and acceptor beads (Figure 2A). Proteins that have been thermally denatured precipitate and cannot be recognized by protein-specific antibodies; thus, only those proteins that have been thermally protected by drug engagement are able to bring the donor bead and acceptor bead in close proximity to elicit an alpha signal at 615nm upon excitation at 680nm.

We first optimized the antibody pairs to be used in the assay (Figure 2B) and found that the most sensitive antibody pair was Novus's mouse anti-GLS (which paired with the donor bead anti-mouse IgG) and Abcam's rabbit anti-GLS (which paired with the acceptor bead anti-rabbit IgG).

To confirm the optimal temperature for the AlphaLISA-based assay, we exposed CB-839-treated cells to varying temperatures. We found that the optimal temperature for this assay was 55ºC (Figure 2C). Similar to the Western blot-based assay, the AlphaLISA-based assay showed concentration-dependent thermal protection of GLS, with maximal binding at 10µM (Figure 2D), suggesting that this assay may be employed to quantitate the degree of drug binding to GLS. To test the optimal heating time for the assay, we heated cells for varying duration and showed that after 1 hour of heating, GLS was maximally denatured (Figure 2E). To be sure that the drug-binding was specific to CB-839-engagement with GLS, we tested the assay with several small molecule inhibitors of other enzymes (Figure 2F), and found that only CB-839 protected GLS from thermal denaturation.



AlphaLISA-based CETSA can detect target engagement of GLS in limited quantity samples

To better simulate the sensitivity of the assay using the minuscule amounts of tissue that are frequently obtained in human therapeutic clinical trials, we tested the AlphaLISA-based assay using only ~10 mg of mouse xenograft tumor harvested after 5 days' of treatment with CB-839. Remarkably, we found that the assay very robustly detected CB-839 engagement of GLS in even this very small amount of tumor tissue (Figure 3).

Finally, we wanted to see if the AlphaLISA-based assay could tell us if CB-839 engages GLS in human patient samples. Whole blood was collected from patients on our clinical trial before starting treatment with CB-839 and after two weeks of continuous twice-daily dosing of CB-839. Platelets were isolated and lysed. Lysates were heated and analyzed using the AlphaLISA-based CETSA. We saw a marked increase in alpha signal in platelets collected from patients while they were receiving treatment compared to platelets collected prior to starting therapy

(Figure 4A), suggesting that CB-839 specifically engages GLS in human platelets. Moreover, this assay was highly reproducible, as three independent experiments, which were performed over a three-month time frame, generated nearly identical Alpha readings for each sample (Figure 4B).

## DISCUSSION

We have successfully developed a highly sensitive, reproducible, specific, fast, and easy assay to assess the pharmacodynamics of CB-839 in patients. First, as we showed in Figures 2 and 3, only 10 mg of tissues are needed for the AlphaLISA-based CETSA, which is a fraction of a needle biopsy. Thus, this assay is sensitive enough to measure the pharmacodynamics of CB-839 in tumor biopsies. In fact, we will utilize this method to assess the pharmacodynamics of CB-839 in tumor biopsies obtained from our ongoing phase II clinical trial (NCT02861300). Second, this assay is highly reproducible. We performed three independent AlphaLISA-based CETSA assays over a three-month period for six human platelet samples, and the AlphaLISA readings are nearly identical for each sample (Figure 4B). The high reproducibility enables the comparison of patient samples harvested at different times. Third, the assay is highly specific. As we showed in Figure 2F, only CB-839, but not any of the other ten unrelated small molecules, can protect GLS from precipitation. Fourth, the method takes only 3 hours to finish the entire procedure and thus is fast. Fifth, this assay requires very few steps of pipetting and is amenable for high-throughput analysis.

It is worth noting that the actin proteins are thermal stable in cell lysates (Figure 1 A to C). However, the actin proteins in tumors aggregate and precipitate as the heating temperature increased (Figure 1D), which may be due to the complexity of the tumor tissues. Nonetheless,

this feature does not hinder the quantitation of our method, as we normalize GLS amounts in heated samples to the unheated samples for the AlphaLISA-based CETSA assay.

