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ABCG2 transporter expression impacts Group3 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 if these transporters are expressed or functional in less therapeutically tractable cancers such as Group3 medulloblastoma (G3 MB). Herein we show that among this class of drug transporters only ABCG2 was expressed at highly increased levels in human G3 MB and a mouse model of this disease. In the mouse model, Abcg2 protein was expressed at the plasma membrane where it functioned as expected based on export of prototypical substrates. By screening ABC substrates against mouse G3 MB tumorspheres in vitro, we found that Abcg2 inhibition could potentiate responses to the clinically employed drug topotecan, producing a >9-fold suppression cell proliferation. Extended studies in vivo in this model confirmed that Abcg2 inhibition was sufficient to enhance antiproliferative responses to topotecan, producing a significant survival advantage compared to 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 Group3 medulloblastoma.

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ATP Binding Cassette (ABC) Transporters; Abcg2; Group 3 (G3) medulloblastoma; chemotherapy; MYC; Trp53; topotecan; Ko143

Introduction

Medulloblastoma (MB) is the most common malignant pediatric brain tumor arising in the posterior fossa. Current treatment that cures ~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 based on 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, Group3 (G3) and Group4 (G4), each of which respond differently to the current therapeutic regimen (1–3). Human G3 MBs are characterized by overexpression of the c-MYC (MYC) oncogene, with amplification of the gene in ~17% of cases, and large cell/anaplastic tumor histology. Overall, human G3 MBs have the worst prognosis and are the most difficult to treat (1). Two mouse models that recapitulate human G3 MBs were recently developed (4,5). Our mouse model was generated by orthotopic transplantation of granule neural progenitors (GNPs) isolated from the cerebella of 5 to 7 day old *Trp53*^{-/-}; *Cdkn2c*^{-/-} mice in which Myc is overexpressed by retroviral gene transfer (4). Murine G3 MB 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 naïve 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 biological membranes (6). Within the mouse, ABC transporters comprise 48 distinct proteins arranged within families based on 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 c-MYC (MYC) or MYCN transcriptional activation in neuroblastoma to the upregulation of ABCC1, ABCC4, and ABCG2 (12–14). Unexpectedly, ABCB1 was not a direct MYC target, suggesting its up-regulation is indirect (12). Despite this knowledge that MYC, directly or indirectly, regulates ABC transporters, it is unknown if the therapeutic

response of tumors with increased MYC expression is impacted by ABC transporter expression.

Due to the aforementioned poor prognosis and treatment difficulties with human G3 MB, 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 MB to current therapy.

Material and Methods

Cell culture

Medulloblastoma cells and GNPs were purified by Percoll density gradient from primary mouse G3 MB (4) and from the cerebella of 7 days old *Trp53*^{-/-}; *Cdkn2c*^{-/-} mice, respectively, and cultured as neurospheres, as described previously (4). MB#689 (Myc1) MB# 15486 (Myc2), MB# 9728 (Myc3) and MB# 9730 (Myc4) are tumorsphere lines derived from two independently arisen mouse G3 MB. All mouse Group3 medulloblastoma tumor cells were purified from tumors growing into the cortices of mice and used immediately for experiments. Primary tumor lines were tested for their ability to recapitulate Group3 medulloblastoma when re-injected into the brain of naïve recipient mice. Tumors were subjected to Affymetrix gene chip array to confirm that they were Group3 tumors. MB# 2416 (Myc1-luc) is a tumorsphere line derived from Myc1 infected with a virus co-expressing luciferase and the yellow fluorescent protein (YFP) (VCL20SF2-Luc2aYFP). 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 tested. Bone marrow was isolated from the femur of *ABCG2*-null mice bred in our facility, as described previously.

Microarray gene analysis

Microarray data were extracted from microarray data sets 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 gel and immunoblotted with the following primary antibodies: Abcc4 (purified rabbit polyclonal antibody raised against mouse Abcc4 1:1,000) (19), Abcg2 (1:1,000) (Abcam, Cambridge, MA), Abcc1 (1:1,000) (Santa Cruz, CA), Abcb1 (purified rabbit polyclonal antibody raised against human ABCB1, 1:10,000), transferrin receptor (1:10,000) (Abcam, Cambridge, MA). The secondary antibody was HRP-conjugated goat anti-rabbit (1:1,000) (GE Healthcare). Proteins were detected by chemiluminescence using reagents from Bio-Rad Laboratories, CA. An antibody to β -Actin (1:2000) (clone AC-13, Sigma-Aldrich) was used as control.

