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ORIGINAL ARTICLE

Novel Glutamine Antagonist JHU395 Suppresses MYC-Driven Medulloblastoma Growth and Induces Apoptosis

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Abstract

Medulloblastoma is the most common malignant pediatric brain tumor. Amplification of c-MYC is a hallmark of a subset of poor-prognosis medulloblastoma. MYC upregulates glutamine metabolism across many types of cancer. We modified the naturally occurring glutamine antagonist 6-diazo-5-oxo-l-norleucine (DON) by adding 2 promoeities to increase its lipophilicity and brain penetration creating the prodrug isopropyl 6-diazo-5-oxo-2-(((phenyl (pivaloyloxy) methoxy) - carbonyl) amino) hexanoate, termed JHU395. This prodrug was shown to have a 10-fold improved CSF-to-plasma ratio and brain-to-plasma ratio relative to DON. We hypothesized that JHU395 would have superior cell penetration compared with DON and would effectively and more potently kill MYC-expressing medulloblastoma. JHU395 treatment caused decreased growth and increased apoptosis in multiple human high-MYC medulloblastoma cell lines at lower concentrations than DON. Parenteral administration of JHU395 in Nu/Nu mice led to the accumulation of micromolar concentrations of DON in brain. Treatment of mice bearing orthotopic xenografts of human MYC-amplified medulloblastoma with JHU395 increased median survival from 26 to 45 days compared with vehicle control mice ($p < 0.001$ by log-rank test). These data provide preclinical justification for the ongoing development and testing of brain-targeted DON prodrugs for use in medulloblastoma.

Key Words: 6-diazo-5-oxo-l-norleucine, Cancer metabolism, DON, Pediatric brain tumor, Prodrug.

INTRODUCTION

Abnormal metabolism is a hallmark of cancer. High-level expression of MYC increases glutamine transport and use (1). Human and mouse studies using FDG-glutamine demonstrated increased glutamine uptake in cancer cells compared with normal brain (2). MYC-amplification defines a subtype of medulloblastoma, which is the most common pediatric brain tumor. MYC-amplified medulloblastoma has a worse outcome compared with non-MYC amplified medulloblastoma (3). MYC-amplification is found in large cell/anaplastic medulloblastoma, and this histologic feature is associated with increased risk of death (4). A magnetic resonance spectroscopy study found that medulloblastoma patients with high levels of glutamate in their tumors, potentially indicative of increased glutamine use, had significantly worse survival than patients with low glutamate levels (5).

Drugs that antagonize glutamine are increasingly being tested against MYC-driven malignancies (6–8). The naturally occurring compound DON (6-diazo-5-oxo-l-norleucine) was tested in adult and pediatric clinical trials, but was never systematically tested against MYC-driven brain tumors (9). To increase DON's applicability to clinical use, we modified DON to improve oral bioavailability and showed that the DON prodrug JHU083 had activity against MYC-amplified medulloblastoma in vitro and in vivo (10). We have subsequently further modified DON to improve brain penetration, and we hypothesized that this new brain-targeted DON prodrug called JHU395 would kill MYC-driven medulloblastoma cells.

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Under a license agreement between Dracen Pharmaceuticals, Inc. and the Johns Hopkins University, Dr Slusher, Dr Rais, and Mr Alt are entitled to royalty distributions related to technology used in the research described in this publication. In addition, Dr Slusher and Dr Rais are founders of and hold equity in Dracen Pharmaceuticals, Inc. This arrangement has been reviewed and approved by the Johns Hopkins University in accordance with its conflict of interest policies.

Supplementary Data can be found at http://academic.oup.com/jnen.

MATERIALS AND METHODS

JHU395

The synthesis of JHU395 prodrug (Dracen Pharmaceuticals, New York, NY), isopropyl 6-diazo-5-oxo-2-(((phenyl (pivaloyloxy) methoxy) - carbonyl) amino) hexanoate (JHU395), was conducted as detailed previously (11). For in vitro and in vivo testing, JHU395 was dissolved in 100% sterile dimethyl sulfoxide (DMSO) (Millipore Sigma, Burlington, MA).

Cell Culture

The patient-derived medulloblastoma cell lines D283MED and D425MED, first established at Duke University, were grown in MEM media (Gibco, Waltham, MA) supplemented with 5% fetal bovine serum (FBS) (Gibco) and 1% non-essential amino acids (NEAA) (Gibco) (12). The MED211 patient-derived xenograft was obtained from the Brain Tumor Resource Lab and has been previously described (10, 13). A cell line was derived from the MED211 PDX model by removing tumor tissue from tumor-bearing mice at the time of death and passing tumor through a P1000 pipette. Cells were grown in epidermal growth factor (EGF) and fibroblast growth factor (FGF) (Peprotech, Rocky Hill, NJ) media. We used lentivirus containing MYC, R248W TP53 or BMI1 to immortalize human neural stem cells, as previously described (14, 15). We subsequently transduced these cells with constitutively active AKT and hTERT creating immortalized cells harboring MYC/DNp53/AKT/hTERT or BMI1/DNp53/AKT/hTERT. Expression of oncogenes was confirmed by Western blot (15). All cells were verified to be mycoplasma-free by PCR testing. Cell line identity testing was performed by the Johns Hopkins Genetic Resources Core Facility and is included in Supplementary Data Figure S1.

