ABCorticosis 2 Transporter Expression Impacts Group 3 Medulloblastoma Response to Chemotherapy

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Abstract

While a small number of plasma membrane ABC transporters can export chemotherapeutic drugs and confer drug resistance, it is unknown whether these transporters are expressed or functional in less therapeutically tractable cancers such as Group 3 (G3) medulloblastoma. Herein we show that among this class of drug transporters, only ABCG2 was expressed at highly increased levels in human G3 medulloblastoma and a mouse model of this disease. In the mouse model, Abcg2 protein was expressed at the plasma membrane where it functioned as expected on the basis of export of prototypical substrates. By screening ABC substrates against mouse G3 medulloblastoma tumorspheres in vitro, we found that Abcg2 inhibition could potentiate responses to the clinically used drug topotecan, producing a more than 9-fold suppression of cell proliferation. Extended studies in vivo in this model confirmed that Abcg2 inhibition was sufficient to enhance anti-proliferative responses to topotecan, producing a significant survival advantage compared with subjects treated with topotecan alone. Our findings offer a preclinical proof of concept for blockade of ABCG2 transporter activity as a strategy to empower chemotherapeutic responses in G3 medulloblastoma.

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Introduction

Medulloblastoma is the most common malignant pediatric brain tumor arising in the posterior fossa. Current treatment that cures about 80% of patients includes resection, radiation, and chemotherapy (1). However, patients who survive often suffer long-term sequelae due to the toxicity of the current therapy. While chemotherapeutic regimens vary amongst treatment centers, current therapy is tailored on the basis of patient’s age at diagnosis, tumor histology, presence of metastasis, and, most recently, molecular subgroup. The current chemotherapeutic regimen for medulloblastoma includes cyclophosphamide, vincristine, and cisplatin, but recurrent tumors are further treated with topotecan (1).

Recent studies identified four major molecular subgroups of medulloblastoma: WNT, SHH, Group 3 (G3), and Group 4 (G4), each of which responds differently to the current therapeutic regimen (1–3). Human G3 medulloblastomas are characterized by overexpression of the c-MYC (MYC) oncogene, with amplification of the gene in about 17% of cases, and large cell/anaplastic tumor histology. Overall, human G3 medulloblastomas have the worst prognosis and are the most difficult to treat (1). Two mouse models that recapitulate human G3 medulloblastomas were recently developed (4, 5). Our mouse model was generated by orthotopic transplantation of granule neural progenitors (GNP) isolated from the cerebella of 5- to 7-day-old Trp53−/−;Cldn2c−/− mice in which Myc is overexpressed by retroviral gene transfer (4). Murine G3 medulloblastoma tumor cells grow as tumorspheres that can be passaged indefinitely in culture and form secondary medulloblastoma similar to the primary tumors when transplanted into the cortices of naive recipient animals, therefore providing a unique opportunity to test novel therapies (4).

ATP-binding cassettes (ABC) transporters are specialized membrane proteins (localized to either plasma membranes or membranes of intracellular organelles) that use ATP hydrolysis to move diverse substances across biologic membranes (6). Within the mouse, ABC transporters comprise 48 distinct proteins arranged within families on the basis of sequence and organization of their ATP-binding domain(s) (7). Some plasma membrane ABC transporters export an array of structurally diverse cancer chemotherapeutic drugs, thereby reducing cytotoxicity. ABCB1, ABCC1, and ABCG2 are among the most highly studied drug-exporting ABC transporters, perhaps because they were first identified secondary to their overexpression in cells that, after sustained chemotherapeutic drug exposure, acquired strong resistance to multiple...
drugs. These changes in ABC transporter expression might impact therapeutic response. Expression of some ABC transporters can be modulated, either up or down, by oncogenic changes within tumor cells (8–11). Recent studies have directly linked either MYC or MYCN transcriptional activation in neuroblastoma to the upregulation of ABCG1, ABCC4, and ABCG2 (12–14). Unexpectedly, ABCB1 was not a direct MYC target, suggesting that its upregulation is indirect (12). Despite this knowledge that MYC, directly or indirectly, regulates ABC transporters, it is unknown whether the therapeutic response of tumors with increased MYC expression is impacted by ABC transporter expression.

Because of the aforementioned poor prognosis and treatment difficulties with human G3 medulloblastoma, we hypothesized that overexpression of ABC transporters might provide a potential mechanism for the failure of some current chemotherapy treatments (15–17). We here report that ABCG2 could be, in part, responsible for the resistance of G3 medulloblastoma to current therapy.