ABC transporter localization

Cell-surface proteins were biotinylated with cell-impermeable biotin sulfo-succinimidyl-2-(biotinamido) ethyl-1, 3-dithiopropionate (sulfo-NHS-SS-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 from the assay. The plasma membrane levels of the proteins were represented as percentage of total input protein used in the pull down assay and transferrin receptor (TFR) was used as internal control for plasma membrane proteins. Briefly, tumorspheres were rinsed with ice-cold PBS and incubated with 10 mM EZ-Link sulfo-NHS-SS-biotin solution in PBS (pH 8.0) (Pierce) for 30 min at room temperature. Unreacted biotin molecules were quenched with ice-cold PBS containing 100mM glycine for 10 min before cell lysis and streptavidin-agarose capture.

PMEA accumulation assay

Bis(POM)-PMEA accumulation was performed to assess Abcc4 function, using mouse G3 MB tumorspheres. Briefly, single cells were re-suspended in serum-free media and incubated with Bis(pivaloyloxymethyl)-9-[2-(phosphonmethoxy)ethyl]-adenine [Bis (POM)-PMEA] (with a trace amount of [³H] Bis (POM)-PMEA) at a final concentration of 10 μ M for 6 hr. Tumorspheres were treated with the Abcc4 inhibitor MK571 (5–50 μ M) for 30 minutes prior to adding PMEA. After 6hrs, tumorspheres were lysed with 0.5 M 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 fluorescence-activated cell sorting (FACS). In brief, cells were incubated with either vehicle (DMSO) or 5 μ M fumitremorgin C (FTC, Enzo Life Sciences, Plymouth Meeting, PA), an inhibitor of porphyrin synthesis, for 15 min at 37°C, followed by addition of vehicle or Hoechst 33342 (1 μ M). After 30 min of incubation with the dye, cells were washed and re-suspended in fresh media with or without FTC for one 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 mM) for 2 hr 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 twelve chemotherapeutic drugs that are known ABCG2 substrates on the mouse G3 MB Myc1 neurosphere, as previously described (22). Briefly, 1000 cells per well were plated in 384-well plates. After 24 hr, half of the plates were treated with 10 μ M of FTC (Alexis Biochemical, San Diego, CA, USA), 10 μ M of Ko143 (Tocris Bioscience, Bristol, UK), or 10 μ M of Tariquidar (Sigma-Aldrich, USA), incubated for 30 minutes, and all plates were treated with 28 nl of compound in a dose-response (final drug concentration 9.3 μ M to 0.5 nM). After 72 hr, 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 prior to calculating the percentage of inhibition using the following equation: $100 \times (\text{negative control mean} - \text{compound value}) / (\text{negative control mean} - \text{positive control mean})$. Cells treated with 1% DMSO served as negative control with 0% inhibition. Cells treated with 35 μM cycloheximide were used as positive control with 100% inhibition.

Apoptosis measurement

5×10^5 mouse G3 MB tumorspheres were treated with 10 μM FTC or/and 200 nM topotecan for 24, 48 or 72 hr. Apoptosis was measured on 3×10^5 cells per treatment condition stained with Annexin V (Pharmingen) and DAPI and analyzed by FACS.

In vivo efficacy of topotecan in combination with an Abcg2 inhibitor, Ko143

1×10^5 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 (IP) in Myc1-luc-bearing mice 4 days post-tumor implantation, when the luminescence signal reached 5×10^5 photons. Ko-143 (Tocris Bioscience, Bristol, UK) was dissolved in saline at a concentration of 2.5 mg/mL and injected IP at 10 mg/kg. Ko143 is the non-neurotoxic, but equally effective derivative of the specific ABCG2 inhibitor, FTC (23). Topotecan (Sigma, St Louis, MO, USA) was prepared in saline and injected IP at 0.75 mg/kg. Control animals received saline IP. 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 immunohistochemistry

When animals were moribund, brains were collected and fixed in 10% formalin, sectioned in 4 micron sections and immunostained with antibodies to cleaved caspase 3 (CP229C, BioCare Medical, Concord, CA, 1/500), Ki67 (TA-125-MH, ThermoShandon, Fremont, CA, 1:500), and Abcg2 (BXP53, Enzo Life Sciences, Farmingdale, NY, 1:10,000).

