Brain Distribution and Efficacy of the Brain Penetrant PI3K Inhibitor GDC-0084 in Orthotopic Mouse Models of Human Glioblastoma


Departments of Drug Metabolism and Pharmacokinetics (LS, SSL, JC, JP, EGP, CQ, XZ), Discovery Chemistry (TPH, AGO), Cancer Signaling and Translational Oncology (BA, MN, MNP, LBL, SEG, HSP), Biomedical Imaging (TC, RAC, JG) and Pathology (HK, SL, LR), Genentech Inc, South San Francisco, CA, 94080, USA.
Running Title: GDC-0084 Brain Penetrant PI3K Inhibitor Against GBM

Corresponding author:
Laurent Salphati, Pharm.D., Ph.D.
Genentech, Inc.
1 DNA Way, South San Francisco, CA 94080
Phone: 650-467-1796
Fax: 650-467-3487
Email: salphati.laurent@gene.com

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Abbreviations: BCRP, Breast cancer resistance protein; GBM, glioblastoma multiforme; GDC-0084, 5-(6,6-dimethyl-4-morpholino-8,9-dihydro-6H-[1,4]oxazino[4,3-e]purin-2-yl)pyrimidin-2-amine; MALDI, matrix-assisted laser desorption ionization; Pictilisib (GDC-0941; 2-(1H-Indazol-4-yl)-6-(4-methanesulfonyl-piperazin-1-ylmethyl)-4-morpholin-4-yl-thieno[3,2-d]pyrimidine; PI3K, Phosphatidylinositol 3-kinase; PO, oral; MCT, 0.5% methylcellulose/0.2%Tween 80; P-gp, P-glycoprotein.
Abstract

Glioblastoma (GBM) is the most common primary brain tumor in adults. Limited treatment options have only marginally impacted patient survival over the past decades. The PI3K pathway, frequently altered in GBM, represents a potential target for the treatment of this glioma. GDC-0084 is a PI3K inhibitor that was specifically optimized to cross the blood-brain barrier. The goals of our studies were to characterize the brain distribution, pharmacodynamic (PD) effect and efficacy of GDC-0084 in orthotopic xenograft models of GBM. GDC-0084 was tested in vitro to assess its sensitivity to the efflux transporters P-glycoprotein (P-gp) and breast cancer resistance protein (BCRP) and in vivo in mice to evaluate its effects on the PI3K pathway in intact brain. Mice bearing U87 or GS2 intracranial tumors were treated with GDC-0084 to assess its brain distribution by MALDI imaging and measure its PD effects and efficacy in GBM orthotopic models. Studies in transfected cells indicated that GDC-0084 was not a substrate of P-gp or BCRP. GDC-0084 markedly inhibited the PI3K pathway in mouse brain, causing up to 90% suppression of the pAkt signal. MALDI imaging showed GDC-0084 distributed evenly in brain and intracranial U87 and GS2 tumors. GDC-0084 achieved significant tumor growth inhibition of 70% and 40% against the U87 and GS2 orthotopic models, respectively. GDC-0084 distribution throughout the brain and intracranial tumors led to potent inhibition of the PI3K pathway. Its efficacy in orthotopic models of GBM suggests that it could be effective in the treatment of GBM. GDC-0084 is currently in Phase I clinical trials.
Introduction

Glioblastoma (GBM) is the most frequently diagnosed primary brain tumor in adults, with more than 10,000 patients affected each year in the US (CBTRUS, 2015). Characterized by its invasiveness and aggressive progression, GBM, once diagnosed, presents a median survival of less than 2 years (Adamson et al., 2009). Treatment options have remained limited with few advances or success in the past decades (Levin et al., 2015), and despite an aggressive standard therapy that includes surgery, radiation and temozolomide, with the recent addition of bevacizumab at recurrence (Stupp et al., 2005; Friedman et al., 2009; Kreisl et al., 2009), only 5% of patients survive longer than 5 years. Molecular as well as regional and micro-environmental heterogeneity of GBM can be invoked to explain this lack of improvement (Phillips et al., 2006; Cloughesy et al., 2014). However, often overlooked is the significant challenge represented by the blood-brain barrier (BBB), constituted by tightly joined endothelial cells and efflux transporters such as P-glycoprotein (P-gp) and breast cancer resistance protein (BCRP) (de Vries et al., 2006). These transporters further reduce the permeability and delivery of drugs to the brain and prevent them from reaching therapeutic concentrations. While disruption of the BBB often occurs in GBM (Oberoi et al., 2016), and delivery of drugs to the tumor core has been shown (Hofer and Frei, 2007; Holdhoff et al., 2010), infiltrative regions of the tumor present an intact BBB and can only be reached by drugs able to cross the BBB and bypass efflux transporters.

Recent reports by TCGA Research Network (Brennan et al., 2013) have identified major signaling pathways dysregulated in GBM. Among those, the PI3K pathway emerged as one of the most frequently altered, with mutation, amplification or loss of key signaling proteins such as EGFR, PTEN or PI3K, detected in more than 80% of GBM. Thus, this pathway represents a
compelling target for the treatment of high grade glioma. However, most, if not all, drugs acting on the PI3K pathway that have been tested, and have achieved disappointing results (Agarwal et al., 2011b), are substrates of P-gp and/or BCRP, the two main efflux transporters expressed at the BBB. It is then expected that these compounds, including erlotinib (de Vries et al., 2012), everolimus (Chu et al., 2009), gefitinib (Agarwal et al., 2010) or lapatinib (Polli et al., 2009), will not be able to cross the BBB and are unlikely to maintain uniform and adequate concentrations throughout the brain.

GDC-0084 (Fig. 1A) is a selective inhibitor of PI3K ($\alpha$Ki 2 nM) also able to potently inhibit mTOR (K$_i$ 70 nM) (Heffron et al., 2016). This compound, possessing physicochemical properties optimized for brain penetration, was specifically developed as a potential treatment for GBM and is currently being evaluated in Phase I in patients with high grade glioma. GDC-0084 was shown to inhibit the proliferation of several glioma cells in vitro with IC$_{50}$ ranging from 0.3 to 1.1 µM (U87: 0.74 µM, (Heffron et al., 2016); GS2: 0.61 µM, unpublished data). In addition, this compound was also able to inhibit the phosphorylation of Akt in U87 human glioma implanted as a subcutaneous xenograft in nude mice, leading to significant tumor growth inhibition (Heffron et al., 2016).