Materials and Methods

Cell culture

Medulloblastoma cells and GNPs were purified by Percoll density gradient from primary mouse G3 medulloblastoma (4) and from the cerebella of 7-day-old Tpr53+/-;Cdh2c2-/- mice, respectively, and cultured as neurospheres, as described previously (4). MB#689 (Myc1), MB# 15486 (Myc2), MB# 9728 (Myc3), and MB# 9730 (Myc4) are tumor spheres derived from two independently arisen mouse G3 medulloblastoma. All mouse G3 medulloblastoma tumor cells were purified from tumors growing into the cortices of mice. Primary tumor spheres were tested for their ability to recapitulate G3 medulloblastoma when reinjected into the brain of naive recipient mice. Tumors were subjected to Affymetrix gene chip array to confirm that they were G3 tumors. MB# 2416 (Myc1-luc) is a tumor sphere derived from Myc1 infected with a virus coexpressing luciferase and the yellow fluorescent protein (YFP; pCL20-SF2-Luc2a-YFP). Murine erythroleukemia cells (Mel) were cultured, as previously described (18). The Mel cell line was a gift from Paul Ney (formerly at St. Jude) and has been maintained at low passage and has not been frozen previously. The Mel cell line was used as control.

Microarray gene analysis

Microarray data were extracted from microarray datasets deposited with the NCBI Gene Expression Omnibus (GEO) and designated with the following accession numbers: Mouse Affymetrix chip (HT430PM), GSE33200 and Human Affymetrix chip (U133v2), GSE37418.

Immunoblotting

Proteins were separated on a 10% SDS-PAGE and immunoblotted with the following primary antibodies: Abcc4 (purified rabbit polyclonal antibody raised against mouse Abcc4 1:1,000; ref. 19), Abcg2 (1:1,000; Abcam), Abcc1 (1:1,000), Abcb1 (puriﬁed rabbit polyclonal antibody raised against human ABCB1, 1:10,000), and transferrin receptor (1:10,000; Abcam). The secondary antibody was horseradish peroxidase-conjugated goat anti-rabbit (1:1,000; GE Healthcare). Proteins were detected by chemiluminescence using reagents from Bio-Rad Laboratories. An antibody to β-actin (1:2,000; clone AC-13, Sigma-Aldrich) was used as control.

ABC transporter localization

Cell surface proteins were biotinylated with non-cell-impermeable biotin sulfo-NHS-Lc-biotin (sulfo-NHS-Lc-biotin), as described earlier (20). The surface biotinylated proteins were captured on streptavidin agarose beads and immunoblot analysis performed on total lysates, streptavidin-captured surface proteins, as well as the supernatants. The plasma membrane levels of the proteins were represented as percentage of total input protein used in the pull-down assay, and transferrin receptor was used as internal control for plasma membrane proteins. Briefly, tumorspheres were rinsed with ice-cold PBS and incubated with 10 mmol/L EZ-Link sulfo-NHS-Lc-biotin solution in PBS (pH 8.0; Pierce) for 30 minutes at room temperature. Unreacted biotin molecules were quenched with ice-cold PBS containing 100 mmol/L glycine for 10 minutes before cell lysis and streptavidin agarose capture.

PMEA accumulation assay

Bis(POM)-PMEA accumulation was performed to assess Abcg2 function, using mouse G3 medulloblastoma tumorspheres. Briefly, single cells were resuspended in serum-free media and incubated with Bis(pivaloyloxymethyl)-9-(2-(phosphonomethoxy)ethyl)-adenine [Bis (POM)-PMEA] (with a trace amount of [3H]Bis (POM)-PMEA) at a final concentration of 10 μmol/L for 6 hours. Tumorspheres were treated with the Abcg4 inhibitor MK571 (5–50 μmol/L) for 30 minutes before adding PMEA. After 6 hours, tumorspheres were lysed with 0.5 mol/L NaOH, and the amount of radioactivity in the lysate was measured by scintillation counting. Intracellular PMEA levels were normalized to the protein concentration in the lysate measured by Bradford assay.

Flow cytometric analysis of Abcg2 function and porphyrins biosynthesis

Abcg2 function in mouse tumorspheres was measured by Hoechst 33342 exclusion assays using FACS. In brief, cells were incubated with either vehicle (DMSO) or 5 μmol/L fumitremorgin C (FTC; Enzo Life Sciences), an inhibitor of Abcg2, for 15 minutes at 37°C, followed by addition of vehicle or Hoechst 33342 (1 μmol/L). After 30 minutes of incubation with the dye, cells were washed and resuspended in fresh media with or without FTC for 1 hour at 37°C to allow efflux of Hoechst 33342. Cells were washed and analyzed by FACS. For protoporphyrin IX (PPIX) analysis, neurospheres were incubated with the PPIX precursor, δ-aminolevulinic acid (ALA:1 mmol/L) for 2 hours at 37°C, and intracellular PPIX levels were determined by FACS, as previously described (21).

