Substrate Overlap between Mrp4 and Abcg2/Bcrp Affects Purine Analogue Drug Cytotoxicity and Tissue Distribution

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Abstract

The use of probe substrates and combinations of ATP-binding cassette (ABC) transporter knockout (KO) animals may facilitate the identification of common substrates between apparently unrelated ABC transporters. An unexpectedly low concentration of the purine nucleotide analogue, 9-(2-(phosphonomethoxy)ethyl)-adenine (PMEA), and up-regulation of Abcg2 in some tissues of the *Mrp4* KO mouse prompted us to evaluate the possibility that Abcg2 might transport purine-derived drugs. Abcg2 transported and conferred resistance to PMEA. Moreover, a specific Abcg2 inhibitor, fumitremorgin C, both increased PMEA accumulation and reversed Abcg2-mediated PMEA resistance. We developed *Mrp4* and *Abcg2* double KO mice and used both single KOs of *Abcg2* and *Mrp4* mice to assess the role of these transporters *in vivo*. Abcg2 contributed to PMEA accumulation in a variety of tissues, but in some tissues, this contribution was only revealed by the concurrent absence of Mrp4. Abcg2 also transported and conferred resistance to additional purine analogues, such as the antineoplastic, 2-chloro-2′-deoxyadenosine (cladribine) and puromycin, a protein synthesis inhibitor that is often used as a dominant selectable marker. Purine analogues interact with ABCG2 by a site distinct from the prazosin binding site as shown by their inability to displace the substrate analogue and photoaffinity tag [^{125}I]iodoarylazidoprazosin. These studies show that Abcg2, like Mrp4, transports and confers resistance to purine nucleoside analogues and suggest that these two transporters work in parallel to affect drug cytotoxicity and tissue distribution. This new knowledge will facilitate an understanding of how Abcg2 and Mrp4, separately and in combination, protect against purine analogue host toxicity as well as resistance to chemotherapy. [Cancer Res 2007;67(14):6965–72]

Introduction

The concept of substrate overlap among ATP-binding cassette (ABC) transporters is well known in cell culture models (1, 2). However, direct extrapolation of these findings to *in vivo* models is difficult because of an inability to attain expression levels comparable with *in vivo*. One approach to test for functional interactions among ABC transporters is to evaluate both substrate and transporter tissue distribution in knockout (KO) animals. We speculated that if two transporters showed similar tissue distribution and patterns of up-regulation in each others absence, then it was likely they shared similar endogenous or drug substrates. Mrp4 (also known as Abcc4) and Abcg2 have similar patterns of tissue distribution (3, 4). Mrp4 was first identified as a transporter of nucleoside monophosphates [primarily purine nucleoside monophosphates; e.g., 9-(2-(phosphonomethoxy)ethyl)-adenine (PMEA); ref. 5], but more recent studies have indicated that Mrp4 transports substrates in common with Abcg2, most recently camptothecin analogues (6, 7). Because Abcg2 also transports and confers resistance to camptothecin analogues (8, 9) as well as steroid-like compounds and antifolates (e.g., methotrexate), we postulated that the substrate specificities of Mrp4 and Abcg2 may overlap to a greater extent than previously appreciated.

In our *Mrp4* KO mouse, Mrp4 absence had no effect on PMEA accumulation in the brain and prompted us to look for compensatory changes in other ABC transporters. We discovered up-regulation of Abcg2 and then showed that ABCG2 transported PMEA and conferred resistance to the cytotoxic effects of PMEA. These studies were extended to test if absence of either or both Abcg2 and Mrp4 altered the tissue concentrations of PMEA (10). These experiments were conducted in the *Mrp4* KO (*Mrp4*$^{-/-}$; ref. 6), the *Abcg2* KO (*Abcg2*$^{-/-}$; ref. 11), and the *Mrp4* and *Abcg2* double KO (DKO) mouse developed recently in our laboratory. We then tested if other purine analogues were Abcg2 substrates. One purine analogue, 2-chloro-2′-deoxyadenosine (2-CdA), is of particular relevance. 2-CdA is frequently used for treatment of leukemia (12) and this is notable because Abcg2 (Bcrp) is expressed at higher levels in certain subtypes of leukemia (13). These studies show, using KO mice and cells engineered to express ABCG2, that Mrp4 and Abcg2 affect not only the *in vivo* drug tissue concentrations of purine analogues but also the sensitivity of cells to the cytotoxic effects of purine analogue antiviral and cancer chemotherapeutic agents. The therapeutic implications of these results are 2-fold. First, both Abcg2 and Mrp4 may reduce the antiviral and cytotoxic effects of these drugs. Second, the inhibition of Mrp4 and Abcg2 may increase the therapeutic efficacy of purine-derived chemotherapeutics.

Materials and Methods

Materials. [^{3}H]PMEA (12.3 μCi/nmol), unlabeled PMEA, [^{3}H]bis(pivaloxymethyl)-9-(2-phosphonylmethoxyethyl) adenine [Bis(POM)PMEA; 11 μCi/nmol], unlabeled Bis(POM)PMEA, [^{3}H]2-CdA, and [^{3}H]puromycin were purchased from Moravek Chemicals. 2-CdA, puromycin, and prazosin were purchased from Sigma-Aldrich, Inc. The prazosin analogue [^{125}I]iodoarylazidoprazosin ([^{125}I]IAAP) was obtained from Perkin-Elmer Lifesciences.