Topotecan plasma pharmacokinetics in the presence or absence of Ko143

Pharmacokinetics studies for topotecan with and without Ko143 were performed in non-tumor bearing mice, and are described in detail in Supplementary Material and Methods.

Results

ABC transporter expression in human and mouse G3 MB

RNA expression of 48 ABC transporters was analyzed in human G3 MB using the Pediatric Cancer Genome Project (PCGP) database (Supplementary Fig. 1A). These were classified as

either ABC transporters that transport drugs or those with a defined biological function based upon knockout models or human insufficiency. Among the ten ABC transporters that export drugs, *ABCC10*, *ABCC5*, *ABCG2* and *ABCC1* were the most highly expressed RNAs in human G3 MB (Fig. 1A). Affymetrix gene chip array analysis of purified tumor cells from seven independently-derived mouse G3 MB and GNPs purified from the cerebella of four mice of different litters revealed elevated RNA expression of six of the ten 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 to GNPs (Fig. 1B, Supplementary Fig. 1B).

We confirmed protein expression of three ABC transporters by immunoblotting from total cell lysates from five independently-derived mouse G3 MB neurospheres, Myc1-5 (Figure 1C, lanes 1, 2, 3, 4, 5), and tumor chunks from 3 independently-derived mouse G3 MB (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 MB

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 MB-derived tumorspheres, we performed surface membrane biotinylation with non-cell permeable NHS-SS-biotin, as previously described (Figure 2) (20). We found greater than 50% of total *Abcc1* and *Abcc4* were expressed at the plasma membrane in Myc1 tumorspheres (Figure 2A, left panel). In contrast, in the same cells, we detected less than 12% of *Abcg2* and 10% of *Abcb1* (Fig. 2A, right panel). We confirmed the localization of *Abcg2* by immunohistochemistry (IHC) on Myc1 tumorspheres and found *Abcg2* staining on the plasma membrane and in the cytoplasm (Supplementary Fig. 2, right panel).

We assessed the efflux function of *Abcc4* by measuring PMEA drug accumulation in the presence and absence of an *Abcc4* inhibitor, MK571. Using 10 μ M of radiolabelled Bis (POM) PMEA along with increasing concentrations of MK571 (5–50 μ M), we found radiolabeled PMEA accumulation increased with the dose of MK571 (Fig. 2B). Taken together, these results further confirm that in mouse G3 MB 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, 2D). Mel cells were used as positive control (Fig. 2C, top panel). Tumorspheres Myc1-luc and Myc2, incubated in the presence of FTC, an *Abcg2* specific inhibitor (23), accumulated more intracellular Hoechst 33342 compared to tumorspheres incubated with the Hoechst dye alone (Fig. 2C, middle and bottom panels, respectively). These data were quantified as shown in Figure 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 MBs and GNP (Fig. 3A and Supplementary Table 1). Mouse G3 MBs had strong up-regulation of this set of heme/porphyrin biosynthetic genes. We tested if these genes were functional by briefly incubating neurospheres with the PPIX precursor, δ -aminolevulinic acid (ALA), followed by FACS analysis to determine cellular PPIX concentrations (Figs. 3B, 3C) (21). Mel cells incubated with ALA (Fig. 3B, top left panel) and tumorspheres Myc1 (Fig. 3B, bottom left panel), Myc1-luc (Fig. 3B, top right panel) and Myc2 (Fig. 3B, bottom right panel) had increased PPIX mean fluorescence intensity compared to control. PPIX levels were quantified in Figure 3C. Inhibition of Abcg2, by FTC during the ALA incubation, strongly increased the intracellular PPIX indicating Abcg2 is highly functional in tumorspheres.

