ABSTRACT
Metabolic changes induced by oncogenic drivers of cancer contribute to tumor growth and are attractive targets for cancer treatment. Here, we found that increased growth of PTEN-mutant cells was dependent on glutamine flux through the de novo pyrimidine synthesis pathway, which created sensitivity to the inhibition of dihydroorotate dehydrogenase, a rate-limiting enzyme for pyrimidine ring synthesis. S-phase PTEN-mutant cells showed increased numbers of replication forks, and inhibitors of dihydroorotate dehydrogenase led to chromosome breaks and cell death due to inadequate ATR activation and DNA damage at replication forks. Our findings indicate that enhanced glutamine flux generates vulnerability to dihydroorotate dehydrogenase inhibition, which then causes synthetic lethality in PTEN-deficient cells due to inherent defects in ATR activation. Inhibition of dihydroorotate dehydrogenase could thus be a promising therapy for patients with PTEN-mutant cancers.

SIGNIFICANCE: We have found a prospective targeted therapy for PTEN-deficient tumors, with efficacy in vitro and in vivo in tumors derived from different tissues. This is based upon the changes in glutamine metabolism, DNA replication, and DNA damage response which are consequences of inactivation of PTEN. Cancer Discov; 7(4); 380–90. © 2017 AACR.

INTRODUCTION
The Warburg effect, describing heightened aerobic glycolysis in tumors, played a key role in launching the field of cancer metabolism. Subsequent studies have found that glutamine is also vital for growth by fueling tricarboxylic acid cycle intermediates, phospholipid and nucleotide synthesis, and NADPH (1). Oncogenic signaling pathways have been shown to play a major role in reprogramming glucose and glutamine metabolism, thus connecting genetic mutations with metabolic alterations (2–5). PTEN is one of the most commonly mutated tumor suppressors and is a fulcrum of...
PTEN Inactivation Creates Vulnerability to DHODH Inhibition

multiple cellular functions (6, 7). PTEN’s canonical role is as a lipid phosphatase for phosphatidylinositol-3,4,5-trisphosphate, central to the PI3K pathway, limiting AKT, mTOR, and RAC signaling (8–11). Inactivation of PTEN enhances glucose metabolism and diminishes DNA repair and DNA damage checkpoint pathways (12–14). Furthermore, deficient homologous recombination in PTEN-mutant cells leads to sensitivity to γ-irradiation and PARP inhibitors (13, 15). The role of PTEN in metabolism, however, is incompletely understood, and in this study we examine the metabolic consequences of PTEN loss and the resulting vulnerability of PTEN-mutant tumors.

RESULTS

To better understand the relationship between PTEN, cell growth, and cellular metabolism, we generated Pten flox/flox primary mouse embryonic fibroblasts (MEF). Pten−/− MEFs proliferated at a higher rate than wild-type (WT) MEFs but showed no difference in cell death (Fig. 1A; Supplementary Fig. S1A–S1C). This increased proliferation was associated with an increase in the proportion of cells within S-phase and higher numbers of replication forks per S-phase cell (Fig. 1B–D; Supplementary Fig. S1D). There was no difference in mitochondrial function between Pten−/− and WT MEFs, suggesting a different source of altered replication (Supplementary Fig. S1E and S1F).