Glutamine addiction is well recognized as a targetable metabolic driver in some malignancies. CB-839 is a drug developed to target GLS, which is one of the critical enzymes that regulate glutamine metabolism in cancer cells, and is currently being investigated in several clinical trials. Our study develops a much-needed assay to quantify the interaction of CB-839 with GLS in patient samples. This method will be invaluable for clinical investigators seeking to show that the drug is on target in their patient population. Furthermore, in addition to CB-839, at least two more GLS inhibitors are entering clinical trials [NCT03894540; (Leone et al., 2019)]. Our newly developed assay is applicable to assess the pharmacodynamics of these compounds as well. Lastly, this method has the potential to be expanded in a high-throughput fashion to screen for novel inhibitors of glutaminase to develop new therapies for patients.



## MATERIALS AND METHODS:

### Cell culture and chemicals

Colorectal cancer (CRC) cell lines, HCT116, DLD1 were obtained from ATCC. These cell lines were cultured in McCoy's 5A medium containing 10% fetal bovine serum and 1% penicillin/streptomycin. Cells were cultured in a humidified incubator at 37°C, 5% $CO_2$. All cell lines were confirmed as Mycoplasma free. CB-839 was kindly provided by Calithera Biosciences, Inc. 5-Fluorouracil (5-FU), and aminooxyacetic acid (AOA) were purchased from Sigma-Aldrich. Ibrutinib, neratinib, sorafenib, LY294002, Sch772984, PF-04691502, MG132,

OSU-03012, and TG100-115 were purchased from Selleck Chemicals and used at a concentration of 10μM.

**Drug treatment of xenograft tumors**

Animal experiments were approved by the Case Western Reserve University Animal Care and Use Committee and performed in accordance with relevant guidelines and regulations. Xenograft tumors were established by injecting $3x10^6$ cells subcutaneously into the flanks of 6 to 8-week-old female athymic nude mice (Jackson Laboratory, USA #002019). When tumors reached approximately $200mm^3$, mice were randomly divided into groups (10 tumors for each group). Mice were treated with CB-839 (200 mg/kg) or an equivalent volume of vehicle once daily by oral gavage. Formulated CB-839 and vehicle were generously gifted by Calithera Biosciences, Inc. Mice were sacrificed after being treated for five days.

**Human samples**

Patients with metastatic colorectal cancer were enrolled in our Phase II clinical trial (registered on ClinicalTrials.gov NCT02861300) to receive CB-839 800mg orally twice daily continuously in addition to standard chemotherapy of capecitabine 1000mg/m2 twice daily by mouth for 14 days every 21 days. Blood was drawn for correlative studies prior to starting treatment and again when patients had received 14 days of treatment. Whole blood was drawn in EDTA-filled phlebotomy tubes using standard phlebotomy techniques. Tubes were centrifuged at 200 x *g* x 20 minutes, and platelet-rich plasma separated and frozen for later use. The trial was conducted in accordance with institutional regulatory requirements and the International Conference on Harmonisation Good Clinical Practice guidelines. All patients provided written informed consent.

**Western blot-based cellular thermal shift assay**

The cellular thermal shift assay (CETSA) was performed as described(Almqvist et al., 2016; Martinez Molina et al., 2013). For cultured cells, HCT116 cells were plated in 6-well plates at 2 × $10^5$ cells/well. When the cells reached ~70% confluence, they were treated with DMSO or CB-839 (10 µM) for the indicated time. Cells were trypsinized, washed with PBS, and resuspended in PBS supplemented with protease/phosphatase inhibitors. The cell suspension was then heated at 52℃ for 3 minutes, then cooled for 3 minutes at room temperature. Cells were then lysed in 200 µl kinase buffer (25mM Tris-HCl (pH 7.5), 5mM β-glycerophosphate, 2mM DTT, 0.1mM $Na_3VO_4$, 10mM $MgCl_2$) by using 3 cycles of freeze-thaw. The lysates were centrifuged at 20,000 x *g* for 20 minutes at 4°C to remove precipitated cell debris. The supernatants were run through 10% SDS-PAGE and analyzed by Western blot using a 1:10,000 rabbit anti-GLS antibody (Proteintech, Inc, USA, 20170-1-AP) with Horseradish peroxidase-conjugated secondary antibody (Jackson ImmunoResearch, Inc, USA, #711-035-152). Equal amounts of protein (10 µg) were loaded into each well.