Cell Growth Assay

Experiments were performed in 96-well plates. Equal numbers of cells were plated in triplicate for each treatment. 20 µL of CellTiterBlue (Promega, Madison, WI) was added to each well for every 100 µL of media and incubated at 37°C for 3 hours. Absorbance was measured on a plate reader with TECAN read Infinite M1000 PRO on days 0, 3, and 5. Growth between vehicle and drug-treated cells was statistically analyzed on day 5 with 1-way analysis of variance (ANOVA) with Dunnet's multiple comparison test using GraphPad Prism.

Western Blots

Protein was extracted from cell pellets using RIPA buffer (Millipore Sigma) and quantified using a Bradford Assay. Antibody against cleaved-PARP is from Cell Signaling Technologies (no. 9541) (Danvers, MA). Antibody against Beta ACTIN (sc-47778) is from Santa Cruz Biotechnologies (Dallas, TX). The following dilutions were used cleaved-PARP (1:1000), Beta ACTIN (1:1000). Peroxidase-labeled secondary antibodies are from Cell Signaling Technologies and used at a 1:3500 dilution. Bands were quantified using ImageJ.

BrdU and Cleaved Caspase-3 Immunofluorescence

Following 72-hour drug treatment, cells were incubated with bromo-deoxyuridine (BrdU) (Cell Signaling Technologies) for 6 hours and fixed in cytospin fluid and spun onto glass slides using a cytocentrifuge as described previously (16). Cells were permeabilized in 0.1% TritonX, blocked in 5% normal goat serum, and incubated with anti-Brdu and anti-cleaved caspase-3 antibody diluted 1:400 (Cell Signaling Technology, clone 5A1E), followed by a secondary antibody conjugated to Cy3 diluted 1:500 (Jackson ImmunoResearch, West Grove, PA). 4′,6-diamidino-2-phenylindole (DAPI) (Roche Diagnostics, Mannheim, Germany) was used as a counterstain. Five DAPI and the corresponding Cy3 images were taken for each slide. The number of DAPI- and Cy3-positive cells were counted using Imagescope. For each pair of images, the percent of Cy3-positive cells was calculated. The average Cy3 positivity was determined by calculating the average of at least 5 pairs of images for each treatment. Images were blinded before counting. Vehicle and drug-treated cells were compared by Student t-test.

Tumor Cell-to-Plasma Partitioning

D425MED cells grown under standard conditions were centrifuged and washed with Dulbecco's phosphate buffered saline (PBS) (Gibco, Cat. no. 14-190-144) and resuspended in human plasma (Innovative Research, Inc., Novi, MI) to obtain a cell density of 10 million cells/mL of plasma. For analysis of cell partitioning, 1 mL of the cell-plasma suspension was pre-incubated at 37°C for 5 minutes; then, JHU395 was added to a final concentration of 20 µM. Cells were reincubated at 37°C for 1 hour. Following incubation, the cell suspension was centrifuged at 10 000 g for 10 minutes at 4°C and supernatant plasma was collected and stored at −80°C until bioanalysis. The cell pellet was washed once with ice-cold Dulbecco's PBS (Gibco), followed by centrifugation and stored at −80°C for intact JHU395 and DON bioanalysis. Briefly, the calibration curves for quantification of analytes were prepared using naïve D425 medulloblastoma cells or human plasma. Calibration curves were constructed over the range 0.03–100 nmol/mL for DON and 10–20 µmol/mL for JHU395. For extraction of analytes, pellets were weighed and suspended in a known

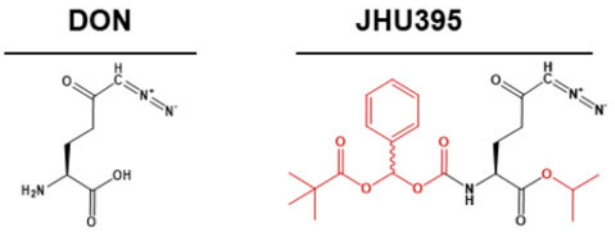

FIGURE 1. Chemical structure of 6-diazo-5-oxo-l-norleucine and (isopropyl 6-diazo-5-oxo-2-(((phenyl (pivaloyloxy) methoxy) - carbonyl) amino) hexanoate).