Upon testing the potential role of glutamine for explaining the increased growth of Pten−/− cells, we found that the growth advantage of Pten−/− MEFs was dependent on glutamine: Depletion of glutamine or the addition of the glutaminase inhibitor CB-839 collapsed the growth difference between Pten−/− and WT MEFs (Fig. 1E and F). To better understand the relationship between PTEN and glutamine, we performed steady-state metabolomic profiling of 292 aqueous metabolites to determine whether the loss of PTEN triggers abnormal cellular metabolism to increase growth. Unbiased global metabolic assessment of WT and Pten−/− MEFs revealed that seven of the ten most upregulated pathways in Pten−/− MEFs involved nucleotide synthesis and DNA metabolism, including a higher concentration of pyrimidine 2-deoxyribonucleotides in Pten−/− MEFs (Fig. 1G; Supplementary Fig. S2A and S2B). Because glutamine contributes both nitrogen and carbon to pyrimidines (16), we performed metabolic flux analysis with heavy isotope 15N or 13C-labeled glutamine, which showed increased synthesis of dihydroorotate to orotate, and other and other components of the de novo pyrimidine synthesis pathway in Pten−/− MEFs relative to WT (Fig. 1H and I; Supplementary Fig. S2C). In addition, the pyrimidine metabolism gene set was upregulated in mRNA from Pten−/− MEFs (Supplementary Fig. S2D). Although Pten−/− fibroblasts had somewhat elevated steady-state glucose metabolism and glycolytic flux relative to WT, depletion of glucose from the medium did not rescue the differences in cell growth, suggesting that glutamine was more critical for the growth advantage of Pten−/− cells (Supplementary Fig. S2A, S2E, and S2F).

Nucleotide synthesis is a prerequisite for cellular growth, and Pten−/− MEFs appear to channel glutamine for this purpose.

The fourth step of de novo pyrimidine synthesis in mammals is the conversion of dihydroorotate to orotate, catalyzed by dihydroorotate dehydrogenase (DHODH; ref. 17). To see whether orotate contributes to the growth effects observed, the effect of DHODH inhibitors on cell proliferation was examined. Pten−/− MEFs were about 3-fold more sensitive to leflunomide, a DHODH inhibitor (18), than WT MEFs were (Fig. 2A; Supplementary Fig. S3A and S3B). Pten−/− MEFs were also more sensitive to A771726, the active metabolite of leflunomide (19, 20), as well as another DHODH inhibitor, brequinar (18), indicating that the observed effects were likely through DHODH (Fig. 2A).

To determine whether PTEN genotype is predictive of sensitivity to DHODH inhibition in cancer cells, we tested human breast cancer, glioblastoma, and prostate cancer cell lines with DHODH inhibitors. Consistently, the GI50 of the Pten−/− mutant lines was lower than that of WT (Fig. 2B; Supplementary Fig. S3C). The mouse cancer lines MCCL-357 (Myc, Pten−/−) and CaP8 (Pten−/−) were also more sensitive than the mouse cancer lines MCCL-278 (Myr, Pik3caH1047R) and Myc-CaP (Myc) were (Fig. 2C; Supplementary Fig. S3D and S3E; refs. 20, 21). Moreover, Pten−/− MEFs, PTEN-mutant human breast cancer cell lines, and Pten−/− mouse breast cancer cell lines displayed increased cell death over time upon treatment with leflunomide (Fig. 2D and E; Supplementary Fig. S3F). It is important to note that sensitivity to leflunomide was not associated with the proliferation rates of human breast, mouse breast, or mouse prostate tumor cell lines (Fig. 2F; Supplementary Fig. S3G and S3H).

In addition, consistent with previous reports (22), we found that Pten homozygous deletion caused greater AKT phosphorylation than Pik3ca missense mutation did. This was particularly prominent in the nuclear fractions, where AKT may phosphorylate nuclear substrates (Fig. 2G; Supplementary Fig. S3I and S3J).

