

Inhibition of Glutaminase Preferentially Slows Growth of Glioma Cells with Mutant IDH1

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Abstract

Mutation at the R132 residue of isocitrate dehydrogenase 1 (IDH1), frequently found in gliomas and acute myelogenous leukemia, creates a neoenzyme that produces 2-hydroxyglutarate (2-HG) from α -ketoglutarate (α -KG). We sought to therapeutically exploit this neoreaction in mutant IDH1 cells that require α -KG derived from glutamine. Glutamine is converted to glutamate by glutaminase and further metabolized to α -KG. Therefore, we inhibited glutaminase with siRNA or the small molecule inhibitor bis-2-(5-phenylacetamido-1,2,4-thiadiazol-2-yl)ethyl sulfide (BPTES) and found slowed growth of glioblastoma cells expressing mutant IDH1 compared with those expressing wild-type IDH1. Growth suppression of mutant IDH1 cells by BPTES was rescued by adding exogenous α -KG. BPTES inhibited glutaminase activity, lowered glutamate and α -KG levels, and increased glycolytic intermediates while leaving total 2-HG levels unaffected. The ability to selectively slow growth in cells with *IDH1* mutations by inhibiting glutaminase suggests a unique reprogramming of intermediary metabolism and a potential therapeutic strategy. *Cancer Res*; 70(22); 8981–7. ©2010 AACR.

Introduction

Despite the availability of oxygen, cancer cells exhibit high glycolytic rates and increased lactate production, known as the Warburg effect or aerobic glycolysis, rather than high oxidative phosphorylation. Over the past decade, oncogenes (*MYC*, *PI3K*, *RAS*, and *AKT*) and tumor suppressors (*VHL* and *p53*) have been documented to reprogram cancer cell metabolism to aerobic glycolysis (1, 2). Cancer cells also consume glutamine for energy or as a carbon skeleton or nitrogen donor (3, 4). Recently, the oncogene *MYC* was found to induce mitochondrial biogenesis and increase glutamine metabolism, indicating that *MYC* stimulates both aerobic glycolysis and glutamine oxidation (5).

Before the discovery of isocitrate dehydrogenase 1 (*IDH1*) and 2 (*IDH2*) mutations, succinate dehydrogenase and fumarate hydratase mutations, which cause hypoxia-inducible factor 1, alpha subunit stabilization and hereditary cancer syndromes, were the only known oncogenic

mutations of metabolic enzymes. *IDH1* and *IDH2* mutations occur frequently in malignant low-grade gliomas, secondary glioblastomas, and acute myelogenous leukemias (AML; refs. 2, 6–8). These discoveries underscore the importance of metabolic alterations in oncogenesis and suggest the possibility of targeting genetically altered cancer metabolism.

Although wild-type (WT) *IDH1* converts isocitrate and NADP⁺ to α -ketoglutarate (α -KG) and NADPH, mutated amino acids in *IDH1* and *IDH2* reside in the catalytic pocket and result in a neoenzymatic activity: α -KG + NADP⁺ → D-2-hydroxyglutarate (2-HG) + NADP⁺ (9). *IDH1* mutations in gliomas and AML have, thus far, only been found at residue R132, which is most commonly mutated to a histidine (7). Mutations of *IDH2* have been found at both R140 and R172 (10). All documented *IDH1* or *IDH2* mutations result in the ability to produce 2-HG from α -KG (9, 10).

Although the role of *IDH1* mutation in tumorigenesis has not been determined, changes in enzymatic function that result from *IDH1* mutation likely contribute to tumor formation. Decreased NADPH production from loss of *IDH1* WT function coupled with increased 2-HG levels could lead to oxidative stress (11, 12). Secondly, 2-HG interferes with the electron transport chain and could alter mitochondrial physiology and drive cells toward aerobic glycolysis (13). Due to structural similarity between 2-HG and α -KG, 2-HG could also interfere with the function of enzymes that utilize α -KG (e.g., histone demethylases). Lastly, 2-HG is produced in inborn errors of metabolism (L-2- or D-2-hydroxyglutaric aciduria) where the enzyme that metabolizes 2-HG (L- or D-2-hydroxyglutarate dehydrogenase) is nonfunctional (14, 15). Individuals with L-2-hydroxyglutaric aciduria have been documented to