**AlphaLisa-based CETSA**

For AlphaLisa-based CETSA, cells were lysed in CETSA Cell Lysis Buffer 2 (PerkinElmer, USA, CETSA-BUF2-100ML). Reactions were completed in 384-well plates; each reaction was 9 µL final volume: 3µL of lysate, 1:3000 rabbit anti-GLS (Abcam, USA, AB156876), 1:3000 mouse anti-GLS (Novus Biologicals, USA, NBP2-42763), 1:500 anti-mouse IgG donor beads (PerkinElmer, AS104D), 1:125 anti-rabbit IgG acceptor beads (PerkinElmer, AL104C), and AlphaLISA Immunoassay buffer (PerkinElmer, AL000F). Other antibodies were attempted, with suboptimal performance in our AlphaLISA-based assay (Proteintech mouse anti-GLS, 66264-1-

Ig, and Santa Cruz mouse anti-GLS, sc-100533). AlphaLISA assays were performed using Synergy Neo2 Multi-Mode Reader (BioTek Instruments).

Mouse xenograft tumors were harvested and frozen at -80ºC. Approximately 10mg of tissue was homogenized in 500µl cold PBS containing protease/phosphatase inhibitors using a glass tissue grinder, then lysed using three freeze-thaw cycles. Lysates were separated from cell debris via centrifugation at 20,000 x *g* for 20min at 4ºC. Lysates were then heated and used for CETSA assays.

For human samples, approximately $1x10^7$ frozen platelets from 3 patients enrolled on a clinical trial with CB-839 and capecitabine before starting treatment and two weeks after starting treatment. Platelets were thawed and lysed with 500 µL CETSA Lysis Buffer. Platelet lysates were heated to 42ºC, then used for the AlphaLISA-based CETSA.

**Statistical analysis**

The data are plotted as mean $\pm$ SEM. The student t-test was used for the statistical analyses.

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**Conflict of Interest:** The authors declare no conflict of interest.

