A High-Throughput Fluorimetric Assay for 2-Hydroxyglutarate Identifies Zaprinast as a Glutaminase Inhibitor

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ABSTRACT

Recently identified isocitrate dehydrogenase (IDH) mutations lead to the production of 2-hydroxyglutarate (2HG), an oncometabolite aberrantly elevated in selected cancers. We developed a facile and inexpensive fluorimetric microplate assay for the quantitation of 2HG and performed an unbiased small-molecule screen in live cells to identify compounds capable of perturbing 2HG production. Zaprinast, a phosphodiesterase 5 inhibitor, was identified as an efficacious modulator of 2HG production and confirmed to lower 2HG levels in vivo. The mechanism of action was not due to cGMP stabilization, but rather, profiling of metabolites upstream of mutant IDH1 pointed to targeted inhibition of the enzyme glutaminase (GLS). Zaprinast treatment reversed histone hypermethylation and soft-agar growth of IDH1-mutant cells, and treatment of glutamine-addicted pancreatic cancer cells reduced growth and sensitized cells to oxidative damage. Thus, Zaprinast is efficacious against glutamine metabolism and further establishes the therapeutic linkages between GLS and 2HG-mediated oncogenesis.

SIGNIFICANCE: Gain-of-function IDH mutations are common events in glioma, acute myelogenous leukemia, and other cancer types, which lead to the accumulation of the oncometabolite 2HG. We show that the drug Zaprinast is capable of reducing cellular 2HG levels by inhibiting the upstream enzyme GLS, thus identifying a new strategy to target 2HG production in selected IDH-mutant cancers.

INTRODUCTION

Altered glutamine metabolism can maintain oncogenic transformation and support rapid growth in some cancer cells (1). Oncogenic MYC regulates glutamine metabolism by increasing both the uptake of glutamine and its catabolism through miRNA-driven regulation of glutaminase (GLS). As such, MYC-transformed cells are dependent on glutamine for growth (2, 3). In addition, RAS-driven reprogramming of cellular metabolism shunts glutamine toward NADPH-generating reactions to maintain oxidative balance (4). Glutamine, through glutamate, is also a precursor for cellular α-ketoglutarate (αKG), which can undergo further metabolism through the Krebs cycle or, of particular interest to our study, can be further metabolized to 2-hydroxyglutarate (2HG) by mutant isocitrate dehydrogenase (IDH; ref. 5).
Heterozygous somatic mutations in IDH enzymes are present in over 80% of grade 2 and 3 gliomas as well as secondary glioblastomas (6, 7). Mutations have also been detected in acute myeloid leukemia, chondrosarcomas, and cholangiocarcinoma, among others (8–10). Nearly all identified mutations are in arginine residues 100 and 132 of IDH1 or residues 140 and 172 of IDH2, all of which are located in the active sites of the enzymes (11). Instead of inhibiting the enzymatic activity of IDH, these mutations alter the catalytic activity such that the normal product, αKG, is metabolized to R-2HG in a reaction that consumes NADPH. Although endogenous levels of 2HG are normally low, gliomas harboring mutant IDH1 or IDH2 accumulate millimolar quantities of R-2HG (5).

Structural similarities between 2HG and αKG suggested that 2HG could modulate the function of αKG-dependent dioxygenases to promote transformation and alter differentiation. Indeed, 2HG was found to inhibit the TET family of methylcytosine dioxygenases, as well as several members of the JmjC family of histone demethylases (12, 13). In addition, R-2HG stimulates EGLN, driving the degradation of hypoxia-inducible factor (HIF; ref. 14). Modulating the activity of these various dioxygenases drives DNA and histone hypermethylation, blocks differentiation, and promotes transformation (15–17). Importantly, recent findings indicate that some of these events are reversible. Withdrawal of cell-permeable 2HG or treatment with a small-molecule inhibitor targeting IDH2 R140Q restores differentiation of leukemia cells, while inhibition of IDH1 R132H in transformed cells reduces histone methylation and soft-agar growth (16, 18, 19). Thus, reducing 2HG could provide therapeutic benefit in patients with malignancies harboring gain-of-function IDH mutations.

