Impaired anaplerosis is a major contributor to glycolysis inhibitor toxicity in glioma

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Abstract

Reprogramming of metabolic pathways is crucial to satisfy the bioenergetic and biosynthetic demands and maintain the redox status of rapidly proliferating cancer cells. In tumors, the tricarboxylic acid (TCA) cycle generates biosynthetic intermediates by oxidation of anaplerotic substrates, such as glucose-derived pyruvate and glutamine-derived glutamate. We have previously documented that a subset of tumors with 1p36 homozygous deletion exhibit co-deletion of ENO1, in turn becoming extremely dependent on its redundant isoform ENO2 and sensitive to an overall enzymatic deficiency of enolase. Metabolomic profiling of ENO1-deleted glioma cells treated with an enolase inhibitor revealed a profound decrease in TCA cycle metabolites, which correlated with
cell-line specific sensitivity to enolase inhibition, highlighting the importance of glycolysis-derived pyruvate for anaplerosis. Correspondingly, the toxicity of the enolase inhibitor was significantly attenuated by exogenous supplementation of supraphysiological levels of anaplerotic substrates including pyruvate. These findings led us to hypothesize that cancer cells with ENO1 homozygous deletions treated with an enolase inhibitor might show exceptional sensitivity to inhibition of glutaminolysis because of reduced anaplerotic flow from glycolysis. We found that ENO1-deleted cells indeed exhibited selective sensitivity to the glutaminase inhibitor CB-839, and this sensitivity was also attenuated by exogenous supplementation of anaplerotic substrates including pyruvate. Despite these promising in vitro results, the antineoplastic effects of CB-839 as a single agent in ENO1-deleted xenograft tumors in vivo were modest in both intracranial orthotopic tumors, where the limited efficacy could be attributed to the blood brain barrier (BBB), and subcutaneous xenografts, where BBB penetration is not an issue. This contrasts with the enolase inhibitor HEX, which, despite its negative charge, achieved antineoplastic effects in both intracranial and subcutaneous tumors. Together, these data suggest that at least for 1p36-deleted gliomas, tumors in vivo—unlike cells in culture—show limited dependence on glutaminolysis and instead primarily depend on glycolysis for anaplerosis. Our findings reinforce the previously reported metabolic idiosyncrasies of the in vitro and in vivo environments as the potential reasons for the differential efficacy of metabolism-targeted therapies in in vitro and in vivo systems.

**Keywords:** [3-10 keywords]
Cancer Metabolism, Anaplerosis, Collateral Lethality, Glycolysis, Glutaminolysis, Enolase Inhibitor, POMHEX, CB-839
Background

Tumor cells must undergo significant alterations in their metabolic pathways to support their bioenergetic and biosynthetic demands in their mostly nutrient- and oxygen-deficient microenvironment (1-3). One such metabolic adaptation is the Warburg phenomenon, a bioenergetically inefficient process characterized by an increased reliance of cancer cells on glycolysis for ATP production, making glycolysis a targetable metabolic vulnerability in diverse cancers (4, 5). However, therapeutically targeting glycolysis specifically in cancer cells remains aspirational because glycolysis is an indispensable metabolic pathway for some nonmalignant cells (6); thus, blockade of glycolysis is incompatible with life. However, synthetically lethal interactions supported by glycolysis can be exploited to specifically target cancer cells. Building on this idea, we conceived the framework of collateral lethality, whereby incidental loss of passenger metabolic genes—genes that are lost along with driver tumor suppressor genes—can be exploited therapeutically by targeting the redundant isoforms of the passenger genes (7, 8). Using this concept of collateral lethality, we validated that in a subset of gliomas with 1p36 deletions, passenger deletion of the glycolytic gene ENO1 selectively renders cancer cells sensitive to inhibition of the redundant isoform ENO2 (8). Thus, to exploit this therapeutic opportunity in this subset of tumors, we developed a specific inhibitor of enolase, HEX and a pro-drug thereof, POMHEX. Both POMHEX and HEX exerted dramatic antineoplastic effects on ENO1-deleted tumor cells in culture and in ENO1-deleted xenograft tumors. Metabolomic and biochemical data strongly indicated both specific and dose-dependent inhibition of glycolysis by these enolase inhibitors (9).
Besides glucose, cancer cells also exhibit a predilection for glutamine, a nonessential amino acid that cancer cells consume disproportionately to support their biosynthetic requirements (10, 11). Multiple studies have shown that cancer cells are “addicted” to glutamine, at least in vitro (10, 11). Therefore, glutamine metabolism has emerged as another promising therapeutic target, and telaglenastat (CB-839), a glutaminase inhibitor targeting glutamine metabolism is currently being investigated in randomized clinical trials against a range of malignancies. (https://www.clinicaltrials.gov/, NCT03428217, NCT04265534) (12).

Pyruvate derived from glycolysis and glutamate derived from glutaminolysis converge to replenish tricarboxylic acid (TCA) cycle intermediates at different steps. The TCA cycle is strategically situated at the center of cellular metabolism, where it serves as an anabolic hub for the synthesis of macromolecules such as fatty acids, cholesterol, and amino acids that are crucial to support rapidly growing tumors. Carbon atoms are constantly drained from the TCA cycle for biosynthetic reactions and for CO₂ release by the NAD-dependent dehydrogenases to supply reducing equivalents to oxidative phosphorylation. The TCA cycle must thus be constantly replenished by carbon atoms, a process termed anaplerosis (13). The coupling of anaplerosis and cataplerosis—the removal of TCA cycle intermediates—is critical to maintaining the balance of the TCA cycle metabolites that are essential for supporting crucial biosynthetic reactions. Additionally, this coupling also ensures that redox equilibrium and ATP production via oxidative phosphorylation are maintained. The current dogma in biochemistry is that the main anaplerotic substrates are pyruvate (from glucose) and glutamate (from glutamine), with lesser contributions from precursors of propionyl-CoA, such as odd-chain fatty acids,
amino acids, and C5 ketone bodies that feed into succinate (14). With a view to exploiting both the increased dependence of cancer cells on glucose and glutamine for their growth and the roles of these molecules in TCA cycle anaplerosis, we surmised that impairing the use of these two key nutrients can be exploited as a therapeutic strategy to induce bioenergetic and anaplerotic nutrient stress and suppress tumor growth.