The goals of the studies presented here were to characterize the PI3K pathway modulation and the efficacy of GDC-0084 in orthotopic models of human GBM and to investigate the brain and tumor distribution of GDC-0084 in these intracranial tumor models.
Materials and Methods

Chemicals
All Genentech compounds (>99% pure), including GDC-0084, [D6]GDC-0084 and pictilisib, were synthesized by Genentech, Inc. (South San Francisco, CA). All solvents used in analytical assays were purchased from Thermo Fisher Scientific (Watham, MA) and were of analytical or high-performance liquid chromatography grade. All other chemicals and reagents were purchased from Sigma-Aldrich (St Louis, MO) unless specified.

In Vitro Studies

Transport Assays in Cell Monolayers
Madin-Darby canine kidney (MDCK) cells expressing human P-gp, human BCRP or mouse Bcrp1 and LLC-PK1 cells transfected with mouse P-gp (mdr1a) were used to determine whether GDC-0084 was a substrate of these transporters. MDR1-MDCKI cells were licensed from the NCI (National Cancer Institute, Bethesda, MD) and Bcrp1-MDCKII, BCRP-MDCKII and Mdr1a-LLC-PK1 cells were obtained from the Netherlands Cancer Institute (Amsterdam, The Netherlands). For transport studies, cells were seeded on 24-well Millicell plates (Millipore, Billerca, MA) 4 days prior to use (polyethylene terephthalate membrane, 1 µm pore size) at a seeding density of 2.5x10^5 cells/mL (except for MDR1-MDCKI, 1.3x10^5 cells/mL). GDC-0084 was tested at 5 µM in the apical to basolateral (A-B) and basolateral to apical (B-A) directions. The compound was dissolved in transport buffer consisting of Hank’s balanced salt solution (HBSS) with 10 mM HEPES (Invitrogen Corporation, Grand Island, NY). Lucifer Yellow (Sigma-Aldrich, St. Louis, MO) was used as the paracellular and monolayer integrity marker. GDC-0084 concentrations in the donor and receiving compartments were determined by LC-
MS/MS analysis. The apparent permeability ($P_{\text{app}}$), in the apical to A-B and B-A directions, was calculated after a 2-hour incubation as:

$$P_{\text{app}} \ (10^{-6} \text{ cm/sec}) = \frac{\text{d}Q/\text{d}t}{A} \cdot \frac{1}{C_0}$$

Where: $\text{d}Q/\text{d}t = \text{rate of compound appearance in the receiver compartment} \ (\text{fmol/sec})$; $A \ (\text{cm}^2) = \text{Surface area of the insert}$; $C_0 \ (\mu\text{M}) = \text{Initial substrate concentration at T0}$.

The efflux ratio (ER) was calculated as ($P_{\text{app}, \text{B-A}}/P_{\text{app}, \text{A-B}}$).

**Determination of Plasma Protein and Brain Binding**

GDC-0084 protein binding was determined *in vitro*, in mouse plasma (Bioreclamation, Inc., Hicksville, NY) by equilibrium dialysis using a RED device (Thermo Scientific, Rockford, IL) as described previously (Salphati et al., 2012). Incubations were performed in triplicate. Parameters are presented as mean ± standard deviation (SD).

The binding of GDC-0084 to mouse brain was determined as described by Kalvass et al. (Kalvass et al., 2007). Brain tissue was prepared and dialyzed as described previously (Salphati et al., 2012). Following dialysis, tissues and buffer samples were analyzed as outlined for the plasma protein binding studies.

**In vivo Studies**

All studies performed were approved by the Institutional Animal Care and Use Committee at Genentech, Inc. (South San Francisco, CA) and are described in Table 1.
Female CD-1 mice (Charles River Laboratories, Hollister, CA) received a 25 mg/kg oral (PO) dose of GDC-0084 in 0.5% methylcellulose/0.2% Tween 80 (MCT). Two blood samples (0.15 mL each) were collected from 3 mice per timepoint by retro-orbital bleed or terminal cardiac puncture while the animals were anesthetized with isoflurane. Blood samples were collected (with K2EDTA as anticoagulant) predose and at 0.083, 0.25, 0.5, 1, 3, 6, 9 and 24 hours post-dose. Samples were centrifuged within 1 hour of collection and plasma was separated and stored at -80ºC until analysis. Total concentrations of GDC-0084 were determined by a non-validated LC-MS/MS assay. Back-calculated concentrations of calibration curve (accuracy of calibration standards) were within 20% of the theoretical values. The linearity of the calibration curve was assessed and deemed acceptable if R² was greater than 0.98. Following plasma protein precipitation with acetonitrile, the supernatant was injected onto the column, a Phenomenex Kinetex C18 column (50 × 2 mm, 2.6 μm particle size). A CTC HTS PAL autosampler (LEAP Technologies, Chapel Hill, NC) linked to Thermo Accela UPLC pumps, coupled with an AB Sciex API 5500 Qtrap mass spectrometer (AB Sciex, Foster City, CA) were used for the LC-MS/MS assay. The aqueous mobile phase was water with 0.1% formic acid and the organic mobile phase was acetonitrile with 0.1% formic acid. The lower and upper limits of quantitation of the assay were 1 ng/ml and 20000 ng/ml, respectively. The total run time was 1.5 min and the ionization was conducted in the positive ion mode. Brains were collected at 1 and 6 h post-dose from 3 different animals at each time point, rinsed with ice-cold saline, weighed and stored at -80ºC until analysis. For GDC-0084 quantitation, mouse brains were homogenized in 3 volumes of water. The homogenates were extracted by protein precipitation with acetonitrile. LC-MS/MS analysis was conducted as described for the plasma. The lower and upper limits of quantitation of the brain homogenate assay were 5 ng/ml and 11000 ng/ml, respectively. Brain homogenate
concentrations were converted to brain concentrations for the calculations of brain-to-plasma ratios.