With this in mind, we developed a fluorimetric microplate assay for measuring 2HG with the intention to screen for compounds capable of perturbing 2HG production in live cells. We reasoned that this approach could identify novel drug interactions that ultimately lead to diminished mutant IDH1 activity, and perhaps perturb other related vital pathways, such as glutamine metabolism, with the potential to repurpose drugs originally developed for alternative disorders. After validation and optimization of assay conditions, we performed an unbiased screen of a small-molecule library using HT1080 cells, which harbor an endogenous IDH1 R132C mutation, and identified Zaprinast, a phosphodiesterase 5 (PDE5) inhibitor, as a candidate modulator of the 2HG metabolic pathway. Rather than being cGMP mediated, the mechanism of action of Zaprinast centered on the inhibition of GLS upstream of mutant IDH1.

RESULTS

Novel Coupled Fluorescence Assay for Quantitation of 2HG

Conventional methods to measure 2HG in cell culture rely on time-consuming and costly chromatography platforms that are not amenable to high-throughput screening experiments. Instead, we developed an enzymatically coupled fluorescence assay to directly quantify the total levels of 2HG in cultured cells. Our strategy involved a primary enzymatic reaction that is specific to 2HG and produces NADH, which is coupled to a second reaction that consumes NADH with mitochondrial diaphorase. The Escherichia coli K-12 enzyme 3-phosphoglycerate dehydrogenase (PHGDH) is known to have dehydrogenase activity against human R-2HG and uses NAD⁺ as a cofactor (20). Published reaction conditions for the 2HG dehydrogenase activity of E. coli PHGDH provided a basis for its use in our fluorescence assay. The NADH produced by E. coli PHGDH is subsequently oxidized by the diaphorase enzyme to reduce resazurin to resorufin, a highly fluorescent red-shifted molecule with an emission peak at 587 nm, thereby minimizing autofluorescence (Fig. 1A). In the process, NADH is recycled back to NAD⁺, and resorufin is produced in stoichiometric proportion to the amount of 2HG present in the sample (21). The assay is rapid, inexpensive, quantitative, and nondestructive if measuring secreted
metabolites, making it ideal for large-scale screening applications.

After optimization of assay conditions, we detected a change in fluorescent signal in direct proportion to added 2HG concentration (Fig. 1B and C). Exogenous 2HG was detected when dissolved in culture media in a concentration-dependent manner, indicating that secreted 2HG could not only be detected, but also be quantified (Fig. 1C). However, signal background was measurably higher in the fluorescent assay when compared with mass spectrometry. By assaying a 20-μL aliquot of culture media, we determined that assay activity was linear to 100 μmol/L and saturated at 2HG concentrations of 1 mmol/L or higher; the lower limit of detection was approximately 4 μmol/L (Supplementary Fig. S1). We then determined the specificity of the assay for 2HG by testing the effect of metabolites within the IDH metabolic pathway, including glutamine, glutamate, αKG, and isocitrate, and found that they do not interfere with assay function (Supplementary Fig. S2). Next, to determine whether the assay could detect physiologic changes in 2HG production by cultured cells, we stably transduced immortalized human astrocytes with vector, wild-type (WT) IDH1, or R132H IDH1. Accumulation of 2HG in astrocytes expressing R132H IDH1 was first confirmed by conventional gas chromatography–mass spectroscopy (GC-MS; Fig. 1D). Then, by the fluorimetric assay, media from astrocytes expressing mutant IDH1 showed significantly higher signal when compared with cells expressing either vector or WT IDH1 (Fig. 1E). This was also verified in HEK293T cells (Fig. 1F). In addition, to determine whether the fluorimetric assay could detect a reduction in 2HG levels, we performed shRNA knockdown of IDH1 in the human fibrosarcoma cell line HT1080, which harbors an endogenous heterozygous gain-of-function R132C IDH1 mutation. Using two different hairpin sequences, we assayed 2HG levels in the culture media and showed a significant reduction in signal in proportion to IDH1 protein knockdown (Fig. 1G).

High-Throughput Screen for Modulators of 2HG Metabolism

Using the assay to quantify 2HG in media, we screened a library of 480 bioactive compounds targeting a wide variety of cellular processes in IDH1-mutant HT1080 cells to
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**Figure 2.** Zaprinast lowers 2HG levels in IDH1-mutant cells in culture and tumors in vivo. A, HT1080 high-throughput screen results plotted as 2HG versus viability. Data points represent the average of screens performed in triplicate. Linear fit and 95% predictive interval (P.I.) show a correlation between 2HG and viability. Compounds in the top left quadrant outside the 95% predictive interval produced a reduction in 2HG that was not predicted to be a consequence of general drug toxicity. B, HT1080 cells were treated with the indicated concentrations of Zaprinast for 48 hours and secreted 2HG was measured in the media using the fluorescent assay (n = 3; data are plotted as fold-viability over fold-unreated). HT1080 cells (C) and NHA cells (D) ectopically expressing IDH1 R132H treated with Zaprinast for 48 hours were analyzed for intracellular 2HG by GC-MS (n = 3). E, GC-MS quantification of 2HG in HT1080 tumor xenografts treated with vehicle (Veh) or Zaprinast (Zap; n = 6 for vehicle and n = 7 for drug; error bars, SEM; *, P < 0.05).