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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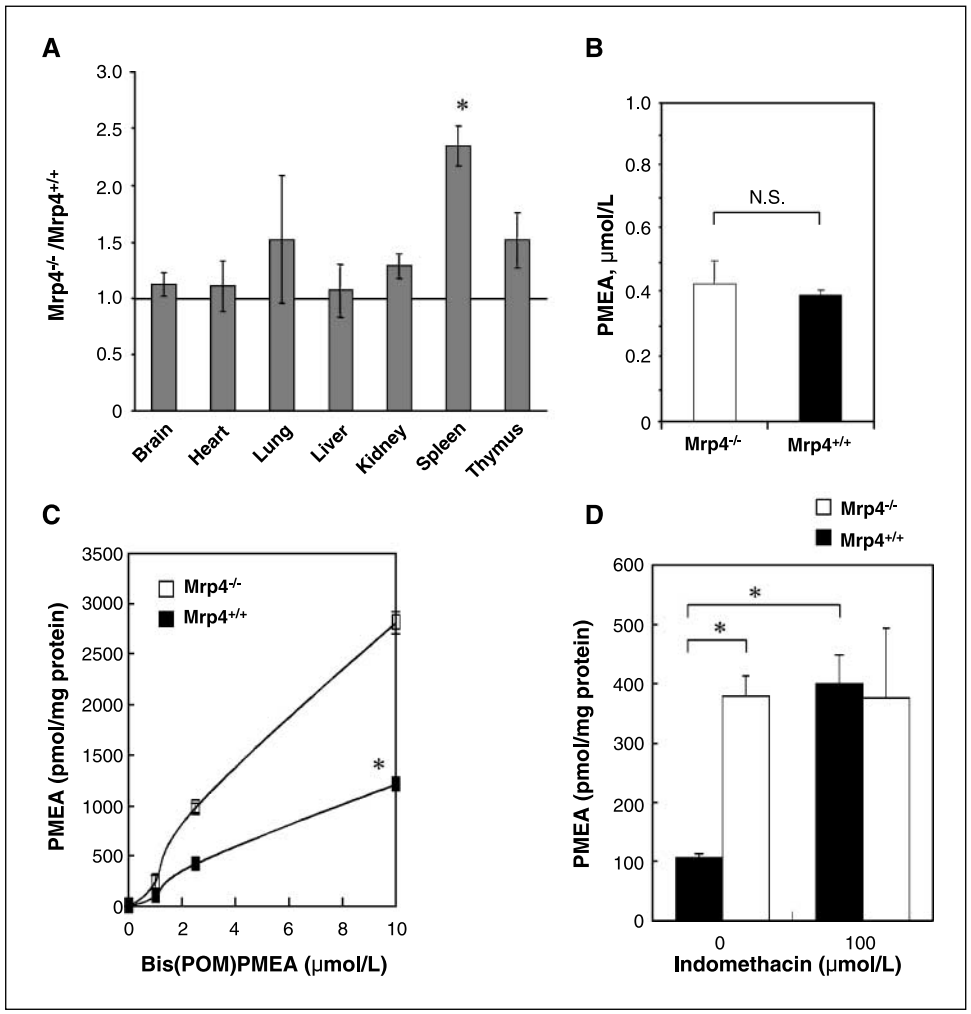

Figure 1. Mrp4 absence selectively affects the tissue distribution of PMEA. *A,* comparison of PMEA accumulation in selected tissues of *Mrp4* KO mice with WT mice. Values represent the ratio of mean PMEA accumulation level in *Mrp4*$^{-/-}$ mice to that in WT mice. *B,* plasma level of PMEA 6 h after i.v. administration of 20 mg/kg [^{3}H]PMEA. *C,* accumulation of [^{3}H]Bis(POM)PMEA in splenocytes obtained from *Mrp4*$^{-/-}$ mice and WT mice after incubation for 6 h. *Columns* and *points,* mean of three independent experiments, each done in triplicate; *bars,* SE. *, $P < 0.01$, relative to splenocytes from WT mice. *D,* effect of 100 μmol/L indomethacin on [^{3}H]Bis(POM)PMEA accumulation in splenocytes. *Columns,* mean of three independent experiments, each done in triplicate; *bars,* SE. $P < 0.01$.

Fumitremorgin C (FTC) was kindly provided by Dr. Susan Bates (National Cancer Institute, NIH, Bethesda, MD).

Cell culture. Human osteosarcoma cell line, Saos2, was cultured in DMEM containing 10% FCS and 100 units/mL penicillin, 100 μg/mL streptomycin, and 2 mmol/L L-glutamine. ABCG2-overexpressing Saos2 cells (Saos2-ABCG2) and the empty vector control cell line, Saos2 pcDNA3.1 cells, have been reported previously (14). The MCF-7/FLV1000 cells were kindly provided by Dr. Susan Bates.

Animals. Female wild-type (WT), *Mrp4*$^{-/-}$ (6), *Abcg2*$^{-/-}$ (11), and *Mrp4*/*Abcg2* DKO mice were used. DKO mice were obtained by crossbreeding of *Mrp4*$^{-/-}$ and *Abcg2*$^{-/-}$ mice. All mice were from a mixed C57BL/6 129SVJ background and were bred and maintained at St. Jude Children's Research Hospital. All procedures were reviewed and approved by the Institutional Animal Care and Use Committee of St. Jude Children's Research Hospital.

Isolation of splenocytes from mice. Spleens were isolated from WT and *Mrp4*$^{-/-}$ mice followed by isolation and culture of splenocytes as described previously (15). Briefly, fresh isolated organs were meshed and passed through a strainer (70 μmol/L) to separate fibrous tissue. The splenic cells were kept in culture medium composed of RPM1 1640, 10% fetal bovine serum, 10 mmol/L HEPES (pH 7.0), 2 mmol/L L-glutamine (1×) nonessential amino acids, 1 mmol/L sodium pyruvate, and 50 mmol/L 2-mercaptoethanol. After lysis of RBC, the splenic cells were incubated with radiolabeled drug for 6 h.