**Modulation of pAkt and pS6 in Brain**

Female CD-1 mice were dosed PO with GDC-0084 at 25 mg/kg. Brains and plasma were collected at 1 and 6 hours post-dose, from 3 animals at each time point. Each brain was split in half for PD analysis and GDC-0084 concentration measurement. The samples were stored at -80°C and analyzed for GDC-0084 total concentration. For PD analysis, cell extraction buffer (Invitrogen, Camarillo, CA) containing 10 mM Tris pH 7.4, 100 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM NaF, 20 mM Na₃P₂O₇, 2 mM Na₃VO₄, 1% Triton X-100, 10% glycerol, 0.1% SDS, and 0.5% deoxycholate was supplemented with phosphatase, protease inhibitors (Sigma, St. Louis, MO) and 1mM PMSF and added to frozen brain biopsies. Brains were homogenized, sonicated on ice, and centrifuged at 20,000 g for 20 minutes at 4°C. Protein concentration was determined using BCA protein assay (Pierce, Rockford, IL). Proteins were separated by electrophoresis and transferred to NuPage nitrocellulose membranes (Invitrogen, Camarillo, CA). Licor Odyssey Infrared detection system (Licor, Lincoln, NE) was used to assess and quantify protein expression. PI3K pathway markers were evaluated by immunoblotting using antibodies against pAkt⁰⁴⁷³, total Akt, pS6⁰²³⁵/²³⁶ and total S6 (Cell Signaling, Danvers, MA). These antibodies cross-react between the human and the mouse proteins, allowing detection of pathway markers in mouse brain as well as in the intracranially implanted human tumors. The differences in marker levels between the treated and control mice were evaluated using the Student’s t-test (Prism 5, GraphPad).
Brain and Tumor Distribution by MALDI Imaging

Six female CD-1 Nude mice (Charles River Laboratories) were implanted with either the U-87 MG/M (U87) glioblastoma cancer cells (a Genentech variant of U-87 MG cells from American Type Culture Collection (Manassas, VA)) or GS2 tumor cells (Gunther et al., 2008), injected via stereotactic surgery into the right striatum in a volume of 3 to 5 µL (250K U87 cells and 100K GS2 cells). A single oral dose of 15 mg/kg GDC-0084 was administered 19 to 21 days post-implantation. The formulation was 0.5% methylcellulose/0.2% Tween 80 (MCT). Mice were euthanized at 1 and 6 hours post-dose via exsanguination by perfusion under anesthesia. Brains were excised, flash frozen in liquid N2 and stored in a -80°C freezer until analyzed. Fresh frozen tissue sections were obtained on a cryomicrotome (Leica CM3050S, Buffalo Grove, IL) at 12 µm thickness and thaw-mounted onto indium tin oxide coated glass slides (Bruker Daltonics, Billerica, MA). Tissue sections were analyzed by imaging MALDI MS, providing signal intensities (and not absolute quantitation), followed by cresyl violet staining for histological interrogation.

Imaging MALDI Mass Spectrometry

A 40 mg/mL solution of 2,5-dihydroxybenzoic acid (Sigma-Aldrich, St. Louis, MO) was prepared in methanol:water (70:30 v/v). A stable-labeled internal standard, [D6]GDC-0084, was spiked into the MALDI matrix solution at 2 µM prior to deposition onto the tissue sections. Matrix solution was homogenously spray-coated onto the tissue using a HTX TM-Sprayer (HTX technologies, Chapel Hill, NC). Matrix-coated tissue sections were transferred to the MALDI mass spectrometer (SolariX 7T FT-ICR, Bruker Daltonics, Bremen, Germany) for imaging analysis. Imaging data were collected at 100 µm pixel resolution in positive ionization mode,
under continuous accumulation of selected ions (CASI) windows optimized for a 50 Da window centered on \( m/z \) 383 (\( m/z \) 358-408). Laser intensity and number of shots were optimized for sensitivity of the parent drug (1200 shots) with ion detection collected over the mass range of \( m/z \) 150-3000. Drug images were generated based on accurate mass of the parent drug (GDC-0084 \( m/z \) 383.1938) using FlexImaging v4.0 64-bit (Bruker Daltonics, Billerica, MA) with a mass tolerance of ± 2 mDa and normalized to internal standard response.

Histology

Following completion of the imaging experiments, matrix coating was removed by rinsing the glass slide in 100% methanol for 30 seconds or until the entire matrix was visibly removed. Tissue sections were stained utilizing a freshly prepared 0.5% cresyl violet staining solution (Chaurand et al., 2004) by submerging the glass slide for 30 seconds, then rinsed for an additional 30 seconds in two cycles of 100% ethanol. Microscope images were obtained on an Olympus BX51 (Tokyo, Japan) at 10x magnification and stitched using MicroSuite Analytical v3.0 software (Olympus, Tokyo, Japan). Subsequently, stained images were co-registered to the optical images in FlexImaging for visualization and annotation of tumor and non-tumor regions for the drug images.

Intra-Tumor and Healthy Brain Distribution

To assess drug distribution, imaging MALDI MS data from the U87 and GS2 tumor models were coregistered to the cresyl violet stained microscope images in FlexImaging. Regions of interest (ROIs) were selected based on the anatomical features defined in the histological image, including tumor and non-tumor regions. Drug intensity for each pixel within the defined ROI
was extracted and exported. Drug intensities were binned in 0.1 increments over a range of 0.0 to 2.0. Histogram plots were created in GraphPad Prism 5 to visualize the distribution of pixel intensity frequencies.