To determine whether Zaprinast could lower 2HG concentrations in vivo, we generated flank tumor xenografts of HT1080 cells in nu/nu mice. Because the pharmacokinetics of Zaprinast for this application have not been characterized, we performed direct intratumoral injection to ensure delivery of the compound. Tumors treated with Zaprinast showed a modest but significant reduction of 2HG levels relative to vehicle, as quantified by GC-MS (Fig. 2E).

**Mechanism of Action of Zaprinast**

The reported IC_{50} for Zaprinast against PDE5 is approximately 0.15 μmol/L. However, approximately 100 to 300 μmol/L were required to observe a significant reduction in 2HG. This, along with the finding that the Zaprinast-mediated effects seemed to be cGMP-independent and not mimicked by other PDE5 inhibitors, suggested that Zaprinast blocks 2HG hydrolysis by elevating intracellular cGMP (22). However, we observed no change in levels of 2HG when treating HT1080 cells with sildenafil or tadalafil, two clinically approved PDE5 inhibitors (Fig. 3A). We then asked if either cAMP or cGMP was sufficient to reduce 2HG levels in HT1080 cells. Treatment with the cell-permeable analogues 8-bromo-cAMP and 8-bromo-cGMP did not cause a reduction in 2HG (Fig. 3B). To confirm that the lack of 2HG reduction upon treatment with cell-permeable cGMP was not due to structural differences between the analogue and its endogenous counterparts, we sought to elevate endogenous cGMP by expressing constitutively active soluble guanylyl cyclase (sGC) (ref. 23). Coexpression of the WT α1 subunit and the constitutively active β1 C105H mutant that compose the sGC heterodimer resulted in a significant increase in basal cGMP levels, as measured by ELISA (Fig. 3C and D). 2HG levels, however, were unaffected by the elevation of intracellular cGMP (Fig. 3E), indicating that the effects of Zaprinast on 2HG production were likely not a consequence of altered cGMP levels.
production through an off-target effect. To define the mechanism of action of Zaprinast, we examined changes in metabolites upstream of 2HG. The predominant source of 2HG is cellular glutamine. GLS metabolizes glutamine to glutamate, which is then converted to αKG by glutamate dehydrogenase, followed by mutant IDH1 metabolism of αKG to 2HG (Fig. 4A; ref. 5). We reasoned that by measuring upstream metabolites, we could identify a candidate target enzyme whose activity was either directly or indirectly inhibited by Zaprinast. Using mass spectrometry, we measured cellular levels of glutamine, glutamate, αKG, and 2HG in vehicle- and Zaprinast-treated HT1080 cells. Unexpectedly, in addition to 2HG, we found that the levels of αKG and glutamate were also reduced in HT1080 cells treated with Zaprinast, but intracellular levels of glutamine were unaffected. We repeated this in NHA-immortalized astrocytes expressing R132H IDH1 and detected the same pattern of metabolite level changes (Fig. 4B). These data suggested that Zaprinast directly or indirectly inhibited the activity of GLS, causing a reduction in the level of downstream metabolites. Treatment of HT1080 cells with BPTES, a known GLS inhibitor, produced a similar pattern of metabolite modulation (Fig. 4C). Furthermore, in cells treated with Zaprinast, adding back a cell-permeable form of αKG restored 2HG back to baseline levels (Fig. 4D), consistent with traversing a block at the level of GLS. To determine whether Zaprinast modulates 2HG by directly inhibiting GLS, we purified human GLS and used an in vitro assay to measure its enzymatic activity. The enzymatic activity of GLS was directly inhibited by Zaprinast with an IC$_{50}$ of approximately 200 μmol/L, which corresponded to the concentrations at which a significant reduction in 2HG was observed in cells (Fig. 4E). GLS kinetic analysis determined that Zaprinast acted as a noncompetitive inhibitor ($K_i = 220$ μmol/L; Fig. 4F and G). In a control experiment, cGMP had no direct effect on GLS enzymatic activity (Supplementary Fig. S3).