To independently test whether DHODH inhibition is detrimental to PTEN-deficient cells, we performed a rescue experiment with orotate, the metabolite directly downstream of DHODH, as well as with uridine. Increasing concentrations of orotate or uridine rescued growth inhibition by leflunomide (Fig. 2H–K; Supplementary Fig. S4A–S4C). In addition, siRNA against DHODH preferentially killed Pten−/− mutant cells, verifying that DHODH was the target of the small-molecule inhibitors (Supplementary Fig. S4D). There was no difference in endogenous DHODH protein level between Pten−/− and WT MEFs, and A771726 did not affect PI3K signaling (Supplementary Fig. S4E and S4F). Consistent with prior reports, CAD phosphorylation downstream of mTORC1 was increased in Pten−/− cells, likely contributing to the push of glutamine flux into the pyrimidine synthesis pathway (Supplementary Fig. S4G and S4H; ref. 5). Pten−/− cells were more sensitive than WT cells to the mTOR inhibitor RAD001, as expected, but RAD001 did not synergize with leflunomide (Supplementary Fig. S4I–S4K; refs. 10, 23). Interestingly, treatment with nucleotide analogue inhibitors, 5-fluorouracil or mercaptopurine, did not show a differential sensitivity, demonstrating that Pten−/− MEFs are selectively vulnerable to inhibition of de novo pyrimidine synthesis (Supplementary Fig. S4L and S4M).
Figure 1. A, Growth of Pten WT and knockout (KO) MEFs (one-way ANOVA, *, \( P < 0.0001, n = 3 \)). B, MEFs labeled with EdU. Representative confocal microscopy images. C, Quantification of B (Student t test, *, \( P < 0.05, n = 6 \)). D, MEFs labeled with EdU; flow cytometry determined the mean fluorescence intensity (MFI) among cells positively stained (Student t test, *, \( P < 0.01, n = 6 \)). E, Pten WT and KO MEFs in media containing full glutamine (6 mmol/L) or no added glutamine (one-way ANOVA, *, \( P < 0.0001, n = 3 \)). F, MEFs treated with 12.5 nmol/L CB-839 or control (one-way ANOVA, *, \( P < 0.0001, n = 3 \)). G, Relative metabolite concentrations of DNA nucleotide precursors (dGMP was unable to be measured so dGTP was used; Student t test, *, \( P < 0.05, n = 3 \)). H, Relative metabolite levels of glutamine-labeled de novo pyrimidine synthesis intermediates (Student t test, *, \( P < 0.05, n = 3 \)). Data were also analyzed with IMPalA: \( 13^C \) glutamine-derived pyrimidine metabolism enrichment in PTEN−/− MEFs \( q \) value = 3.92 \( \times 10^{-09} \). I, Schematic of the de novo pyrimidine synthesis pathway. Not every intermediate was measured in our mass spectrometry panel. Data, means ± SD. TCA, tricarboxylic acid.
PTEN Inactivation Creates Vulnerability to DHODH Inhibition

**Figure 2.** A, Pten WT and knockout (KO) cells treated with dose titrations of leflunomide, A771726, or brequinar to determine GI50 (Student t test, *P < 0.05, n = 3). B and C, Cells treated with dose titrations of leflunomide to determine GI50 (Student t test, *P values on figures, n = 3). D and E, Cells treated with 100 μmol/L leflunomide and DRAQ7 to monitor accumulation of cell death, in intervals of 6 hours (one-way ANOVA, *, P values on the figures). F, Human breast cancer cell line growth rates. G, Immunoblots of pAKT in nuclear fractions of Pten−/− and Pik3ca mutant MEFs. H, Cells treated with 50 μmol/L leflunomide in combination with 0 or 640 μmol/L orotate. Confluence of cells after 5 days of treatment was measured (Student t test, *P < 0.05, n = 3). I, Cells treated with 50 μmol/L leflunomide in combination with 0, 31.25, 62.5, or 125 μmol/L orotate. Confluence of cells after 5 days was measured (Student t test, *P < 0.05, n = 3). J, Cells treated with 100 μmol/L leflunomide in combination with 0 or 3 125 mmol/L uridine. Confluence of cells after 5 days was measured (Student t test, *P < 0.05, n = 3). Data, means ± SD.
MYC activation is known to cause glutamine addiction (4). CaP8 (Pten<sup>−/−</sup>) cells were nearly as sensitive to glutamine deprivation as Myc-CaP (Myc oncogene transformed) cells were, substantiating that a notable level of glutamine dependency is also elicited by PTEN loss (Supplementary Fig. S4N). As Myc-CaP cells were resistant to leflunomide, it seems it is not the entry of glutamine alone but its flux into pyrimidines that is important (Supplementary Fig. S3D). Although MYC is known to largely direct glutamine to the tricarboxylic acid cycle and phospholipid synthesis (4), our data suggest that Pten loss in MEFs causes glutamine to cascade through the de novo pyrimidine synthesis pathway, creating the point of vulnerability to DHODH inhibition.

