ABCG2 and ABCB1 Limit the Efficacy of Dasatinib in a PDGF-B–Driven Brainstem Glioma Model


Abstract

Dasatinib is a multikinase inhibitor in clinical trials for glioma, and thus far has failed to demonstrate significant efficacy. We investigated whether the ABC efflux transporters ABCG2 and ABCB1 expressed in the blood–brain barrier (BBB), are limiting the efficacy of dasatinib in the treatment of glioma using genetic and pharmacologic approaches. We utilized a genetic brainstem glioma mouse model driven by platelet-derived growth factor-B and p53 loss using abcg2/abcb1 wild-type (ABC WT) or abcg2/abcb1 knockout mice (ABC KO). First, we observed that brainstem glioma tumor latency is significantly prolonged in ABC KO versus WT mice (median survival of 47 vs. 34 days). Dasatinib treatment nearly doubles the survival of brainstem glioma-bearing ABC KO mice (44 vs. 80 days). Elacridar, an ABCG2 and ABCB1 inhibitor, significantly increases the efficacy of dasatinib in brainstem glioma-bearing ABC WT mice (42 vs. 59 days). Pharmacokinetic analysis demonstrates that dasatinib delivery into the normal brain, but not into the tumor core, is significantly increased in ABC KO mice compared with ABC WT mice. Surprisingly, elacridar did not significantly increase dasatinib delivery into the normal brain or the tumor core of ABC WT mice. Next, we demonstrate that the tight junctions of the BBB of this model are compromised as assessed by tissue permeability to Texas Red dextran. Finally, elacridar increases the cytotoxicity of dasatinib independent of ABCG2 and ABCB1 expression in vitro. In conclusion, elacridar improves the efficacy of dasatinib in a brainstem glioma model without significantly increasing its delivery to the tumor core. Mol Cancer Ther; 15(5): 819–29. © 2016 AACR.

Introduction

Brain stem glioma, also known as diffuse intrinsic pontine glioma (DIPG), is a rare and incurable brain tumor with poor survival rates due to lack of effective therapeutic options (1–3). DIPG predominantly arises in children and accounts for about 15%–20% of all pediatric brain tumors (1). It is the leading cause of death for patients with pediatric brain tumors; the median survival is <1 year, and <10% of children survive >2 years after diagnosis (1, 2, 4). This poor prognosis has remained unchanged over the past 30 years (1, 4). The infiltrative nature and anatomic location of this disease make surgical resection impossible (4). Therefore, there is an urgent need for the development of effective strategies to treat this devastating disease.

Until recently, knowledge concerning the molecular biology of DIPG was limited due to lack of availability of tumor tissue (4). However, in recent years, there has been a paradigm shift toward obtaining DIPG tissue, which has resulted in improved understanding of DIPG tumor biology. These studies have demonstrated that DIPGs harbor both genetic alterations common to gliomas that arise in other parts of the central nervous system, including adult gliomas, as well as unique genetic alterations, such as gain-of-function histone mutation, K27M, in H3.1 or H3.3 and activating mutations in ACGV1 (5–10). With regard to receptor tyrosine kinase signaling, platelet-derived growth factor (PDGF) signaling, in particular PDGFRA, is thought to play a role in brainstem gliomagenesis as multiple studies indicate that approximately 30% of human DIPGs harbor gain or amplification in PDGFRA (11–15). Dasatinib, a multikinase inhibitor that inhibits PDGFRA and SRC among others has recently been demonstrated to have antitumor activity in vitro in four DIPG cell lines (16). In contrast, dasatinib has recently been evaluated in a clinical trial for children with DIPG with disappointing results, citing only limited efficacy of the drug (3). Evaluation of the cerebrospinal fluid (CSF) of two patients suggested insufficient drug delivery as a potential mechanism of resistance, although CSF is not a good surrogate for brain penetration for a drug like dasatinib, a substrate of both ABCB1 (also known as P-glycoprotein, P-gp, or MDR) and ABCG2 (also known as BCRP; refs. 3, 17). With this perspective, we sought to evaluate the efficacy of dasatinib in a genetically engineered mouse model of brainstem glioma driven by PDGF-B signaling (18, 19).

One of the major hurdles in the treatment of brain-related disorders is the delivery of therapeutic agents across the blood–brain barrier (BBB). The BBB, formed by capillary endothelial cells
joined together by tight junction proteins, limits the paracellular movement of large molecules (20). While tight junctions present a physical barrier to the drugs, efflux transporter proteins found within the brain primarily on endothelial cells, such as ABCB1 and ABCC2, present an additional functional barrier by pumping drugs back into blood circulation (21). Recent studies have shown that several molecularly targeted agents, including dasatinib, are substrates of ABCB1 and ABCC2 and as such have limited brain distribution (21–26). One potential strategy to improve delivery of substrate drugs across the BBB is simultaneous inhibition of these efflux transporters. Elacridar is a potent inhibitor of both ABCB1 and ABCC2 and coadministration of elacridar with molecularly targeted agents results in improved brain distribution (24, 26, 27). However, the use of elacridar in preclinical setting for chronic administration is limited by its poor oral absorption and lack of injectable formulations (27–29). Recently, we reported a novel microemulsion formulation of elacridar, which has improved bioavailability and can be used for chronic administration in preclinical models (29).