**Zaprinast-Mediated Modulation of the 2HG Phenotype**

Having shown that Zaprinast reduces 2HG levels by blocking flux through the pathway at the level of GLS, we next explored whether Zaprinast treatment could reverse the effects of 2HG on cells. 2HG is structurally similar to αKG, differing only in the oxidation state of the C2 carbon (11). Because of this similarity, 2HG has been shown to act as either an inhibitor or activator of various αKG-dependent enzymes. The Jmjd family of histone demethylases has been shown to be inhibited by 2HG, and expression of mutant IDH1 was shown to cause an elevation in methylated histone lysine residues and lead to a block in cellular differentiation (12, 15). As expected, expression of R132H IDH1 in human astrocytes caused an increase in histone methylation marks (H3K9me2, H3K9me3, H3K27me3, and H3K79me2), and treatment with Zaprinast caused a marked reduction toward baseline methylation levels (Fig. 5A and B). In contrast, Zaprinast treatment of IDH1 WT–expressing astrocytes did not produce such a marked reduction in methylation levels of either H3K9me2 or H3K9me3 (Supplementary Fig. S4). In addition, mutant IDH1 was recently shown to promote soft-agar colony formation of immortalized human astrocytes (14). As expected, IDH1 R132H expression increased colony formation of human astrocytes when compared with vector or IDH1 WT. Importantly, Zaprinast treatment reduced colony formation in IDH1 R132H astrocytes down to the level of vector and WT cells (Fig. 5C and D).

**Zaprinast Inhibits Glutamine-Dependent Tumor Cell Proliferation**

Because of the requirement for glutamine in 2HG production, we hypothesized that disruption of glutamine metabolism with Zaprinast could abrogate the growth of
glutamine-addicted cells with normal IDH activity. In cells undergoing the Warburg effect, whereby glucose is shunted toward lactate production in the presence of oxygen, glutamine often functions in anaplerotic reactions to replenish citric acid cycle intermediates (24). A recent report by Son and colleagues (4) showed that HRAS-driven pancreatic ductal adenocarcinoma (PDAC) cells use glutamine in a different manner. PDAC cells are dependent on glutamine metabolism to maintain redox balance by shuttling glutamine carbon through the transaminase GOT1 and ultimately malic enzyme (ref. 4). Using two PDAC cell lines, Panc1 and MiaPaca2, which are glutamine-addicted but show minimally detectable levels of 2HG (data not shown), we sought to determine whether Zaprinast could perturb redox balance in a manner similar to glutamine deprivation. First, we confirmed that Panc1 and MiaPaca2 cells were dependent on extracellular glutamine for their growth, saturating at glutamine concentrations of 2 mmol/L (Fig. 6A). Furthermore, treatment with Zaprinast led to significant reductions in cellular pools of glutamate and αKG and an increase in glutamine as measured by mass spectrometry, indicating that Zaprinast blocks GLS activity in live PDAC cells (Fig. 6B). Interestingly, glutamine levels are increased more dramatically in PDAC cells treated with Zaprinast when compared with NHA or HT1080 cells treated with Zaprinast (Fig. 4B). This is likely a consequence of RAS-driven metabolic reprogramming in PDAC cells, wherein glutamine metabolic fluxes are high to maintain oxidative balance and promote transformation. Growth in the presence of 100 and 300 μmol/L Zaprinast was significantly reduced (Fig. 6C). To confirm that growth inhibition in the presence of Zaprinast was due to GLS inhibition, glutamate supplementation of the media was performed, producing a restoration of growth (Fig. 6D). Consistent with previous findings that glutamine metabolism maintains oxidative homeostasis in PDAC, Zaprinast treatment of PDAC cells caused an increase in reactive oxygen...
species (ROS) in a concentration-dependent manner (Fig. 6E and F). This resulted in increased susceptibility to oxidative damage, as cells pretreated with Zaprinast were significantly more susceptible to hydrogen peroxide (H$_2$O$_2$) stress than cells treated with vehicle (Fig. 6G). Moreover, adding back glutamate to Zaprinast-treated cells nearly completely abolished the increased sensitivity to oxidative damage (Fig. 6G), again consistent with blockade at the level of GLS. These findings suggest that Zaprinast could increase the sensitivity of PDAC to oxidative stress, as might be therapeutically induced by radiotherapy or chemotherapy.