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

Myc1 was treated with twelve chemotherapeutic drugs, known ABCG2 substrates (18), in a dose response (1 nM–10 μ M), with or without 10 μ M of the specific ABCG2 inhibitor FTC (Fig. 4A) (23). Cell proliferation was measured by CellTiter Glo 72 hr after addition of the drug. FTC either did not or minimally changed (<3.5 fold) the EC₅₀ value of eleven of the chemotherapeutic drugs, but enhanced the potency of topotecan from 110 nM to 12 nM when tumor cells were pre-treated with FTC (Table 1). A similar screen was performed on Myc1 neurospheres with 10 μ M of Ko143 or Tariquidar (Supplementary Fig. 3). Addition of Ko143 (green curves) or Tariquidar (red curves) improved the potency of topotecan from 130 nM to 39 nM and 46 nM, respectively. Similar results were observed for mitoxanthrone or methotrexate (Supplementary Fig. 3 and Supplementary Table 2). 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 if topotecan and FTC increased apoptosis of mouse G3 MB *in vitro*, Myc1 cells were treated with 10 μ M of FTC, followed by 10 nM of topotecan. Cells were pelleted 24, 48 or 72 hr 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 to control at 24 hr (Fig. 4B, green and blue bars, respectively), but each compound appeared to produce a slight increase in apoptosis at 48 or 72 hr, with a strongest effect for topotecan as single agent. In contrast, topotecan and FTC treatment increased apoptosis, modestly at 24 hr and strongly at 48 or 72 hr (Fig. 4B, red bars).

ABCG2 inhibition increases topotecan cytotoxicity on mouse G3 MB *in vivo*

To determine the effect of inhibiting ABCG2 function on topotecan plasma disposition, we used Ko143 since it is the non-neurotoxic, but equally effective derivative of the specific ABCG2 inhibitor, FTC (23). We first performed a study in non-tumor bearing CD1 nude mice by IP 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 IP dosing, followed by biphasic disposition (Figs. 5A and 5B). Pharmacokinetic parameters

representing plasma disposition of topotecan were listed in Supplementary Table 3. 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 MB *in vivo*, we stereotactically transplanted 1×10^5 purified mouse G3 MB luciferase marked tumor 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 post-transplant, and that the resulting tumors faithfully recapitulate the primary tumors (4,22).

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

The *in vivo* sensitivity of mouse G3 MB 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 MB growth curves revealed that the rate of tumor expansion was profoundly reduced over 240% 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 to 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 to saline treated animals (Fig. 6B). Mice tolerated the treatments well and we saw no changes in their weight regardless of the regimen used (Supplementary Figure 4A).

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, 6D) and caspase 3 to measure apoptosis (Fig. 6E, 6F). Tumors from animals treated with Ko143 alone had a high mitotic index (91% $\pm$ 2.54) and few areas of active caspase 3 (6% $\pm$ 2.1), which is similar to tumors from vehicle treated animals (mitotic index 5.2% $\pm$ 1.22, caspase 3 85.03% $\pm$ 2.52). In contrast, tumors from mice treated with

topotecan alone displayed a decrease in mitotic index (77% $\pm$ 10.6) and a slight increase in caspase 3 staining (12% $\pm$ 2.1). Tumors from animals treated with both drugs shared the same mitotic index as the topotecan-treated tumors (76% $\pm$ 9.8) but displayed almost a 3-fold increase in caspase 3 staining (33% $\pm$ 16.1, p-value=0.0319).

Topotecan therapy might only eradicate tumors that express ABCG2. To determine if topotecan therapy affected Abcg2 protein levels in mouse G3 MB, tumors were isolated from moribund animals and immunoblotting performed on total cell lysates (Fig. 6G, 6H). 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. 4B).

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 MB 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 MB increased when Abcg2 was specifically inhibited and suggested that Abcg2 expression might account for the resistance of G3 MB to chemotherapy regimens, especially those including drugs that are substrates for ABCG2 like topotecan.

ABC transporter expression and function in G3 MB 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 CNS, with increased expression of some related to increased resistance to chemotherapy and radiotherapy (32). A RNA expression survey demonstrated that out of ten recognized drug-transporting ABC transporters in human G3 MB, *ABCC10*, *ABCC5*, *ABCC1* (*MRP1*) and *ABCG2* (*BRCP*) were the most highly expressed, whereas in the mouse G3 MB model, *Abcc4*, and *Abcg2* were consistently expressed and, to a lesser extent, *Abcc1* and *Abcb1*, 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 MBs. Because ABCG2 was the most highly expressed in both mouse and human G3 MB, it was of particular interest for therapeutic regimens that might employ its substrates.