Immunoblot analysis. As described previously, cell and tissue homogenates were analyzed by immunoblotting with the following antibodies: Mrp4 (M4$_I$-10), Abcg2 (BXP-53, Alexis Biochemicals), and β-actin (6). Protein concentration was quantified by using the Bio-Rad protein assay with bovine serum albumin (BSA) as standard. Whole-cell lysates and homogenates (100 μg) were separated on 7.5% or 10% polyacrylamide gels. The membrane was blocked and incubated with the primary antibody at the appropriate dilution and then with an anti-rat IgG, horseradish peroxidase–linked whole antibody (Amersham Bioscience). Signals were developed using the Amersham enhanced chemiluminescence detection system. Densitometry analysis of bands was done by Scion image (Scion Corp.),[5] and the ratio of KO to WT was determined after normalization by the actin signal.

Intracellular accumulation of drugs. Saos2-ABCG2 cells and vector control cells were seeded at 5×10^5 per well in six-well plates and grown overnight. Cells were incubated with labeled drugs for the indicated time and at the indicated concentrations. Radioactivity in the samples was measured by liquid scintillation counting. Values were normalized to total protein content as described (16).

Cytotoxicity assay. Cells were seeded at 5,000 cells per well in 96-well plates and cultured at 37°C for 20 h. The cells were incubated with drugs for 6 h, the medium was removed, and the cells were incubated without drugs for 3 to 4 days. The viability of cells was determined by CellTiter 96 Nonradioactive Cell Proliferation assay (Promega). The percentage of growth for treated cells, compared with untreated cells, was calculated. An inhibitory concentration was determined and represented an amount of drug that inhibited cell proliferation by 50% (IC_{50}). Online software was used to calculate the IC_{50} using Java Applets & Servlets for Biostatistics (programmed by H. Ono, Nagoya City University, Nagoya City, Japan). Each

[5] http://www.scioncorp.com

assay included duplicate samples for each drug concentration, and all experiments were done at least twice.

Photoaffinity labeling of ABCG2. [^{125}I]IAAP (specific activity, 2,200 Ci/mmol) labeling was done as described previously (17). Briefly, a mixture of 30 μg crude membrane protein from MCF-7/FLV1000 cells (expressing the R482wt form of human ABCG2) containing (18 nmol/L) [^{125}I]IAAP in the absence or presence of PMEA, puromycin, or prazosin was photo cross-linked with UV light (365 nm). The samples were subjected to SDS-PAGE, and gels were dried followed by exposure to X-ray film overnight at −80°C.

Pharmacokinetic experiments of PMEA. Mice were injected with [^{3}H]PMEA (12 μCi and 20 mg/kg of body weight) via the tail vein. Mice were sacrificed at 6 h after injection. Blood was obtained by cardiac puncture, and plasma was separated by centrifugation at 13,000 rpm for 5 min. Selected tissues (brain, heart, lung, liver, spleen, kidney, ovary, and thymus) were collected and homogenized in a 4% (w/v) BSA solution. The level of radioactivity was determined in the homogenates by liquid scintillation counting.

Statistical analysis. A two-sided Student's *t* test was used to determine the statistical significance between experimental groups and differences were considered significant when P was <0.05.

Clinical chemistries and biochemical analysis. A sample of blood was collected from anesthetized mice by retro-orbital sinus puncture, and serum was obtained after centrifugation. The blood cells were analyzed by using an automated blood cell analyzer, a Hemavet 3700 from Drew Scientific. Biochemical analyses were done by using a chemistry analyzer, a Vet Scan from Abaxis.

Results

Mrp4 absence increases PMEA concentrations in select tissues. The amount of MRP4 expressed is inversely correlated with the PMEA concentration in cells *in vitro* (18). To evaluate the contribution of Mrp4 to PMEA tissue concentration *in vivo*, *Mrp4*$^{-/-}$ and WT mice received an i.v. dose of 20 mg/kg [^{3}H]PMEA. Subsequently, plasma and tissues were evaluated for the amount of radioactivity. Relative to WT mice, the *Mrp4*$^{-/-}$ mice accumulated greater levels of radioactivity in spleen, thymus, lung, and kidney (the average ratio of *Mrp4*$^{-/-}$ to WT mice was 2.3, 1.5, 1.4, and 1.3, respectively. Fig. 1*A*). The accumulation of PMEA was also dose related because at 40 mg/kg, the average ratio of *Mrp4*$^{-/-}$ to WT was 3.3 in the spleen. In particular, PMEA accumulation in spleen of *Mrp4*$^{-/-}$ mice was significantly higher than that of WT mice in accordance with the *in vivo* accumulation of topotecan in the spleen of the *Mrp4*$^{-/-}$ mouse (6). Notably, the plasma levels of PMEA were not significantly different between *Mrp4*$^{-/-}$ and WT mice (Fig. 1*B*).

To directly test the role of Mrp4 in PMEA accumulation, we isolated splenocytes and incubated them with various concentrations of the PMEA prodrug, [^{3}H]Bis(POM)PMEA. Bis(POM)PMEA has been used by us and others to bypass the PMEA uptake carrier OAT1 (5, 18, 19). As shown in Fig. 1*C*, splenocytes from *Mrp4*$^{-/-}$ mice accumulated significantly higher concentrations of PMEA (2.5-fold) compared with splenocytes from WT mice (P < 0.01). In addition, 100 μmol/L indomethacin, an inhibitor of Mrp4, dramatically elevated PMEA accumulation in splenocytes of WT mice to levels achieved in splenocytes from the *Mrp4*$^{-/-}$ mice (Fig. 1*D*). These studies showed that Mrp4 strongly contributes to PMEA accumulation in the spleen.