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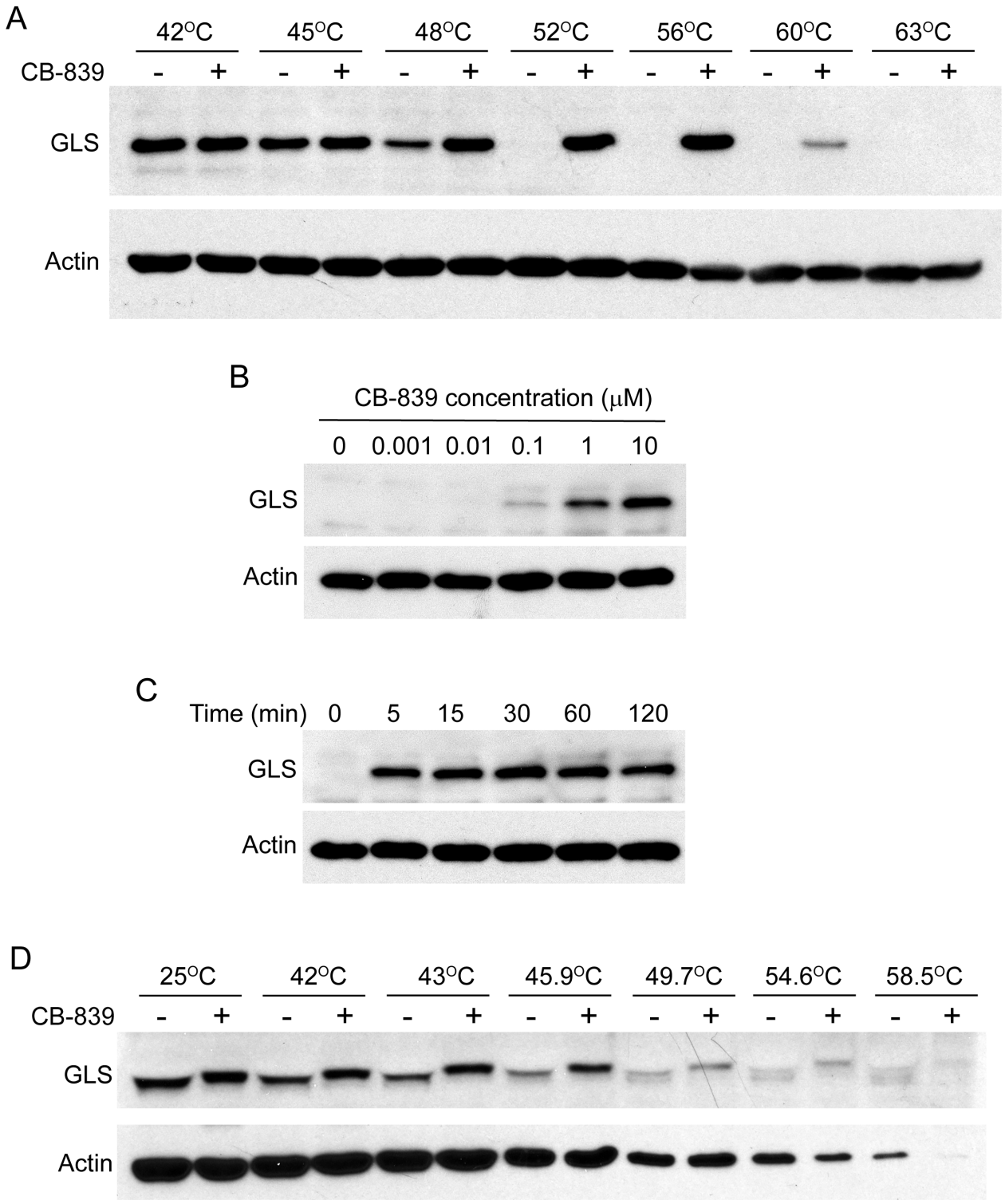
**Figure Legends**

**Figure 1: CB-839 protects GLS from thermal denaturation. (A)** HCT116 colon cancer cells were treated with 10 µM CB-839 for 2 hours. Cells were then trypsinized and resuspended in PBS, heated at the noted temperatures for 3 minutes, then lysed and subjected to SDS-PAGE and immunoblotted for GLS or actin. **(B) CB-839 engages GLS in a concentration-dependent manner.** HCT116 cells were treated with varying concentrations of CB-839 for 2 hours, then trypsinized and heated 52ºC for 3 minutes before being lysed and run through SDS-PAGE and immunoblotted for GLS or actin. (**C) CB-839 rapidly engages GLS.** HCT116 cells were treated with 10 µM CB-839 at varying time points; heating conditions were 52ºC for 3 minutes. (**D**) **CB-839 engages GLS in mouse xenograft tumors.** Western blot-based assay of shows similar levels of GLS in unheated HCT116 tumor homogenates with CB-839 treatment. In heated tumor samples, CB-839 engages GLS to protect it from thermal denaturation. Equal amounts of protein (10µg) were loaded into each well.

**Figure 2: Development of AlphaLISA-based CETSA to measure CB-839 engagement of GLS. (A) Schematic of AlphaLISA-based CETSA.** GLS engagement by CB-839 protects it from thermal denaturation, which allows it to be recognized by GLS antibodies in 384-well plates. Secondary antibodies conjugated to donor and acceptor beads recognize the Fc domain of GLS antibodies, and all together form a complex. When donor beads are excited at 680nm, they emit singlet oxygen; the close complex formed by donor bead-antibody-GLS-antibody-acceptor bead allows the singlet oxygen to excite the acceptor bead, and an Alpha signal is emitted at 615nm. In contrast, if no CB-839 is present to engage with GLS, GLS is thermally denatured and precipitates, and cannot be recognized by the anti-GLS antibodies to generate the close complex necessary for singlet oxygen to excite the acceptor bead, and no Alpha signal is emitted. **(B) Optimization of Antibodies**. HCT116 cells were trypsinized, resuspended in PBS, and heated for 3 minutes at 54ºC prior to being lysed. Lysates were added to the noted anti-GLS antibodies in 384-well plates with a mixture of donor and acceptor beads. **(C) Optimization of temperatures.** HCT116 cells were treated with 10 µM CB-839 for 2 hours, then exposed to the noted temperatures for 3 minutes. **(D): Characterization of concentration-dependent engagement of CB-839 to GLS.** Unheated samples have baseline alpha signal, because GLS remains intact. This alpha signal does not significantly change with increasing concentrations of CB-839. However, heated samples have no alpha signal with very low CB-839 concentrations; the alpha signal increases with increased CB-839 concentrations, suggesting thermal protective effect with drug engagement of GLS. **(E) Optimal duration of cell treatment.** Cells were treated with 10 µM CB-839 for the noted durations; heating conditions: 52ºC for 3 minutes. **(F) CB-839 specifically engages GLS to provide thermal stability.** HCT116 cells were treated with 10uM of compounds noted, all known to inhibit other enzymes; only CB-839 provided thermal protection of GLS.