**DISCUSSION**

The accumulation of 2HG in tumors harboring IDH1 and IDH2 mutations modulates the activity of several αKG-dependent dioxygenases and leads to histone and DNA hypermethylation, blocked differentiation, and cellular transformation (11). Herein, we have developed a fluorimetric assay that is capable of detecting changes in 2HG levels and allows for rapid, high-throughput quantification when compared with mass spectrometry. Our objective was to identify alternative cellular targets or mechanisms for reducing total cellular 2HG levels. An assay using (D)-2HG dehydrogenase as the driver enzyme was recently published by Balss and colleagues (25) to quantify 2HG in patient serum and tumor tissues with mutant IDH. Compared with E. coli PHGDH, whose primary substrate is 3-phosphoglycerate (3PG), (D)-2HG dehydrogenase is thermodynamically more specific for 2HG and could provide an alternative driver enzyme for future experiments. Nonetheless, GLS inhibition with either BPTES or Zaprinast did not cause an elevation in cellular 3PG, thus reducing the likelihood of interference within the context of our PHGDH-based assay signals (Supplementary Fig. S5). The development and optimization of our fluorimetric assay was focused on high-throughput robotic screening and led to the successful screening of a library of 480 bioactive compounds against live cells overproducing 2HG. We identified Zaprinast, a PDE5 inhibitor, as a modulator of 2HG production. The ease of the fluorimetric assay in the high-throughput setting, coupled with its quantitative capacity and low cost, is encouraging from a drug-discovery perspective. Although we screened a relatively modest library of 480 compounds, interrogation of much larger compound libraries as well as siRNA libraries is also feasible by this method and provides important strategies to identify targets that reduce 2HG for therapeutic purposes and define mechanisms of regulation.

Among the other compounds identified were a calcium channel modulator and a PKC inhibitor. We chose to further follow up on Zaprinast because the drug showed the most dramatic reduction in 2HG levels, and several other PDE5 inhibitors have undergone clinical approval. Additional follow-up will be required to characterize the mechanism and efficacy of other promising screen hits. Specifically, several
PKC inhibitors have undergone clinical trial development for use in cancer, heart failure, coronary artery disease, and diabetic retinopathy (26). Further characterization of their effects on 2HG and metabolism may allow for drug repurposing or generation of more potent analogues. We determined that the effects of Zaprinast are likely not mediated by cGMP, as neither the cell-permeable cGMP analogue, 8-bromo-cGMP, nor expression of constitutively active sGC reduced 2HG levels. Instead, we identified a previously unknown off-target effect of Zaprinast against GLS, the first enzyme of glutaminolysis that ultimately supplies αKG for mutant IDH reactions. Losman and colleagues (16) recently showed that withdrawal of cell-permeable 2HG following transformation induced by treatment with cell-permeable 2HG restores dependence on
growth factors and differentiation of leukemia cells. Similarly, treatment with small-molecule inhibitors specifically targeting IDH1 R132H and IDH2 R140Q showed that many of the effects of mutant IDH1, including histone hypermethylation, colony formation, and differentiation, were indeed reversible (18, 19). Here, we show that indirectly blocking 2HG production by inhibiting GLS (and thus flux through mutant IDH1) is capable of reversing histone hypermethylation and soft agar growth in human astrocytes, indicating that indirectly blocking mutant IDH1 activity by inhibiting GLS may serve as an alternative therapeutic strategy in appropriately programmed cells. Additional studies are needed to fully validate the therapeutic potential of GLS inhibition in IDH-mutant cancer, including dissecting relevant reprogrammed metabolic fluxes, further characterization of selective GLS inhibitors, and use of improved cell-permeable analogues of R-2HG. This is of particular interest with regard to mutant IDH1-induced histone hypermethylation, because inhibition of GLS simultaneously reduced both cellular 2HG and αKG. In the face of opposing metabolite actions (2HG inhibits αKG-dependent dioxygenases such as the JmC histone demethylases, while αKG activates), histone hypermethylation states were nonetheless reversed overall by Zaprinast treatment, suggesting that the activity of histone demethylases could be more sensitive to reductions in 2HG than αKG. How the ratio of 2HG to αKG determines the activity of these enzymes or regulation of histone methylation through other routes requires further study. Zaprinast is among the first small-molecule inhibitors not directly targeting mutant IDH to show this effect and broadens the targets in the pathway amenable to modulation. Selective targeting of GLS may be important for subsets of patients with IDH1 or IDH2 mutations not affected by small-molecule inhibitors developed against target-specific IDH mutations or patients displaying resistance to IDH1-targeted therapies.