Mrp4 and Abcg2 expression in mice. Previous studies in ABC transporter KO animals reveal that the absence of an ABC transporter can elicit, in some tissues, compensatory changes in

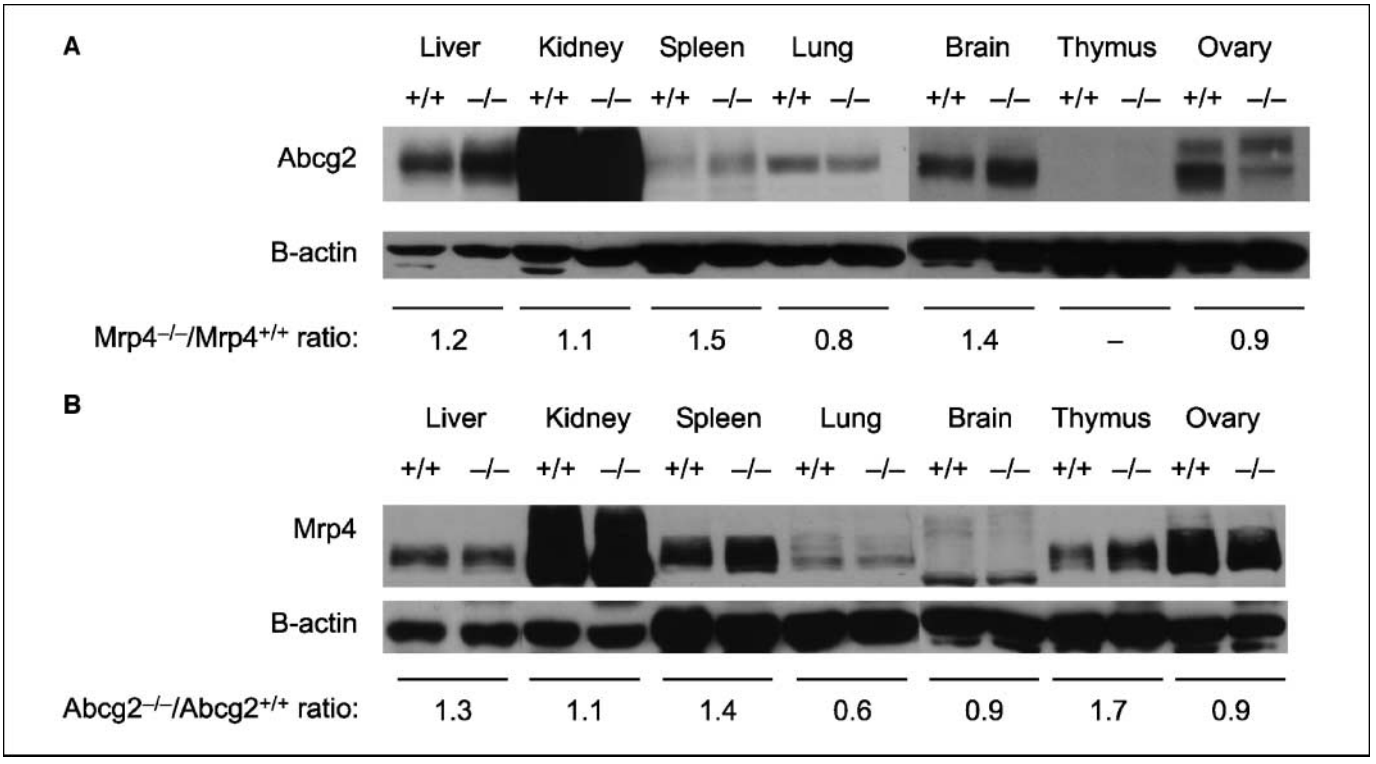

Figure 2. Immunoblot analysis of Mrp4 and Abcg2 expression. Tissue homogenates (100 μg of protein/well) were obtained from *Mrp4*$^{-/-}$ mice and *Abcg2*$^{-/-}$ mice. The Mrp4 and Abcg2 antibodies were used as described in Materials and Methods. Representative immunoblot from two independent experiments. Densitometry analysis of bands was done by Scion image. The density of each band was normalized to the β-actin signal and the ratios represented an average ratio of the normalized values from the two experiments. *A,* Abcg2 expression in *Mrp4*$^{-/-}$ mice. Values represent the ratio of mean density of bands in *Mrp4*$^{-/-}$ mice to that in WT mice. *B,* Mrp4 expression in *Abcg2*$^{-/-}$ mice. Values represent the ratio of mean density of bands in *Abcg2*$^{-/-}$ mice to that in WT mice.