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Note: Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

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doi: 10.1158/0008-5472.CAN-10-1666

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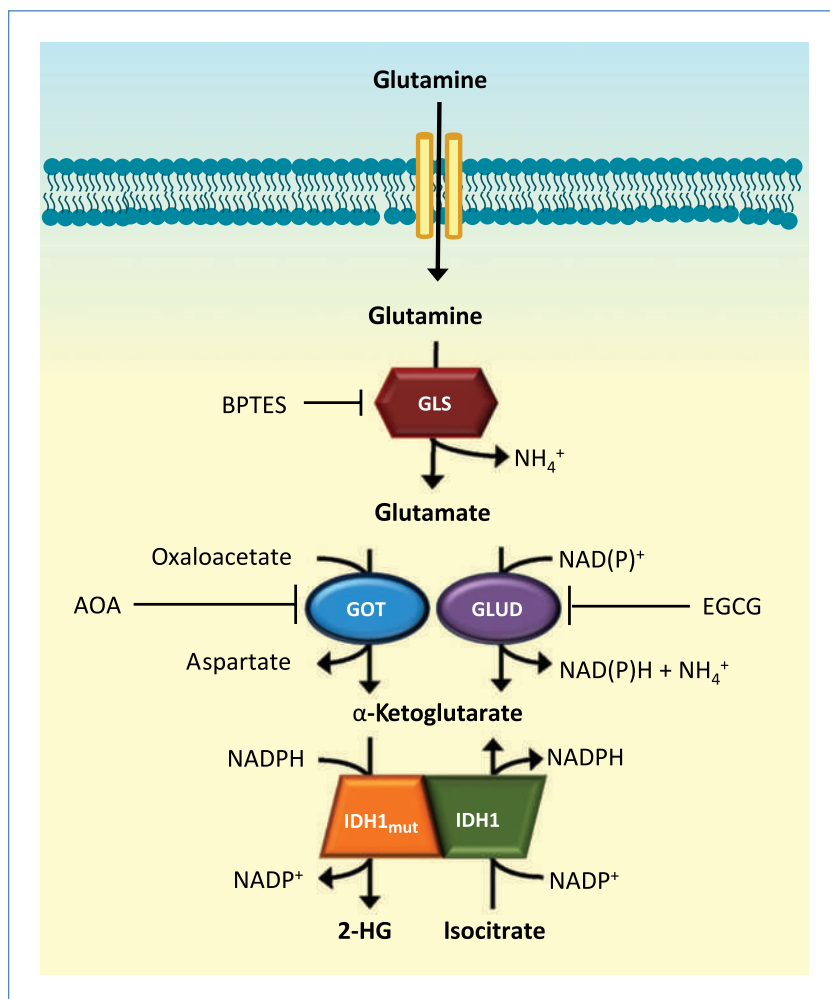


Figure 1. Pathway showing production of 2-HG from glutamine and inhibitor targets. GLS, glutaminase; GOT, glutamate oxaloacetate transaminase; GLUD, glutamate dehydrogenase; IDH1, isocitrate dehydrogenase; IDH1_{mut}, mutant IDH1.

develop gliomas (16), but not those with D-2-hydroxyglutaric aciduria (the enantiomer produced by mutant IDH1/2).

It is not clear if blocking IDH1 mutant activity would be an effective therapy, particularly if the mutant protein is only involved in tumor initiation. However, because mutant IDH1 tumors require α -KG to produce 2-HG, they could consequently be susceptible to alteration of α -KG homeostasis. It has been shown that 2-HG is primarily derived from glutamine (Fig. 1; ref. 9). Glutamine is hydrolyzed by glutaminase to produce glutamate that is subsequently converted to α -KG. α -KG is then converted to 2-HG by mutant IDH1. Thus, we sought to determine whether inhibiting glutaminase might perturb α -KG homeostasis and yield a selective response in cancer cells bearing IDH1 mutant enzymes.