Drug screen for Abcg2 substrates

We performed a high-throughput screen of 12 chemotherapeutic drugs that are known ABCG2 substrates on the mouse G3 medulloblastoma Myc1 neurosphere, as previously described (22). Briefly, 1,000 cells per well were plated in 384-well plates. After 24 hours, half of the plates were treated with 10 μmol/L of FTC (Alexis Biochemical), 10 μmol/L of Ko143 (Tocris Bioscience), or 10 μmol/L of Tariquidar (Sigma-Aldrich), incubated for 30 minutes, and all plates were treated with 28 μL of compound in a dose response (final drug concentration, 9.3 μmol/L–0.5 nmol/L).
After 72 hours, the cell number was determined in each well using CellTiter-Glo (Promega). The luminescence signal was measured in an automated Envision plate reader (Perkin-Elmer). Luminescence data were normalized by log₁₀ transformation before calculating the percentage of inhibition using the following equation: \( \frac{100 \times (\text{negative control mean} - \text{compound value})}{(\text{negative control mean} - \text{positive control mean})} \). Cells treated with 1% DMSO served as negative control with 0% inhibition. Cells treated with 35 \( \mu \)mol/L cycloheximide were used as positive control with 100% inhibition.

Apoptosis measurement
A total of \( 5 \times 10^5 \) mouse G3 medulloblastoma tumorspheres were treated with 10 \( \mu \)mol/L FTC or/and 200 nmol/L topotecan for 24, 48, or 72 hours. Apoptosis was measured on \( 3 \times 10^5 \) cells per treatment condition stained with Annexin V (Pharmingen) and 4',6-diamidino-2-phenylindole (DAPI) and analyzed by FACS.

In vivo efficacy of topotecan in combination with an ABCG2 inhibitor, Ko143
A total of \( 1 \times 10^5 \) mouse Myc1-luc tumorspheres were implanted in the cortices of recipient CD-1 nude mice, as previously described (4). For drug efficacy studies, compounds were injected intraperitoneally in Myc1-luc–bearing mice 4 days posttumor implantation, when the luminescence signal reached \( 5 \times 10^5 \) photons. Ko143 (Tocris Bioscience) was dissolved in saline at a concentration of 2.5 mg/mL and injected intraperitoneally at 10 mg/kg. Ko143 is the non-neurotoxic, but equally effective derivative of the specific ABCG2 inhibitor, FTC (23). Topotecan (Sigma) was prepared in saline and injected intraperitoneally at 0.75 mg/kg. Control animals received saline intraperitoneally. Tumor growth was monitored by measuring bioluminescence, as previously described (4). Mice displaying signs of morbidity including head dome, slow moving, seizure, or >20% weight loss were euthanized, the tumor removed, and fixed in 10% formalin. Histopathology was performed to determine tumor grade and presence of necrotic area. All animal studies were conducted according to the NIH guidelines and approved by the St. Jude Children’s Research Hospital Animal Care and Usage Committee.

Expression of Ki67, caspase-3, and Abcg2 by IHC
When animals were moribund, brains were collected and fixed in 10% formalin, sectioned in 4-\( \mu \)m sections, and immunostained with antibodies to cleaved caspase-3 (CP229C, BioCare Medical; 1:500), Ki67 (TA-125-MH, ThermoShandon; 1:500), and Abcg2 (BXP53, Enzo Life Sciences; 1:10,000).

Topotecan plasma pharmacokinetics in the presence or absence of Ko143
Pharmacokinetics studies for topotecan with and without Ko143 were performed in nontumor-bearing mice and are described in detail in Supplementary Materials and Methods.

Results
ABC transporter expression in human and mouse G3 medulloblastoma
RNA expression of 48 ABC transporters was analyzed in human G3 medulloblastoma using the Pediatric Cancer Genome Project database (Supplementary Fig. S1A). These were classified as either ABC transporters that transport drugs or those with a defined biologic function based upon knockout models or human insufficiency. Among the 10 ABC transporters that export drugs, ABCC10, ABCC5, ABCG2, and ABCC1 were the most highly expressed RNAs in human G3 medulloblastoma (Fig. 1A). Affymetrix gene chip array analysis of purified tumor cells from 7 independently derived mouse G3 medulloblastoma and GNP's purified from the cerebella of four
mice of different litters revealed elevated RNA expression of 6 of the 10 ABC transporters with consistently high expression of Abcc4 and Abcg2 but much lower and more variable levels of Abcb1 and Abcc1 in purified tumor cells compared with GPNPs (Fig. 1B and Supplementary Fig. S1B).