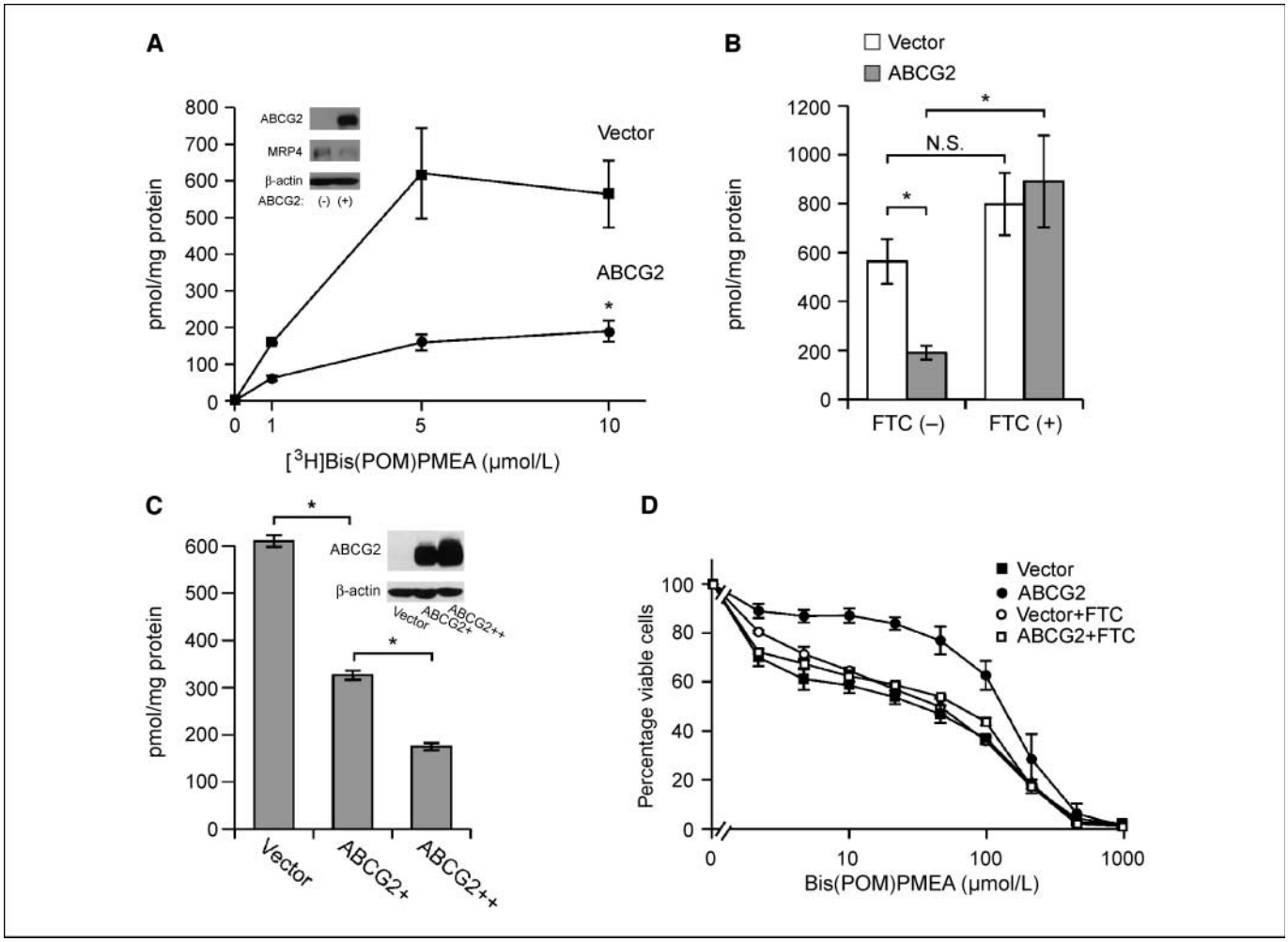

Figure 3. ABCG2 transports and confers resistance to PMEA. *A,* immunoblot analysis of Mrp4 and ABCG2 expression in vector and Saos-ABCG2 cells (*inset*). Bis(POM)PMEA uptake by Saos2 cells transfected with either empty vector or ABCG2. Cells were incubated at the indicated concentrations of Bis(POM)PMEA for 20 h. *Points,* mean of three independent experiments, each done in duplicate; *bars,* SE. *, $P = 0.013$, relative to vector control. *B,* effect of 10 μmol/L FTC on accumulation of Bis(POM)PMEA (10 μmol/L) in Saos2 cells transfected with ABCG2. *Columns,* mean of three independent experiments, each done in duplicate; *bars,* SE. *, $P < 0.01$. *C,* amount of ABCG2 affects intracellular accumulation of PMEA. Soas2 cells expressing either low (ABCG2+) or high (ABCG2++; *inset*) were incubated with 1 μmol/L Bis(POM)PMEA for 6 h. *Columns,* mean of two independent experiments, each done in triplicate; *bars,* SE. *D,* cell survival after 4 d of incubation including 6 h of Bis(POM)PMEA exposure in Saos2 cells transfected with vector control or ABCG2. *Points,* mean of two to three independent experiments, each done in triplicate; *bars,* SE. *, $P < 0.01$.

functionally related ABC transporters (20). We used the *Abcg2*$^{-/-}$ and *Mrp4*$^{-/-}$ mice to test this possibility because Mrp4 and Abcb2 have similar tissue distribution patterns. Compared with WT mice, *Mrp4*$^{-/-}$ mice showed higher expression of Abcg2 in the spleen and brain (Fig. 2*A*). In contrast, spleen and thymus from *Abcg2*$^{-/-}$ mice had higher Mrp4 expression compared with WT mice (Fig. 2*B*), suggesting that Mrp4 is up-regulated in an apparent attempt to compensate for the loss of Abcg2 expression.