Using the collateral lethality paradigm, we previously reported that a subset of glioblastoma tumors with collateral homozygous deletion of ENO1 along with the 1p36 tumor suppressor locus is extraordinarily sensitive to the inhibition of ENO1’s redundant paralog ENO2 (7, 8). Here, we show that a major mechanism of toxicity of glycolysis inhibition by the enolase inhibitor POMHEX is depletion of TCA cycle intermediates via inhibition of the formation of anaplerotic pyruvate from glucose. In support of this model, metabolomic data indicate significant decreases in the TCA cycle intermediates malate and fumarate in response to enolase inhibition. We show that enolase inhibitor toxicity can be partially rescued by high-dose exogenous pyruvate supplementation in the media. Furthermore, inhibition of anaplerotic reactions through glutaminase by the clinical-grade glutaminase inhibitor CB-839 is synergistically toxic with the enolase inhibitor POMHEX, in a manner also modulated by the availability of exogenous pyruvate. Furthermore, CB-839 displays selective toxicity towards cells with homozygous deletions of ENO1 but not isogenic rescued cells even in the absence of the enolase inhibitor, and this toxicity can be completely alleviated by pyruvate supplementation in the media. The in vitro data strongly indicated that anaplerosis could be targeted for cancer therapy, provided that one arm (glycolysis or glutaminolysis) is inactivated or deficient in a specific cancer cell. However, despite mechanistically promising in vitro data, the synergistic effect endowed
by the enolase and glutaminase inhibitors could not be entirely recapitulated in vivo in both orthotopic (intracranial) and subcutaneous tumor models. Our observation that cancer cells in culture may depend on both glycolysis and glutaminolysis, with glutamine oxidation being essentially dispensable for tumor sustenance in vivo, echoes previously reported discrepancies in tumor metabolism in in vitro vs. in vivo systems (15-17).

Methods

Cell lines and enolase and glutaminase inhibitor toxicity testing in vitro

The cell lines used in the experiments were LN319 (CVCL_3958, glioblastoma), H423/D423-MG (CVCL_1160, glioblastoma), H502/D502-MG (CVCL_1162, glioblastoma), U-343MG (CVCL_S471, glioblastoma), and ENO1 isogenic rescue, D423 ENO1. LN319 has been previously described (18) and is ENO1 wild-type (19); it has been identified as a subclone of LN992 (20), but for our experiment—as an ENO1 wild-type control—this was considered acceptable. The H423/D423-MG cell line, referred to as D423 here, features a 1p36 homozygous deletion encompassing ENO1 (8). The H502/D502-MG cell line, referred to as D502 throughout the paper, has a 1p36 homozygous deletion but with ENO1 excluded and not deleted. Both D423 and D502 were generously by D. Binger (18). U343-MG is heterozygous for ENO1 and has been described previously (8). D423 ENO1 is an isogenic cell line generated by our lab for previous experiments and engineered for constitutive and ectopic expression of ENO1 (18). All cell lines were cultured at 37 °C in a 5% CO2 atmosphere in Dulbecco’s modified Eagle medium (DMEM). The DMEM used contained 4.5 g/L glucose, 110 mg/L pyruvate, and 584 mg/L glutamine (Cellgro/Corning #10-013-CV) and contained 10% fetal bovine serum, 1% penicillin/streptomycin, and 0.1% Amphotericin B. Cell lines were validated by
the Characterized Cell Line Core (CLCC) at The University of Texas MD Anderson Cancer Center using the Promega 16 High Sensitivity STR KIT. Short tandem repeat profiles were compared to the CLCC database and external cell databases (ATCC/DSMZ/RIKEN/JCRB). Cell lines were tested on a regular basis for mycoplasma contamination using the MycoAlert PLUS detection kit (Lonza #LT07-118).

To test the sensitivity of each cell line to enolase and glutaminase inhibitors, we seeded 2,000 cells per well in 96-well plates in appropriate media. After incubating for 24 h, cells in columns 3-10 of the 96-well plate were treated with fresh media containing an enolase inhibitor in a twofold dilution series. Columns 1, 2, 11, and 12 were given fresh media and left as controls. For tests of combination treatments with POMHEX and CB-839, a uniform dose of 500 nM CB839 was administered in rows 3-12, while POMHEX dilution treatments were administered in rows 3-10. After 7 days, cells were washed with phosphate-buffered saline (PBS) and fixed in 10% formalin. Fixed cells were stained with 0.05% crystal violet and extracted with 0.1% acetic acid. Cell viability was quantified by spectrophotometric absorption at 595 nm using a microplate reader.

Experimental animals

All in vivo experiments with mice were approved by MD Anderson’s Institutional Animal Care and Use Committee (IACUC) and performed in an AAALAC-accredited facility at MD Anderson. Mice used in this experiment were immunocompromised female nude Foxn1nu/nu mice bred at the Experimental Radiation Oncology Breeding Core at MD Anderson.

Establishment of orthotopic intracranial tumors
Intracranial glioma tumors were established in mice aged 4 to 6 months. First, bolts were inserted into the skulls of the mice by drilling a hollow plastic screw into the skull (21). Mice were given 2 weeks to recover and monitored for signs of morbidity. Next, using a Hamilton syringe, 200,000 glioma cells were injected into the brain of the mice through the bolt. Both intracranial bolt implantation and tumor injection were performed by the MD Anderson Intracranial Injection Fee-for-Service Core (Dr. Fred Lang, Director). Throughout the experiment, any animals exhibiting any severe neurological morbidities were euthanized according to IACUC regulations.