Topotecan and G3 medulloblastoma

ABCG2, initially cloned from a drug resistant cell line that did not express either ABCB1 (P-glycoprotein) or ABCC1 (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 vivo* (37). Topotecan treatment of mice harboring G3 MB enhanced survival when Abcg2 was inhibited by the highly selective inhibitor Ko143 (23). Strikingly, these *in vivo* findings extend the *in vitro* results demonstrating Abcg2 inhibition in G3 MB 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. 4D). 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 MBs 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 MB 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 the BBB (39). However, we have previously demonstrated, in this 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 MB cell 9-fold. Moreover, the overall reduction in Abcg2 expression in tumor samples post-treatment suggests the treatment of topotecan and Ko143 eradicates medulloblastoma expressing Abcg2. While these findings suggests this combination therapy is efficacious, it is unknown if 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 MB 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 MBs 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 MB 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) (19). In addition, ABCG2 and ABCC4 have some similar substrates (44). Among the ABCC4 substrates tested in the tumorspheres were an antifolate (methotrexate), (19) KI (dasatinib) (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 MBs was not evaluated. ABCG2 was of particular interest in G3 MB 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 non-differentiating state (47,48). Further, solid tumor cells harboring functional ABCG2 exhibit intrinsic properties such as pluripotency, greater growth capacity, improved survival rate after cytotoxic drug exposure and enhanced tumorigenicity (49,50). G3 MB 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 MB express several markers of stemness, including Nestin and Sox2 (4). In addition, overexpression of MYC, the hallmark of G3 MBs and MYCN in recurrent SHH-subgroup MB, 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 MB suggests that potent and selective inhibitors of ABCG2 could be used to improve response to chemotherapeutic drug as substrates of ABCG2, including topotecan.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

ABC transporters in G3 MB. A, Log2 mRNA expression of ten *ABC* transporters in human G3 MB. B, Relative RNA expression of eight *ABC* transporters and specific genes comparing mouse G3 MB and GNP (4): after z-score normalization, high (red) and low (green) RNA expression. C, Immunoblots of Abcc4, Abcc1 and Abcg2 expression in lysates from five mouse G3 MB tumorspheres (Myc1, lane 1; Myc2, lane 2; Myc1-luc, lane 3; Myc3, lane 4; and Myc4, lane 5), and three tumor chunks from independently derived tumors (lanes 6, 7, and 8). Actin was used as loading control.

Figure 2.

Functional analysis of ABC transporters in G3 MB. A, Immunoblotting of surface biotinylated streptavidin-bound lysates (100 μ g/lane) from the mouse G3 MB neurosphere Myc1 (Total: input, Surf: plasma membrane, Sup: supernatant from the pull-down assay). TFR: transferrin receptor used as internal control for plasma membrane proteins. Plasma membrane protein levels are depicted in the bar graph as percentage of total input protein used in the pull down assay). B, Bis (POM)-PMEA uptake was determined in Myc2 in the presence of increasing concentrations (5–50 μ M) of the ABCC4 inhibitor MK571.

Intracellular PMEA levels are represented as pmol/mg of the protein, with error bars showing standard deviation. C, Intracellular Hoechst levels from Mel cells (top panel), tumorspheres (Myc1, middle panel; Myc2, bottom panel) measured by FACS in the absence (blue) or presence (red) of an ABCG2 inhibitor, FTC. D, Hoechst levels were quantified by FACS analysis from Fig. 1C.

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Figure 3.

ABCG2 function and porphyrin biosynthesis. A, Relative RNA expression of genes involved in the porphyrin pathway for mouse G3 MBs and GNPs (4): high (red) and low (green) expression. List of genes and values for each sample in Supplementary Table 1. B, PPIX levels measured by FACS analysis of Mel cells (top left panel) and three independent tumorspheres (Myc1, bottom left panel; Myc2, bottom right panel, and Myc1-luc, top right panel) incubated with ALA in the presence (pink) or absence of FTC (green). Tumorspheres

incubated with ALA accumulated more intracellular PPIX than when incubated with vehicle (blue). C, PPIX levels were quantified by FACS analysis from Fig. 1F.