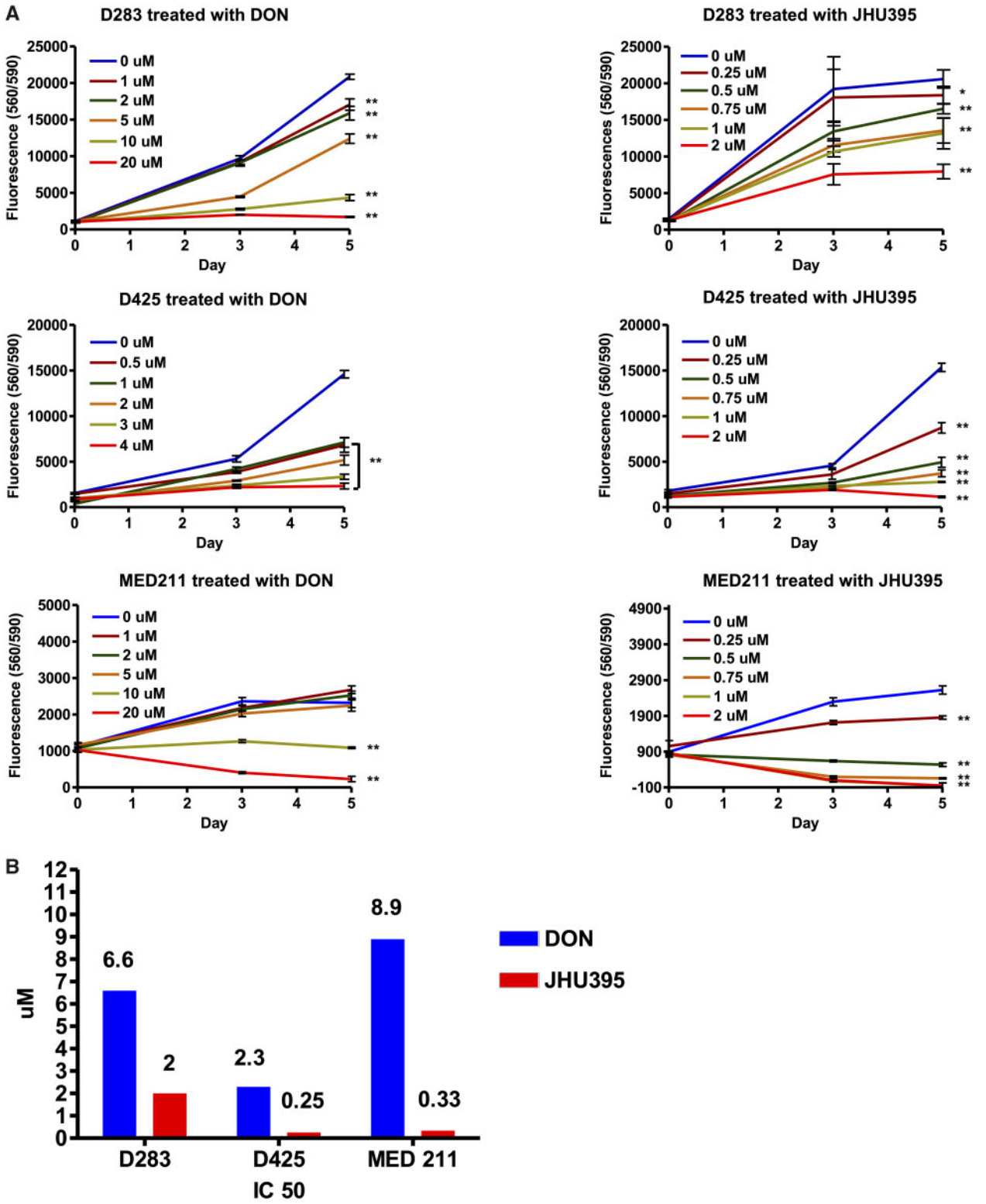

FIGURE 2. Isopropyl 6-diazo-5-oxo-2-(((phenyl (pivaloyloxy) methoxy) - carbonyl) amino) hexanoate (JHU395) decreases growth of multiple high MYC-expressing medulloblastoma cell lines. (**A**) CelltiterBlue growth assays showing JHU395 decreased growth of multiple cell lines expressing MYC at lower concentrations compared to 6-diazo-5-oxo-l-norleucine (DON). Asterisks indicate $p < 0.05$, double asterisks indicate $p < 0.01$ by 1-way ANOVA with Dunnet's multiple comparison test compared with control on day 5. Representative graphs are shown. Each experiment was repeated a minimum of 3 times with similar results. (**B**) IC50 of DON and JHU395 were calculated by GraphPad Prism on different high MYC-expressing medulloblastoma cell lines.