Given the feasibility of chronic administration of elacridar in the microemulsion formulation, and the current ongoing clinical trials with dasatinib in childhood high-grade gliomas including DIPG, the aim of the current study was to evaluate the efficacy of dasatinib alone and in combination with the novel microemulsion formulation of elacridar in a preclinical model of brainstem glioma. We hypothesized that dasatinib delivery would be significantly improved by coadministration with the elacridar microemulsion formulation, ultimately resulting in improved efficacy. Using a PDGF-B–driven brainstem glioma model, we showed that genetic deletion of ABCB1 and ABCC2 or treatment with elacridar significantly enhances the efficacy of dasatinib. Surprisingly, the observed increased efficacy of elacridar in combination with dasatinib relative to dasatinib alone was not due to improved drug delivery to the tumor core. Furthermore, elacridar treatment significantly enhances the cytotoxicity of dasatinib of both ABC WT and ABC KO brainstem glioma cell lines suggesting an ABCB1/ABCC2–independent mechanism for elacridar. Our data provide a preclinical foundation for future trials using this novel combination (elacridar microemulsion with dasatinib) in the hopes of improving survival for children suffering from this devastating disease.

Materials and Methods

Chemicals
Dasatinib was purchased from either LC labs or from Selleck Chemicals. Elacridar [GF120918; N-(4-[2-(2,6-dimethoxy-3,4-dihydro-1H-isouquinolin-2-yl)ethy]-5-methoxy-9-oxo-10H-acridine-4-carboxamide] was purchased from Toronto Research Chemicals, Inc. for in vitro studies and obtained from GLaxoSmithKline for in vivo studies. Cremophor EL and Capstex 355 were obtained from Abitech. Carbitol [2-(2-ethoxyethoxy) ethanol] was purchased from Sigma-Aldrich. Texas Red Dextran 3000 MW (TRD) was purchased from Molecular Probes, Invitrogen. All other chemicals used were reagent grade or HPLC grade from Sigma-Aldrich.

Animals
Ntv-α; p53 heterozygous mice in the C57BL/6j background (Jackson Laboratory) have been previously described (18). FVB-abcb1a1+/−; abcb1b−/−; abcg2−/− (ABC KO) and control Ntv-α;p53+/+, abcb1a1+/−; abcb1b+1+/−; abcg2+/− (ABC WT) mice of the same genetic background. The generations were kept similar between the two models (to maintain the same genetic mixtures between the two models). For genotyping, tail DNA was obtained using tissue DNA extraction kit XNAT (Sigma Aldrich) according to the manufacturer’s instructions. All work with mice was done in accordance with the Duke University Animal Care and Use Committee and the Guide for the Care and Use of Laboratory Animals.

DF1 cell culture and generation of PDGF-B–induced glioma in ABC KO and ABC WT mice
DF1 cells were cultured and transfected with RCAS-PDGFB and RCAS-Cre plasmids as described previously (18). Combination of viruses at a 1:1 ratio was injected to generate in vivo brainstem glioma as described previously (18). Injected mice were monitored daily for symptoms and euthanized with CO2 upon observing sure signs of brain tumors (enlarged head, ataxia, lethargic, weight loss up to 25%) or 16 weeks postinjection in the absence of symptoms.

Histologic evaluation of tumor-bearing ABC WT and ABC KO mice
We induced tumors in ABC WT (n = 20) and KO (n = 15) mice as described above using RCAS-PDGF-B and RCAS-Cre. Mice were sacrificed when they reached euthanasia criteria as described above or at the 16-week endpoint without presentation of symptoms. Brain tissues were removed and fixed in 10% formalin followed by paraffin embedding, and sectioning at a thickness of 5 μm using a rotary microtome (Leica, RM 2235). Slides were then stained with hematoxylin and eosin (H&E) and digital images of microscopic fields of brain tissue sections were acquired with a Leica DMLB microscope, Leica digital camera, and Leica Application Suite Version 3.7 (Leica) at 40× magnification (high powered field). IHC was also performed for tumor characterization using Discovery XT (Ventana Medical Systems). Sections were immunostained for cleaved caspase-3 (CC3; Asp175; Cell Signaling Technology; #9661), antiphosphohistone H3 (Ser10; Millipore; 06-570), Nestin (BD Pharmingen; 556309), and Olig2 (Millipore; AB9610). Tumor grade was determined by microscopic examination of the tumor H&E sections with the following grading scheme: a grade 4 glioma was defined by the presence of pseudopalisading necrosis, microvascular proliferation, and increased proliferation. A grade 3 glioma was defined by the presence of microvascular proliferation and increased proliferation. A grade 2 glioma did not harbor pseudopalisading necrosis, or microvascular proliferation, but harbored increased proliferation (grade 2 glioma diagnosis was a diagnosis of exclusion).