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Figure 4.

Synergy between ABCG2 inhibition and known chemotherapeutic agents. **A**, Dose-response curves (1 nM to 10 μ M) to chemotherapeutic agents that are known ABCG2 substrates revealed distinct patterns of relative sensitivity between tumorsphere Myc1 treated with DMSO (blue curves) or 10 μ M of FTC (green curves). **B**, Myc1 tumorspheres treated with 0.1% DMSO (white bars), 200 nM topotecan [EC_{50} obtained from the HTS screen at 72 hrs for Group3 tumor cells (blue bars)], 10 μ M FTC (green bars), or topotecan and FTC (red bars). Percentage of apoptosis measured by annexin V staining and FACS, 24, 48 or 72hr after addition of the drug to the medium: DMSO, 24 hr 4.6%, 48 hr 5.2%, 72 hr 2.7% ; topotecan, 24 hr 6.1%, 48 hr 9.2%, 72 hr 14.5% ; FTC, 24hr 4.6%, 48 hr 7.4%, 72 hr 4.1%; topotecan with FTC, 24 hr 11.4%, 48 hr 23.8%, 72 hr 44.6% (p-value <0.001 by t-test).

Figure 5.

Plasma pharmacokinetics of topotecan alone or combined with Ko143. Topotecan plasma concentrations plotted against time from G3 MB-bearing mice treated A, with 0.75 mg/kg given intraperitoneally (IP) as single agent, or B, in combination with 10 mg/kg of Ko143 given IP. Open circles represent observed concentrations whereas dotted lines represent model predicted mean concentrations. C, Comparison of topotecan plasma AUC₀₋₆ between the two groups.

Figure 6.

Inhibition of Abcg2 increases topotecan efficacy *in vivo*. Mice injected at day 0 with 1×10^5 luciferase positive G3 MB tumorsphere (Myc1-luc) were treated when luciferase reached 5×10^5 photons. Mice were treated daily for 5 days for two consecutive weeks. A, Luciferase measurement and B, survival 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 -value=0.0016). Ko143 and topotecan treatment increased survival by 11 days (from 17 to 28 days, p -value=0.0002).

compared to vehicle; p -value=0.0165 compared with topotecan alone). Drug treated group $n=10$; saline treated group $n=7$. C, Immunostaining and D, quantification of Ki67 positive cells in tumors from mice treated with vehicle (black bar), Ko143 (green bar), topotecan (blue bar), and both (red bar). E, Immunostaining and F, quantification 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 versus topotecan and Ko143, $p=0.0319$). G, Immunoblotting and H, quantification 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, 9 9)).

Table 1
Chemotherapeutic drugs tested on mouse Group3 medulloblastoma tumorspheres

EC50 (with upper and lower bound on EC50 95% confidence interval) for 12 chemotherapeutic drugs in the absence (–) or presence (+) of FTC, an ABCG2 inhibitor; classes of drugs (in bold). NaN, no activity.

Chemotherapeutic Drugs	FTC EC50 (μM)	
	–	+
Topoisomerase inhibitors		
Mitoxantrone (Topo II)	0.0061 (0.0047–0.008)	0.0019 (0.0013–0.0027)
Daunorubicin	0.92 (0.22–1.1)	0.77 (0.21–1.1)
Doxorubicin	1 (0.8–1.5)	0.83(0.094–1)
Camptothecin analogs		
Topotecan	0.11 (0.092–0.16)	0.012 (0.0085–0.013)
Tyrosine kinase Inhibitors		
Dasatinib	7.1 (5.4–7.1)	NaN
Elrotinib	NaN	NaN
Imatinib	3.8 (3–5.1)	NaN
Antimetabolites		
Methotrexate	0.00092 (0.0006–0.0012)	0.00026 (0.00018–0.00035)
Trimetrexate	0.12 (0.034–0.58)	0.11 (0.044–0.38)
Cladribine	0.086 (0.077–0.11)	0.063 (0.056–0.072)
Other anti-cancer drugs		
Teniposide	0.0067 (0.0045–0.012)	0.0047 (0.0027–0.0081)
Flavopiridol	1 (0.11–1.1)	0.64 (0.53–0.88)