ABCG2 transports and confers resistance to the purine prodrug, Bis(POM)PMEA. The expression of Abcg2 in *Mrp4*$^{-/-}$ mouse brain was elevated, but penetration of PMEA was no different between *Mrp4*$^{-/-}$ and WT mice (Fig. 1*A*). These findings led us to test the hypothesis that Abcg2 transports PMEA. To determine if PMEA is an Abcg2 substrate, an uptake assay was conducted using either Saos2 cells expressing human WT ABCG2 (Saos2-ABCG2) or empty vector (Saos2 vector). Mrp4 levels were low in these cells as determined by immunoblot analysis (Fig. 3*A*, *inset*). We used [^{3}H]Bis(POM)PMEA as described above and previously (5, 21) to determine intracellular PMEA accumulation. Depending on the concentration of Bis(POM)PMEA used, the Saos2-ABCG2 cells showed 3- to 4-fold lower intracellular drug accumulation compared with the Soas2 vector cells [10 μmol/L Bis(POM)PMEA; $P = 0.03$; Fig. 3*A*]. Moreover, the specific Abcg2 inhibitor, FTC (at 10 μmol/L), increased [^{3}H]PMEA accumulation ~5-fold in Saos2-ABCG2 cells (Fig. 3*B*). We further tested if the intracellular concentration of PMEA was related to the amount of ABCG2 (Fig. 3*C*). We used two cell lines with different amounts of ABCG2 (Fig. 3*C*, *inset*) and discovered that the concentration of PMEA was related to the amount of ABCG2. Subsequently, we evaluated whether ABCG2 affected PMEA cytotoxicity using a lactate dehydrogenase release assay. Saos2-ABCG2 cells were >9-fold less sensitive to Bis(POM)PMEA compared with vector transfectants [IC_{50}, 219 μmol/L (Saos2-ABCG2) versus 26.1 μmol/L (vector); Fig. 3*D*]. Increasing the PMEA incubation from 6 to 20 h resulted in a modest reduction in the IC_{50} for vector cells (15.7 μmol/L) and a 4-fold reduction for the ABCG2 cells

(53.4 μmol/L). Furthermore, FTC reversed ABCG2-mediated PMEA resistance.

Mrp4 and Abcg2 in combination and separately contribute to tissue distribution of PMEA. We next determined how the absence of either Abcg2 or both Mrp4 and Abcg2 affected tissue distribution of PMEA *in vivo*. DKO mice were developed by interbreeding *Mrp4*$^{-/-}$ and *Abcg2*$^{-/-}$ mice. The DKO mice are viable, fertile, and grow as expected. Clinical chemistry and hematologic analysis revealed no gross disruption of mature myeloid or lymphoid elements and no organ dysfunction (Supplementary Table S1). Thus, under the normal housing conditions at this institution, the DKO had normal physiologic variables. The mice received an i.v. dose of 20 mg/kg [^{3}H]PMEA, and 6 h later, plasma and tissues were harvested. Plasma concentrations of PMEA were no different among WT, *Abcg2*$^{-/-}$, and DKO mice (data not shown) and similar to the concentrations for the *Mrp4*$^{-/-}$ animals (Fig. 1*B*). However, the absence of Abcg2 alone increased the PMEA concentration in the liver (1.4×), kidney (1.4×), brain (1.25×; see Supplementary Fig. S1), and ovary (2.5×; Fig. 4; see Supplementary Fig. S1). DKO mice showed higher PMEA concentration than *Abcg2*$^{-/-}$ mice among the liver (1.3×), kidney (1.3×), and heart (1.25×; see Supplementary Fig. S1), indicating that, in these tissues, Mrp4 and Abcg2 exert a combined effect on PMEA accumulation. In contrast, PMEA accumulation in the spleen and lungs seems unaffected by Abcg2 absence (Fig. 4), whereas, in DKO mice (relative to *Agcg2*$^{-/-}$), the spleen (1.6×) and lung (1.4×) showed higher concentration of PMEA. These data indicate that Mrp4 predominantly affects PMEA concentration in these tissues (Fig. 4). This interpretation agrees with our finding in *Mrp4*$^{-/-}$ mice shown above (Fig. 1). Notably, these data indicated that Abcg2 influences PMEA brain concentration to an extent similar to mitoxantrone, a known Abcg2 substrate (22). Together, these data show that both Mrp4 and Abcg2 affect PMEA tissue distribution in peripheral tissues, but only the absence of Abcg2 increases PMEA brain penetration.

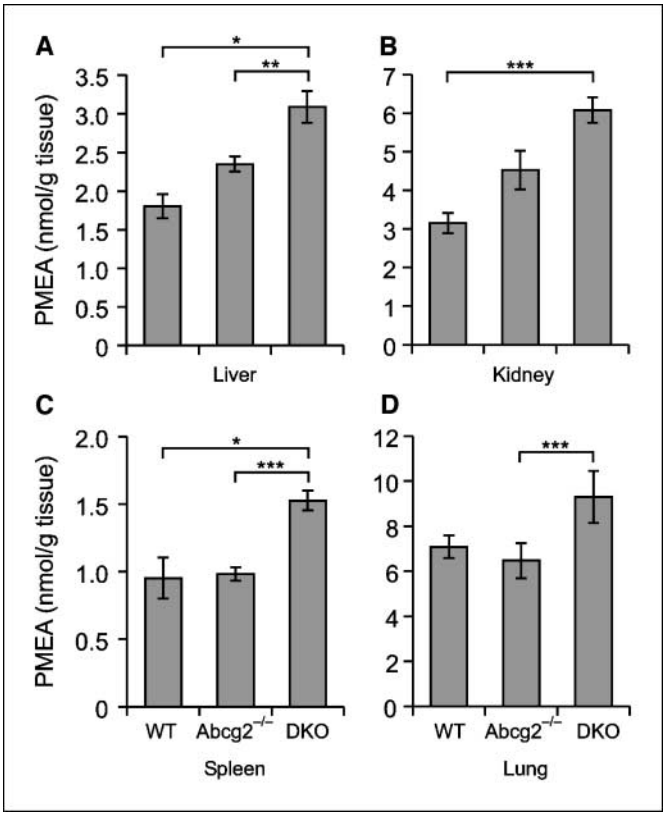

Figure 4. Abcg2 and Mrp4 individually and separately contribute to PMEA tissue concentrations. [^{3}H]PMEA (20 mg/kg) was given by tail vein into mice. At 6 h, mice were sacrificed and radioactivity in the indicated tissues was determined by scintillation counting. Accumulation in liver (*A*), kidney (*B*), spleen (*C*), and lung (*D*) of WT, Abcg2$^{-/-}$ mice, and DKO mice. *Columns,* mean (n = 3); *bars,* SE. *, P < 0.05; **, P = 0.05; ***, P < 0.01.