Pharmacodynamic effect of dasatinib in tumor-bearing ABC WT and ABC KO mice
Tumor-bearing WT (vehicle n = 8; drug n = 8) and KO (vehicle n = 6; drug n = 6) mice were administered a single dose of either dasatinib (25 mg/kg) or vehicle (50 mmol/L sodium acetate buffer pH 4.6) intraperitoneally. Mice were then sacrificed 24 hours postinjection, and brain tissues were formalin fixed and paraffin embedded for immunohistochemical analysis. The effect of dasatinib was determined by staining the tumor-bearing...
sections for CC3. CC3 levels were quantified using MetaMorph software (version 7.6) and are graphed as CC3-positive pixels per nuclear area within the tumor.

**Plasma and brain distribution of dasatinib in combination with elacridar in ABC WT and ABC KO mice**

Tumor-bearing WT mice were randomly assigned to treatment with dasatinib in combination with either elacridar microemulsion (ABC WT – elacridar + dasatinib group, n = 14) or blank microemulsion (ABC WT - dasatinib alone group, n = 10). The composition of microemulsion used in this study was described previously (29). Treatments began 28 days after injection of tumor-initiating viruses at a time point where tumors are already present but mice are asymptomatic (19). The treatments entailed once daily dosing of either elacridar or blank microemulsion intraperitoneally (10 mg/kg) 1 hour before the administration of dasatinib intraperitoneally (25 mg/kg) for total seven doses (7-day treatment). The 1-hour interval between elacridar or blank microemulsion intraperitoneal administration and dasatinib intraperitoneal administration was on the basis of a previous study from our group demonstrating that the time to maximal plasma concentration of elacridar intraperitoneal administration with this formulation is 1 hour (29). The same treatment regimen was followed for tumor-bearing KO mice treated with dasatinib in combination with either elacridar microemulsion (ABC KO – elacridar + dasatinib, n = 7) or blank microemulsion (ABC KO – dasatinib alone, n = 10). The treatment of ABC KO mice started at 34 days postinfection to account for the difference in tumor latency between the strains. One hour after the last dose of the 7-day treatment, the animals were euthanized, and the brainstem (tumor tissue if tumor visible by eye or half-of-the-brainstem if tumor not visible by eye with pathologic confirmation for the presence of tumor with the other half-of-the-brainstem) and cortex (nontumor tissue) were isolated and snap frozen. Blood samples were also collected from facial vein into heparinized tubes 1 hour after the last dose of the 7-day treatment before euthanasia. Plasma was obtained by centrifuging the blood at 3,500 rpm for 10 minutes. The brain and plasma concentrations of dasatinib and elacridar in tissue specimens were determined using a sensitive and specific liquid chromatography/tandem mass spectrometry assay as follows: briefly, frozen samples were homogenized with three volumes of 5% BSA in acetate buffer pH 4.6; for analysis, the tissue samples were thawed to room temperature and homogenized with three volumes of deionized (DI) water. The TRD concentrations in total tissue homogenates were determined using BioteK plate reader. Briefly, a stock solution (1 mg/mL) of TRD was prepared by dissolving 1 mg of TRD in 1 mL of DI water. Brain homogenate was prepared by homogenizing the brain tissue (blank) with three volumes of DI water. To 450 μL of blank brain homogenate, 50 μL of 1 mg/mL TRD solution was added to get a stock TRD brain homogenate (100 μg/mL). From this TRD stock brain homogenate, serial dilutions were made with blank brain homogenate to get concentration range from 50 ng/mL to 50 μg/mL. The fluorescent intensity at different concentrations was measured using the Biotek plate reader at an excitation/emission wavelength of 590/645 nm to generate a calibration curve. The concentrations of TRD in unknown samples were determined using the calibration curve.