To determine how clinically relevant leflunomide may be as a targeted cancer therapy, we grew patient-derived glioblastomas as three-dimensional neurospheres. Formation of neurospheres was inhibited at lower concentrations of leflunomide in PTEN-deficient samples (Fig. 3A; Supplementary Fig. S5A). In addition, we treated two PTEN-mutant triple-negative breast cancer xenografts with leflunomide, dosing orally as is done clinically. Tumors slowed or regressed upon treatment; remarkably, even very large tumors (4 × 10<sup>7</sup> photons) regressed after only 1 week of treatment, indicating that leflunomide may have use for neoadjuvant therapy (Fig. 3B and C; Supplementary Fig. S5B). To ensure the effect in vivo is specific to PTEN loss, MCCL-357 and MCCL-278 xenografts were treated with leflunomide; MCCL-357 xenografts had a 4-fold better response than MCCL-278 xenografts did (Supplementary Fig. S5C).

It is logical that a blockade of pyrimidine synthesis would stop cells from dividing, and leflunomide has been previously established as a cytostatic drug (18). What is more enigmatic, however, is why it would cause PTEN<sup>−/−</sup> cells to die. Consistent with prior reports (24), Pten<sup>−/−</sup> MEFs had a higher level...
PTEN Inactivation Creates Vulnerability to DHODH Inhibition

of γH2AX, an indicator of DNA damage (Fig. 4A). We hypothesized that the dearth of pyrimidine deoxynucleotides caused by DHODH inhibition would exacerbate this defect, and discovered that leflunomide (or A771726) augmented DNA damage to a significantly greater degree in PTEN-deficient cells and that this damage colocalized with replication forks labeled with EdU (Fig. 4B–D; Supplementary Fig. S6A and S6B). Leflunomide-induced DNA damage was rescued by uridine, demonstrating that damage is likely instigated by pyrimidine depletion (Fig. 4E). The greater number of replication forks we described in Pten−/− MEFs remained intact after 24 hours of treatment with leflunomide, showing that the cells continue to replicate despite the presence of DNA damage (Figs. 1B and 4F; Supplementary Fig. S6C and S6D).

Depletion of nucleotide pools normally activates the ATR checkpoint at replication forks in S-phase cells (25). ATR checkpoint activation at stalled forks requires two signals, one through single-strand DNA-binding protein [replication

Figure 4. A, Cells were labeled with γH2AX antibody. Flow cytometry determined the mean fluorescence intensity (MFI; Student t test, *P < 0.05, n = 3). B and C, Cells treated with 100 μmol/L leflunomide or A771726 were labeled with a γH2AX antibody. Flow cytometry determined the mean fluorescence intensity (Student t test; *P < 0.05, n = 3). D, MEFs treated with 150 μmol/L A771726 for 24 hours, labeled with EdU and γH2AX. Left, representative confocal microscopy images; right, quantification of the number of foci per cell (Student t test; *P < 0.05, n = 3). E, Cells treated with 100 μmol/L leflunomide with or without uridine and labeled with a γH2AX antibody. Flow cytometry determined the mean fluorescence intensity (Student t test; *P < 0.05, n = 3). F, MEFs treated with 100 μmol/L leflunomide or control for 48 hours and labeled with EdU. Left, representative confocal microscopy images; right, quantification of the number of foci per cell (Student t test; *P < 0.05, n = 6). G, Cells were labeled with a pTOPBP1 S1159 antibody. Flow cytometry determined the mean fluorescence intensity (Student t test; *P < 0.05, n = 3)(continued on next page)
The presence of A771726, followed by a shift toward both RPA action of RPA and checkpoint at replication forks. Activation of CHK1 in MCCL-278 cells declined as RPA declined, suggesting that Pten WT cells eventually recovered from DHODH inhibition, whereas Pten−/− cells instead accumulated damage at 18 hours (Fig. 4I). By 48 hours, this genomic stress manifested in a greater number of chromosome gaps, breaks, and multiradial formations in MCCL-357 cells treated with A771726 compared with MCCL-278 cells (Fig. 4J and K; Supplementary Fig. S6J and S6K). These findings are consistent with the sensitivity to hydroxyurea that occurs in the setting of an ATR inhibitor (30). Furthermore, we were able to rescue DNA damage and cell death in leflunomide-treated Pten−/− cells by transfecting cells with TOPBP1 and CHK1 mutants incapable of being phosphorylated by AKT and, labeled with a γH2AX antibody after 100 μmol/L leflunomide treatment. Flow cytometry determined the mean fluorescence intensity (Student t test; *, P < 0.05, n = 3). M. Pten-mutant cells were transfected with either WT TOPBP1 and CHK1, or mutants incapable of being phosphorylated by AKT, and DRAQ7 was used to monitor accumulation of cell death in intervals of 6 hours (one-way ANOVA; *, P < 0.05, n = 3). Data, means ± SD.