**Efficacy Studies in Brain Tumor Models**

The U-87 MG/M (U87) glioblastoma cancer cells (a Genentech variant of U-87 MG cells from American Type Culture Collection (Manassas, VA) and the GS 2 (GS2) glioblastoma cells (Gunther et al., 2008) were selected to test the efficacy of GDC-0084. These two models are PTEN-deficient, with the GS2 cell line having a copy number loss at the PTEN locus (Gunther et al., 2008), and no detectable PTEN protein by western blot. (Carlson et al., 2011) This glioblastoma stem-like (GS) cell line, derived from an adult patient, long term survivor who had been subjected to two surgeries, radiation and chemotherapy, presents mutations in the TP53 gene, no amplification of the EGFR gene and forms spheres that grow semi-adherently *in vitro* (Gunther et al., 2008). The identity of the two cell lines was confirmed by STR profiling (DNA Diagnostics Center) using cells within 5 passages of those utilized for *in vivo* studies. The U87 (250K) and GS2 (100K) tumor cells were injected via stereotaxic surgery into the right striatum in a volume of 3-5 µl. For each experiment, mice were randomized into groups of 10 to obtain comparable mean tumor volumes between treatment and control groups for each model. Treatments were initiated at a time that ensured that the BBB had recovered from surgical disruption and tumors were expanding. Previous studies performed in our laboratories in intracranial models (and including sham surgery) indicated that the BBB had recovered from the disruption caused by the surgery after 7 days. Mice bearing intracranial U87 or GS2 xenografts were administered GDC-0084 (15 mg/kg), or vehicle (MCT) PO daily for 2 or 4 weeks,
respectively, starting 7 days (U87) or 14 days (GS2) post tumor cell inoculation. Mouse body weights were recorded twice per week during the study and animals were euthanized if body weight loss was greater than 20% from their initial body weight. Tumor volumes were monitored by ex vivo micro-computed tomography (micro-CT) imaging and T2 MRI for the GBM models U87 (on Day 14 of dosing) and GS2 (pre-dose and on Day 28 of dosing), respectively. The differences between treatment groups were evaluated using Student’s t test in Prism (Prism 5, GraphPad). MRI was performed on a Varian 9.4T MRI system with a 30 mm quadrature volume coil. During the imaging, animals were kept under anesthesia with 2% isoflurane in air. Body temperature was continuously monitored using a rectal probe and was maintained at 37°C by a heated-air flow system regulated by in-house LabVIEW controller software. A T2-weighted fast spin echo, multi-slice (FSEMS) sequence was used to detect lesions by MRI. 12-20 axial 0.5-0.8 mm-thick slices were acquired with a 20 x 20 mm field of view (FOV), and 128 x 128 matrix, zero-filled to 256 x 256 images. TR=3500-4000 ms, TE=9-10 ms, ETL=8, k-zero=4, NEX=8. Tumor volumes were calculated from the T2-weighted FSEMS images using an intensity threshold based region-growing tool in MRVision software. Brain sample preparation, micro-CT scanning, and image analysis for ex-vivo micro-CT imaging were performed as described previously (de Crespigny et al., 2008).

In the studies conducted with the GS2 tumor-bearing mice, plasma and brains were also collected at the end of treatment to measure GDC-0084 concentrations and assess PI3K pathway modulation in the tumor. Each brain was dissected to separate the tumor from the healthy tissues. Plasma and normal brains were processed and analyzed by LC-MS/MS. The GS2 tumors isolated
from the brains were processed and the PI3K pathway markers pAkt, pS6 and p4EBP1 were measured as described previously.

The effects of GDC-0084 on the PI3K pathway in GS2 orthotopic tumors were also evaluated by immunohistochemistry following the last PO dose of GDC-0084 (15 mg/kg) to mice after the 4-week treatment. Brains were collected 2 hours following the last dose, after the animals had been anesthetized with pentobarbital, perfused first with heparinized phosphate-buffered saline and subsequently with 4% paraformaldehyde.

IHC for detection of pAkt and pPRAS40 with antibodies D9E and C77D7, respectively (Cell Signaling Technologies, Danvers, MA), was performed on 4µ thick paraffin-embedded tissue sections using a Discovery XT autostainer and CC1 standard antigen retrieval (Ventana Medical Systems; Tucson, AZ). Specifically bound primary antibody was detected using OmniMap detection (Ventana) followed by hematoxylin counterstain. The antibodies used react with the human and the mouse proteins, allowing detection of pathway markers in mouse brain as well as in the intracranially implanted human tumors.
Results

In Vitro Studies

Transport Studies in Transfected Cell Lines

The permeability and bidirectional transport of GDC-0084 were measured in transfected cell lines over-expressing human or mouse P-gp or BCRP. The apparent permeability (P_{app}) was high and comparable to that of metoprolol, the high P_{app} marker used in the same experiments (Table 2). The efflux ratios (P_{app,B-A}/P_{app,A-B}) did not markedly differ from 1 in the MDCK or LLC-PK1 transfected cells (Table 2), indicating that GDC-0084 was a poor substrate of these efflux transporters.

Plasma protein and Brain Tissues Binding

GDC-0084 binding to plasma proteins was low, with a free fraction (%) of 29.5 ± 2.7 (n=3) in CD-1 mouse plasma, when tested at 5 µM. Binding to brain tissues from CD-1 mice was higher, with a free fraction of 6.7% (± 1; n=3).

In Vivo Studies

Pharmacokinetics of GDC-0084 in Mouse

The plasma concentrations-time profile of GDC-0084 following a single oral dose (25 mg/kg) to mice is presented in Fig. 1B. Brain concentrations measured at 1 and 6 hours post-dose were similar to plasma levels. Total and free brain-to-free plasma ratios of approximately 1.4 and 0.4, respectively, were consistent at the two time points (Table 3).

Modulation of pAkt and pS6 in Brain
Inhibition of the PI3K pathway was assessed in the brain of healthy mice through measurement of two markers, pAkt and pS6. Following a single oral dose of GDC-0084 (25 mg/kg), pAkt and pS6 levels were significantly lower than those detected in the control animals (Fig. 1C). Suppression of pAkt and pS6 reached 90% 1 hour post dose and stayed greater than 70% 6 hours after dosing (Fig. 1D).