**BBB integrity in brainstem glioma**

Tumor-bearing ABC WT and ABC KO mice (n = 10 per group) were injected with TRD (1.5 mg/animal body weight) via tail vein. Ten minutes postinjection of TRD, each animal was perfused by a brief cardiac washout for 30 seconds using 1× DPBS (Gibco, 14190-144; ref. 30). After perfusion, the tumor tissue in the brainstem was isolated from normal tissue in the cortex and was separately snap frozen and stored at −80°C. For analysis, the tissue samples were thawed to room temperature and homogenized with three volumes of deionized (DI) water. The TRD concentrations in total tissue homogenates were determined using Biotek plate reader. Briefly, a stock solution (1 mg/mL) of TRD was prepared by dissolving 1 mg of TRD in 1 mL of DI water. Brain homogenate was prepared by homogenizing the brain tissue (blank) with three volumes of DI water. To 450 μL of blank brain homogenate, 50 μL of 1 mg/mL TRD solution was added to get a stock TRD brain homogenate (100 μg/mL). From this TRD stock brain homogenate, serial dilutions were made with blank brain homogenate to get concentration range from 50 ng/mL to 50 μg/mL. The fluorescent intensity at different concentrations was measured using the Biotek plate reader at an excitation/emission wavelength of 590/645 nm to generate a calibration curve. The concentrations of TRD in unknown samples were determined using the calibration curve.

**Cell lines**

Each cell line was generated in the Becher laboratory in 2014 from one of our murine brainstem gliomas induced with PDGF-B and p53 loss in either ABC WT mouse background or ABC KO mouse background (ABC KO 1 = 14-801-1, ABC KO 2 = 12-801-2, ABC WT 1 = 14-410-1, ABC WT 2 = 14-506-2) as described previously (31). ABC transporter status of each cell line was confirmed with genotyping in May 2015 and early passage cells were used for cell viability assays below (passage 3–5).

**In vitro cell viability assays with dasatinib ± elacridar**

Cells (10,000/well) were plated in a 96-well plate for 24 hours before drug treatment. After 24 hours, cells were exposed to dasatinib at various concentrations with or without 5 μmol/L elacridar for 72 hours. Cell viability was measured by CellTiter-Glo (Promega). The dose–response curves were generated with Prism software and analyzed using nonlinear regression. The values of half-maximal inhibitory concentration (IC50) were calculated using log (agonist) versus response including variable slope (four parameters) statistics and normalized in GraphPad Prism.
qRT-PCR
Total RNA was isolated from ABC KO or ABC WT tumor neurospheres or brainstem nestin progenitors sorted from nestin-CFPnuc mice (32) using the RNeasy Kit (Qiagen). The RNA was quantified in a NanoDrop spectrophotometer. cDNA synthesis was started with 1 μg of RNA with Superscript II reverse transcriptase (Invitrogen). qRT-PCR was performed using SYBR green assay for target genes and controls (β-actin) on 25× diluted cDNA template.

Statistical analysis
Survival curves were compared using Mantel–Cox (log-rank) statistical analysis. The significant differences of CC3 levels, TRD levels, and dasatinib distribution were determined by Mann–Whitney statistical test.

Results
Absence of abcg2 and abcb1 significantly increases tumor latency
We observed a significant difference in the tumor latency between ABC KO and ABC WT mice (47 days for ABC KO mice, n = 15 vs. 34 days for ABC WT mice, n = 20; P = 0.006; Fig. 1A). However, the tumor incidence in both ABC KO and ABC WT models was the same (80%). Incidence of grade IV gliomas (40% vs. 60%, P = 0.31 Fisher exact test), grade III gliomas (33% vs. 15%, P = 0.25 Fisher exact test), and grade II gliomas (7% vs. 5%, P = 1.0 Fisher exact test) was not significantly different between ABC KO and ABC WT tumor models, respectively. ABC KO and ABC WT tumors were characterized as gliomas by staining for H&E, Nestin, Olig2, and phospho-H3 (Fig. 1B). Thus, these data show that absence of abcg2 and abcb1 increases tumor latency without affecting tumor incidence or grade and without significantly impacting the level of nestin, olig2, or phospho-H3 expression. The mechanism for how abcg2 and abcb1 may impact tumor latency is unknown.

As this model arises in nestin-expressing progenitors residing in the neonatal brainstem, we examined abcg2 and abcb1 expression in these progenitors by dissecting out the neonatal brainstem of nestin-CFPnuc mice at postnatal day 3, sorting for CFP-expressing cells, and using real-time PCR. We observed that abcg2, abcb1a, and abcb1b transcripts are expressed in nestin-expressing brainstem progenitors (Supplementary Fig. S2).