Figure 4. (Continued) H. Cells treated with 150 μmol/L A771726 for times indicated and labeled with antibodies to RPA and γH2AX. Flow cytometry determined the percentage of the cell population positively stained for RPA alone or both RPA and γH2AX (Student t test; *, P < 0.05, n = 4). J, pCHK1 immunoblot after 150 μmol/L A771726 treatment for times indicated. J and K. Quantified chromosomal breaks and multiradial formations per haploid genome (Student t test; *, P < 0.05, n = 100). L, Pten-mutant cells were transfected with either WT TOPBP1 and CHK1, or mutants incapable of being phosphorylated by AKT, and labeled with a γH2AX antibody after 100 μmol/L leflunomide treatment. Flow cytometry determined the mean fluorescence intensity (Student t test; *, P < 0.05, n = 3).
PTEN Inactivation Creates Vulnerability to DHODH Inhibition

Inhibiting DHODH has the advantage of affecting a specific pathway of glutamine flux downstream of glutaminase, thus preserving glutamine's other important functions in the cell. This increases the specificity of DHODH inhibitors to cells which are dependent on glutamine's role in pyrimidine synthesis per se, and is perhaps why their toxicity is low enough to be taken as a daily medication by patients with rheumatoid arthritis or multiple sclerosis (19). It has been shown that leflunomide inhibits B-cell and T-cell proliferation, contributing to its immunomodulating effects (33, 34). It is possible that preexisting B and T cells can still function in the presence of DHODH inhibitors, thus potentially arguing for the benefit of immunotherapy in combination with a DHODH inhibitor, perhaps in a metronomic therapy pattern. Here, we show that high activation of AKT toward TOPBP1 and CHK1 that downregulates ATR activation at replication forks, compounded with enhanced pyrimidine flux that both occur as a consequence of PTEN inactivation contribute to the observed synthetic lethality between PTEN mutation and DHODH inhibition. We hope that DHODH inhibitors will be a promising therapy for patients with PTEN-deficient cancers.

METHODS

Immunoblotting

Samples were lysed in 2× Laemelli sample buffer before separation by SDS-PAGE and transferring to PVDF membranes, blocked with 10% milk in TBST for 1 hour, and incubated with primary antibodies overnight. The following antibodies were used: PTEN 6H2.1 (Millipore 04-035; 1:1,000), DHODH (Protein Tech 14877-1-AP; 0.5 ng/μl), vinculin (Sigma; 1:50,000), pCHK1 (Cell Signaling Technology, 2341; 1:500), CHK1 G-4 (Santa Cruz Biotechnology, sc-8408; 0.4 ng/μl), pCAD (Cell Signaling Technology, 12662; 1:500), CAD (Cell Signaling Technology, 11933; 1:500). HRP-conjugated secondary antibodies were used to detect protein signals.