Materials and Methods

Standard techniques were used to introduce the R132H mutation into human IDH1 engineered with a 6X-His-tag and produce lentivirus containing either WT or mutant *IDH1*. D54 cells or transformed normal human astrocytes (TNA) were transduced with virus, and lines were grown

from individual cells using limiting dilution. IDH1 expression in response to 0.04 $\mu\text{g}/\text{mL}$ doxycycline was confirmed using a 6X-His tag antibody (Millipore). Liquid chromatography/mass spectrometry was conducted as previously described (9). siRNA was used to knock down glutaminase, and cells were assessed for knockdown using Western blotting (5). Cell growth assays using alamarBlue were carried out as described following DMSO or bis-2-(5-phenylacetamido-1,2,4-thiadiazol-2-yl)ethyl sulfide (BPTES) treatment (17). Glutaminase activity was measured after treatment with DMSO or BPTES by incubating cell extracts with [^3H]-glutamine. [^3H]-glutamate was isolated from the reaction mixture using anion exchange and measured using a scintillation counter. All effects of BPTES were assayed 48 hours after treatment. Data were evaluated using a two-tailed Student's *t*-test. $P \leq 0.05$ was considered significant. Detailed materials and methods are available as Supplementary Information.

Results

Currently, no established glioblastoma cell lines are reported to possess *IDH1* or *IDH2* mutations, and our attempts

to derive cell lines from patients with an *IDH1* mutation have been unsuccessful. Therefore, we created tet-inducible, stable D54 glioblastoma cell lines that overexpress WT or R132H IDH1 (Fig. 2A). Expression of R132H IDH1 decreased total IDH activity by 50% and 25% compared with cells overexpressing WT IDH1 and parental D54 cells, respectively (Supplementary Fig. S1). This observation corroborates decreased IDH activity in cell culture models and glioblastoma tumor sections (7, 11, 18). 2-HG levels were elevated in mutant IDH1 cells compared with WT IDH1-expressing cells, whereas α -KG levels were not significantly different (Fig. 2B and C).

Because previous studies had shown that α -KG consumed by mutant IDH1 was derived from glutamine (Fig. 1; ref. 9), we targeted glutaminase with siRNA to determine if mutant IDH1 cells exhibited decreased growth compared with WT IDH1 cells. siRNA against glutaminase specifically slowed the growth of mutant IDH1 cells but not parental cells or cells overexpressing WT IDH1 (Fig. 3A). Consistent with the effects of anti-glutaminase siRNA, BPTES, a glutaminase inhibitor (19), preferentially slowed growth of mutant IDH1 cells without inducing apoptosis (Fig. 3B, Supplementary Figs. S2 and S3). We also treated TNA, which overexpressed R132H or WT IDH1, with BPTES and found that mutant IDH1 cells were also more sensitive to BPTES (Supplementary Fig. S4).

We then sought to restore α -KG levels in BPTES-treated cells to determine whether this would ameliorate growth inhibition. Exposing cells to dimethyl- α -ketoglutarate, a cell permeable α -KG precursor, significantly reduced growth inhibition of mutant IDH1 cells by BPTES (Fig.

3B, Supplementary Fig. S2). These observations suggest that glutaminase inhibition in mutant IDH1 cells decreased α -KG levels, potentially altering intermediary metabolism and consequently inhibiting cell proliferation. The effects of BPTES at higher concentrations, however, were not blocked by 1 mmol/L dimethyl- α -ketoglutarate. Although the reasons for this are unclear, it is possible that 1 mmol/L dimethyl- α -ketoglutarate is not sufficient to rescue the effects of 50 or 100 μ mol/L BPTES, or BPTES may have additional growth inhibitory effects at elevated concentrations.