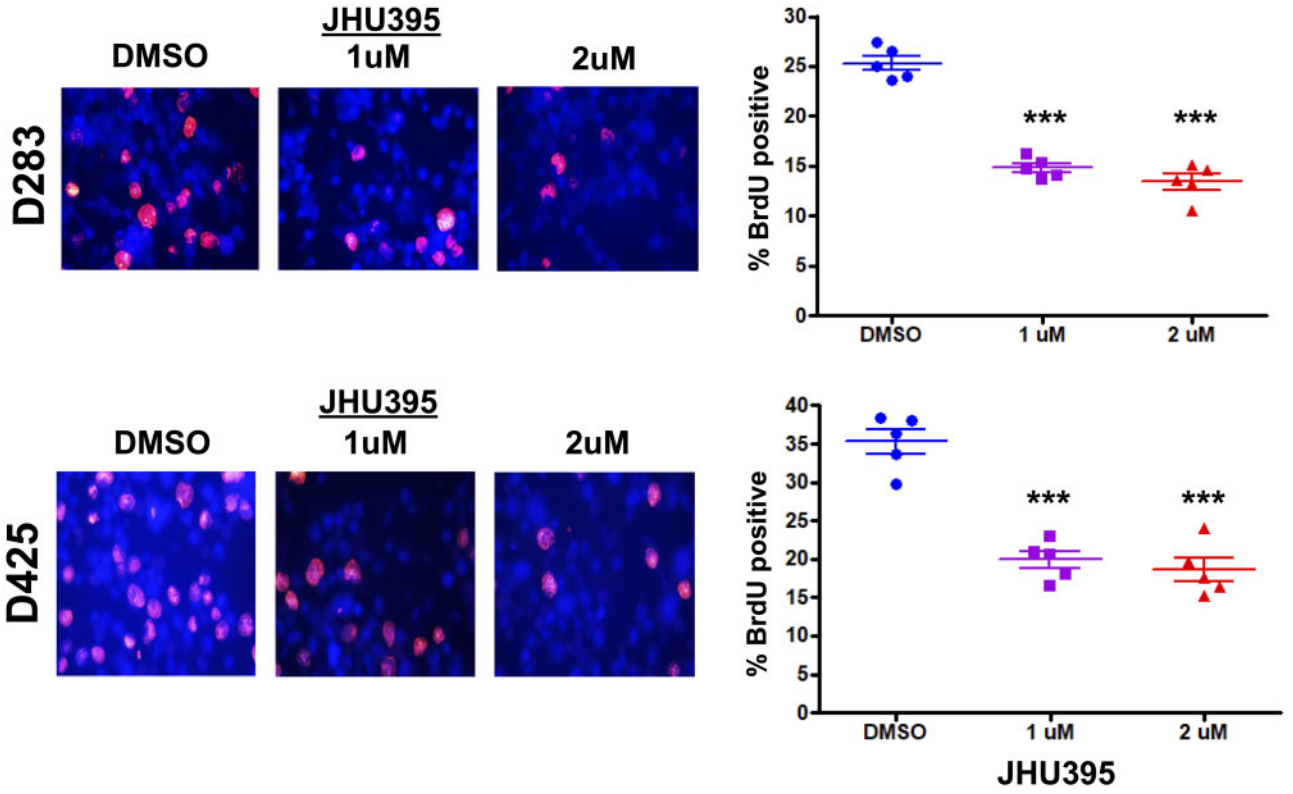

FIGURE 3. Isopropyl 6-diazo-5-oxo-2-(((phenyl (pivaloyloxy) methoxy) - carbonyl) amino) hexanoate (JHU395) decreases cell proliferation. Cells treated with 1 or 2 μM JHU395 for 72 hours show decreased incorporation of BrdU by immunofluorescence. Top: 200× photomicrographs of BrdU immunofluorescence (red) of D283 medulloblastoma cells treated with JHU395 for 72 hours. DAPI counterstains nuclei blue. Bottom: BrdU immunofluorescence of D425 medulloblastoma treated with JHU395 for 72 hours. Graphs at right show quantification. Bars represent range with each replicate result represented by a solid dot. ***$p < 0.001$ by Student t-test ($n = 5$ biological replicates).

quantity of water. To 50 μL of cell suspension or plasma, ice-cold methanol containing internal standards (losartan: 0.5 μM and glutamate-d5: 10 μM) was added in a 5:1 ratio. Samples were vortex-mixed, and centrifuged at 10 000*g* for 10 minutes at 4°C. The supernatant was collected and analyzed for both DON and intact prodrug as previously described (17, 18).

Animal Studies

After induction of general anesthesia with ketamine/xylazine in Nu/Nu mice, a burr hole was made in the skull of female Nu/Nu mice Charles River (Wilmington, MA) 1 mm to the right of and 2 mm posterior to the lambdoid suture with an 18-gauge needle. The needle of a Hamilton syringe was inserted to a depth of 2.5 mm into the cerebellum using a needle guard, and 100,000 D425MED cells in 3 μL of media were injected. JHU395 was prepared DMSO and administered by IP injection on day 10 after surgery with 15 mg/kg in a 100 μL bolus twice weekly on Tuesdays and Fridays. Animals were monitored daily. Symptomatic individuals were killed, brains removed and fixed in formalin. Survival curves were analyzed using a Log-rank test. For the JHU395 brain penetration pharmacokinetic study, athymic nude mice without brain tumors were given a single DON-equivalent dose of 20 mg/kg JHU395 dissolved in DMSO by IP. Exactly 1-hour postdose, mice were killed and the brain regions manually dissected and flash frozen. Dosing of the animals was staggered to ensure each animal was only exposed to the drug for 1 hour. Extraction and quantification of DON was performed as previously described (19). For animal care and anesthesia, "Principles of laboratory animal care" (NIH publication No. 8623, revised 1985) was followed, using a protocol approved by the Johns Hopkins Animal Care and Use Committee, in compliance with the United States Animal Welfare Act regulations and Public Health Service Policy.

RESULTS

Structure of JHU395

To enhance brain penetration of DON, we created a prodrug with increased lipophilicity. Figure 1 shows the structure of this prodrug, JHU395. The intracellular environment converts JHU395 to active DON (11, 17).

DON Prodrug JHU395 Suppresses MYC-Amplified Medulloblastoma Growth

Treatment with JHU395 significantly inhibited the growth of multiple MYC-amplified medulloblastoma cell