**In vivo tumor volume measurement**

Prior to undergoing magnetic resonance imaging (MRI), the mice were briefly anesthetized with isoflurane. Throughout the imaging protocol, the animal’s body temperature was maintained with a heating blanket, and a stereotactic holder was used to restrain the mouse and hold its head in place. The animals’ heart and breathing rates were monitored throughout the imaging procedure. Weekly T2-weighted MRI scans of intracranial tumors were performed with a 7T Biospec USR70/30 MRI system (Bruker Biospin MRI, Billerica, MA) in MD Anderson’s Small Animal Imaging Facility (SAIF).

To center the image, a low-resolution axial scan was first taken. After calibration, one high-resolution coronal scan was taken with a slice thickness of 0.750 mm and a slice spacing of 1.000 mm. Next, two high-resolution axial scans, each offset from the other by 0.500 mm, were taken, with a slice thickness of 0.500 mm and slice spacing of 1.000 mm. The offset of 0.500 mm was chosen to allow better coverage of the tumor, as the scans individually had noncontiguous slices. For each scan, the number of slices varied based on tumor size.
MRI scans were analyzed with the open-source software 3D slicer (v4.10, http://www.slicer.org) by 4 independent members of our labs (22). For each slice, the Draw tool in the Editor module was used to manually select tumor tissue. Areas of edema and concave regions of the tumor where the bolt was implanted were excluded from the selections. Using the Label Statistics module, tumor volumes from each scan were calculated automatically by summing the selected pixel area from each slice, converting it to cm² in accordance with the DICOM metadata, and multiplying it by the slice spacing of 1.000 mm. Each lab member then calculated a final volume for the entire MRI series as the mean of the 3 volumes (1 coronal, 2 axial) they determined. For final data analysis purposes, the volume for each MRI series was the average of the volumes calculated by the 4 lab members.

**Establishment of subcutaneous tumors**

Subcutaneous tumors were established in mice aged 2-4 months. ENO1 deleted cells were first trypsinized and washed with PBS twice. The cells were then resuspended in PBS and mixed with MatrigelBD in a 1 to 1 ratio and left on ice to prevent Matrigel solidification. 200 µl (5 million cells total) of Matrigel-cell suspension was then administered to each anesthetized mouse on its right flank. The mice were monitored for a week for any sign of inflammation.

Tumors were measured three times a week using Vernier’s Calipers. Two dimensions were measured, and the volume was determined as the product of two dimensions and the average of two dimensions. When the tumors reached approximately 200 mm³, the mice were grouped into different treatment groups for drug administration.

**Xenografted mouse drug treatment with HEX and CB-839**
A solution of HEX in ddH₂O (250 mg/ml) was made and adjusted to 7.2-7.4 pH, then sterile-filtered and stored at -20 °C. HEX was injected subcutaneously into mice in drug volumes adjusted according to their weight.

CB-839 (20mg/ml) formulation was provided by Calithera Biosciences along with the vehicle control. Both CB-839 and vehicle controls were aliquoted and frozen in -20 °C and administered 200 mg/kg BID by oral gavage. Per Calithera’s instructions, the thawed drug was used one-time only and the left-over drug was discarded.

**Polar metabolite profiling of cells and extracted tumors**

To profile polar metabolites, we used the Johan Asara Metabolomics Platform at the Beth Israel Deaconess Medical Center (BIDMC) (23). Polar metabolite samples were analyzed by liquid chromatography-tandem mass spectrometry using a 5500 QTRAP hybrid triple quadrupole mass spectrometer (SCIEX) coupled to a high-performance liquid chromatography column (Shimadzu) with an amide hydrophilic interaction chromatography column (Waters; pH = 9.0 at 400 mL/min). Selection reaction monitoring with polarity switching tracked a total of 300 polar metabolites of interest from both cells and tumor tissue. Q3 peaks were integrated using MultiQuant 2.1 software.

Glioma cells grown in 10cm dishes were harvested at roughly 90% confluency. First, the medium was removed, and the cells were washed once with cold PBS. Then, 4 mL of 80% methanol precooled to -80 °C was added, and the cells were incubated for 20 minutes on -80 °C dry ice. Cells were harvested using a scraper, and lysates were collected into precooled tubes. Cell lysates were spun down for 5 minutes at 18,000 × g at 4 °C to precipitate cell debris and nonpolar metabolites. The polar metabolites collected into the supernatant, which was aliquoted into 1.5-mL Eppendorf tubes and dried with a
Thermo Fisher SpeedVac. The concentrated and dried polar metabolites were then sent for profiling to the BIDMC Metabolomics Platform.

Tumors were harvested when they grew to approximately 1000 mm$^3$. First, animals were euthanized under a standard IACUC protocol, usually after 4-6 hours of final drug dose administration. Subcutaneous tumors were then extracted, weighed, and cut in half. One half was promptly snap-frozen in liquid N$_2$ for further metabolomic analysis, while the other half was fixed in 10% formalin for immunohistochemical analyses. Snap-frozen tumor chunks were cut into pieces weighing roughly 50 mg and transferred to chilled microcentrifuge tubes (Fisher Scientific, Cat. 02-681-291) containing Qiagen steel beads. After 1 mL of -80 °C 80% methanol was added to tubes, tumor tissue was bead-mill homogenized at 28 Hz for multiple durations of 45 s using a Qiagen TissueLyser. We next added 80% methanol to the homogeneous tumor lysate to reach a final 50 mg/2 mL composition. Tumor lysates were then incubated for 15 minutes on dry ice and homogenized once more for 1 minute with a vortex mixer. Samples were then promptly centrifuged for 5 minutes at 14,000 × $g$ at 4 °C. The supernatant containing polar metabolites was submitted to BIDMC for analysis.