**Brain and Tumor Distribution by MALDI Imaging**

Distribution of GDC-0084 in the brain and intracranial U87 and GS2 tumors following administration of a single PO dose (15 mg/kg) was investigated by MALDI imaging. Brains were collected 1 hour post dose and images presented in Figure 2 show that GDC-0084 distributed readily and quite evenly throughout the brain, including in the GS2 (Fig. 2A) and U87 (Fig. 2B) tumors. In addition, the homogeneity and pattern of distributions of GDC-0084 in the tumors and non-tumored regions of the brains were further analyzed. In order to assess in a more quantitative manner, less prone to visual bias, the homogeneity of the brain distribution, the intensity of each pixel (signal) was extracted in the entire tumor region or in the whole non-tumored, healthy area, of the brains. The frequency of signal intensities (frequency of pixel intensities), binned by increment of 0.1 as described in the Methods, appeared to follow a normal distribution in healthy brain, superimposed (mean pixel intensity 0.54) to that observed in U87 tumors (Fig. 3A; mean pixel intensity 0.54). The similar mean pixel intensities in the two regions of the brain along with the superimposable Gaussian distributions indicated that the compound was able to reach the tumor and the non-diseased brain to a similar extent. A Gaussian distribution of signals was observed as well in GS2 tumors (Fig. 3B), also indicating a homogeneous tumor penetration, however with a slightly lower mean pixel intensity (0.34 vs.
0.55), suggesting an overall lower GDC-0084 concentration in GS2 tumors than in normal brain. Comparisons of the GDC-0084 signal homogeneity in non-tumored brain regions between the U87 and GS2 tumor-bearing mice showed identical distribution (Fig. 3C) and mean pixel intensities, confirming the reproducible and consistent brain penetration properties of GDC-0084. Similar results were obtained in brains collected at 6 hours post dose. Furthermore, to contrast the distribution of GDC-0084 to that of a non-brain penetrant compound, MALDI images previously obtained with pictilisib in the U87 tumor model (Salphati et al., 2014) were re-analyzed by extracting pixel intensity frequencies as described here. While signal intensities in the U87 tumor for GDC-0084 could be fit to a Gaussian curve, signals from pictilisib were concentrated in the low intensity bins, with a distribution that appeared more heterogeneous (Fig. 3D). This can be interpreted as poor brain penetration, with localized hot spots of signal, due to leaky BBB.

Efficacy in Brain Tumor Models

The efficacy of GDC-0084 was tested in the U87 and GS2 intracranial models. GDC-0084 was administered PO at 15 mg/kg daily for 2 and 4 weeks to U87 and GS2 tumor-bearing mice, respectively. The effect of the treatment on the U87 and GS2 tumor volumes was assessed at the end of the dosing period. Images of U87 tumor obtained by micro-CT are presented in Figure 4A. The U87 tumor volumes were reduced by approximately 70%, when compared to the vehicle control, (Fig. 4B) following treatment with GDC-0084. Similarly, the GS2 tumors measured by MRI (Fig. 4C) in the treated mice were significantly (p<0.01) smaller (~40%) than those in the control group (Fig. 4D). Plasma and healthy brain concentrations of GDC-0084 were measured at the end of the study in the GS2 tumor-bearing mice and are presented along with brain-to-
plasma ratios in Table 4. Brain concentrations in the normal part of the brain and brain-to-plasma ratios were comparable to those obtained previously (Table 3). Modulation of the PI3K pathway in the GS2 tumors was assessed by western blot at the end of the dosing period, 2 and 8 hours after the final administration of GDC-0084 (Fig. 5A). Levels of pAkt were significantly reduced at 2 and 8 hours, by 90 and 70%, respectively. Suppression of pS6 and p4EBP1 was less pronounced at 2 hours, reaching 35 and 43%, respectively. These two markers were back to baseline levels 8 hours post-dose (Fig. 5B). In addition, inhibition of the PI3K pathway was also measured at tumor cell level by immunohistochemistry. The tissue was probed with antibodies against the PI3K pathway markers pAkt and pPRAS40. GDC-0084 caused a marked reduction in staining when compared to the vehicle-treated animal (Fig. 5C), confirming inhibition of the pathway in the tumor.
Discussion