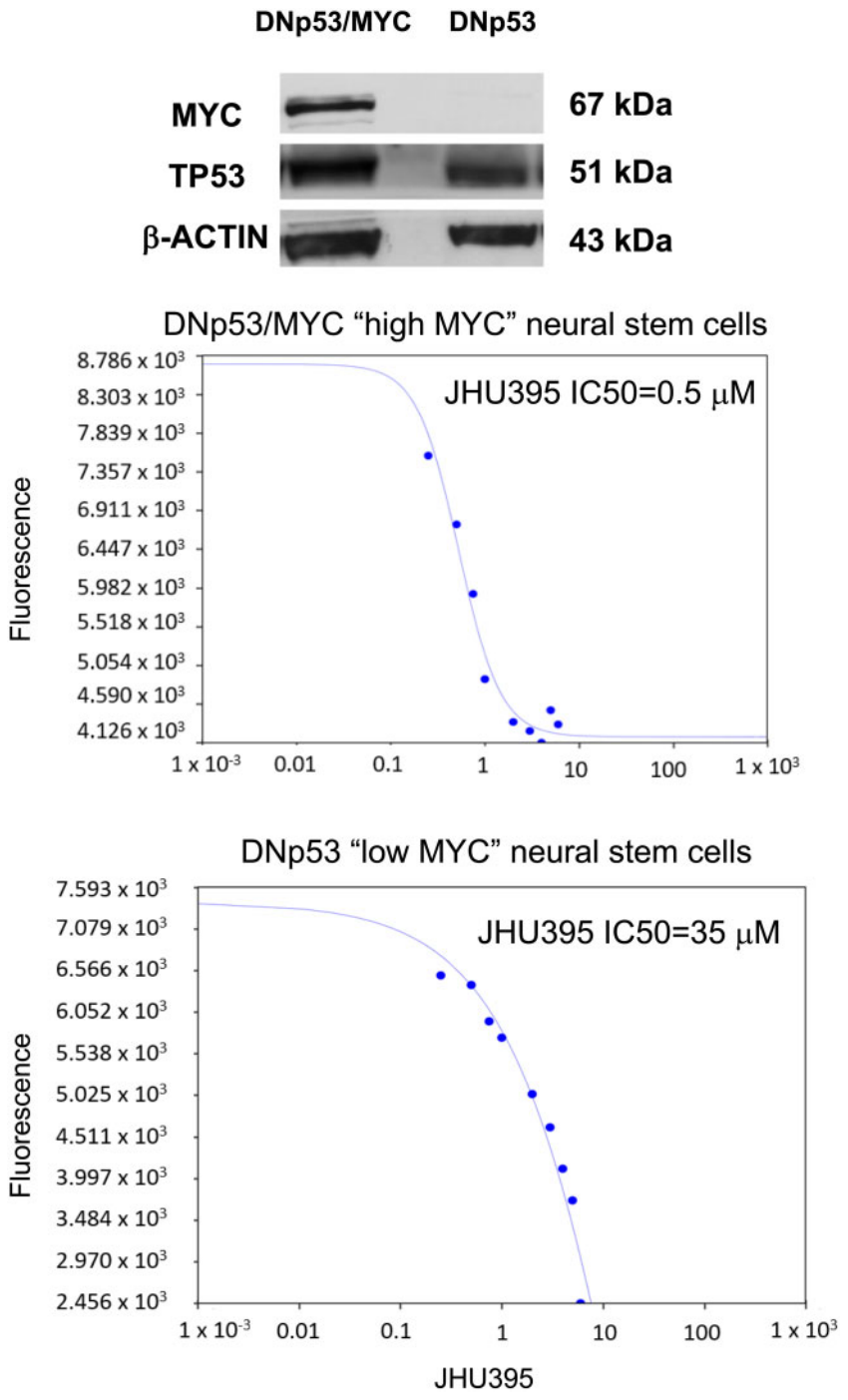

FIGURE 4. MYC transformed cells are more sensitive to isopropyl 6-diazo-5-oxo-2-(((phenyl (pivaloyloxy) methoxy) - carbonyl) amino) hexanoate (JHU395). JHU395 inhibited the growth of neural stem cells transduced with MYC and R248W p53 (high MYC cells) to a greater extent than neural stem cells transduced with BMI1 and R248W p53 (low MYC cells). The IC50 of JHU395 was 0.5 μM for high MYC cells as compared with 35 μM for low MYC cells.

lines at lower concentrations compared with DON, as analyzed on day 5 with 1-way ANOVA with Dunnet's multiple comparison test. In D283MED, DON inhibited growth at 1 μM and above ($p < 0.01$ compared with vehicle control). In contrast, 0.25 and 0.5 μM JHU395 suppressed growth in this cell line ($p < 0.05$ and 0.01 respectively; Fig. 2A). Similar results were seen when we treated D425MED cells with DON, with suppression of growth at 0.5 μM ($p < 0.01$) while JHU395 suppressed growth starting at 0.25 μM ($p < 0.01$). In MED211, DON inhibited growth at 10 μM and above ($p < 0.01$), while JHU395 suppressed growth starting at 0.25 μM ($p < 0.01$).