Immunohistochemistry

Mice were euthanized either when their subcutaneous tumors reached 1000 mm$^3$ or earlier if they exhibited any morbidities. The tumors were harvested and promptly fixed in 10% paraformaldehyde. The fixed tumors were then submitted to the Veterinary Pathology Core at MD Anderson, where tissues were dehydrated, embedded in paraffin, and sectioned. We dried the sectioned tissue slides overnight at 60 °C before deparaffinization with xylene. Deparaffinized slides were rehydrated via a dilution series
of aqueous ethanol. For antigen retrieval, slides were boiled in citrate buffer for 10 minutes and cooled for 30 minutes. Slides were then incubated for 1 h in 2% goat serum for blocking. Blocked slides were incubated evenly with primary antibodies diluted to 1:1000 in PBS containing 2% goat serum for 24 h at 4 °C. The primary antibodies used were anti-phospho S10 histone H3 (rabbit anti-phospho histone H3 (S10) IHC antibody, affinity purified; Bectyl Laboratories, IHC-00061) and monoclonal anti-cleaved caspase 3 rabbit (cleaved caspase-3 (Asp175) (5A1E) rabbit mAb; CST# 9664T, Cell Signaling Technology). Following 3-5 minute washes in PBS with shaking, slides were incubated with 1x goat anti-rabbit Poly HRP secondary antibody (Thermo Fisher, #B40962) for 30 minutes and then washed in PBS with Tween (3 × 5 min) with shaking before developing. Sections were developed using EnzMet (Nanoprobes # 6001-30 ML, yields a black stain) were counterstained with hematoxylin and eosin. Stained slides were mounted using Thermo Scientific Cytoseal 60 and Ultra Microscope Cover Glass and dried for 24 h at room temperature.

**Quantification of PH3 and CC3 signals**

Aperio Images scope (Leica Biosystem) was used to snap pictures of the slides. Pictures of 10X (100X with objective) sections of 1.6*10^6 µm^2 surface area, were taken and the black stains for PH3 and CC3 were counted. Results are expressed as positive nuclei per 10X section.

**Statistical analysis**

Statistical analyses reported in this study were performed using either Microsoft Excel or Graph Pad Prism 8. Unpaired Student’s test and 1- or 2-way ANOVA were used where appropriate. Tukey’s post hoc analysis was used to determine statistical significance following ANOVA. P<0.05 was used as a threshold to determine statistical significance.
Results

Profound anaplerotic inhibition by enolase inhibition and reversal of toxicity by exogenous supplementation of anaplerotic substrates

We previously demonstrated that glioma cells with ENO1 passenger deletions are selectively susceptible to inhibition of ENO1’s redundant paralog ENO2 through the collateral lethality paradigm (8). To test the therapeutic utility of this concept in tumors with ENO1 deletions, we developed small-molecule inhibitors of the glycolytic enzyme enolase. HEX, a phosphono-hydroxamate, as well as its prodrug, pivaloyloxymethyl (POM)-adduct POMHEX, both potently inhibit glycolysis and display four-fold greater specificity against ENO2 than ENO1 (9). In the current study, we found that glioma cell lines that have either homozygous (D423) or heterozygous (D502, U343) deletions of ENO1 are selectively more sensitive to the enolase inhibitor POMHEX than are ENO1 rescued (D423 ENO1) and wild-type cells (LN319) (Figure 1A-C). The dose-response curves and the half maximal inhibitory concentration (IC$_{50}$) data showed that the enolase inhibitor sensitivity of the cancer cell lines correlated with their ENO1 status. Cells with ENO1 homozygous deletions incurred the most significant toxicity from POMHEX, followed by cells with ENO1 heterozygous deletions and ENO1 wild type cells (Figure 1A-C). Notably, D502 and U343 cells, despite both having ENO1 heterozygous deletions, displayed differential sensitivities to inhibition of enolase. We surmised that differences in the basal levels of carboxylesterase and phosphodiesterase, the enzymes that cleave the POM adduct from POMHEX, may explain the differences in enolase inhibitor sensitivity between D502 and U343.
Next, we sought to elucidate the mechanistic basis of the toxicity induced by enolase inhibition by performing comprehensive metabolomic profiling. Cells treated with POMHEX showed elevation of the levels of metabolites upstream of enolase and a concurrent decrease in lactate, indicating effective target engagement by the inhibitor (Figure 1D-E, and supplemental Figure S1 for other glycolytic intermediates). Additionally, across glioma cell lines treated with POMHEX in vitro, we consistently observed dose-dependent depletion of major TCA cycle metabolites such as citrate, fumarate, and malate (Figure 1E, and supplemental Figure 2 for other TCA cycle metabolites). Interestingly, the extent of disruption in the TCA cycle metabolites corresponded with the sensitivity of the cell line to the inhibitor. This was particularly evident in D502 and U343 cells; D502 cells were more sensitive than U343 cells to POMHEX (Figure 1A and B) and displayed more substantial depletion of TCA cycle intermediates in response to POMHEX than did U343 cells (Figure 1E, and supplemental Figure S2). Abrogation of pyruvate formation by enolase inhibition reduces the number of carbon atoms that feed into the TCA cycle, effectively depleting the TCA cycle intermediates. Thus, these findings suggest that a proximal cause of toxicity by POMHEX is the depletion of the anaplerotic substrate pyruvate.