The PI3K pathway is altered in more than 80% of patients diagnosed with GBM. However, repeated failures of PI3K inhibitors in clinical trials may call into question the validity of this target or the ability to reach it for therapeutic benefit (Nichol and Mellinghoff, 2015). In order to ensure delivery of the drug to its target and address one of the obstacles to effective treatment, the PI3K inhibitor GDC-0084 was specifically optimized to cross the BBB, while maintaining adequate potency (p110α Ki 2 nM; mTOR Ki 70 nM) and selectivity (Heffron et al., 2016) with the treatment of GBM and PI3K-dependent brain tumors as the primary objective. In vitro studies in MDCK and LLC-PK cells over-expressing human or mouse P-gp or BCRP indicated that GDC-0084 was not a good substrate of these two efflux transporters (Table 2), which limit the brain penetration of many compounds (Agarwal et al., 2011a). These in vitro results suggesting that GDC-0084 brain penetration would not be hindered by these transporters were consistent with data obtained in vivo in mice showing that GDC-0084 was able to cross the BBB following PO administration (25 mg/kg), and reach free brain concentrations greater than pAkt inhibition IC50 estimated in PC3 cells (0.13 µM; PTEN-null cells used in our program to test and select PI3K inhibitors) (Heffron et al., 2016). These levels of free drug (i.e., available to interact with the PI3K) at the target site (brain) achieved significant pathway suppression up to 6 hours post-dose (Fig. 1). The total brain-to-plasma ratio exceeded 1 and the ratio of free concentrations in brain and plasma was 0.4 (Table 3). For drug passively permeating the BBB, the equilibrium theory of free drug applies (Tillement et al., 1988), and the ratio of unbound concentrations in the brain and plasma (Cu,br/Cu,p) is expected to equal (or approach) 1. Such ratios have been reported for the CNS drugs fluoxetine (0.41), citalopram (0.52) or caffeine (0.71) (Liu et al., 2006; Liu et al., 2014). For GDC-084, efflux transporters other than P-gp and
bcrp may explain the lower $C_{u,b}/C_{u,p}$. Alternatively, experimental variability in the determination of the free fraction in brain and/or plasma may also have led to underestimation of this ratio. The in vitro determination of binding to brain homogenate has nevertheless been shown to be a reliable method to estimate free brain concentration (Liu et al., 2009). Despite this uncertainty, further analyses by MALDI imaging confirmed that GDC-0084 was able to distribute uniformly throughout the brain and tumors in the two orthotopic models of GBM tested (Fig. 2), the U87 and GS2 glioma cells. The U87 cell line is a widely used and characterized glioma model (Clark et al., 2010) that grows adherently when cultured and forms well-delineated tumors when implanted as intracranial xenograft. The GS2 cells grow in vitro as neurosphere and develop more diffusive tumors in the brain (Gunther et al., 2008). While these two models present striking differences in their BBB status (intact vs. disrupted) (Salphati et al., 2014), the analysis of drug signal intensity revealed a Gaussian distribution, reflective of homogeneous penetration of GDC-0084 in the U87 and GS2 tumors as well as in non-tumor regions of the brain (Fig. 3), independent of the local variations in BBB integrity. In addition, in the U87 tumor model, mean pixel intensities (related to compound concentration) were similar in the tumor and in the healthy part of the brain, suggesting undifferentiated distribution in these two areas (Fig. 3A). In the GS2 tumors, signal intensities also appeared to follow a normal distribution, consistent with homogeneous penetration of the compound. The mean pixel intensity was nevertheless slightly lower in the tumor than in the non-tumor region (0.37 vs. 0.55). This weaker signal in the GS2 tumor may be caused by lower tumor vascularization, which could limit the diffusion of GDC-0084. The nearly superimposed frequencies of signal intensities in the healthy brain region of mice bearing either GS2 or U87 tumors underscore the consistency and reproducibility of GDC-0084 brain penetration (Fig. 3C). In contrast, reanalysis of data previously obtained with the P-
gp and BCRP substrate (efflux ratios of 27 and 80, respectively; Table 2) and non-brain penetrant compound pictilsib (Salphati et al., 2014) showed heterogeneous (non Gaussian) intra-tumor distribution of pixel intensities (Fig. 3D), most likely dependent on BBB disruption within the tumor, skewed towards low signal intensity (low levels of compound). Such a brain/tumor distribution is most likely representative of most of the unsuccessful targeted agents tested so far against GBM. Homogeneous and undifferentiated compound distribution throughout healthy brain tissues and tumor is essential to potentially treat not only the core of the tumor but also invasive glioma cells protected by an intact BBB or blood-tumor barrier. Studies dissecting tumors and adjacent tissues from the rest of the brain in preclinical models have shown a sharp decrease in compound concentration from the tumor core, to the tumor rim, to the normal brain with several targeted agents (Agarwal et al., 2012; Agarwal et al., 2013) that consequently failed to treat the whole brain. Such insufficient brain distribution in preclinical models of GBM is expected to mirror what occurs in patients with non-brain penetrant drugs. Indeed, high concentrations of compounds, substrates of efflux transporters, have been measured in resected human brain tumors (Fine et al., 2006; Pitz et al., 2011), while these drugs had minimal therapeutic effects. Similarly, high levels of erlotinib were shown to co-localize with the contrast-enhancing regions of brain metastases (Weber et al., 2011).

The potent inhibition of the PI3K pathway in the brain, as well as the distribution in healthy tissues and brain tumor, suggested that GDC-0084 could be efficacious in orthotopic models of GBM. Studies in the U87 and GS2 models showed that GDC-0084 could significantly reduce tumor volumes, compared to the vehicle control. While promising, the findings in the U87 tumors were not unexpected as this model presents a disrupted BBB and can be sensitive to compounds with limited brain distribution (Carcaboso et al., 2010; Salphati et al., 2012). This
GBM model may however represent what might occur in the contrast-enhancing regions of a human GBM. The GS2 model, in contrast, possesses an intact BBB (Salphati et al., 2014) and recapitulates better the invasive regions of GBM. In that model, GDC-0084 administration significantly reduced tumor volume, when compared to vehicle treatment, and led to marked suppression of the PI3K pathway (Fig. 5), assessed by western blot and IHC (Fig. 5C). However, this significant efficacy was less pronounced than in the U87 model, despite comparable in vitro potency against the two cell lines. This may be attributed to the slightly lower concentration of GDC-0084 in the GS2 tumor suggested by MALDI imaging, or could also indicate that pathways other than PI3K contribute to the growth of these tumors. This hypothesis may only be tested with the combination of agents able, like GDC-0084, to cross the BBB and distribute throughout the brain. When considering the genetic heterogeneity of GBM, such combination therapies may be necessary to achieve optimal efficacy.

Multiple studies and a recent symposium have emphasized the role of the BBB and efflux transporters (or blood-tumor barrier) in limiting the therapeutic effects of most targeted drugs evaluated in GBM clinical trials (Agarwal et al., 2011b; Levin et al., 2015; Oberoi et al., 2016). While it is not the only challenge, it still appears to be an “underappreciated obstacle” (Mason, 2015) to optimal GBM treatment. In addition, maintaining or improving potency against pathways or targets involved in GBM, as well as ensuring that adequate free concentrations are reached in the brain (Smith et al., 2010) are also key elements in the optimization of potential drugs. The PI3K inhibitor GDC-0084 was purposely designed to cross an intact BBB and distribute throughout the brain. It is being evaluated in patients, and exposures reached at tolerated doses are consistent with those associated with efficacious doses in mouse models. While brain penetrance is a necessary but not sufficient attribute, this compound may provide a
much needed treatment option for GBM and should allow a more informative assessment of pharmacodynamic hypotheses.
Authorship Contributions

Participated in research design: Salphati, Alicke, Heffron, Shahidi-Latham, Nishimura, Pang, Quiason, Gould, Phillips, Olivero

Conducted experiments: Alicke, Nishimura, Cheong, Lau, Lee, Plise

Contributed new reagents or analytical tools: Shahidi-Latham, Cao, Carano, Greve, Quiason, Rangell, Zhang

Performed data analysis: Salphati, Shahidi-Latham, Quiason, Cao, Nannini-Pepe, Pang, Phillips

Wrote or contributed to the writing of the manuscript: Salphati, Shahidi-Latham, Carano, Koeppen
References

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the PI3K pathway in the brain--efficacy of a PI3K inhibitor optimized to cross the blood-brain barrier. *Clin Cancer Res* 18:6239-6248.