Glutaminase activity was significantly reduced in both WT and mutant IDH1 expressing cells (59% and 68% inhibition, respectively) by 10 μ mol/L BPTES (Fig. 4A). Consistent with decreased glutaminase activity, BPTES treatment diminished glutamate and α -KG levels. Lowered α -KG led to decreases in subsequent tricarboxylic acid cycle intermediates (succinate and malate), as well as aspartate, which is derived from transamination of oxaloacetate with glutamate serving as the nitrogen donor. Surprisingly, 2-HG levels remained unchanged in treated mutant IDH1 cells (Fig. 4B, Supplementary Fig. S5). However, levels of glycolytic intermediates (fructose-1,6-bisphosphate, dihydroxy-acetone-phosphate, and 3-phosphoglycerate) increased with BPTES treatment, indicating that glycolytic flux is altered. Intriguingly, citrate levels changed in diametrically opposite directions between WT and IDH1 mutant cells before and after BPTES treatment. Basal citrate levels were lower but increased in treated mutant IDH1 cells as compared with higher basal levels in WT IDH1 cells that decreased with BPTES treatment (Fig. 4C).

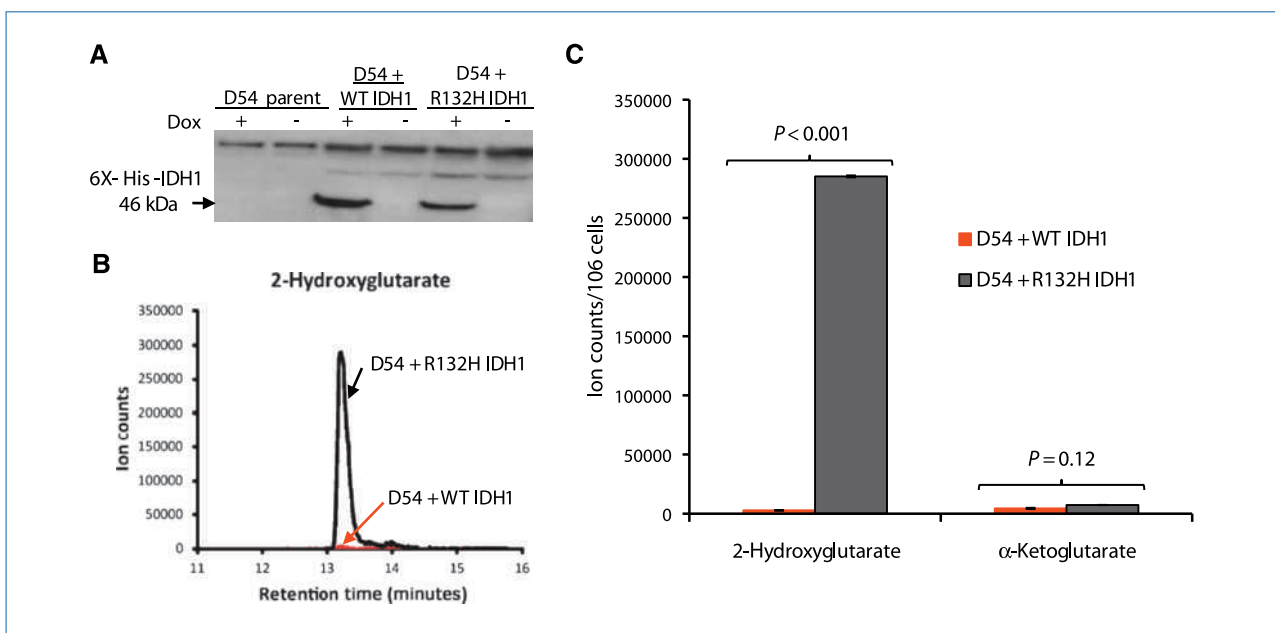


Figure 2. Validation of tet-inducible, stable D54 glioblastoma lines. A, Western blot showing doxycycline (Dox)-induced expression of 6X-His-Tag-IDH1. B, 2-hydroxyglutarate liquid chromatography/mass spectrometry (LC/MS) retention peaks. C, 2-HG and α -KG levels measured by LC/MS.