The pharmacodynamic effects of dasatinib are greater in ABC KO mice compared with those in ABC WT mice
To test whether dasatinib administration in ABC KO mice results in enhanced antitumor activity, tumor-bearing ABC WT and ABC KO mice were treated with a single dose of either dasatinib (25 mg/kg) or vehicle via intraperitoneal injection. Twenty-four hours after treatment, animals were sacrificed and brain tissue sections were obtained. Tumor sections were stained for the apoptosis marker CC3. The amount of CC3 staining was quite variable in the dasatinib-treated ABC WT and ABC KO tumors both within and across tumors (Supplementary Figs. S3 and S4). Despite the intratumor and intertumor heterogeneity, there was greater CC3 staining in ABC KO mouse tumors treated with dasatinib as compared with any other group (Fig. 2A and B). The levels of CC3 staining were quantified using MetaMorph software by measuring the CC3-positive pixels per high-powered field normalized to nuclear area. The mean CC3-positive pixels per nuclear area in ABC KO mice treated with dasatinib (0.47) was significantly greater than both ABC WT mice treated with dasatinib (0.23, P = 0.015; Fig. 2C) and ABC KO mice treated with vehicle
There was a significant difference in CC3 levels between ABC WT mice treated with dasatinib and ABC WT mice treated with vehicle (0.054, \( P = 0.020 \); Fig. 2C). Because we observed significantly greater CC3 immunostaining in tumor-bearing ABC WT mice treated with dasatinib, these results suggest that ABCB1 and ABCG2 may influence the antitumor activity of dasatinib in the PDGF-B–driven brainstem glioma mouse model.

Dasatinib significantly prolongs survival in ABC KO mice

To determine whether the increased apoptotic effect of dasatinib in the ABC KO model relative to the ABC WT model will translate to a survival benefit, tumor-bearing ABC KO mice were treated with either dasatinib (25 mg/kg i.p.) or vehicle once daily for 7 days starting at 28 days postinjection of the virus producing cells. The median survival of the vehicle-treated cohort was 44 days compared with 80 days when ABC KO mice were treated with dasatinib (\( n = 14 \) vehicle group vs. \( n = 11 \) dasatinib group; \( P = 0.0004 \); Table 1; Fig. 3A). These results suggest that dasatinib therapy is efficacious in the PDGF-B–driven brainstem glioma mouse model in ABC KO mice.

Normal brain, but not tumor, distribution of dasatinib in tumor-bearing mice is significantly higher in ABC KO mice versus ABC WT mice

To investigate whether increased drug delivery is the mechanism for the increased apoptosis and survival observed with dasatinib therapy in the ABC KO model, we compared the brain and plasma distribution of dasatinib in ABC WT and ABC KO tumor-bearing mice after a 7-day treatment with dasatinib. Dasatinib concentrations were measured in the tumor-bearing brainstem region and nontumor cortical brain regions 1-hour post dasatinib administration. Dasatinib concentrations were not significantly different in the tumor-bearing brainstem regions of ABC KO and ABC WT mice but were significantly higher in the normal cortical region of ABC KO mice compared with the same respective region of ABC WT mice (\( P = 0.052 \) and 0.0043, respectively; Fig. 3B). Similar results were observed when the dasatinib levels in the tumor-bearing and nontumor brain regions were normalized to the respective plasma concentrations (\( P = 0.18 \) and 0.004, respectively; Fig. 3C). These data suggest that the delivery of dasatinib is significantly improved to the normal brain regions but not to the tumor-bearing region in the absence of the ABCG2 and ABCB1 transporters.

Table 1. Tumor latency after 7-day treatment

<table>
<thead>
<tr>
<th>KO</th>
<th>Treatment</th>
<th>Vehicle</th>
<th>Dasatinib</th>
<th>Dasatinib alone</th>
<th>Elacridar - dasatinib</th>
<th>No treatment</th>
<th>Elacridar only</th>
</tr>
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<td>Mice (n)</td>
<td></td>
<td>14</td>
<td>11</td>
<td>17</td>
<td>17</td>
<td>11</td>
<td>10</td>
</tr>
<tr>
<td>Median survival (days)</td>
<td></td>
<td>44</td>
<td>80</td>
<td>42</td>
<td>59</td>
<td>33</td>
<td>34</td>
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Elacridar microemulsion increases the efficacy of dasatinib in ABC WT tumor-bearing mice

To determine whether the efficacy of dasatinib in ABC WT animals could be improved with ABC transporter inhibition, elacridar (dual ABCB1/ABCG2 inhibitor) was coadministered with dasatinib. ABC WT tumor-bearing mice were randomly assigned to treatment with dasatinib in combination with the elacridar microemulsion (elacridar + dasatinib group) or dasatinib alone (dasatinib alone group) once daily for 7 days starting at 28 days postinjection of PDGF-B and Cre virus–producing cells. There was a significant increase in the median survival time of ABC WT mice treated with dasatinib in combination with elacridar as compared with dasatinib alone group (dasatinib alone group = 42 days, dasatinib + elacridar group = 59 days; \( n = 17 \) in both groups; \( P < 0.0001 \); Table 1; Fig. 4A). Additional control arms evaluated elacridar microemulsion treatment alone and untreated mice. Elacridar microemulsion did not significantly impact survival relative to untreated mice [elacridar microemulsion alone treatment (elacridar microemulsion alone treatment \( n = 10 \)) = 34 days, untreated mice (untreated mice \( n = 11 \)) = 33 days, \( P = 0.42 \); Table 1; Fig. 4A]. Dasatinib + blank microemulsion had a modest but significant effect on survival relative to untreated mice (dasatinib alone group = 42 days, untreated mice = 33 days, \( P < 0.0001 \)). These results demonstrate that elacridar microemulsion significantly improves the efficacy of dasatinib in this brainstem glioma model.