Interestingly, Selzer and colleagues (27) showed that GLS inhibition by BPTES preferentially limits growth of D54 glioblastoma cells expressing IDH1 R132H when compared with WT IDH1. However, 2HG levels were unaffected by BPTES. This may be attributed to differences in metabolic wiring that allow some cells to overcome GLS inhibition. Indeed, Cheng and colleagues (28) showed that some cells use pyruvate carboxylase for anapleurosis under conditions of glutamine deprivation or silencing of GLS. Thus, it is possible that differences in expression of pyruvate carboxylase may dictate responsiveness to pharmacologic inhibition of GLS in IDH1-mutant tumors.

GLS expression has been shown to correlate with tumor growth and tumor grade independent of IDH mutations (29). c-MYC-driven expression of GLS promotes glutaminolysis and glutamine dependence, and, furthermore, inhibition of GLS was shown to cause a marked reduction in growth of several glutamine-dependent cell lines and xenograft models (2, 3, 30). Son and colleagues (4) showed that PDAC cells rely on glutamine metabolism for maintenance of redox state. We examined the effect of Zaprinast on Panc1 and MiaPaca2 cells and showed that Zaprinast inhibits cell growth, causes an increase in ROS levels, and sensitizes cells to oxidative damage by H2O2, all in a manner that can be rescued by extracellular glutamate. Thus, our data indicate that Zaprinast mimics the effects of glutamine deprivation in PDAC cells and point to a targeted small-molecule strategy amenable to translation. Thus, our work and that of others highlight the potential utility of pharmacologically targeting GLS in cancer and further stress the need for potent and safe inhibitors (30, 31). We have identified a new chemical structure capable of directly inhibiting the activity of purified GLS noncompetitively, which typically implies allosteric binding of compound to target enzyme. Importantly, treatment of multiple independent cell lines with Zaprinast altered cellular metabolite levels in a manner most consistent with GLS inhibition as the mechanism of action. In addition, in Zaprinast-treated cells, the addition of cell-permeable glutamate rescued growth and abolished the heightened sensitivity to oxidative damage, providing further evidence for a GLS-targeted mechanism in live cells. However, given the higher IC50 of Zaprinast for GLS relative to PDE5, doses needed to block GLS in vivo may not be achievable clinically without also producing vascular side effects. Although pharmacokinetic modulation and dosing regimens may assist in clinical translation, it is more likely that Zaprinast can serve as a convenient tool compound with improved drug-like molecular properties compared with other candidate GLS inhibitors, such as BPTES. Zaprinast may be useful for preclinical mechanistic studies of the linkages between GLS and 2HG-mediated oncogenesis as well as a new scaffold from which alternate analogues with increased potency and selectively against GLS can be generated. Future structure–activity relationship studies are needed to tease out the binding site and active pharmacophores required for rational drug design and the development of more potent and selective GLS inhibitors.

METHODS

Plasmid Construction

IDH1 cDNA clone (BC0128846.1) was purchased from ATCC in the pCMV-Sport6 backbone. Site-directed mutagenesis was carried out to introduce a G395A mutation (R132H) and sequence verified. The open reading frame (ORF) of both WT and R132H IDH1 was then subcloned into pcDNA 3.1 and pLXV-IRE5-Hyg vectors using standard molecular biology techniques. Histidine-6x-tagged E. coli PHGDH plasmid was a kind gift from Dr. Gregory Grant (Washington University School of Medicine, St. Louis, MO). SGCu and SGCβ,105 plasmids were a kind gift from Dr. Emil Martin (University of Texas Health Sciences Center, Houston, TX). Human GLS cDNA (BC126537) was purchased from Thermo Scientific, and the ORF was subcloned into pSV281 containing an N-terminal 6x-His tag using forward and reverse restriction sites, BamHI and HindIII respectively. pLKO.1-puro shRNA constructs were provided by the Washington University Genome Institute (St. Louis, MO) and used for RNA interference against IDH1. Sequences for the shRNAs were as follows: (i) 5′-CCTTGTGATCGAGACACAAA-3′ and (ii) 5′-GCTGCTTGACATTAAAGGT-3′.

Cell Culture, Transfection, and Generation of Stable Cell Lines

HT1080, HEK293T, Panc1, and MiaPaCa-2 cells were obtained from ATCC. NHAs immortalized with E6/E7/TVTERT were a kind gift from Dr. Russell O. Peiper (University of California San Francisco, San Francisco, CA). All cells tested negative for Mycoplasma infection. HT1080 cells were authenticated by genotyping for IDH1 and were confirmed to harbor a heterozygous IDH1 R132C mutation by Sanger sequencing of genomic DNA products. The remaining cell lines were not further authenticated. HEK293T, NHA, PANC-1, and

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MIA PaCa-2 cells were maintained with complete DMEM containing 10% FBS and 1% penicillin/streptomycin. HT1080 cells were maintained in minimum essential media α (MEM) containing 10% FBS and 1% penicillin/streptomycin. Glutamine-free DMEM (Life Technologies) was supplemented with 10% FBS and 1% penicillin/streptomycin and spiked with varying amounts of l-glutamine (CellGro) for glutamine growth curves. Zaproginast (Sigma-Aldrich) and H$_2$O$_2$ were added to the appropriate fresh media as indicated.