ABCG2 transports and confers resistance to puromycin and 2-CdA. Many purine analogues act as antimetabolites and are potent and effective immunosuppressants and chemotherapeutic agents. Although puromycin is not used clinically, it is used as a dominant selectable marker in some mammalian expression vectors (23). We assessed puromycin uptake to determine if it is a substrate of ABCG2. As shown in Fig. 5*A*, Saos2-ABCG2 cells accumulated 2-fold less [^{3}H]puromycin compared with vector cells (P < 0.01). FTC (10 μmol/L) blocked puromycin efflux and effectively enhanced [^{3}H]puromycin accumulation in Saos2-ABCG2 cells to concentrations similar to those found in Saos-2 vector cells (Fig. 5*A, inset*). These studies were extended to determine if Abcg2 conferred resistance to the cytotoxic effects of puromycin. The Saos2-ABCG2 cells exhibited over a 6-fold increase in puromycin resistance [IC_{50}, 52.7 μmol/L (Saos2-ABCG2) versus 8.0 μmol/L (vector); Fig. 5*B*] and ABCG2-mediated puromycin resistance was completely reversed by FTC (10 μmol/L; Fig. 5*B*), thus indicating that ABCG2 reduced intracellular puromycin concentration, which directly affects puromycin cytotoxicity.

2-CdA is a stable analogue of deoxyadenosine, an agent commonly used to treat lymphoid and other malignancies (24, 25). An evaluation of the effect of ABCG2 expression on 2-CdA uptake revealed that ABCG2 strongly reduced the rate of 2-CdA accumulation in Saos-2-ABCG2 cells (P = 0.01 at 60 min; Fig. 5*C*). We then evaluated if ABCG2 conferred resistance to 2-CdA. As shown in Fig. 5*D*, Saos2-ABCG2 cells were 5-fold more resistant to 2-CdA compared with vector-only transfectants [IC_{50}, 1,273.7 μmol/L (Saos2-ABCG2) versus 261.4 μmol/L (vector)]. Moreover, coaddition of FTC completely reversed the ABCG2-mediated resistance. These studies indicate that ABCG2 reduces the intracellular concentration of purine analogues and this corresponds to a reduction in cellular cytotoxicity.

PMEA does not compete with IAAP for binding to the ABCG2 protein. To determine if the nucleosides interact with known substrate binding sites, we used the substrate analogues [^{125}I]IAAP. Prazosin, a known substrate of WT ABCG2 (1), specifically competes with its photoaffinity analogue [^{125}I]IAAP to reveal one of the ABCG2 substrate binding sites (26). To evaluate whether PMEA and puromycin interact with the [^{125}I]IAAP binding site, we labeled membranes from ABCG2-expressing cells (MCF-7/FLV1000) with [^{125}I]IAAP in the presence of PMEA and puromycin (Fig. 6*A* and *B*). Whereas prazosin readily blocked [^{125}I]IAAP labeling, neither PMEA nor puromycin up to 25 μmol/L had any affect on [^{125}I]IAAP labeling of ABCG2, thus showing that these purine nucleoside analogues do not potently compete with IAAP for binding to ABCG2 (Fig. 6*A* and *B*). The prodrug Bis(POM)PMEA was also ineffective in displacing IAAP (data not shown). These studies indicate that puromycin and PMEA interact with a site on Abcg2 that is distinct from the prazosin binding site described previously (17, 26).