Footnotes

Address correspondence to: Dr. Laurent Salphati, Department of Drug Metabolism and Pharmacokinetics, MS 412A, Genentech, Inc., 1 DNA Way, South San Francisco, CA. 94080. Email: salphati.laurent@gene.com
**Figure Legends**

**Figure 1.** A, Chemical structure of GDC-0084. B, Plasma concentration-time profile and brain concentrations of GDC-0084 following PO administration (25 mg/kg) to CD-1 mice. C, Western blot of mouse brains probed with antibodies against pAkt, total Akt, pS6, total S6 and actin. D, quantitation of pAkt/total Akt and pS6/total S6 at 1 and 6 hours post-dose in CD-1 mice. B and D, Results are presented as the mean ± S.D. of three animals.

* Significantly different from control. p<0.05, t-test.

**Figure 2.** Brain Distribution of GDC-0084 in Orthotopic Models of GBM by MALDI Imaging. Distribution of GDC-0084 in GS2 (A) and U87 (B) intracranial tumors 1 hour following PO administration of 15 mg/kg. Localization of the tumors by cresyl violet staining and drug distribution in MALDI MS images are presented. Dotted contours delineate the tumors.

**Figure 3.** Distribution of MALDI Imaging Signal Intensity of GDC-0084 in U87 and GS2 Intracranial Tumors. A, Distributions of signal intensity of GDC-0084 in tumor (orange bars) and non-tumor regions (gray bars) in the U87 orthotopic GBM model. B, Distributions of signal intensity of GDC-0084 in tumor (orange bars) and non-tumor regions (gray bars) in the GS2 orthotopic GBM model. C, Distributions of signal intensity of GDC-0084 in the non-tumor regions of the U87 (green bars)
and GS2 (gray bars) orthotopic GBM models. D, Distributions of signal intensity of GDC-0084 (orange bars) and pictilisib (gray bars) in the U87 orthotopic GBM model. When appropriate, a Gaussian distribution curve was fitted to the signal intensity data.

**Figure 4.** Efficacy in Orthotopic Models of GBM.

A, Efficacy of GDC-0084 in U87 glioblastoma tumor model following treatment for two weeks. Representative micro-CT images of brain from control and treated mice. B, U87 brain tumor volume in control mice and mice treated with GDC-0084 for 2 weeks. Results are presented as the mean ± S.E. of ten animals.

C, Efficacy of GDC-0084 in GS2 neurosphere tumor model following treatment for four weeks. Representative T2-weighted MRI images of brain from control and treated mice. D, GS2 brain tumor volume in control mice and mice treated with GDC-0084 for 4 weeks. Results are presented as the mean ± S.E. of ten animals.

**Figure 5.** Modulation of PI3K Pathway in Intracranial GS2 Xenograft by GDC-0084.

A, Western blot of the PI3K pathway markers pAkt, pS6 and p4EBP1 in intracranial GS2 xenografts after completion of the 4-week efficacy dosing period. B, quantitation of pAkt/total Akt, p4EBP1/total 4EBP1 and pS6/total S6 at 2 and 8 hours following the last PO dose of GDC-0084 (15 mg/kg). C, immunohistochemical analysis of GS2 tumor-bearing brain section from vehicle-treated mice and mice dosed with GDC-0084 (15 mg/kg). Sections were probed for the PI3K markers pAkt and pPRAS40.
## Tables

**Table 1.** Study Designs, Experimental Conditions and Measured Endpoints for the In Vivo Studies

<table>
<thead>
<tr>
<th>Study</th>
<th>Dose (mg/kg)</th>
<th>Formulation</th>
<th>Mouse Strain</th>
<th>Collected Samples</th>
<th>Measured Endpoints</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pharmacokinetics</td>
<td>25</td>
<td>MCT</td>
<td>CD1, female</td>
<td>Plasma, brain</td>
<td>- GDC-0084 concentrations</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>- PD modulation (pAkt, pS6)</td>
</tr>
<tr>
<td>MALDI imaging</td>
<td>15</td>
<td>MCT</td>
<td>CD-1 Nude, female intracranial tumor-bearing</td>
<td>Brain</td>
<td>- GDC-0084 brain distribution</td>
</tr>
<tr>
<td>Efficacy</td>
<td>15</td>
<td>MCT</td>
<td>CD-1 Nude, female intracranial tumor-bearing</td>
<td>Plasma, brain, tumors</td>
<td>- Tumor volumes</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>- GS2 tumors: GDC-0084 plasma, brain and tumor concentrations, PD modulation</td>
</tr>
</tbody>
</table>

MCT: 0.5% methylcellulose/0.2% Tween 80
Table 2. Apparent Permeability ($P_{\text{app}}$) of GDC-0084, Pictilisib and Metoprolol in Transfected Cells.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Cell Line</th>
<th>$P_{\text{app}}$ (10^{-6} cm/s)</th>
<th>$P_{\text{app}}$ Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>A to B</td>
<td>B to A</td>
</tr>
<tr>
<td>GDC-0084</td>
<td>MDR1-MDCKI</td>
<td>13.5 ± 0.9</td>
<td>11.5 ± 1.6</td>
</tr>
<tr>
<td></td>
<td>Bcrp1-MDCKII</td>
<td>17.6 ± 2.1</td>
<td>18.6 ± 1.1</td>
</tr>
<tr>
<td></td>
<td>BCRP-MDCKII</td>
<td>23.2 ± 5.4</td>
<td>16.0 ± 1.1</td>
</tr>
<tr>
<td></td>
<td>Mdr1a-LLC-PK</td>
<td>13.1 ± 1.3</td>
<td>19.4 ± 1.3</td>
</tr>
<tr>
<td>Pictilisib</td>
<td>MDR1-MDCKI</td>
<td>1.4 ± 0.2</td>
<td>37 ± 5.4</td>
</tr>
<tr>
<td></td>
<td>Bcrp1-MDCKII</td>
<td>0.47 ± 0.16</td>
<td>32 ± 3.2</td>
</tr>
<tr>
<td>Metoprolol</td>
<td>MDR1-MDCKI</td>
<td>14.3 ± 4.9</td>
<td>13.4 ± 0.3</td>
</tr>
</tbody>
</table>