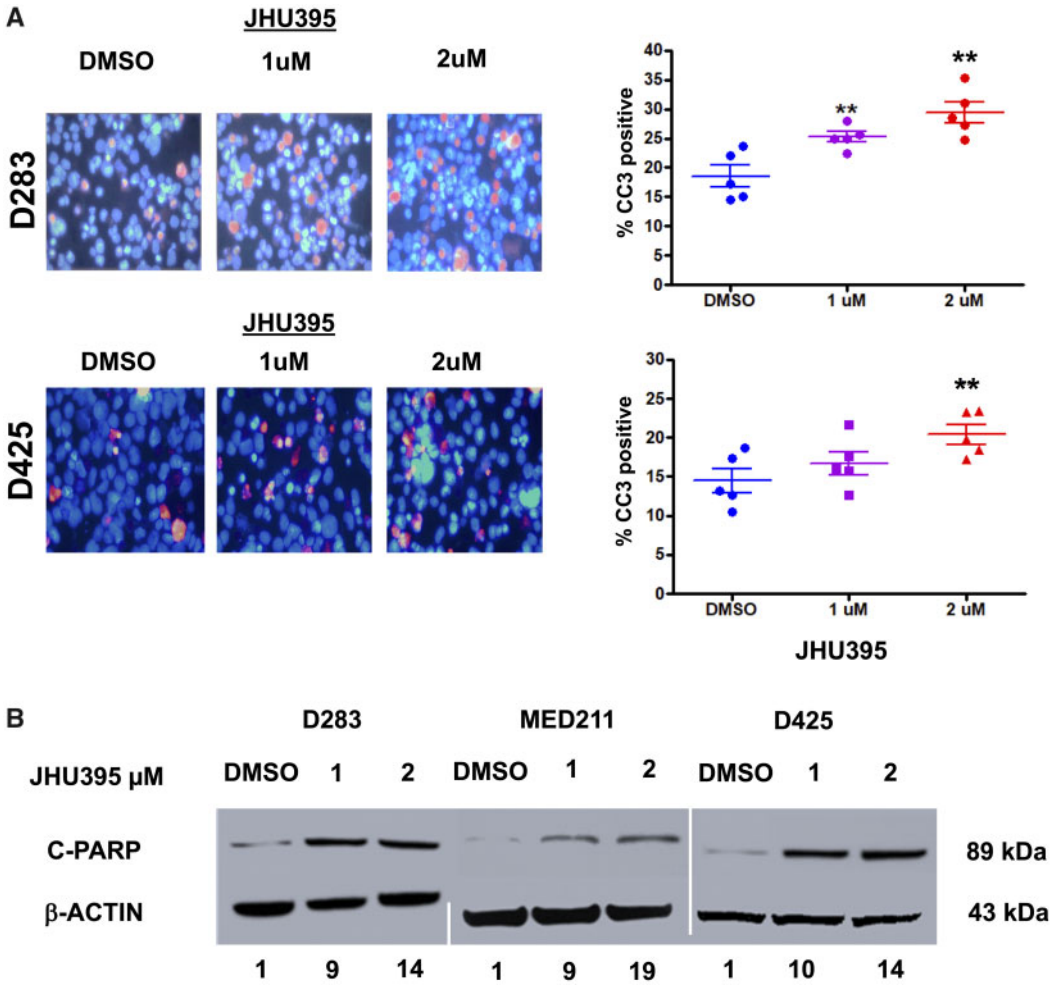

FIGURE 5. Isopropyl 6-diazo-5-oxo-2-(((phenyl (pivaloyloxy) methoxy) - carbonyl) amino) hexanoate (JHU395) treatment induces cell death by apoptosis. (**A**) 200× magnification photomicrographs of cleaved caspase-3 immunofluorescence of medulloblastoma cells treated for 72 hours with 1 and 2 μM JHU395 or DMSO control. DAPI counterstains nuclei blue. Quantification of results is represented in graphs at right. $^{**}p < 0.01$, $^{***}p < 0.001$ by Student t-test compared with vehicle control ($n = 5$ biological replicates). Bars represent range with each replicate result represented by a solid dot. (**B**) Treatment with 1 and 2 μM JHU395 causes increased cleaved-PARP expression as determined by Western blot. Numbers below the blot indicate fold-increase of cleaved-PARP expression compared with vehicle control. Cleaved-PARP expression was normalized to Actin.

Half-maximal inhibitory concentrations (IC50) values were calculated for all 3 MYC-amplified cell lines at day 5 of treatment. For D283MED, the IC50 of DON was 6.6 μM versus 2 μM for JHU395. In D425MED, the IC50 of DON was 2.3 μM while the IC50 of JHU395 was 0.25 μM. In MED211, the IC50 of DON was 8.9 μM and the IC50 of JHU395 was 0.33 μM (Fig. 2B).

Cells treated with JHU395 at 1 and 2 μM for 72 hours showed significantly decreased BrdU expression as measured by immunofluorescence staining, statistically analyzed by Student t-test compared with vehicle control. In D283MED, BrdU positivity decreased from 25% in vehicle-treated cells to 14.8% and 13.3% when treated with 1 and 2 μM JHU395, respectively ($p < 0.01$). Similar results were seen in D425MED, in which vehicle-treated cells were 35.2% BrdU-positive while treatment with 1 and 2 μM JHU395 led to 19.8% and 18.5% BrdU positivity, respectively ($p < 0.01$; Fig. 3).

Because MYC controls many aspects of glutamine metabolism, we hypothesized that JHU395 would have greater effect on high-MYC cells compared with low-MYC cells (1). JHU395 inhibited the growth of neural stem cells transduced with MYC and R248W p53 (high MYC cells) to a greater extent than neural stem cells transduced with BMI1 and R248W p53 (low MYC cells). The IC50 of JHU395 was 0.5 μM for high MYC cells as compared with 35 μM for low MYC cells (Fig. 4).

JHU395 Treatment Increases Apoptosis in MYC-Expressing Medulloblastoma Cell Lines

JHU395 treatment of medulloblastoma cell lines for 72 hours caused an increase in the expression of cleaved caspase-3 (CC3) as measured by immunofluorescence, as analyzed by Student t-test compared with vehicle control

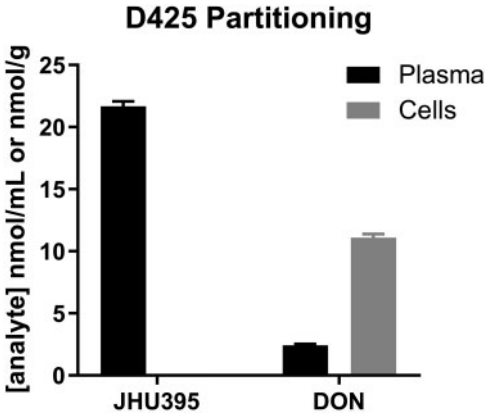

FIGURE 6. Tumor cell-to-plasma partitioning. Intact Isopropyl 6-diazo-5-oxo-2-(((phenyl (pivaloyloxy) methoxy) - carbonyl) amino) hexanoate (JHU395) and 6-diazo-5-oxo-l-norleucine (DON) quantification (nmol/mL plasma or nmol/g cell) in D425 medulloblastoma cells following 1-hour incubation with 20 μmol/L JHU395. After 1-hour incubation with JHU395 in D425 medulloblastoma cells, the intact prodrug was detected in plasma (21 nmol/L) with minimal amount in cells (0.1 nmol/g). DON release from JHU395 was higher in cells (11 nmol/g) versus plasma (2.5 nmol/L) with cell- to-plasma ratio of ~4.5.