We confirmed protein expression of three ABC transporters by immunoblotting from total cell lysates from 5 independently derived mouse G3 medulloblastoma neurospheres, Myc1-5 (Fig. 1C, lanes 1–5) and tumor chunks from three independently derived mouse G3 medulloblastoma (Fig. 1C, lanes 6–8). Abcc4, Abcg2, and Abcc1 proteins were expressed in all samples, with the exception of Abcc1 with lower levels in Myc2 tumorspheres (Fig. 1C, lane 2).

ABC transporters are functional in mouse G3 medulloblastoma

Protein expression of ABC transporters in tumor cell lysates does not consider intracellular localization, an important consideration given ABC transporter plasma membrane localization is affected by oncogenic signals (24). To investigate the localization of Abcc1, Abcc4, Abcg2, and Abcb1 in mouse G3 medulloblastoma–derived tumorspheres, we performed surface membrane biotinylation with non–cell-permeable NHS-SS-biotin, as previously described (Fig. 2; ref. 20). We found that greater than 50% of total Abcc1 and Abcc4 were expressed at the plasma membrane in Myc1 tumorspheres (Fig. 2A, left). In contrast, in the same cells, we detected less than 12% of Abcg2 and 10% of Abcb1 (Fig. 2A, right). We confirmed the localization of Abcg2 by IHC on Myc1 tumorspheres and found Abcg2 staining on the plasma membrane and in the cytoplasm (Supplementary Fig. S2, right).

We assessed the efflux function of Abcc4 by measuring PMEA drug accumulation in the presence and absence of an Abcc4 inhibitor, MK571. Using 10 μmol/L of radiolabeled Bis (POM) PMEA along with increasing concentrations of MK571 (5–50 μmol/L), we found radiolabeled PMEA accumulation increased with the dose of MK571 (Fig. 2B). Taken together, these results further confirm that in mouse G3 medulloblastoma tumorspheres, Abcc4 is localized to the plasma membrane and is functional.

Abcg2 function was evaluated by measuring export of the fluorescent substrate, Hoechst 33342 using FACS analysis (Fig. 2C and D). Mel cells were used as positive control (Fig. 2C, top). Tumorspheres Myc1-luc and Myc2, incubated in the presence of FTC, an Abcg2-specific inhibitor (23), accumulated...
more intracellular Hoechst 33342 compared with tumorspheres incubated with the Hoechst dye alone (Fig. 2C, middle and bottom, respectively). These data were quantified as shown in Fig. 2D.

Protoporphyrin IX (PPIX), the fluorescent penultimate precursor to heme, is a well-documented, specific endogenous ABCG2 substrate (21, 25), and tumors accumulate high levels of PPIX, which might be due to either elevation of heme/porphyrin biosynthetic genes or reduced expression of ABCG2. We evaluated the RNA level of genes related to heme/porphyrin biosynthesis (26, 27), in mouse G3 medulloblastomas and GNPs (Fig. 3A and Supplementary Table S1). Mouse G3 medulloblastomas had strong upregulation of this set of heme/porphyrin biosynthetic genes. We tested whether these genes were functional by briefly incubating neurospheres with the PPIX precursor, ALA, followed by FACS analysis to determine cellular PPIX concentrations (Fig. 3B and C; ref. 21). Mel cells incubated with ALA (Fig. 3B, top left), tumorspheres Myc1 (Fig. 3B, bottom left), Myc1-luc (Fig. 3B, top right), and Myc2 (Fig. 3B, bottom right) had increased PPIX mean fluorescence intensity compared with control. PPIX levels were quantified in Fig. 3C. Inhibition of Abcg2, by FTC during the ALA incubation, strongly increased the intracellular PPIX, indicating that Abcg2 is highly functional in tumorspheres.

**ABC transporters and in vitro drug resistance of mouse G3 medulloblastoma**

Myel was treated with 12 chemotherapeutic drugs, known ABCG2 substrates (18), in a dose response (1 nmol/L–10 μmol/L), with or without 10 μmol/L of the specific ABCG2 inhibitor FTC (Fig. 4A; ref. 23). Cell proliferation was measured by CellTiter Glo 72 hours after addition of the drug. FTC either did not or minimally changed (~3.5-fold) the EC50 value of 11 of the chemotherapeutic drugs but enhanced the potency of topotecan from 110 to 12 nmol/L when tumor cells were pretreated with FTC (Table 1). A similar screen was performed on Myel neurospheres with 10 μmol/L of Ko143 or Tariquidar (Supplementary Fig. S3). Addition of Ko143 (green curves) or Tariquidar (red curves) improved the potency of topotecan from 130 to 39 and 46 nmol/L, respectively. Similar results were observed for mitoxantrone or methotrexate (Supplementary Fig. S3 and Supplementary Table S2). Tariquidar had a more pronounced effect in potentiating the chemotherapeutic drugs, which might be explained by the fact that Tariquidar is a dual inhibitor of ABCG2 and Pgp (28).