Results reported as mean ± S.D. (n=4-6).
Table 3. Plasma Concentrations, Brain Concentrations and Brain-to-Plasma Ratio of GDC-0084 Following PO Administration (25 mg/kg) to CD1 Mice

<table>
<thead>
<tr>
<th>Time post-dose (h)</th>
<th>Total Brain (µM)</th>
<th>Total Plasma (µM)</th>
<th>Brain-to-Plasma Ratio</th>
<th>Free Brain (µM)</th>
<th>Free Plasma (µM)</th>
<th>Free Brain-to-Plasma Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4.72 ± 1.07</td>
<td>3.39 ± 0.72</td>
<td>1.39 ± 0.02</td>
<td>0.32 ± 0.07</td>
<td>0.76 ± 0.16</td>
<td>0.41 ± 0.01</td>
</tr>
<tr>
<td>6</td>
<td>2.46 ± 0.30</td>
<td>1.80 ± 0.09</td>
<td>1.37 ± 0.17</td>
<td>0.16 ± 0.02</td>
<td>0.41 ± 0.02</td>
<td>0.41 ± 0.05</td>
</tr>
</tbody>
</table>

Results reported as mean ± S.D. (n=3).
Table 4. Plasma Concentrations, Brain Concentrations and Brain-to-Plasma Ratio
Measured 2 and 8 hours Following PO Administration of GDC-0084 (15 mg/kg) to GS2 Tumor-Bearing Mice (non-tumored half of the brain).

<table>
<thead>
<tr>
<th>Time post-dose (h)</th>
<th>Total Brain (µM)</th>
<th>Total Plasma (µM)</th>
<th>Brain-to-Plasma Ratio</th>
<th>Free Brain (µM)</th>
<th>Free Plasma (µM)</th>
<th>Brain-to-Plasma Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>5.51 ± 1.58</td>
<td>3.64 ± 2.05</td>
<td>1.67 ± 0.51</td>
<td>0.37 ± 0.11</td>
<td>1.07 ± 0.61</td>
<td>0.38 ± 0.11</td>
</tr>
<tr>
<td>8</td>
<td>2.48 ± 1.25</td>
<td>2.01 ± 1.19</td>
<td>1.29 ± 0.16</td>
<td>0.17 ± 0.08</td>
<td>0.59 ± 0.35</td>
<td>0.29 ± 0.03</td>
</tr>
</tbody>
</table>

Results reported as mean ± S.D. (n=3).
Figure 1

A

B

C

D

GDC-0084 25 mg/kg

Vehicle

1 hr

6 hr

pAkt^{Ser473}
tAkt

pS6^{Ser235/236}
tS6

Actin

pAkt/tAkt

pS6/tS6
Figure 2

A. GS2  
Cresyl Violet Stain | MALDI MS Image
---|---
Animal 1
Animal 2
Animal 3

B. U87  
Cresyl Violet Stain | MALDI MS Image
---|---
Animal 4
Animal 5
Animal 6

Legend:
- **Cresyl Violet Stain**: Shows the staining pattern.
- **MALDI MS Image**: Displays the MALDI mass spectrometry images with color gradients indicating different intensities.
Figure 3

A  
- U87 - Non Tumor Region
- U87 - Tumor Region

B  
- GS2 - Non Tumor Region
- GS2 - Tumor Region

C  
- U87 - Non Tumor
- GS2 - Non Tumor

D  
- U87 - Tumor – Pictilisib
- U87 - Tumor – GDC-0084
Figure 4

A

Vehicle  
GDC-0084

B

Vehicle  
GDC-0084

C

Vehicle control (4-week)  
GDC-0084 (4-week)

D

Vehicle  
GDC-0084  

* *
Figure 5

A

<table>
<thead>
<tr>
<th></th>
<th>Vehicle</th>
<th>GDC-0084 (15 mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2 hr</td>
<td>8 hr</td>
</tr>
<tr>
<td>pAkt</td>
<td><img src="pAkt.png" alt="Image" /></td>
<td><img src="pAkt.png" alt="Image" /></td>
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<tr>
<td>tAkt</td>
<td><img src="tAkt.png" alt="Image" /></td>
<td><img src="tAkt.png" alt="Image" /></td>
</tr>
<tr>
<td>pS6</td>
<td><img src="pS6.png" alt="Image" /></td>
<td><img src="pS6.png" alt="Image" /></td>
</tr>
<tr>
<td>tS6</td>
<td><img src="tS6.png" alt="Image" /></td>
<td><img src="tS6.png" alt="Image" /></td>
</tr>
<tr>
<td>p4EBP1</td>
<td><img src="p4EBP1.png" alt="Image" /></td>
<td><img src="p4EBP1.png" alt="Image" /></td>
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<tr>
<td>4EBP1</td>
<td><img src="4EBP1.png" alt="Image" /></td>
<td><img src="4EBP1.png" alt="Image" /></td>
</tr>
<tr>
<td>Actin</td>
<td><img src="Actin.png" alt="Image" /></td>
<td><img src="Actin.png" alt="Image" /></td>
</tr>
</tbody>
</table>

B

- **pAkt/tAkt**
- **pS6/tS6**
- **p4EBP1/4EBP1**

C

<table>
<thead>
<tr>
<th></th>
<th>Vehicle</th>
<th>GDC-0084</th>
</tr>
</thead>
<tbody>
<tr>
<td>pAkt</td>
<td><img src="pAkt.png" alt="Image" /></td>
<td><img src="pAkt.png" alt="Image" /></td>
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<tr>
<td>pPRAS40</td>
<td><img src="pPRAS40.png" alt="Image" /></td>
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