Because the depletion of TCA cycle intermediates was found to be the most prominent metabolic effect of enolase inhibition with POMHEX and a strong correlate of cells’ sensitivity to enolase inhibition, we sought to determine whether exogenous supplementation with physiological, supraphysiological, or artificial exogenous anaplerotic substrates could modulate enolase inhibitor toxicity in vitro. Different cellular metabolites funnel into the TCA cycle to replenish the carbon atoms at different steps
(Figure 2A); thus, we performed in vitro rescue experiments in pyruvate-free DMEM with a panel of anaplerotic substrates (Figure 2, Table 1 and supplemental Figure S3, S4). We found that the medium supplemented with physiological levels of pyruvate (100 μM) only minimally attenuated POMHEX toxicity (data not shown), but medium with supraphysiological levels of pyruvate (5 mM) resulted in a 2.5-fold increase in IC_{50} of POMHEX in cells with ENO1 homozygous or heterozygous deletions (Figure 2B, and supplemental Figure S3). Consistently, methyl-pyruvate, a cell-permeable analog of pyruvate, and oxaloacetate, a TCA cycle metabolite, dramatically rescued POMHEX toxicity, as evidenced by a 6-fold and a 4-fold increase, respectively, in the IC_{50} of POMHEX in ENO1-deleted cells (Figure 2B, and Supplemental Figure S4C).

Synergy of the enolase inhibitor POMHEX and glutaminase inhibitor CB-839 in vitro

Cancer cells consume disproportionate amounts of the nonessential amino acid glutamine to support myriad biosynthetic reactions in the cells (Supplemental Figure S5C). To test whether ENO1-deleted cells exhibit glutamine auxotrophy, we grew ENO1-deleted, ENO1-rescued, and ENO1 wild type cells in glutamine-deficient and glutamine-supplemented medium. It is important to note that the glutamine-deficient DMEM was not exogenously supplemented with glutamine, but it was not devoid of glutamine since it contained FBS-derived glutamine. We observed that regardless of ENO1 status, glutamine was essential for glioma cell growth as cells grown in glutamine-deficient medium displayed substantially delayed growth, confirming previous findings that cancer cells are “addicted” to glutamine in vitro (Supplemental Figure S5A-B) (11, 24). Notably, the availability of pyruvate in the medium (1 mM in DMEM) did not compensate for the
lack of exogenous glutamine. Interestingly, in glutamine-deficient condition, treatment with the clinical-grade glutaminase inhibitor CB-839 instead of exacerbating cell death, to some extent restored cell proliferation (Supplemental Figure S5A-B). We reasoned that the small amount of FBS-derived glutamine that the cells are exposed to is quickly catabolized, rendering glutamine limiting for protein and nucleotide synthesis. CB-839 treatment can partially promote cell proliferation via glutamine accumulation upstream of glutaminase reaction, which may be preserved for protein and nucleotide synthesis in the cells. This points to a dynamic role of the amino acid glutamine in cancer cells where glutamine-mediated non-anaplerotic reactions may be enough to support cancer cell survival and growth when exogenous supply of glutamine is limiting (Supplemental Figure S5A-C). Considering that glutamine is a highly abundant amino acid in plasma and that cells in vivo are constantly exposed to high amounts of glutamine, we were interested to explore how cancer cells depend on glutamine to support TCA cycle reactions. Thus, we tested whether abrogation of a step in glutamine metabolism that is directly relevant to TCA cycle anaplerosis—i.e., glutaminase-mediated glutamine-to-glutamate conversion—could be modulated by addition of pyruvate to the medium (Figure 3A-B and supplemental Figure S5C). To this end, we tested the efficacy of CB-839 under pyruvate-free conditions and found that CB-839 significantly impaired the growth of ENO1-deleted cells (Figure 3A-B). Additionally, the toxicity of CB-839 was reversed upon exogenous supplementation of high-dose pyruvate and methyl-pyruvate, 2-dimethylxogluturate (2-OG) and oxaloacetate, indicating that the efficacy of CB-839 can be modulated by the availability of anaplerotic substrates other than pyruvate. (Figure 3A-B, and supplemental Figure S6A-D). These findings further supported the
idea that the impairment of ENO1-deleted glioma cell growth upon abrogation of glutamine metabolism by CB-839 is indeed due to defects in TCA cycle anaplerosis. Interestingly, the addition of lactate did not rescue the toxicity of CB-839, reinforcing that cancer cells do not prefer lactate as an anaplerotic substrate (Supplemental Figure S6C).

To mechanistically validate this, we performed metabolomic profiling of cells treated with the glutaminase inhibitor CB-839 in pyruvate-free and pyruvate-supplemented medium. We found that treatment with CB-839 in both pyruvate-free and pyruvate-supplemented conditions caused a substantial accumulation of glutamine (Figure 3D and E). CB-839 treatment also diminished glutamate levels in pyruvate-free conditions, although only in ENO1-deleted cells (Figure 3D). However, in pyruvate-supplemented conditions, CB-839 treatment did not diminish glutamate levels in any of the tested cell lines (Figure 3E). Additionally, under pyruvate-free conditions, CB-839 treatment caused a dramatic depletion of TCA cycle intermediates such as citrate, succinate, fumarate, malate, and oxaloacetate (Figure 3D). However, in pyruvate-supplemented conditions, the reduction in the levels of TCA cycle intermediates was not dramatic, suggesting that pyruvate-mediated anaplerosis attenuated the effect of CB-839 (Figure 3E).

Given the interplay of pyruvate and glutamine to sustain the TCA cycle, we reasoned that these two arms of anaplerosis together present a major targetable metabolic liability in ENO1-deleted glioma cells. More specifically, due to the pronounced depletion of TCA cycle metabolites caused by POMHEX and CB-839 treatment individually, we hypothesized that impairing carbon atom restoration in the TCA cycle
through these two pathways could synergize to specifically kill ENO1-deleted cells. Since CB-839 treatment alone showed a dramatic effect on cell viability in pyruvate-free conditions, we tested whether combined inhibition of glycolysis by POMHEX and glutaminolysis by CB-839 exacerbated toxicity specifically in ENO1-deleted cells. We found that ENO1-deleted cells were indeed dramatically sensitive to combined inhibition of glycolysis and glutaminolysis in pyruvate-free conditions (Figure 4A and C). Interestingly, this effect was partially rescued by addition of pyruvate (Figure 4D). These findings reinforce that glycolysis and glutaminolysis can be targeted together for the synergistic killing of ENO1-deleted cells via exacerbating the impairment of TCA cycle anaplerosis (Figure 4E).