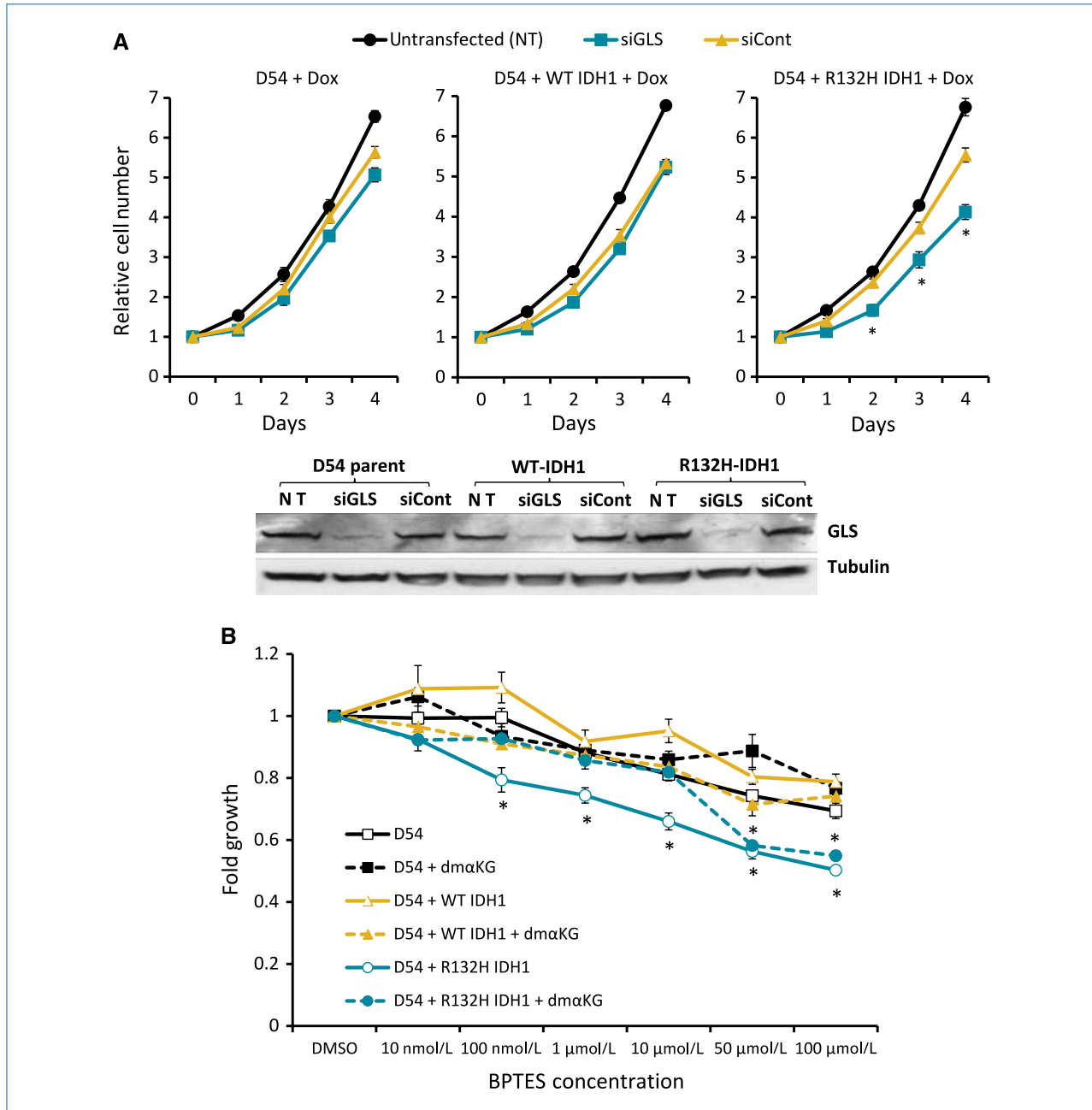


Figure 3. Mutant IDH1 cells depend on glutaminase for cell growth and glutaminase inhibition is negated by dimethyl- α -ketoglutarate. **A**, anti-glutaminase siRNA (siGLS) slows growth of mutant IDH1 cells. Western blot shows decreased levels of glutaminase in response to siGLS. **B**, effects of BPTES in the absence or presence of 1 mmol/L dimethyl- α -ketoglutarate (dmaKG) were measured. Data are from one representative experiment of three with similar trends and the average and SE of four replicates at each concentration. *, $P \leq 0.05$. For **A**, P value is for siGLS compared with siCont. Cell number was normalized to day 0. For **B**, the P value was for D54 + R132H compared with D54 and D54 + WT IDH1. Fold growth represents the ratio of alamarBlue fluorescence units of treated cells to vehicle-treated cells.