Cell Culture

MEFs and mouse breast tumor lines: DMEM (Corning mt01031cv) supplemented with 10% FBS (Atlanta Biologicals), 1% penicillin/streptomycin (Fisher Scientific, 30002ci), and 2 mmol/L L-glutamine (total 6 mmol/L; Fisher Scientific MT25005CI). MDA-MB-468, MDA-MB-231, Myc-CaP (2015), and U87: DMEM supplemented with 10% FBS and 1% penicillin/streptomycin. HCC1919, HCC1806, BT549, ZR75-1, PC3, LNCAP, DBTRG-63MB-7MG (Fisher Scientific, 10040cv) supplemented with 10% FBS and 1% penicillin/streptomycin. CaP8 cells (2015). DMEM with 10% FBS, 1% penicillin/streptomycin, and 5 μg/ml insulin (Sigma 19278). Neurospheres: stem cell media with 10 μg/ml FG (R&D Systems 233-FB-025), 20 μg/ml EGF (Peprotech AF-100-15), and heparin (from R. Yong, May 2015). All cells were cultured in a 37°C incubator with humidity and 5% CO2. Cell lines were obtained from ATCC (which authenticates cell lines using several methods, including DNA fingerprinting) in 2006, with the exception of MEFs, M0CL-278, and M0CL-357, which were produced in our laboratory from mice (2012–2016). Cell lines were clear of Mycoplasma as determined by the Lonza kit (LT07-418) within 6 months of their use. Cell lines were further authenticated in 2015 by LabCorp using a short tandem repeat method.

Mouse Embryonic Fibroblasts

Embryos were harvested 14 days after conception from Pten flx/flx mice from Jackson Labs. MEFs were treated with adenovirus diluted in growth media supplemented with polybrene with or without Cre recombinase and studied passages 2–5 post infection. Please see Supplementary Data for additional details.

Proliferation Assay

Mouse cells (1,500 cells per well) or human cells (3,000 cells per well) were plated in 96-well plates (Corning 720089). Growth rates were determined using the phase-confluency readings on an IncuCyte ZOOM (Essen Biosciences) on live cells over time.

Metabolic Labeling

For glutamine flux, media without added glutamine (Corning 17-207-CV) were supplemented with 13C glutamine (fully labeled) or 15N glutamine (amide labeled; Cambridge Isotope Labs). For glucose flux, media without added glucose (Corning 17-207-CV) were supplemented with 13C glucose (fully labeled; Cambridge Isotope Labs). Cells were plated in 10-cm dishes and grown in normal media. One hour prior to metabolic extraction, media were aspirated and replaced with heavy isotope-labeled media.

Metabolic Extraction

Metabolites were extracted in methanol. Please see Supplementary Data for details.

Targeted Mass Spectrometry

Mass spectrometry was performed by the core facility at Beth Israel Deaconess Medical Center (New York, NY). Please see Supplementary Data for details.

Cell–Cycle Analysis

Instructions for the FlowCellect Bivariate Cell Cycle Kit (Millipore FCCH025102) were followed. Fluorescence was measured on a Guava ZOOM (Essen Biosciences) on live cells over time.

Cell Death

Instructions for the FlowCellect Annexin Red Kit (Millipore FCCH100108) were followed. Fluorescence was measured on a Guava flow cytometer. Bromodeoxyuridine was pulsed for 18 hours.

Seahorse Analysis

A Seahorse XF (Agilent) was used to determine the oxygen consumption and extracellular acidification rates. Cells were plated in the Seahorse cartridge and pH-adjusted media were added. Oligomycin, FCCP, and rotenone (Seahorse XF Kit, Agilent) were injected into
cartridge wells. Controls for calibration and establishing baseline were used.