Co-treatment with HEX and CB-839 attenuates ENO1-deleted tumor growth but does not cause frank tumor regression

We next asked whether the synergistic effect observed in vitro from the combined inhibition of glycolysis and glutaminolysis would also take place in an in vivo tumor model. Since an intracranial orthotopic tumor would be the most faithful model for human glioma, we established ENO1-deleted intracranial tumors in immunocompromised Foxn1nu/nu nude mice. Note that for our in vivo experiments, we used HEX instead of POMHEX to inhibit Enolase. While POMHEX is superior to HEX in cell culture and is more potent (anti-tumor activity) on a molar basis in mice in vivo, the efficacy of POMHEX in mice is limited by its rapid degradation to the monoester form in mouse plasma (t1/2 <1 min) (9). Thus, POMHEX requires administration through intravenous tail vein injections, which are difficult to carry out on a daily basis. At the same time, we wished to observe the interactions of Enolase inhibition and Glutaminase inhibition, which would be easier with
a less potent Enolase inhibitor. Nonetheless, despite HEX’s modest permeability across cell membranes, consistent with our previous data, we observed strong inhibition of intracranial tumors growth in mice bearing in ENO1-deleted intracranial gliomas (9). In contrast, following 3 weeks of drug administration, MRI-based tumor measurements indicated that the antineoplastic effect of CB-839 as a single agent was negligible (Figure 5A-D). Based on our in vitro data, we expected that treatment with both CB-839 and HEX would dramatically potentiate anaplerotic nutrient stress and induce synergistic attenuation of intracranial tumor growth. However, there was no hint that the combination treatment was better than HEX alone. Most likely, the antineoplastic effect we observed with the combination of HEX and CB-839 treatment was induced primarily by HEX. This drastic discrepancy in the efficacy of CB-839 alone and in combination with HEX in in vitro and in vivo settings could be due to CB-839’s poor blood-brain barrier (BBB) penetration, which has been reported previously (12).

In order to rule out the limitations imposed by the BBB in drug delivery to the tumors, we tested the combination of CB-839 and HEX in a subcutaneous tumor model. We subcutaneously injected ENO1-deleted D423 cells into immunocompromised nude Foxn1nu/nu mice. After the tumors reached an approximate size of 200 mm³, we treated the mice with CB-839 and HEX as single agents and in combination. On its own, CB-839 only minimally diminished subcutaneous tumor growth, whereas, consistent with our observations of intracranial tumor growth, HEX on its own had a strong antineoplastic effect (Figure 6A and B). Interestingly, although in the intracranial tumor model the efficacy of the combination of CB-839 and HEX was almost indistinguishable from that of the single-drug treatments, especially HEX, in subcutaneous models, the combination
treatment was modestly more efficacious, indicating a possible additive effect of the two
drugs (Figure 6A and B). Interestingly, immunohistochemical staining for
phosphohistone H3 (PH3) revealed that compared to the control group, there were
significantly fewer actively dividing cells in CB-839, HEX and CB-839+ HEX treated
tumors (Figure 6C, upper panel, and Figure 6D). However, cleaved-caspase 3 (CC3)
signal corroborated the pattern observed on the tumor growth curve, where CB-839
treatment induced only minimal apoptosis, while HEX and combination of CB-839 and
HEX led to considerable tumor apoptosis (Figure 6C, lower panel, and Figure 6E).

Next, to discern the effect of the drugs on the metabolite profiles, we performed
metabolomic analyses of frozen sections of the subcutaneous tumors. Consistent with
our *in vitro* metabolomic data, we found that treatment with HEX, both as a single agent
and in combination with CB-839, led to a dramatic accumulation of glycolytic
intermediates upstream of enolase while significantly diminishing downstream
metabolites such as pyruvate and lactate (Figure 6F and supplemental Figure S7). CB-
839 treatment, however, despite showing modest target engagement as evidenced by
accumulation of glutamine, did not cause notable reductions in TCA cycle metabolites
(Figure 6G). HEX treatment on its own elicited notable reductions in TCA cycle
metabolites such as fumarate and malate, but the addition of CB-839 did not significantly
enhance this effect of HEX (Figure 6G). Moreover, the extent of TCA cycle disruption
corresponded to the extent of tumor growth inhibition in each treatment group. Thus, we
confirmed that even when CB-839’s BBB permeability was not a limiting factor, the
synergy between HEX and CB-839 that we observed in *in vitro* experiments could not be
recapitulated in *in vivo* subcutaneous tumor models and, at best, the combination
treatment exhibited an additive interaction. This suggests that ENO1-deleted glioma cells minimally depend on glutamine as a fuel to support anaplerosis in vivo.

Discussion

Inhibition of central carbon metabolism has long been an aspirational target for the treatment of cancer. As the focal point of central carbon metabolism, the TCA cycle needs to be continuously replenished with carbon atoms that are drained for the synthesis of biosynthetic building blocks and catabolic reactions feeding the mitochondrial electron transport chain (13, 14). It is thought that the main sources of anaplerotic substrates for cancer cells are glutamate derived from glutamine and pyruvate derived from glucose (14). Studies have demonstrated that a compromise in the pyruvate supply to the TCA cycle renders glutamine oxidation essential for TCA cycle anaplerosis, establishing a synthetic lethal interaction that could be exploited for cancer therapy (25, 26). The recent development of high-affinity inhibitors of glutaminase such as CB-839 has led to a flurry of studies on the role of glutaminolysis in sustaining cancer cell growth. Similarly, our lab has recently synthesized and systematically validated high-affinity inhibitors of the glycolytic enzyme enolase and has drawn attention to the existence of subsets of cancers with homozygous deletions of ENO1, which are deficient in overall enolase activity and susceptible to inhibitors of oxidative phosphorylation by virtue of this glycolytic deficiency (9, 27).