The reduction in topotecan EC50 by CellTiter Glo values might be due to either reduced proliferation or increased apoptosis. To determine whether topotecan and FTC increased apoptosis of
mouse G3 medulloblastoma \textit{in vitro}, Myc1 cells were treated with 10 μmol/L of FTC, followed by 10 nmol/L topotecan. Cells were pelleted 24, 48, or 72 hours after and analyzed for apoptosis by annexin V staining followed by FACS analysis. Treatment with FTC alone or topotecan alone did not increase apoptosis compared with control at 24 hours (Fig. 4B, green and blue bars, respectively), but each compound appeared to produce a slight increase in apoptosis at 48 or 72 hours, with a strongest effect for topotecan as single agent. In contrast, topotecan and FTC treatment increased apoptosis, modestly at 24 hours and strongly at 48 or 72 hours (Fig. 4B, red bars).

ABCG2 inhibition increases topotecan cytotoxicity on mouse G3 medulloblastoma \textit{in vivo}

To determine the effect of inhibiting ABCG2 function on topotecan plasma disposition, we used Ko143, as it is the non-neurotoxic, but equally effective derivative of the specific ABCG2 inhibitor, FTC (23). We first performed a study in nontumor-bearing CD1 nude mice by intraperitoneal injection of topotecan at a dosage of 0.75 mg/kg, as a single agent, or in combination with 10 mg/kg of Ko143. Topotecan plasma concentration–time profiles were well represented by a 2-compartment model. Topotecan was rapidly absorbed after intraperitoneal dosing, followed by biphasic disposition (Fig. 5A and B). Pharmacokinetic parameters representing plasma disposition of topotecan are listed in Supplementary Table S3. We found no significant difference ($P = 0.837$) in topotecan plasma exposure when it was administered as a single agent or in combination with Ko143 (Fig. 5C).

To determine how efficacious topotecan and the ABCG2 inhibitor Ko143 were in suppressing proliferation of mouse G3 medulloblastoma \textit{in vivo}, we stereotactically transplanted 1 × 10^5 purified mouse G3 medulloblastoma luciferase marked tumor

Table 1. Chemotherapeutic drugs tested on mouse G3 medulloblastoma tumorspheres

<table>
<thead>
<tr>
<th>Chemotherapeutic drugs</th>
<th>–</th>
<th>FTC (EC_{50}, μmol/L)</th>
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<tbody>
<tr>
<td>Topoisomerase inhibitors</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mitoxantrone (Topo II)</td>
<td>0.0061 (0.0047–0.008)</td>
<td>0.0019 (0.0013–0.0027)</td>
</tr>
<tr>
<td>Daunorubicin</td>
<td>0.92 (0.22–1.1)</td>
<td>0.77 (0.21–1.1)</td>
</tr>
<tr>
<td>Doxorubicin</td>
<td>1 (0.8–1.5)</td>
<td>0.83 (0.94–1)</td>
</tr>
<tr>
<td>Camptothecin analogues</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Topotecan</td>
<td>0.11 (0.092–0.16)</td>
<td>0.032 (0.0085–0.013)</td>
</tr>
<tr>
<td>Tyrosine kinase inhibitors</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dasatinib</td>
<td>7.1 (5.4–7.1)</td>
<td>NaN</td>
</tr>
<tr>
<td>Erlotinib</td>
<td>NaN</td>
<td>NaN</td>
</tr>
<tr>
<td>Imatinib</td>
<td>3.8 (3–5.1)</td>
<td>NaN</td>
</tr>
<tr>
<td>Antimetabolites</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Methotrexate</td>
<td>0.00092 (0.0006–0.0012)</td>
<td>0.00026 (0.00018–0.00035)</td>
</tr>
<tr>
<td>Trimetrexate</td>
<td>0.12 (0.034–0.58)</td>
<td>0.11 (0.044–0.38)</td>
</tr>
<tr>
<td>Cladribine</td>
<td>0.086 (0.077–0.11)</td>
<td>0.065 (0.056–0.072)</td>
</tr>
<tr>
<td>Other anticancer drugs</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Teniposide</td>
<td>0.0067 (0.0045–0.012)</td>
<td>0.0047 (0.0027–0.0081)</td>
</tr>
<tr>
<td>Flavopiridol</td>
<td>1 (0.11–1)</td>
<td>0.64 (0.53–0.88)</td>
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**NOTE:** EC_{50} (with upper and lower bound on EC_{50} 95% confidence interval) for 12 chemotherapeutic drugs in the absence (–) or presence (+) of FTC, an ABCG2 inhibitor.