**Figure 3: AlphaLISA-based CETSA can detect drug engagement of GLS in minute tumor volumes.** Mice implanted with HCT116 or DLD1 colon cancer cells to form xenograft tumors were treated with once-daily CB-839 or vehicle for five days before tumor harvest. Approximately 10 mg of a tumor was used for each sample.

**Figure 4: AlphaLISA-based CETSA is sensitive to measure CB-839-engagement of GLS in human platelets.** Patients with metastatic colorectal cancer on clinical trial with CB-839 and standard chemotherapy capecitabine had blood drawn for correlative studies before starting treatment (pre-treatment) and after two weeks of twice-daily dosing of CB-839 (post-treatment). Platelets were isolated from whole blood and analyzed for CB-839 engagement of GLS. (**A**) Average relative AlphaSignal normalized to pre-treatment values. The statistical significance was determined by Student's t-test. (**B**) To ensure the assay is reproducible, we performed the assay on isolates from three patients' platelet isolates in three independent experiments.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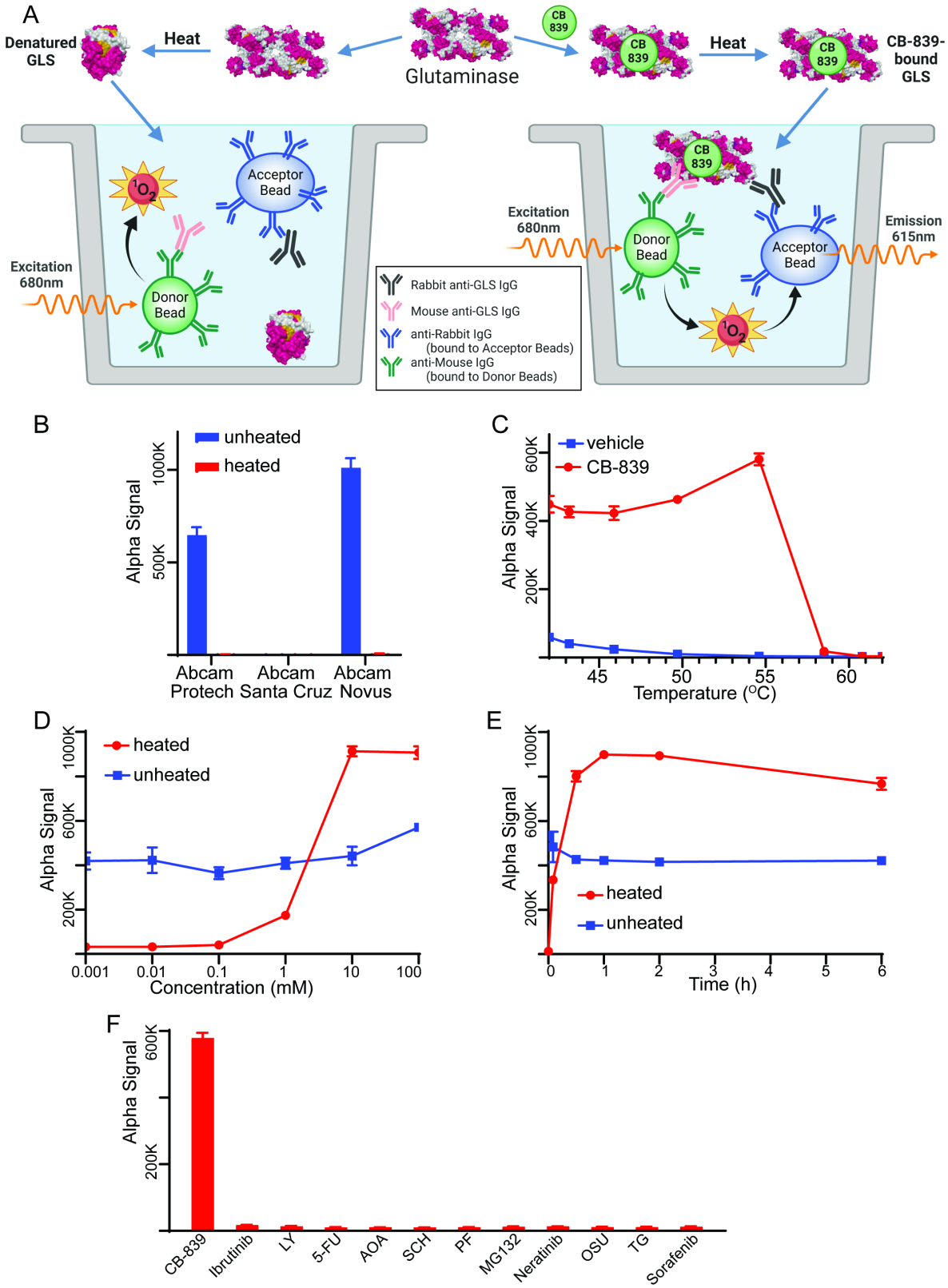
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Alpha Signal