To further show the sensitivity of mutant IDH1 cells to inhibition of α -KG synthesis, we inhibited enzymes that convert glutamate to α -KG. Mutant IDH1 cells were more sensitive to both epigallocatechin gallate (EGCG), a glutamate dehydrogenase inhibitor, and amino-oxyacetic acid (AOA), a pan-transaminase inhibitor. However, a reduced

glucose concentration in the media was required to see the effects of EGCG and AOA, suggesting that reduced glycolytic compensation is necessary to unmask the sensitivity of mutant IDH1 cells to these inhibitors (Supplementary Fig. S6). Mutant IDH1 cells were not more sensitive to glutamine deprivation than WT cells (Supplementary Fig. S7).

Nonetheless, acquisition of mutant IDH1 activity seems sufficient to sensitize cells to inhibition of α -KG synthesis.

Discussion

Therapies that target various aspects of cancer cell metabolism are currently being developed and primarily focused on glucose metabolism (2). The dependence of cancer cells on glutamine for various processes is well documented (2–4) and has also been a target of interest for therapy; however, clinical trials have yielded little success due to a lack of efficacy or undesirable side effects of glutamine analogs (2, 4). Here, we explored an inhibitor of glutamine metabolism, BPTES, which allosterically inhibits glutaminase (but not glutaminase 2) and is not a glutamine analog (19). Specifically, we studied the dependency of cancer cells with IDH1 muta-

tion on glutaminase activity for maintenance of α -KG homeostasis.

The discovery of *IDH1* mutations (6) identified a metabolic genetic alteration present in a large fraction of gliomas and cytogenetically normal AML (6, 8). Genetically, a clustering of heterozygous mutations in IDH1 at a single residue indicates a gain-of-function mutation that is supported by the gain of a new enzymatic activity by mutant IDH1. Rather than converting isocitrate to α -KG, mutant IDH1 consumes α -KG and produces 2-HG. Studies have shown that glutamine serves as a cellular source of α -KG consumed by mutant IDH1 (9). It is not currently understood whether inhibiting mutant IDH1 and reducing 2-HG production would be therapeutically useful, because a role for mutant IDH1 or 2-HG in tumor maintenance has not been established. Thus, we sought to slow mutant IDH1 cell growth by inhibiting glutaminase.