Normal brain and tumor distribution of dasatinib are not significantly increased when used in combination with elacridar in tumor-bearing ABC WT mice

To investigate whether increased drug delivery is the mechanism for the increased efficacy of dasatinib + elacridar in the ABC WT model, we compared the brain and plasma distribution of dasatinib in ABC WT tumor-bearing mice after a 7-day treatment with dasatinib or dasatinib + elacridar. Dasatinib concentrations were measured in tumor-bearing brainstem region and nontumor cortical brain regions 1-hour postdasatinib administration. Surprisingly, dasatinib concentrations were not higher in nontumor cortical and in tumor-bearing brainstem regions of ABC WT tumor-bearing mice after 7-day treatment of dasatinib + elacridar.
compared with dasatinib alone treatment group (P = 0.84 in tumor-bearing regions and 0.11 in normal brain regions; Fig. 4B).

In addition, no statistically significant difference was observed in the brain to plasma ratios of dasatinib (P = 0.47 in tumor-bearing regions and 0.65 in normal brain regions; Fig. 4C). Thus elacridar coadministration does not significantly increase the delivery of dasatinib to the tumor-bearing region or normal brain in this PDGF-B–driven brainstem glioma model. Important controls to these experiments were measurements of elacridar concentrations in the brains of ABC WT and ABC KO mice where we did not observe a significant difference (Supplementary Fig. S5) and measurements of dasatinib concentrations in ABC KO mice treated with dasatinib alone (n = 6) and co-administered with elacridar (n = 8) to observe any significant differences (Supplementary Fig. S6).

**BBB integrity is compromised in brainstem glioma**

To investigate the permeability of BBB in this brainstem glioma model, both tumor-bearing and nontumor-bearing ABC WT/KO mice were injected with TRD and allowed to circulate for 10 minutes. There was no TRD detection in any nontumor-bearing brains (Fig. 5A). Significant TRD levels were detected in the brainstem of tumor-bearing ABC WT (3.06 μg/g; Fig. 5B) and ABC KO animals (5.33 μg/g; Fig. 5B). TRD was not detected in normal cortical brain of tumor-bearing ABC KO mice, minuscule TRD levels were detected (0.32 μg/g; Fig. 5B). Overall, the results suggest that structural integrity of the BBB is compromised in this brainstem glioma model, as well as minimally so in normal cortical brain of tumor-bearing ABC KO mice.

**Elacridar enhances the cytotoxicity of dasatinib in vitro independent of ABCG2 and ABCB1 expression**

As elacridar enhanced the efficacy of dasatinib in ABC WT mice without significantly increasing its delivery into the tumor or normal brain, we evaluated the antitumor activity of dasatinib with or without elacridar in primary serum-free cultures of two ABC KO PDGF-B; p53-deficient tumors and two ABC WT PDGF-B; p53-deficient tumors. Using the Cell Glo assay, we observed that elacridar sensitizes both ABC KO cell lines and ABC WT cell lines to dasatinib (Fig. 6). The dasatinib IC50 of the two ABC KO lines were 0.51 µM and 3.1 µM, respectively.
respectively, and improved to 0.01 μm and 0.009 μm, respectively, with the addition of elacridar (P = 0.007 and 0.005 by paired t test). The dasatinib IC50 of the two ABC WT lines were 0.48 μm and 1.77 μm, respectively, and improved to 0.03 μm and 0.07 μm with the addition of elacridar (P = 0.03 and 0.01 by paired t test). These results suggest that elacridar enhances the antitumor activity of dasatinib independent of ABCG2 and ABCB1 expression.

Figure 5.
Texas Red Dextran (TRD) distribution shows blood–brain barrier (BBB) disruption in tumor-bearing mice. Normal (n = 10 for both ABC KO and WT mice; A) and tumor-bearing (B) ABC KO (n = 10) and ABC WT (n = 9) brain. There is a significant increase in TRD permeability across the BBB in tumor-bearing animals.