**Drug Response Assays**

Cells were plated in 96-well plates at a density of 1,500 or 3,000 cells per well. Leflunomide (Sigma P96137-1G), A771726 (Sigma SML0936), mercaptopurine (Sigma 852678), brequinar (Sigma SML0113), 5-fluorouracil (Millipore 343922), RAD001 and GDC0941 (obtained from Stand Up to Cancer PI3K Dream Team pharmacy), and CB-839 (MedChemexpress HY-12248) were dissolved in DMSO. Sensitivity was determined by a dose–response titration for each cell line, with an equivalent amount of DMSO in each well. 300 μL media with drug was added to one column of wells, and 150 μL media with an equivalent DMSO was added to remaining wells. Serial dilutions of 150 μL resulted in a gradient with half the drug concentration as the previous column while maintaining the same amount of DMSO. GI50 values were calculated by linear interpolation: The maximum growth confluence for a cell line prior to growth plateau was divided by 2 to obtain the 50% confluence value. A linear regression curve was calculated using drug concentrations as x-values and confluence as y-values for points surrounding the 50% value. Linear interpolation using the regression line yielded the GI50 concentration. For cell death assays, DRAQ7 (Cell Signaling Technology, 7406S) was added to the media at a 1:200 dilution and red fluorescence was measured in addition to phase in live-cell imaging to measure accumulation of dead cells. An IncuCyte ZOOM was used.

**γH2AX Measurement**

Instructions for the FlowCollect Cell Cycle Checkpoint H2AX DNA Damage Kit (Millipore FCCH12542) were followed. Briefly, cells were fixed and permeabilized, followed by staining with an anti-phospho-H2AX antibody and propidium iodide. For containing with RPA, an additional step was performed during which the cells were incubated with an RPA antibody (Abcam ab79398) for 1 hour and a secondary antibody for 1 hour (propidium iodide was not used in this setting). Fluorescence was measured on a Guava flow cytometer.

**EdU Detection**

Instructions for the EdU Cell Proliferation Kit (Millipore 17-10525) were followed. Cells were fixed and permeabilized following a 45-minute EdU pulse and a click chemistry reaction was used to add a fluorescent tag. Fluorescence was measured on a Guava flow cytometer or by immunofluorescence.

**Immunofluorescence**

Cells were plated on cover slips in media. For detecting replication forks, following a 45-minute EdU pulse, cover slip–attached cells were fixed and permeabilized, and detected after azide conjugation to EdU. For detecting γH2AX or TOPBP1, cells were incubated with primary antibody (BioLegend and Bethyl A300-111A-M, respectively) overnight at 4°C and with secondary antibody for 2 hours at room temperature. Images were taken using a Zeiss LSM880 Airyscan confocal microscope at 63X, and foci number and colocalization was quantified with ImageJ.

**pTOPBP1 Measurement**

Cells were fixed and permeabilized, followed by incubation with primary antibody (Abgent AP3774a) for 2 hours at room temperature and secondary antibody for 2 hours at room temperature. Fluorescence was measured on a Guava flow cytometer.

**Karyotyping**

Chromosomal analysis was performed on cultured cells treated with colcemid by Dr. Murty Vundavalli at Columbia University (New York, NY). Please see Supplementary Data for details.

**Orotate Rescue**

Orotate (Sigma O2750) was dissolved in DMSO. Cells were plated at fixed-concentration leflunomide with increasing concentrations of orotate, keeping DMSO constant in all wells.

**Uridine Rescue**

Uridine (Sigma U 3750) was dissolved in media. Cells were plated at fixed-concentration leflunomide with increasing concentrations of uridine, keeping DMSO constant in all wells.

**RNA Interference**

siRNA for DHODH was purchased from Qiagen. Cells were transfected using Lipofectamine (Invitrogen 11668-019) and knockdown was confirmed at 48 hours. Scrambled siRNA was used as a control.

**Transfection**

Plasmids were electroporated into cells (1 million) using an Amaza Nucleofector 2b (Lonza) and Cell Line Nucleofector Kit V (Lonza VCA-1003). An mCherry plasmid was cotransfected to determine transfection efficiency and to gate transfected cells for flow cytometry experiments.

**Xenografts**

Six-week-old female nu/nu mice were engrafted orthotopically with either 5 million SUM149, 5 million MDA-MB-468-luciferase, 1 million MCCL-357, or 0.75 million MCCL-278 cells. Mice were treated by oral gavage with 100 mg/kg leflunomide or vehicle (1% carbosymethylcellulose in water). Animal experiments were approved by the Institutional Animal Care and Use Committee of Icahn School of Medicine at Mount Sinai.