For production of lentivirus, 1 x 10$^8$ HEK293T cells were co-transfected with pCMV-VSV-G, pCMVΔR8.2, and either pLKO.1-puro for RNA interference experiments or pLVX-IRES-Hyg constructs for stable infection. Cells transduced with pLKO.1-puro were then selected in batch with 1 μg/mL puromycin for 72 hours, and cells transduced with pLVX-IRES-Hyg were selected in batch with 100 μg/mL hygromycin for 1 week.

**2HG Fluorimetric Assay**

N-terminal 6x-His-tagged PHGDH was expressed and purified from chemically competent BL-21 E. coli using Ni-NTA agarose (Qiagen; ref. 32). Purified protein was then dialyzed in 40 mmol/L α-KG, buffer at 4°C overnight. Conditioned culture media (DMEM or αMEM) containing 2HG was neutralized with 60 mmol/L HCL for 10 minutes followed by 60 mmol/L Tris base. A 20-μL aliquot of neutralized conditioned media was then mixed with 90 μL of optimized assay reaction mix consisting of 5 μg purified PHGDH enzyme, 12.5 μmol/L resazurin (Sigma-Aldrich), 0.125 μL/mL diaphorase (Sigma-Aldrich), 1 mmol/L NAD (Sigma-Aldrich), and 40 mmol/L Tris–HCL pH 8.8, and incubated for 90 minutes in a 96-well plate. Resorufin fluorescence was then quantified using a FLUostar OPTIMA fluoroscent plate reader (BMG Labtech) with excitation and emission peaks of 544 and 590 nm, respectively. Measurements were background subtracted (neutralized fresh media) and normalized to either total protein or viability as measured by Alamar Blue (33).

**High-Throughput Screening**

Screening was carried out using the Beckman Coulter Core Robotics system, including an FX liquid handler, controlled by the Sagian graphical method development tool (SAMI scheduling software). The Institute of Chemistry and Cell Biology (ICCB, Boston, MA) Known Bioactive Library (Enzo Life Sciences; BML-2840-0100) was diluted at 1:200 in αMEM and spiked with varying amounts of L-glutamine (CellGro) for graphical method development tool (SAMI scheduling software). The samples were run by electron ionization (EI), and the source temperature, electron energy, and emission current were 200°C, 70 eV, and 300 μA, respectively. The injector and transfer line temperatures were 250°C. 3-Hydroxy-1,5-pentanedicicoic acid (CDN Isotopes) was used as an internal standard. Extracts were then incubated at −80°C for 20 minutes, sonicated on ice, centrifuged at 14,000 x g for 20 minutes at 4°C to clear precipitates, and supernatants transferred to vials for drying under N$_2$. Derivatizing reagent [MSTFA (N-methyl-N-(trimethylsilyl) trifluoroacetamide); pyridine:acetonitrile 1:12] was added to the vials, which were then heated at 70°C for 15 minutes.

**GC-MS Analysis**

Metabolite extraction from cultured cells was performed as described previously by Figueroa and colleagues (13). Briefly, cells were rinsed in ice-cold PBS and quickly quenched with 80% methanol spiked with 3-hydroxy-1,5-pentanedicicoic acid (CDN Isotopes) and sodium orthovanadate (Roche), sodium azide (1% w/v, and phenylmethylsulfonyl fluoride (PMSF; 1 mmol/L). Whole-cell lysates were normalized for protein content by BCA assay (Pierce). Histone extraction was performed using an EpiQuick Total Histone Extraction Kit (Epigentek), and extracted protein was quantified using a Bradford assay.

Proteins were resolved by SDS-PAGE, transferred to a polyvinylidene fluoride (PVDF) membrane, and probed with the following antibodies: ID1 (Origene; TAA00610), 6x-His (Abcam; ab1187), H3K9me2 (Cell Signaling Technology; 4658p), H3K9me3 (Abcam; ab8898), H3K27me3 (Millipore; 07-449), H3K27me2 (Cell Signaling Technology; 9757p), total H3 (Cell Signaling Technology; 4499p), and actin. Secondary anti-mouse and anti-rabbit horseradish peroxidase-conjugated IgG antibodies were used for detection.