Figure 6.
Elacridar enhances the cytotoxicity of dasatinib in vitro independent of ABCG2 and ABCB1 expression. ABC KO cell lines (A) and ABC WT cell lines (B) were treated with dasatinib alone or in combination with elacridar in 0.1% DMSO for 72 hours. Each drug treatment was performed in triplicate. Cell Glo assay indicates that cell viability was decreased with increasing concentrations of dasatinib. With the addition of elacridar, the IC50 of dasatinib was significantly decreased. **, P < 0.01; *, P < 0.05.
ABC2G and ABCB1 Limit Efficacy of Dasatinib in Brainstem Glioma

Discussion

DIPG is one of the leading causes of cancer-related deaths in children (1). The failure of systemic chemotherapy or targeted agents in DIPG has been attributed to the inadequate delivery across the BBB (4, 23). Therefore, there is an urgent need for effective therapies for this deadly tumor that take into account the issue of drug delivery. In the current study, we have tested a combination therapy of dasatinib with elacridar in a preclinical model of brainstem glioma driven by PDGF-B and p53 loss with the hypothesis that elacridar would increase the efficacy of dasatinib by increasing the brain and tumor distribution of dasatinib. Our data demonstrate that the combination of dasatinib and elacridar does significantly improve the median survival in mice with brainstem glioma relative to dasatinib alone; however, the data also indicate this improvement may not be through increased drug delivery. It should be noted that in this study we did not measure dasatinib concentrations in different regions of the tumor. Measurement of dasatinib concentrations in tumor core and tumor rim would give some additional insights into regional delivery. Even though we observed no significant increase in dasatinib concentrations in the tumor, regional delivery might have had some influence in the resulted increased efficacy. However, measurement of regional dasatinib concentrations in this brainstem glioma model is an extremely difficult task, given its location and size. It remains to be determined exactly how elacridar increases the efficacy of dasatinib in this model.

In our initial experiments, we have evaluated the median survival time for tumor-bearing ABC WT and ABC KO mice. We observed significantly prolonged survival in ABC KO tumor-bearing mice as compared with that in ABC WT tumor-bearing mice. Histologic examination of the tumors revealed that the tumor incidence and grade is not significantly different between ABC WT and ABC KO mice. An intriguing possibility, yet to be confirmed, is that ABCB1 and/or ABCG2 actually promote gliomagenesis. In support of that hypothesis, we did observe that Abcb1a/b and Abcg2 transcripts are present in nestin-expressing progenitors in the neonatal brainstem. These results are consistent with previous observations in a PDGF-B–induced cortical glioma model whereby the authors observed that ABCG2 expression marks a subset of highly tumorigenic glioma stem-like tumor cells (33). In addition, several other groups have reported the expression of ABCG2 and ABCB1 in glioma tumor cells and particularly in glioma cancer stem cells (34–37). In contrast, others did not observe the expression of ABCG2 and ABCB1 in glioma tumor cells but only in tumor endothelial cells, or observed that ABCG2– and ABCB1– glioma cells are similarly tumorigenic (23, 38). Whether the tumor compartment of this brainstem glioma model expresses ABCG2 and ABCB1 and the exact mechanism by which ABCB1 and ABCG2 may promote gliomagenesis will be the subject of future studies.

Previous studies have demonstrated that dasatinib is a dual substrate of ABCB1 and ABCG2 and that its normal brain distribution significantly increases in the absence of ABCB1 and ABCG2 (24, 39). To test the pharmacodynamic effects of dasatinib in this brainstem glioma model induced in ABC KO mice relative to ABC WT mice, we evaluated levels of CC3, a marker of apoptosis, in ABC WT and ABC KO tumor tissue. Significantly higher CC3 staining was observed in ABC KO tumor sections as compared with ABC WT tumor sections. These data suggest that dasatinib is more cytotoxic in a glioma model induced in mice deficient for ABCG2 and ABCB1.

To determine whether the increased cytotoxicity of dasatinib in the ABC KO model results in improved survival, we conducted a survival study, where tumor-bearing ABC WT and ABC KO mice were treated with dasatinib. ABC KO mice treated with dasatinib had a significantly longer survival as compared with the ABC WT mice treated with dasatinib, although a caveat of this comparison is that the ABC KO model inherently has significantly longer tumor latency. In separate animals, the normal brain concentrations of dasatinib were found to be significantly higher in ABC KO mice as compared with those found in ABC WT mice, which is in line with the published literature. Interestingly, tumor dasatinib concentrations were not significantly different in ABC KO mice as compared with those in ABC WT mice suggesting that drug delivery to the tumor may not be an explanation for the increased efficacy of dasatinib in the ABC KO mice.