(Fig. 5A). In D283MED, CC3 expression increased to 25.2% and 29.4% when treated with 1 and 2 μM JHU395, respectively, compared with 18.5% in vehicle ($p < 0.01$). Similar patterns were seen in D425MED. When treated with 1 and 2 μM JHU395, CC3 expression increased to 16.8% and 20.4%, respectively, compared with 14.4% in vehicle control ($p < 0.01$). JHU395 treatment also led to increased apoptotic cell death as measured by cleaved-PARP Western blot. We detected a 9- to-19-fold increase in cPARP as measured by Western blot with JHU395 treatment compared with vehicle control (Fig. 5B).

To understand JHU395 prodrug delivery of DON into high-MYC medulloblastoma cells, we investigated the partitioning of JHU395 into D425MED cells incubated in human plasma as previously described (17). After 1-hour incubation with 20 μM JHU395 in D425MED cells in human plasma, the intact prodrug was detected primarily in plasma (21 nmol/mL), and there was a minimal amount in cells (0.1 nmol/g). In contrast, DON release from JHU395 was higher in cells (11 nmol/g) versus plasma (2.5 nmol/L) with cell-to-plasma ratio of ~4.5 (Fig. 6).

Systemic Administration of JHU395 Achieved Micromolar Concentrations of DON in the Brain

To determine plasma/brain ratio of DON delivered by JHU395, we administered JHU395 intraperitoneally to Nu/Nu mice. After 1 hour, the animals were killed and the brains removed. Intact prodrug and DON were measured by LC/MS-MS as we have previously described (11, 18). IP dosing resulted in an average 0.66 brain-to-plasma ratio of DON (Fig. 7A). We measured 4 μM concentration of DON in the brains of mice that received a dose of 5 mg/kg JHU-395. A higher 20 mg/kg IP dose led to an average concentration of 11.3 μM of DON in the brain.

Because 2 μM JHU395 successfully reduced growth of MYC-expressing medulloblastoma cell lines (Figs. 2 and 3) and induced apoptosis (Fig. 4), we hypothesized that a 15 mg/kg IP dose of JHU395 would be effective in treating orthotropic human medulloblastoma xenografts in mice with an intermittent dosing schedule.

JHU395 Induces Apoptosis in MYC-Driven Medulloblastoma Orthotopic Xenografts

To determine if JHU395 could also induce apoptosis in vivo in high-MYC medulloblastoma, we treated mice bearing orthotopic D425 MED tumors with a single dose of JHU395 (15 mg/kg) or DMSO and killed mice 72 hours later. We extracted protein from tumor-involved cerebellum as well as the uninvolved contralateral cerebellum. Western blot for cPARP showed a single dose of JHU395 induced apoptosis in high MYC-amplified medulloblastoma but not in normal control cerebellum (Fig. 7B).

JHU395 Extends the Survival of Animals with MYC-Driven Medulloblastoma Orthotopic Xenografts

Nude mice treated with 15 mg/kg twice weekly of JHU395 (15 mg/kg twice weekly) had a median survival 45 days compared with 24 days for vehicle-control-treated mice ($p < 0.001$ by Log-rank test; Fig. 7C). Mouse weights were stable during the treatment course. At week 3 of the study, median mouse weight was reduced around 10% in the JHU-395 group, but the mice then gained back the lost weight after 1 week (Fig. 7D). Both groups lost weight when symptomatic from eventual tumor progression, leading to death.

DISCUSSION

The abnormal expression of MYC occurs in many types of cancers (20). MYC drives growth by promoting signal transduction pathways and upregulating the metabolism of both glucose and the amino acid glutamine to accumulate biomass. Glutamine is a nonessential amino acid and is the primary nitrogen source for synthesis of nucleic acids, other amino acids, and hexosamines. Glutamine can also be used to replenish TCA cycle intermediates in an anaplerosis reaction (21, 22). One of the most well-established roles of MYC in cancers is upregulating glutamine metabolism, which occurs across many types of cancers (23–26). This makes cancer cells reliant on glutamine to grow and proliferate and renders them vulnerable to glutamine depletion in vitro (27). We found that the DON prodrug JHU395 killed MYC-driven medulloblastoma at concentrations 450-fold lower (0.25–2 vs 900 μM) than what was recently reported for DON against the non-MYC medulloblastoma cell lines UW228 and DAOY (28), suggesting that the presence of MYC may render cells more sensitive to glutamine inhibitors. We further extended this finding to show that human neural stem cells transduced with MYC and mutant p53 were more sensitive to JHU395 than hu-