Abbreviation: NaN, no activity.
cells (Myc1-luc) in the cortices of naïve recipient mice. We previously determined that this number of tumor cells induced medulloblastoma that kill the animals within 15 days posttransplant and that the resulting tumors faithfully recapitulate the primary tumors (4, 22).

The topotecan schedule and dosage for the efficacy study was determined on the basis of data published by Thompson and colleagues (29), simulation of plasma exposure from previous pharmacokinetic studies in mice (30), and optimal topotecan exposure required for treatment of patients with pediatric medulloblastoma (31). The dosing regimen of topotecan used in the present study of 0.75 mg/kg, administered intraperitoneally daily for 5 d/wk for 2 consecutive weeks was estimated to produce, after a single dose, a topotecan plasma exposure similar to that reported in pediatric patients (140 ng/mL; ref. 31). Tumor development was measured by luminescence imaging (Fig. 6A). Mice bearing mouse G3 medulloblastoma line Myc1-luc were treated by intraperitoneal injections of topotecan at 0.75 mg/kg, Ko143 at 10 mg/kg, or the combination of both drugs 5 d/wk, 2 weeks in a row (Fig. 6A and B). When combined, administration of Ko143 was given 30 min after topotecan to allow Ko143 to take effect before administration of both drugs 5 d/wk, 2 weeks in a row (Fig. 6A and B).

The in vivo sensitivity of mouse G3 medulloblastoma to topotecan has not been previously reported. As single agents, both topotecan and Ko143 restrained tumor growth (Fig. 6A). The combination of topotecan and Ko143 strongly suppressed tumor growth. Log transformation of the G3 medulloblastoma growth curves revealed that the rate of tumor expansion was profoundly reduced more than 2.40% when topotecan was combined with Ko143 (Fig. 6A, insert). In contrast, treatment with topotecan or Ko143 alone only modestly reduced the rate of tumor expansion compared with control by approximately 24% (Fig. 6A).

As expected, mice treated with saline only survived 17 days after transplant (Fig. 6B). While not statistically significant, Ko143-treated mice increased survival from 17 to 20 days. Topotecan alone increased survival of the treated mice by 7 days, from 17 to 24 days. Treatment of mice with topotecan and Ko143 had an 11-day increased survival (from 17 to 28 days) compared with saline-treated animals (Fig. 6B). Mice tolerated the treatments well and we saw no changes in their weight regardless of the regimen used (Supplementary Fig. S4A).

We measured the mitotic index by immunostaining of tumors from animals treated with Ko143, topotecan, or both drugs together with antibodies to Ki67 to measure proliferation (Fig. 6C and D) and caspase-3 to measure apoptosis (Fig. 6E and F). Tumors from animals treated with Ko143 alone had a high mitotic index (91% ± 2.54%) and few areas of active caspase-3 (6% ± 2.1%), which is similar to tumors from vehicle-treated animals (mitotic index, 5.2% ± 1.22%; caspase-3, 85.03% ± 2.52%). In contrast, tumors from mice treated with topotecan alone displayed a decrease in mitotic index (77% ± 10.6%) and a slight increase in caspase-3 staining (12% ± 2.1%). Tumors from animals treated with both drugs shared the same mitotic index as the topotecan-treated tumors (76% ± 9.8%) but displayed almost a 3-fold increase in caspase-3 staining (33% ± 16.1%, P = 0.0319).

Topotecan therapy might only eradicate tumors that express ABCG2. To determine whether topotecan therapy affected Abcg2 protein levels in mouse G3 medulloblastoma, tumors were isolated from moribund animals and immunoblotting performed on total cell lysates (Fig. 6G and H). Abcg2 expression was similar among the mice treated with vehicle, Ko143, or topotecan. The combination of topotecan and Ko143 treatment appeared to increase Abcg2 expression but not significantly. Abcg2 expression was confirmed by IHC on tumors from control or treated animals (Supplementary Fig. S4B).