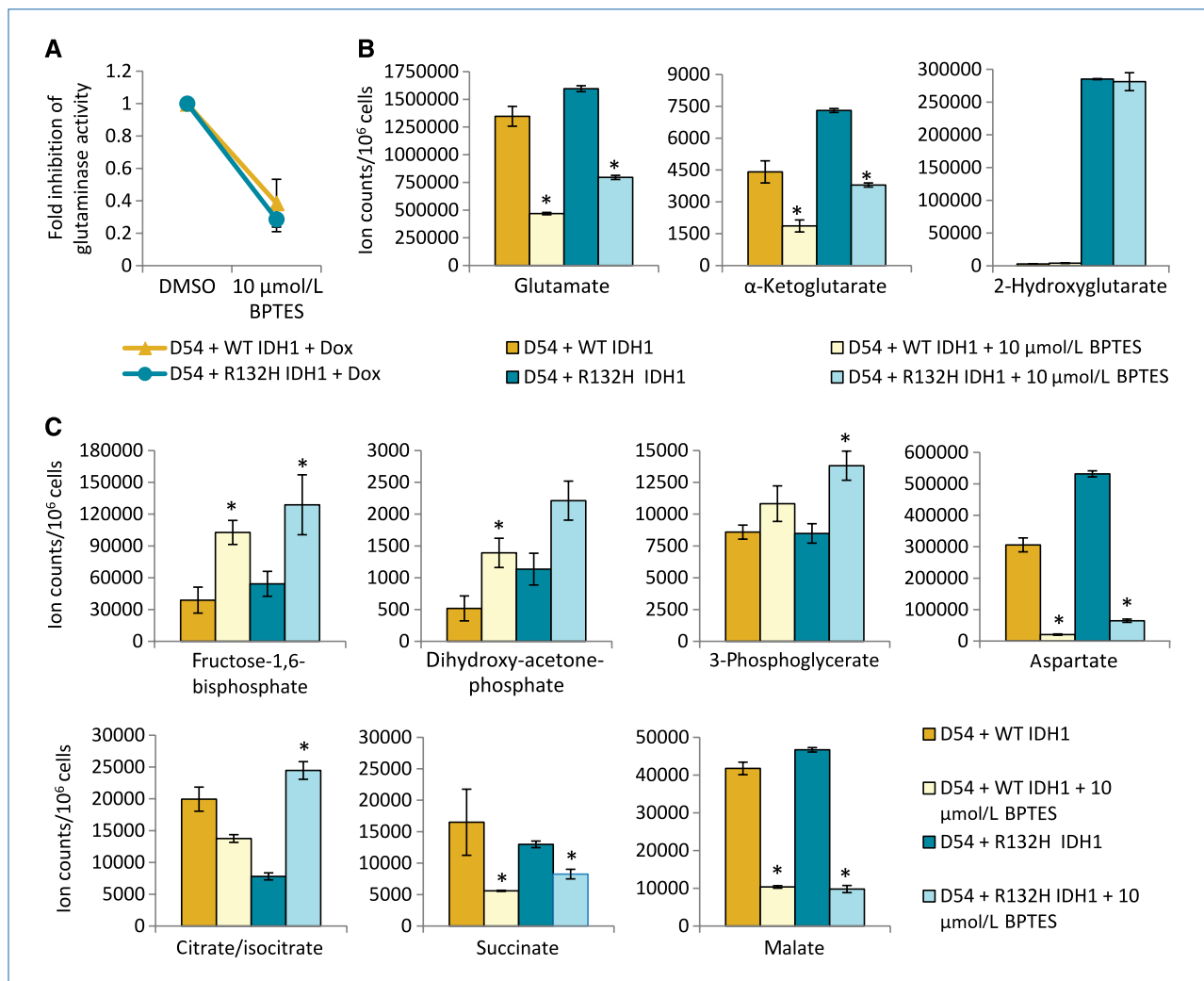


Figure 4. Metabolic changes result from overexpression of mutant IDH1 and 48 hours of treatment with 10 μ mol/L BPTES. Glutaminase activity (A) and glutamate, α -KG, and 2-HG levels (B) were measured in WT and mutant IDH1 cells. C, levels of other metabolites measured using LC/MS in response to BPTES treatment. *, $P \leq 0.05$ for B and C. The P value is for either D54 WT IDH1 DMSO versus 10 μ mol/L BPTES or D54 R132H IDH1 DMSO versus 10 μ mol/L BPTES.

Here we show that BPTES inhibits glutaminase activity and consequently lowers glutamate and α -KG levels in mutant and WT IDH1 cells, but only growth of mutant IDH1 cells is preferentially slowed in response to BPTES treatment. Intriguingly, BPTES treatment was associated with elevated glycolytic intermediates, which may reflect a compensatory increase in glycolysis to produce α -KG and maintain homeostasis. The notion that glutaminase inhibition perturbs α -KG homeostasis and causes growth inhibition is supported by rescue experiments using a membrane permeable α -KG precursor. Further, inhibition of enzymes that convert glutamate to α -KG showed selectivity for mutant IDH1 cells but only under glucose-deprived conditions. Mutant IDH1 cells, however, were not more susceptible than WT IDH1 cells to glutamine deprivation, and withdrawing glutamine altogether will eventually slow the growth of any cell that requires glutamine as an essential nutrient. This result raises the possibility that inhibition of glutaminase may have a different therapeutic result on IDH1 mutant cells compared with inhibition of glutamine uptake.