In this study, we brought together our novel inhibitors of enolase, HEX and POMHEX, as well as the glutaminase inhibitor CB-839, to investigate the redundancies between glycolysis and glutaminolysis for supporting tumor growth and viability in vitro and in vivo in ENO1-deleted gliomas. First, we performed a systematic metabolomic
analysis to determine the effects of the enolase inhibitor prodrug, POMHEX, on cultured glioma cells varying in ENO1-deletion status. We found that even in DMEM with supraphysiological levels of glucose (25 mM, 5-fold higher than physiological), pyruvate (1 mM, 30-fold higher than physiological), and glutamine (4 mM, 8-fold higher than physiological)(28), the inhibition of enolase in multiple glioma cell lines resulted in significant depletion of TCA cycle metabolites (Figure 1E, Supplemental Figure S1 and S2), underscoring the critical role of glucose-derived pyruvate in supporting anaplerosis. The degree of TCA cycle metabolite depletion correlated with the cell lines’ ENO1 status and their sensitivity to enolase inhibitors (Figure 1A-C). Given the close correlation between TCA cycle metabolite depletion (inhibition of anaplerosis) and sensitivity to enolase inhibitors (Figure 1A-E), we determined the effect of experimental manipulation of anaplerotic nutrients, first by supplementing with exogenous anaplerotic substrates and second by inhibition of glutaminolysis with CB-839. We found that omission of exogenous pyruvate in DMEM significantly sensitized all glioma cell lines to the enolase inhibitor POMHEX, irrespective of ENO1 status (Figure 2B, Supplemental Figure S3). Supplementation with physiological levels of pyruvate (100 μM) provided some rescue, with the effect peaking at 1 mM (data not shown) and no further benefit at 5 mM. Similar rescue was obtained with methyl-pyruvate, a cell-permeable pro-metabolite, as well as with oxaloacetate and oxovalerate (Figure 2B and Supplemental Figure S4). These observations draw a metabolic portrait that establishes pyruvate as a major contributor to TCA cycle anaplerosis. However, it is important to note that abrogation of anaplerotic pyruvate by the Enolase inhibitor is not the only mechanism of toxicity, as exogenous supplementation of pyruvate could not completely rescue POMHEX toxicity. This points
to other non-anaplerotic pathways that are altered as a result of glycolysis inhibition and may contribute to cell killing.

Finally, we investigated the effect of glutaminolysis inhibition, given the importance of this pathway for anaplerosis. We found that glioma cells with ENO1 homozygous deletions are selectively sensitive to CB-839; however, this was entirely reversed by exogenous pyruvate supplementation (Figure 3A-B). Metabolomic profiling of CB-839 treated ENO1-deleted, isogenic rescued, and intact glioma cells underscored the importance of exogenous pyruvate, as CB-839 treatment resulted in significant TCA cycle metabolite depletion only in the absence of pyruvate (Figure 3D and E). Strikingly, the selective toxicity of CB-839 was also rescued by the same anaplerotic nutrients as that of enolase inhibition (Figure 3A-B and Supplemental Figure S4 and S6). Similarly, the combination of enolase and glutaminase inhibitors in vitro proved very interesting: the two treatments clearly agonized each other, with their effects being at least additive, and—depending on the drug dose, treatment length, and nutrient composition of the media—synergistic.

With these encouraging results in hand, we tested the antitumor and metabolic effects of enolase and glutaminase inhibitors in vivo. In an intracranial tumor orthotopic model, we saw no effects with CB-839 alone—and no additive effects with the enolase inhibitor HEX—against tumors with ENO1 homozygous deletions (Figure 5A-D). The lack of an antitumor effect of CB-839 could be ascribed to its inability to cross the BBB, though we have noted that D423 tumors do in fact have a breached BBB (9). We therefore repeated these experiments with the same cell line but implanted the tumor subcutaneously. Unexpectedly, we found that the synergy was not conserved in vivo, as
HEX's antineoplastic efficacy was comparable to the efficacy achieved by combining HEX and CB-839, indicating that any antineoplastic effect obtained by combining HEX and CB-839 could largely be attributed to the impairment of glycolysis by HEX. Thus, we argue that bioenergetic collapse, together with reduced pyruvate flux to the TCA cycle, accounts for the dramatic antineoplastic efficacy of HEX against ENO1-deleted gliomas and that glutamine oxidation may not be obligatory for tumor sustenance in vivo.

Our study addresses one of the outstanding controversies in the field: What is the predominant source of carbon atoms for the TCA cycle in cancer cells? Although multiple studies have offered unquestionable evidence that excessive glucose flux and oxidation are near-universal characteristics of cancer cells, the net contribution of different carbon sources such as glucose, glutamine, lactate, and acetate is still an unresolved question (29, 30). Isotope-based labeling studies have attempted to delineate the source of carbon atoms to the TCA cycle in both in vitro and in vivo model systems. For example, some studies have reported that certain tissues and tumors prefer lactate over other substrates as a source of energy (31-33), while other studies have pinpointed glucose rather than lactate as the predominant contributor to the TCA cycle in most tissues (30, 34). Our data do not support that lactate or acetate can be utilized by cells instead of pyruvate to support TCA cycle anaplerosis (Supplemental Figure S4H). This is probably because lactate isotope exchange at equilibrium has been reported to overestimate actual lactate flux and net lactate contribution to the TCA cycle (30, 34). Additionally, in an in vivo setting, the stroma-tumor metabolic coupling may allow lactate extruded by highly glycolytic tumor cells to be used as fuel by stromal cells. In return, stromal cells may reciprocally support the metabolic demands of tumor cells by sharing pyruvate with the tumor cells (35, 36).
Similarly, while other groups have shown that tumor cells can directly use acetate as a source of carbon to replenish TCA cycle intermediates, we did not see any significant reversal of toxicity of glycolysis inhibition with acetate supplementation (37). There are several possible explanations for the differences between acetate and pyruvate. The most obvious is that the transition from pyruvate into the TCA cycle is rapid, energetically favorable, and carried out by high-abundance enzymes, pyruvate dehydrogenase as well as pyruvate carboxylase. The incorporation of acetate into the TCA cycle requires ATP hydrolysis and is predominantly cytosolic. We speculate that anaplerosis by this pathway is simply too slow to compensate for the diminished pyruvate production from inhibited glycolysis.