**Discussion**

We here report the expression and function of ABC transporters, ABCC4 and ABCG2, in G3 medulloblastoma. We demonstrate that, among ABC transporters of drugs, ABCG2 RNA was highly expressed in both human and mouse G3 medulloblastoma and was functional by using a combination of specific substrates and inhibitors. Screening of chemotherapeutic drugs that are known ABCG2 substrates with the specific ABCG2 inhibitor, FTC (23), revealed that topotecan was the most readily modulated in vitro. These studies were extended in vivo to show that topotecan-induced apoptosis of mouse G3 medulloblastoma increased when Abcg2 was specifically inhibited and suggested that Abcg2 expression might account for the resistance of G3 medulloblastoma to chemotherapy regimens, especially those including drugs that are substrates for ABCG2 like topotecan.
Figure 6. Inhibition of Abcg2 increases topotecan efficacy in vivo. Mice injected at day 0 with $1 \times 10^5$ luciferase-positive G3 medulloblastoma tumorsphere (Myc1-luc) were treated when luciferase reached $5 \times 10^5$ photons. Mice were treated daily for 5 days for 2 consecutive weeks. Luciferase measurement (A) and survival (B) of mice treated with vehicle (black lines), 10 mg/kg Ko143 (green lines), 0.75 mg/kg topotecan (blue lines), or both drugs (red lines). Topotecan treatment increased mouse survival by 7 days (from 17 to 24 days, $P = 0.0016$). Ko143 and topotecan treatment increased survival by 11 days (from 17 to 28 days, $P = 0.0002$ compared with vehicle; $P = 0.0165$ compared with topotecan alone). Drug-treated group, $n = 10$; saline-treated group, $n = 7$. Immunostaining (C) and quantification (D) of Ki67-positive cells in tumors from mice treated with vehicle (black bar), Ko143 (green bar), topotecan (blue bar), and both (red bar). Immunostaining (E) and quantification (F) of caspase-3-positive cells in tumors from mice treated with vehicle (black bar), Ko143 (green bar), topotecan (blue bar), and both (red bar; control vs. topotecan and Ko143, $P = 0.0319$). Immunoblotting (G) and quantification (H) of Abcg2 protein expression in tumors from animals treated with vehicle (black bar, lanes 1, 2), Ko143 (green bar, lanes 3–5), topotecan (blue bar, lanes 6, 7), and both (red bar, lanes 8 and 9).
ABC transporter expression and function in G3 medulloblastoma versus other subgroups of medulloblastoma, brain tumors, and other cancers

Since the identification of the first ABC transporter P-glycoprotein (Pgp/MDR1), a total of 48 ABC transporters have been identified, found to be differentially expressed in multiple tissues, including the central nervous system (CNS), with increased expression of some related to increased resistance to chemotherapy and radiotherapy (32). A RNA expression survey demonstrated that of 10 recognized drug-transporting ABC transporters in human G3 medulloblastoma, ABCC10, ABCC5, ABCC1 (MRP1), and ABCG2 (BRCP) were the most highly expressed, whereas in the mouse G3 medulloblastoma model, Abcc4 and Abcg2 were consistently expressed and, to a lesser extent, Abcc1 and Abcl1, but not Abcc10 or Abcc5. Cell surface expression and functional efflux studies with specific inhibitors identified Abcg2 and Abcc4 as the ABC transporters with potential functional roles in chemotherapeutic resistance in mouse G3 medulloblastomas. Because ABCG2 was the most highly expressed in both mouse and human G3 medulloblastoma, it was of particular interest for therapeutic regimens that might use its substrates.

Topotecan and G3 medulloblastoma

ABCG2, initially cloned from a drug resistant cell line that did not express either ABCB1 (P-glycoprotein) or ABCB1 (MRP1) protein (33), was subsequently found to be strongly expressed at the apical membrane of polarized cells including the blood–brain barrier (BBB), limiting penetration of some drugs to the brain (34, 35). Previous studies showed, in tumor cell lines, that ABCG2 conferred resistance to various chemotherapeutic drugs, including topotecan (36). However, established tumor cell lines do not always recapitulate ABC transporter expression and function in vitro (37). Topotecan treatment of mice harboring mouse G3 medulloblastoma enhanced survival when Abcg2 was inhibited by the highly selective inhibitor Ko143 (23). Strikingly, these in vitro findings extend the in vivo results demonstrating that Abcg2 inhibition in G3 medulloblastoma tumorspheres enhanced both topotecan-induced apoptosis and suppression of proliferation. In mice, topotecan treatment with the Abcg2 inhibitor, Ko143, was well tolerated with no evidence of enhanced gastrointestinal toxicity or weight loss (Supplementary Fig. S4D). Topotecan, an inhibitor of topoisomerase activity, is currently used in the treatment of recurrent medulloblastoma (1, 38). However, the high expression of ABCG2 in human G3 medulloblastomas suggests that concurrent addition of a potent and selective inhibitor of ABCG2, like Ko143, might be warranted to improve outcomes to topotecan therapy without increasing toxicity.