Our metabolomic studies revealed a number of interesting findings. First, although BPTES treatment lowered glutamate and α -KG levels, 2-HG levels were not significantly decreased. Accordingly, if 2-HG competes with α -KG for binding sites on α -KG-dependent enzymes occupancy of these sites with α -KG would fall with BPTES treatment. Although the effect would likely not be significant in wild-type cells, where α -KG presumably fills most sites, it could contribute to impaired cell growth upon BPTES treatment of cells expressing mutant IDH1. Second, BPTES treatment decreased subsequent tricarboxylic acid cycle intermediates, succinate and malate. Additionally, levels of glycolytic intermediates were increased, showing that metabolism is perturbed even far from the tricarboxylic acid cycle. We hypothesize that glycolytic intermediates are increased because of increased glycolytic flux to compensate for lowered α -KG levels; however, we have not ruled out the possibility that intermediates build up due to decreased glycolytic flux. One intriguing observation is the diametrically opposite changes in citrate levels between treated WT and R132H IDH1 cells. Currently, the reasons for these differences are not fully understood, and the dissection of these mechanisms is beyond the scope of this article. Nonetheless, differences in intermediary metabolism between WT and mutant IDH1 cells are sufficient to provoke a gain of sensitivity to glutaminase inhibition in cells with mutant IDH1.

Although reduction of glutaminase activity is significant in mutant IDH1 cells, the effects of BPTES on growth are modest (about 20% growth reduction). The modest growth reduction is not particularly surprising, because D54 cells have a genetic background that can tolerate significant

siRNA-mediated reduction of glutaminase level. However, the simple overexpression of mutant IDH1 alters intermediary metabolism sufficiently to sensitize mutant IDH1 cells to glutaminase inhibition. We speculate that glioma cells with a naturally occurring IDH1 mutation would show increased susceptibility to glutaminase inhibition; however, no such cell lines exist at present.

Additionally, cellular metabolism is incredibly dynamic and seems to compensate for changes in intermediary metabolism, such as increased glycolysis, upon BPTES treatment. As a result, we propose that glutaminase inhibition will not be effective as a single-arm therapy but will be a part of a more complex strategy that may involve simultaneous inhibition of glycolysis.

Like all treatments, there could be potential disadvantages to this therapeutic strategy because glioblastoma cells engineered with mutant IDH1 or IDH2 could have a pseudohypoxic phenotype (18). Previous studies documented that exogenous α -KG could reactivate prolyl hydroxylase, decrease hypoxia-inducible factor 1, alpha subunit levels, and inhibit cell growth resulting from pseudohypoxia elicited by mutations in succinate dehydrogenase and fumarate hydratase (20). As such, reducing α -KG by glutaminase inhibition could hypothetically enhance pseudohypoxia and favor tumor growth.

Our study shows that glutaminase could be a potential therapeutic target in mutant IDH1 cancer cells. However, further work is needed to investigate the metabolic consequences and biochemical specificity for mutant IDH1 cells in response to glutaminase inhibition. An understanding of these effects will be useful in developing combination therapies to augment the effects of glutaminase inhibition and provide insights into how IDH1 mutation and 2-HG production affect cellular physiology.

Disclosure of Potential Conflicts of Interest

C.V. Dang and J.D. Rabinowitz are consultants for Agios Pharmaceuticals, Inc. G.J. Riggins is a coinventor on IDH1-related intellectual property managed by Johns Hopkins University. The other authors disclosed no potential conflicts of interest.

Grant Support

Support provided by the Virginia and D.K. Ludwig Fund for Cancer Research, NIH Grants R01NS052507, R01CA57341, R01CA051497; the AACR Stand-Up-to-Cancer grant; Bayer Schering Grants for Targets; the JHU Brain Science Institute; the Irving Sherman Neurosurgery Professorship (G.J. Riggins); and the Johns Hopkins Family Professorship in Oncology Research (C.V. Dang).

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Received 05/10/2010; revised 08/19/2010; accepted 09/15/2010; published OnlineFirst 11/02/2010.

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