**Neurosphere Sensitivity Assay**

Neurospheres were disrupted by manual pipetting until single-cell suspension was achieved, and 10,000 cells/well were plated in low-attachment 6-well plates (Fisher Scientific 3471). After 5 days, neurospheres were counted; sphere-forming ability is an indicator of tumorigenicity. Dense clusters >0.05 mm in diameter were counted as true tumor spheres. Spheres were measured on ImageJ and quantified.

**Statistical Analysis**

ANOVA or Student t tests were used to test means between groups. Correction for multiple comparisons was added where needed. Analysis was done using GraphPad Prism 6 or Microsoft Excel.

**Disclosure of Potential Conflicts of Interest**

L.C. Cantley is a member of the board of directors of Agios and the scientific advisory board at Petra, reports receiving commercial research grants from Astellas and Takeda, has ownership interest (including patents) in Agios and Petra, and is a consultant/advisory board member for Enilibrium. No potential conflicts of interest were disclosed by the other authors.

One of the Editors-in-Chief is an author on this article. In keeping with the AACR’s editorial policy, the peer review of this submission was managed by a senior member of Cancer Discovery’s editorial team;
PTEN Inactivation Creates Vulnerability to DHODH Inhibition

Authors’ Contributions

Conception and design: D. Mathur, S. Schoenfeld, R. Parsons
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): J. Mathur, E. Stratikopoulos, S. Ozturk, S. Schoenfeld, R. Yong, V.V. Murty, J.M. Asara, R. Parsons
Analysis and interpretation of data (e.g., statistical analysis, bio-statistics, computational analysis): D. Mathur, E. Stratikopoulos, S. Ozturk, S. Schoenfeld, J.M. Asara, R. Parsons
Writing, review, and/or revision of the manuscript: D. Mathur, E. Stratikopoulos, N. Steinbach, S. Pegno, R. Yong, J.M. Asara, L.C. Cantley, R. Parsons
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): D. Mathur
Study supervision: D. Mathur, R. Parsons

Acknowledgments

We would like to thank the members of the Parsons laboratory for providing feedback on this manuscript, and D. Mathur’s thesis committee for helping shape the project. Confocal experiments were performed at the Microscopy core facility at Icahn School of Medicine at Mount Sinai. We thank Min Yuan and Susanne Breitkopf for help with mass spectrometry work. We also thank Dr. Gerard Karsenty, Columbia University Medical Center, for use of his Seahorse XF and Dr. Grzegorz Sumara for training us to use it. TOPBP1 WT and mutant plasmids were graciously supplied by Dr. Wei-Chin Lin, Baylor College of Medicine.

Grant Support

This work was supported by R01CA082783, R01CA155117, and P01CA94703 (to R. Parsons), and partially supported by NIH grants 5P01CA120964 and 5P30CA006516 (to J.M. Asara), and R01 GM041890 and the Breast Cancer Research Foundation (to L.C. Cantley).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received June 2, 2016; revised December 2, 2016; accepted January 17, 2017; published OnlineFirst March 2, 2017.

REFERENCES

PTEN Regulates Glutamine Flux to Pyrimidine Synthesis and Sensitivity to Dihydroorotate Dehydrogenase Inhibition

Deepti Mathur, Elias Stratikopoulos, Sait Ozturk, et al.


Updated version
Access the most recent version of this article at:
doi:10.1158/2159-8290.CD-16-0612

Supplementary Material
Access the most recent supplemental material at:
http://cancerdiscovery.aacrjournals.org/content/suppl/2017/02/03/2159-8290.CD-16-0612.DC1

Cited articles
This article cites 34 articles, 19 of which you can access for free at:
http://cancerdiscovery.aacrjournals.org/content/7/4/380.full#ref-list-1

Citing articles
This article has been cited by 16 HighWire-hosted articles. Access the articles at:
http://cancerdiscovery.aacrjournals.org/content/7/4/380.full#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, use this link http://cancerdiscovery.aacrjournals.org/content/7/4/380. Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.