Abcam Protech
Abcam Santa Cruz
Abcam Novus
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Temperature (°C)
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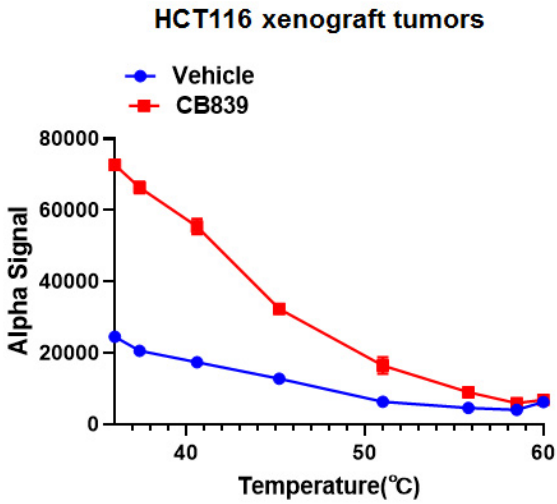
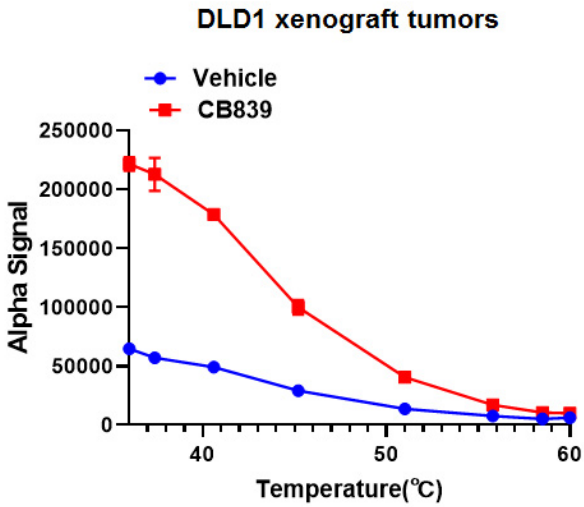
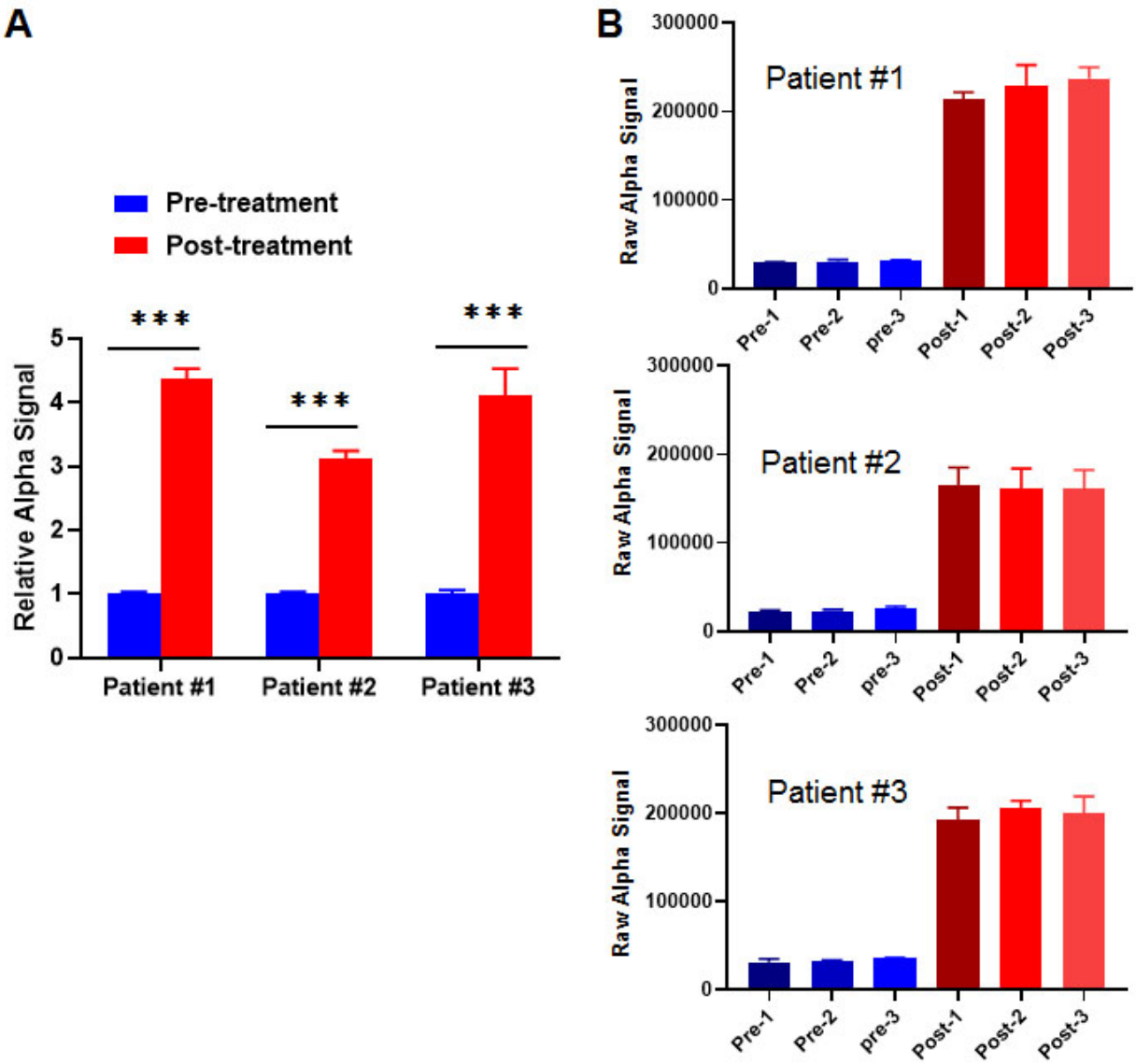
Time (h)
F
CB-839
Ibrutinib
LY
5-FU
AOA
SCH
PF
MG132
Neratinib
OSU
TG
Sorafenib




A
Pre-treatment
Post-treatment
Relative Alpha Signal
0
1
2
3
4
5
***
***
***
Patient #1
Patient #2
Patient #3
B
Raw Alpha Signal
300000
200000
100000
0
Patient #1
Pre-1
Pre-2
pre-3
Post-1
Post-2
Post-3
Patient #2
Patient #3