Furthermore, we observed that the anaplerotic and non-anaplerotic functions of glutamine are dynamic and are dictated by the availability of exogenous glutamine. More specifically, when exogenous glutamine was limiting, we found that cell survival, regardless of ENO1 status, was severely compromised (Supplemental Figure S5). However, addition of CB-839, which under glutamine replete conditions suppressed tumor growth in vitro, but in glutamine free conditions, led to partial rescue of tumor growth. This indicates that anaplerotic functions of glutamine are supported only when glutamine levels are abundant, and CB-839 treatment can indeed impair tumor growth. However, when exogenous glutamine levels are limiting as would be the case in poorly perfused hypoxic regions of a tumor, CB-839 treatment may in fact promote tumor growth via accumulation of endogenous glutamine which is now available for nucleotide and amino acid synthesis. The delineation of the anaplerotic and non-anaplerotic roles of glutamine and the contexts in which they may be predominant, is therefore critical.
especially given that CB-839 is currently being investigated in Phase II clinical trials for multiple malignancies.

Yet, despite a promising observation with CB-839 in vitro (Figure 3A-B), we found that in vivo, glutamine may not be a prominent anaplerotic substrate and that glucose-derived pyruvate predominantly drives anaplerosis, at least in ENO1-deleted gliomas (Figure 5 and 6). Despite its abundance in cell culture medium (4 mM in DMEM) and in human plasma (0.5 mM), the generalizable extent to which cancer cell lines in culture require glutamine for their survival remains controversial (28, 38-41). Additionally, the switch of cancer cells from auxotrophy in vitro to independence from glutamine catabolism in vivo, as reported previously and reinforced in our study, compounds this question (16).

Similarly, if tissue culture media are supplemented with non-physiological levels of nutrients including glutamine, multiple confounding parameters are introduced that do not resemble an in vivo metabolite profile. For example, Muir et al. (42) reported that high levels of cystine in tissue culture medium render cells sensitive to glutaminase inhibition through cystine’s role in glutamate transport via the cystine/glutamate antiporter xCT/SLC7A11. Glutamine catabolism independence has also been reported in in vivo tumor models (38). In the context of glioblastoma, some studies have maintained that pyruvate-derived carbons dominate those coming from glutamine in the TCA cycle (38, 39), while another recent study showed that the origin of the glioblastoma cells influences their preference of anaplerotic substrate (43). Overall, dependence on glutamine oxidation appears to be heterogenous, and dictated by underlying genetics (16, 44), tissue origin (44), and tumor microenvironment (42, 45) and glutamine auxotrophy may not be a universal characteristic of cancer cells in vivo.
Conclusions

In conclusion, we established that ENO1-deleted gliomas rely primarily on glycolysis-derived pyruvate for anaplerosis and that glutamine plays a marginal and nonessential role in anaplerosis. Our study also emphasizes that tumor microenvironmental nutrient availability can affect the net contributions of nutrients to central metabolic pathways; thus, efforts to identify medium conditions that reflect physiological nutrient conditions are warranted. Our findings also have direct relevance for the clinical use of the glutaminase inhibitor CB-839, which is currently being investigated in clinical trials across a broad range of malignancies.

List of abbreviations:

2-OG: 2-dimethyloxoglutarate
BBB: blood-brain barrier
BIDMC: Beth Israel Deaconess Medical Center
CCLC: Characterized Cell Line Core
IACUC: Institutional Animal Care and Use Committee
IC50: half maximal inhibitory concentration
MRI: magnetic resonance imaging
PBS: phosphate-buffered saline
TCA: tricarboxylic acid
ATP: adenosine tri-phosphate
PH3: phosphor-histone H3
CC3: cleaved caspase-3
Declarations

Ethics approval and consent to participate
All animal (mice) involving experiments were approved by MD Anderson’s Institutional Animal Care and Use Committee (IACUC).

Consent for Publication
All authors give consent for publication of the manuscript.

Availability for data materials
All raw metabolomics data are made available through Figshare.

Competing Interests
The authors declare no competing financial interests. F.L.M. is inventor on a patent covering the concept of targeting ENO1-deleted tumors with inhibitors of ENO2 (US patent 9,452,182 B2) and inventor on a patent application describing the synthesis and utility of novel pro-drug inhibitors of enolase US 62/797,315 (Filed Jan 27, 2019). F.L.M. and Y-H.L. are inventors on a patent application for the use of enolase inhibitors for the treatment of ENO1-deleted tumors (US patent 10,363,261 B2).

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Author's contributions
SK and FLM conceived the study, SK designed the experiments and analyzed data. SK, PS and KA performed all in vitro experiments, and SK and FLM analyzed in vitro data. SK and KA designed, performed and analyzed all in vivo experiments. SK and MW performed intracranial MRI scans, and SK, MW, EB and KC analyzed intracranial tumors volumes. SK, Y-HL and JA performed in vitro metabolomics experiments and SK and YB performed in vivo metabolomics experiments and JA performed mass-spectrometry analysis. SK and JA did the IHC experiments and SK, EB and KC did the analysis. CDP helped in MS characterization of the drug. SK wrote the manuscripts with assistance from EB, DKG and FLM. All authors approved the final manuscript.

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