Many studies have indicated that pharmacologic inhibition of ABCB1 and ABCG2 with elacridar may overcome the inadequate drug delivery to the brain (40–42). Until recently, chronic multi-dose regimens in preclinical setting were problematic because of poor bioavailability of elacridar (26, 43, 44). Recent development of a microemulsion formulation of elacridar enabled us to test chronic administration of elacridar at clinically relevant doses either orally or intraperitoneally (29). First, to determine whether elacridar in a microemulsion formulation in combination with dasatinib improves the survival of DIPG-bearing mice, tumor-bearing ABC WT mice were treated with either dasatinib alone or in combination with elacridar. It should be noted that this chronic dosing regimen of elacridar microemulsion was well tolerated, in contrast to prior studies (4). It should be noted that we did not measure any active metabolites of dasatinib and the influence of elacridar on the brain or tumor delivery of dasatinib active metabolites (45). This phenomenon of increased efficacy without significant increase in delivery was observed with mebendazole in xenograft models of glioma and medulloblastoma (46). An alternative site besides the BBB for the synergy of elacridar and dasatinib in this model may be at the tumor cell level. In support of this, we also observed that elacridar can sensitize both ABC WT and ABC KO brainstem glioma neurosphere cultures to dasatinib, while others have shown that elacridar can sensitize human glioma cell-lines to dasatinib in vitro (21). In addition, Lin and colleagues have recently noted synergy between elacridar and a promising combination for patients with glioma (ABT888/Temozolomide) at both the BBB and the tumor cell level (47). The reason for the discrepancy between the two studies may be the dosing regimen of elacridar as we used a lower dose of elacridar and that different glioma models were used. Our in vitro results suggest that elacridar may have ABCG2/ABCB1–independent mechanisms to increase the efficacy of dasatinib.
Several investigators have reported that the BBB is compromised in the presence of a tumor (48, 49). To see whether the integrity of the BBB plays a role in regulating dasatinib levels in tumor, we investigated the permeability of 3 kDa TRD in our brainstem glioma model. In a normal BBB, TRD should be completely excluded from the brain on the basis of its size. Indeed, TRD levels were not detected in either brain stem or cortex of nontumor-bearing ABC WT and ABC KO mice. However, significantly higher TRD levels were observed in brainstem of ABC WT and both cortex and brainstem of ABC KO tumor-bearing mice. These data show that BBB is compromised in this brainstem glioma model, providing an explanation as to why dasatinib levels in the tumor-bearing brainstem region do not change between ABC WT and ABC KO mice. It is noteworthy to mention that the BBB remains relatively intact in the cortex of the tumor-bearing mice. Whether the BBB in this brainstem model is more compromised than in the human disease remains to be determined, as TRD measurements cannot be done in humans. Recently, we have used dynamic contrast enhanced MRI to evaluate the BBB in our brainstem glioma model and compared it to genetically identical gliomas induced in the cerebral cortex. Interestingly, we observed that the BBB in our brainstem glioma model is significantly less permeable than genetically identical gliomas induced in the cerebral cortex, although some contrast enhancement in the brainstem gliomas was observed (50).

Finally, it is worth noting that the PDGF-B; p53-deficient brainstem glioma model used in this study does not include the K27M H3.3/3.1 histone mutations recently described in the majority of human DIPGs (5). In addition, although PDGF-B amplifications have been reported in DIPG, it is more common for the PDGFR-α receptor to be gained or amplified in DIPG (13). As PDGF-B activates both PDGFRα and PDGFRβ, the latter of which is present in the vasculature, this model may be particularly sensitive to angiogenesis inhibition. Therefore, the combination of dasatinib + elacridar should also be evaluated in DIPG xenograft models and in genetically engineered mouse models that also include the K27M H3.3/3.1 mutations.

In summary, this study highlights the effect of elacridar on the efficacy of dasatinib in a preclinical model of brainstem glioma. Our data suggest that elacridar increases the efficacy of dasatinib in brainstem glioma without a significant increase in its delivery to the tumor and potentially through ABCB1- and ABCG2-independent mechanisms. Future studies need to investigate the mechanism behind the increased efficacy of dasatinib in the presence of elacridar microenrollment.

Disclosure of Potential Conflicts of Interest
O.J. Becher's spouse is employed as Director, Global Legal Training, in GlaxoSmithKline. No potential conflicts of interest were disclosed by the other authors.

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Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): K.G. Halvorson, G. Hu
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Acknowledgments
The authors thank Taconic Biosciences, Inc. as the source of the ABC KO mice and Dr. Alfred Schnikel of the Netherlands Cancer Institute as the creator of these mice. The authors also thank GlaxoSmithKline for providing elacridar for the in vitro studies.

Grant Support
This work was supported by NIH, National Cancer Institute Grant R01 CA138437 (to W.F. Elmquist), by a St. Baldrick’s Foundation Scholar Award (to O.J. Becher), and by the Rory David Deutsch Foundation (to O.J. Becher). K.G. Parrish was supported by the Ronald J. Sawchuk, Edward G. Rippie, Rowell, American Foundation for Pharmaceutical Education Pre-Doctoral, and the University of Minnesota Doctoral Dissertation Fellowships. 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 February 2, 2015; revised January 13, 2016; accepted February 10, 2016, published OnlineFirst February 16, 2016.

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Molecular Cancer Therapeutics

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