The almost 3-fold increase in apoptosis of G3 medulloblastoma tumors in vivo, produced by the combination of topotecan and Ko143, is compatible with the suppression of ABCG2-mediated export of topotecan. However, the salutary effect of ABCG2 inhibition in vivo is complicated because Ko143 can have an impact on ABCG2, both in the tumor and in the BBB (39). However, we have previously demonstrated, in a SHH intracranial transplant model, that the BBB is compromised (40). Under these circumstances, Ko143 is likely blocking tumor-catalyzed Abcg2-mediated export of topotecan. This interpretation is consistent with both the increased tumor apoptosis in vivo and our findings in vitro showing Ko143 increased the topotecan sensitivity of G3 medulloblastoma cell 9-fold. Moreover, the overall reduction in Abcg2 expression in tumor samples posttreatment suggests that the treatment of topotecan and Ko143 eradicates medulloblastoma-expressing Abcg2. While these findings suggest that this combination therapy is efficacious, it is unknown whether this would be effective in the treatment of brain tumors because it is unclear whether the integrity of the BBB is disrupted in all children with medulloblastoma (41). Indeed, recent data suggest that the structure and function of the BBB varies markedly among the various subtypes of medulloblastoma (Richard Gilbertson, personal communication). Nonetheless, these findings suggest more favorable outcomes with topotecan might be achieved by inhibition of both BBB and ABCG2 function.

Surprisingly, in vitro cytotoxicity screening of the G3 medulloblastoma mouse line (Myc1) for sensitivity to a dozen ABCG2 chemotherapeutic drug substrates (42) revealed that tumor sensitivity; for a small subset of these drugs (i.e., mitoxantrone, topotecan, and methotrexate), was only enhanced by the specific ABCG2 inhibitor, FTC (23). A variety of potential explanations might account for this unexpected finding, including chemotherapeutic drug concentrations below the affinity of ABCG2 for its drug substrate. However, a simpler explanation might be that another transporter highly expressed at the plasma membrane in G3 medulloblastomas might also transport these drugs, thereby accounting for the unanticipated lack of a cure when ABCG2 was inhibited. ABCC4 is a good candidate because it is functional and highly expressed in both the cultured G3 medulloblastoma lines and the freshly isolated tumors. ABCC4, originally identified as a nucleotide exporter (43), was subsequently shown to confer resistance to a broader range of chemotherapeutic substrates (e.g., camptothecins; ref. 19). In addition, ABCG2 and ABCC4 have some similar substrates (44). Among the ABCC4 substrates tested in the tumorspheres were an antifolate (methotrexate; ref. 19), kinase inhibitor (dasatinib; ref. 45), and camptothecin (topotecan). Topotecan is readily transported by human and murine ABCC4 (19); thus, it is conceivable that a dual inhibitor, producing combined inhibition of both ABCC4 and ABCG2, might yield greater overall improvements in survival.

MYC, ABC transporters, and resistance to therapy

Some ABC transporters were previously shown to be overexpressed in primary human pediatric medulloblastoma (32), but ABCG2 expression in human G3 medulloblastomas was not evaluated. ABCG2 was of particular interest in G3 medulloblastoma because it is overexpressed in both mouse and human tumors, is implicated as a cancer stem cell marker (46), and is responsible for maintaining a nondifferentiating state (47, 48). Furthermore, solid tumor cells harboring functional ABCG2 exhibit intrinsic properties such as pluriptocity, greater growth capacity, improved survival rate after cytotoxic drug exposure, and enhanced tumorigenicity (49, 50). G3 medulloblastomas display stem cell behavior that is recapitulated in the mouse model, which might be one reason for its resistance to current treatment. Indeed, mouse G3 medulloblastomas express several markers of stemness, including Nestin and Sox2 (4). In addition, overexpression of MYC, the hallmark of G3 medulloblastomas and MYCN in recurrent SHH subgroup medulloblastoma, contributes to the
coordinated transcriptional regulation of a large set of ABC transporters, including ABCG2 (12). Therefore, ABCG2 might be added as a prognostic marker to identify potentially recurrent or aggressive medulloblastoma.

**Conclusion**

The identification of ABCG2 as a potential factor of chemoresistance in G3 medulloblastoma suggests that potent and selective inhibitors of ABCG2 could be used to improve response to chemotherapeutic drug as substrates of ABCG2, including topotecan.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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