**GLS Assay**

N-terminal 6x-His-tagged GLS was expressed and purified from chemically competent BL-21 E. coli using Ni-NTA agarose beads (Qiagen). Purified protein was then dialyzed in a 50 mmol/L Tris-phosphate buffer containing 1 mmol/L dithiothreitol at 4°C overnight. A previously described two-step assay was used to measure GLS activity (34). Briefly, 30 μL of purified GLS (660 μg/mL) was incubated with various concentrations of Zaproginast at 37°C for 15 minutes. Zaproginast was dissolved in DMSO and 3 μL was added to each GLS aliquot to maintain a constant concentration of DMSO across samples. Initial reaction mix (30 μL) containing 20 mmol/L glutamine, 0.15 mol/L KPO$_4$, 0.2 mmol/L EDTA, and 50 mmol/L Tris-acetate (pH 8.6) was then added and samples were incubated at 37°C for 10 minutes, after which the reaction was rapidly quenched with 6 μL of 3 N HCl. To generate blank samples, GLS and Zaproginast solutions were inactivated with 3 N HCl before the addition of initial reaction mix. For the second dimensions obtained by caliper measurements. On day 15, tumors were extracted and snap-frozen for follow-up GC-MS analysis.

**Histone Extraction and Western Blot Analysis**

For extraction of total cellular protein, cells were lysed in RIPA buffer (150 mmol/L NaCl, 50 mmol/L Tris, 5 mmol/L EDTA, 1% NP-40, 0.1% SDS, 0.5% sodium deoxycholate, pH 7.4), supplemented with protease inhibitor cocktail (Roche), sodium orthovanadate (1 mmol/L, and phenylmethylsulfonyl fluoride (PMSF; 1 mmol/L). Whole-cell lysates were normalized for protein content by BCA assay (Pierce). Histone extraction was performed using an EpiQuick Total Histone Extraction Kit (Epigentek), and extracted protein was quantified using a Bradford assay.

Proteins were resolved by SDS-PAGE, transferred to a polyvinylidene difluoride (PVDF) membrane, and probed with the following antibodies: ID1 (Origene; TAA00610), 6x-His (Abcam; ab1187), H3K9me2 (Cell Signaling Technology; 4658p), H3K9me3 (Abcam; ab8898), H3K27me3 (Millipore; 07-449), H3K27me2 (Cell Signaling Technology; 9757p), total H3 (Cell Signaling Technology; 4499p), and actin. Secondary anti-mouse and anti-rabbit horseradish peroxidase-conjugated IgG antibodies were used for detection.
step of the assay, 20 μL of completed and quenched initial reaction was transferred to a new plate and 200 μL of secondary reaction mix containing 0.4-mg bovine liver glutamate dehydrogenase (Sigma-Aldrich), 0.09 mol/L Tris-acetate (pH 9.4), 0.2 mol/L hydrazine, 0.25 mmol/L ADP, and 2 mmol/L NAD was added. Samples were incubated for 40 minutes at room temperature and absorbance of NADH (340 nm) was measured using a microplate spectrophotometer.

Quantification of Intracellular cGMP
HT1080 cells were rinsed with ice-cold PBS, lysed with 0.1 mmol/L HCl, and centrifuged at 1,000 × g for 10 minutes. Supernatant was then assayed for cGMP using a cGMP Enzyme Immunoassay Kit (Cayman Chemical, 581021) according to the manufacturer’s protocol.

ROS Quantification
ROS levels in PDAC cells were quantified using 2,7-dichlorofluorescein diacetate (DCFDA). Following 48 hours of drug treatment in a 96-well plate, cells were washed with PBS, stained for 30 minutes at 37°C with 20 μmol/L DCFDA, and imaged using an InCell Analyzer 1000. A 10% objective was used to collect 16 fluorescent and bright-field images per well with a 10% overlap to allow image stitching. Stained cells were then quantified using the GE InCell Investigator software package with Developer Toolbox.

Soft-Agar Assay
A bottom layer of 0.6% soft agar with complete DMEM and 10% FBS was set in 6-well plates and allowed to solidify. Approximately 8,000 cells were then suspended in 0.4% soft agar and plated on top. Colonies were allowed to develop over 3 to 4 weeks and stained with crystal violet.

Statistical Analysis
Statistical significance was evaluated using the Student t test or two-way ANOVA as indicated. Error bars represent SD unless otherwise indicated.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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