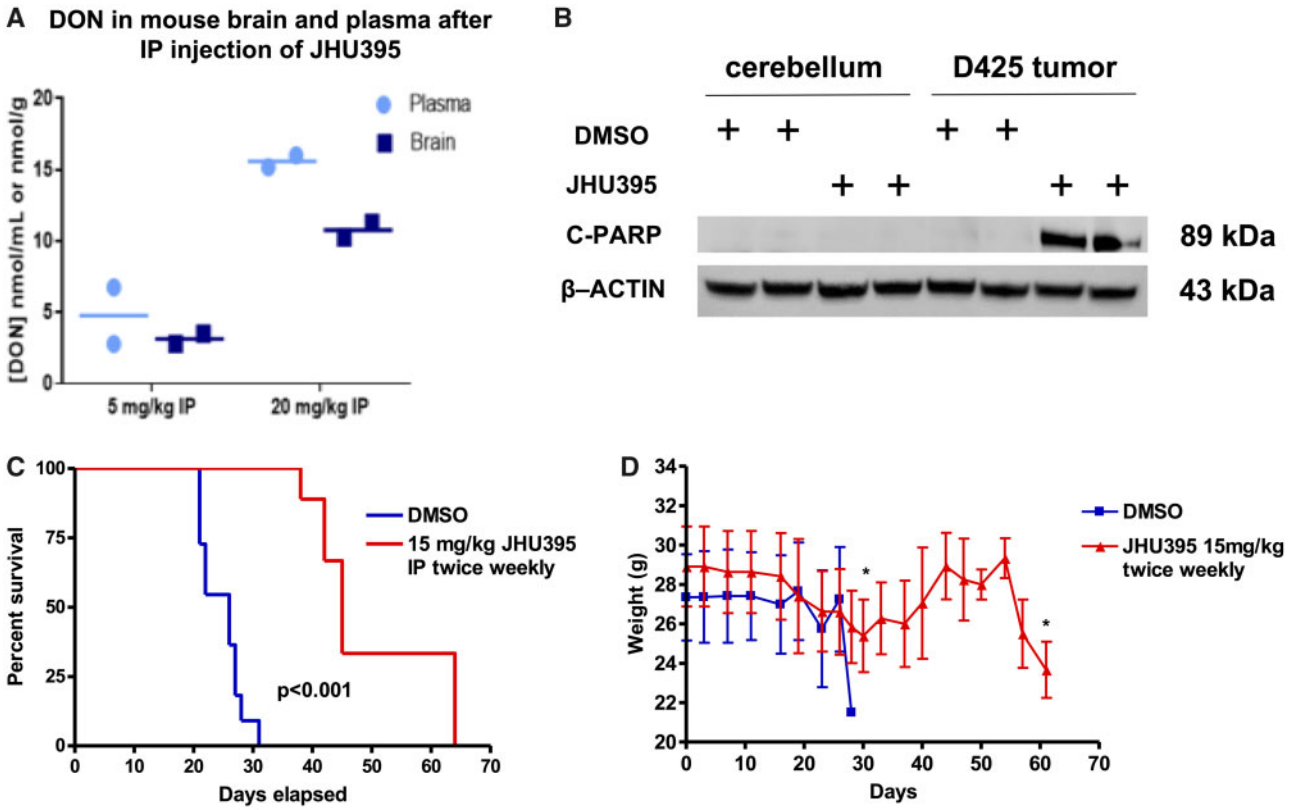

FIGURE 7. Isopropyl 6-diazo-5-oxo-2-(((phenyl (pivaloyloxy) methoxy) - carbonyl) amino) hexanoate (JHU395) administration leads to micromolar levels of 6-diazo-5-oxo-l-norleucine (DON) in brain as measured by mass spectrometry with resulting increase in survival of mice bearing medulloblastoma orthotopic xenografts. (**A**) Parenteral administration of JHU395 dosing at 5 or 20 mg/kg in Nu/Nu mice led to the accumulation of micromolar concentrations of DON in brain as measured by mass spectrometry. IP dosing resulted in an average 0.66 brain-to-plasma ratio. The brain had an average concentration of 11.3 nmol/ml of DON after 20 mg/kg JHU395 intraperitoneal administration. (**B**) Single treatment of JHU395 after 72 hours showed significant effect in inducing apoptotic cells death specifically on D425MED but not on normal cerebellum. (**C**) Twice weekly 15 mg/kg dosing of JHU395 significantly extended the survival of athymic nude mice with D425MED tumors. Red line, JHU-395; blue line, vehicle. $p < 0.001$ comparing treated vs vehicle control as determined by Log-rank test $n = 10$ animals in the control group and $n = 9$ animals in the treatment group). (**D**) Representative graph shows the mouse weights were stable during the treatment course. At week 3 of the study, mean mouse weight reduced around 10% but then subsequently recovered back to baseline. Both control and treatment mice lost weight in the few days immediately before death due to progression of tumor.

man neural stem cells transduced with BMI1 and mutant p53 (Fig. 4).

MYC-amplified medulloblastoma is highly resistant to our current therapy, with <50% of patients having long-term survival (29). New therapies are desperately needed. Targeting abnormal metabolism downstream of MYC may disrupt key metabolic pathways in MYC-driven medulloblastoma that may provide a significant therapeutic index. We showed that a single dose of JHU395 induces apoptosis as measured by cPARP in D425MED orthotopic xenografts but does not induce apoptosis in normal brain from the same animal, highlighting the on-target effects of JHU395 on tumor cells in vivo (Fig. 7B).

We here demonstrate that a novel prodrug of DON has increased potency against MYC-amplified medulloblastoma and suppresses the growth of MYC-amplified medulloblastoma orthotopic xenografts. We find that JHU395 has higher potency compared with DON in suppressing and killing MYC-driven medulloblastoma. We have previously noted the efficacy of another DON prodrug, JHU083, in high-MYC medulloblastoma (10). JHU395 differs from JHU083 in that it is significantly more hydrophobic, which leads to improved penetration into cells and into the brain (11).

The increased potency of JHU-395 against MYC-amplified medulloblastoma compared with DON suggests that lower doses may be efficacious in the clinic. Although DON was generally well tolerated in pediatric early clinical trials (9), gastrointestinal toxicity was a dose-limiting finding in adult clinical trials (30). The brain penetration of DON in humans is unclear, so engineering prodrugs that can deliver DON efficiently to the brain and limit GI exposure may enhance applicability to the clinic. We anticipate that on-going engineering of glutamine antagonist prodrugs will enhance brain penetration and reduce systemic exposure, thereby further broadening the therapeutic index.

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