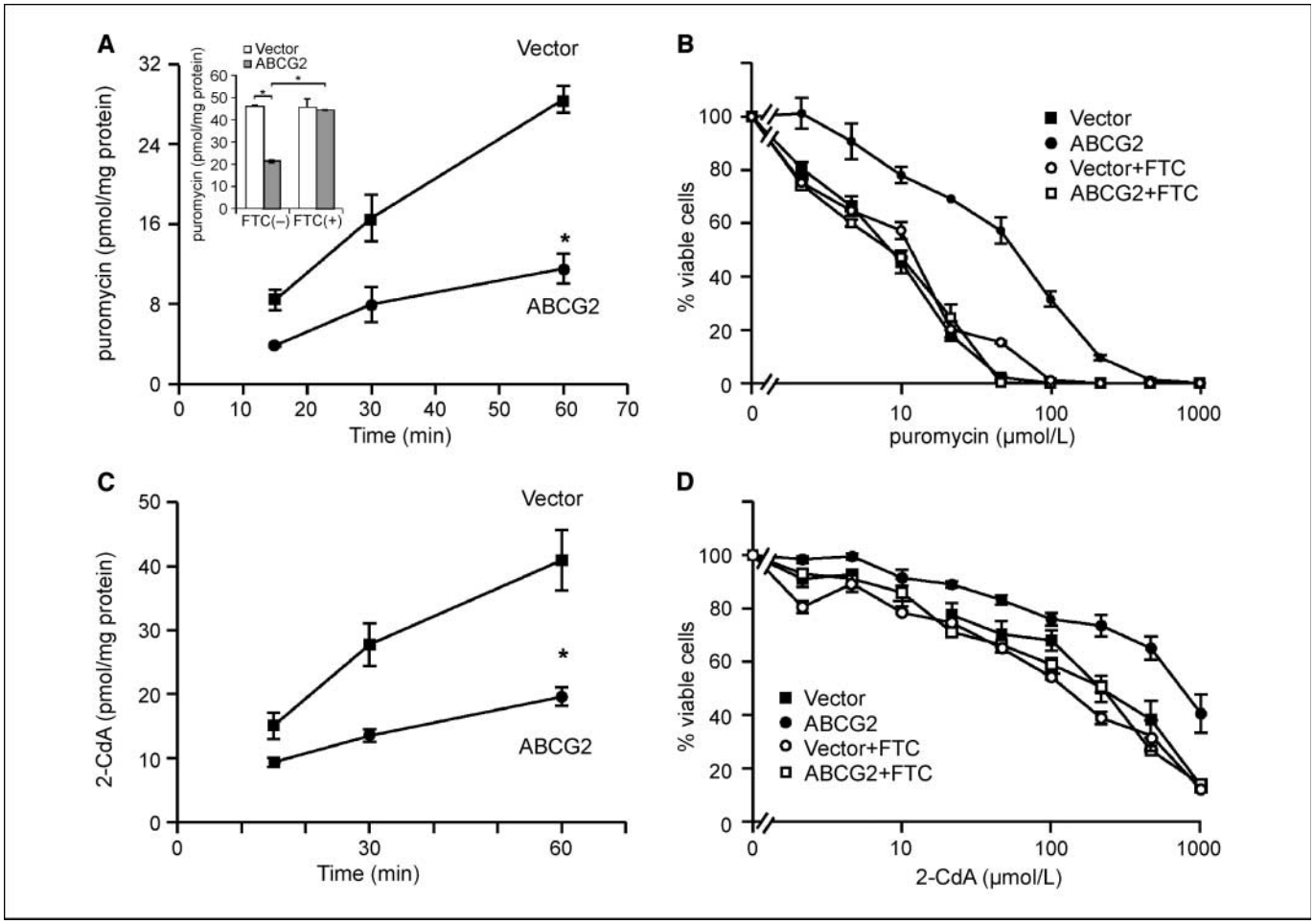

Figure 5. ABCG2 reduces accumulation and cytotoxicity of puromycin and 2-CdA. *A,* time course of [^{3}H]puromycin (10 μmol/L) uptake by either ABCG2-overexpressing cells or empty vector cells. *Points,* mean of three independent experiments, each done in duplicate; *bars,* SE. *, $P < 0.01$, relative to vector control. *Inset,* accumulation of [^{3}H]puromycin (10 μmol/L) in Saos2 cells transfected with ABCG2 was reversed by 10 μmol/L FTC. *Columns,* mean of three independent experiments, each done in duplicate; *bars,* SE. *, $P < 0.01$. *B,* cell survival after 4 d of incubation including 6 h of puromycin exposure in Saos2 cells transfected with vector control or ABCG2. *Points,* mean of two to three independent experiments, each done in duplicate; *bars,* SE. *C,* time course of [^{3}H]2-CdA (10 μmol/L) uptake by Saos2 cells transfected with vector control or ABCG2. *Points,* mean of three independent experiments, each done in duplicate; *bars,* SE. *, $P = 0.01$ relative to vector control. *D,* cell survival after 4 d including 6 h of incubation with 2-CdA. *Points,* mean of two to three independent experiments, each done in duplicate; *bars,* SE.

Discussion

Purine nucleosides are highly effective chemotherapeutic agents, but their effectiveness may be limited by the concentration achieved in a target cell or organ. One mechanism to reduce cellular accumulation and retention of the purine nucleotide analogues was first attributed to the efflux capabilities of the ABC transporter Mrp4 (ABCC4; refs. 5, 18, 27, 28). The current study reveals, for the first time, that, in addition to Mrp4, the ABC transporter and hematopoietic stem cell marker Abcg2 (11, 29) also regulates the intracellular concentration of purine analogues (e.g., PMEA). Moreover, the up-regulation of *Abcg2* in the *Mrp4* KO mouse and the recent findings that both Mrp4 and Abcg2 transport cGMP (30) support the proposal that these genes functionally overlap and compensate for one another. This functional overlap and compensation has important therapeutic implications for the therapeutic use of purine analogues because they are effective as both antivirals and cancer chemotherapeutic agents.

From a practical standpoint, it is notable that puromycin is transported by ABCG2. We propose, based on the puromycin resistance of our ABCG2-expressing cells, that the recommended range of concentrations of puromycin used to select for resistant cells (100–1,000 μmol/L) might lead to the adventitious overexpression of endogenous Abcg2 and circumvent the usual mode of acquiring puromycin resistance (i.e., inactivation by puromycin *N*-acetyltransferase). Consequently, it is possible that cells, selected for puromycin resistance, might acquire overexpression of endogenous Abcg2 and express a phenotype due to Abcg2. Abcg2 can protect against cell stresses (e.g., hypoxia) and cytotoxic agents (e.g., topoisomerase inhibitors as well as methotrexate and purine analogues). Therefore, we offer a caveat that the cell phenotypes obtained after puromycin drug selection may be related to acquired capabilities attributable to Abcg2 expression rather than the gene under selection.

PMEA is an acyclic purine nucleoside phosphonate analogue that is versatile and highly effective against various viruses, such as herpes, EBV, hepatitis B (HBV), retroviruses, and cytomegalovirus, and some cancers (31–36). The PMEA derivatives, Bis(POM)PMEA and tenofivir, are effective in treating patients infected with HBV (37, 38). Resistance to PMEA in HBV-infected patients is associated with viral rebound (39), which in rare cases results from mutations

in the HBV polymerase gene (40). The mechanism of cellular PMEA resistance is complex. However, extrusion of nucleotide analogues by ABC transporters has been reported as a mechanism of acquired drug resistance in cell lines selected for PMEA resistance (5). Our current studies extend this to show that both Abcg2 and Mrp4 (independently and together) reduce intracellular PMEA concentrations. These findings have implications for the treatment of HBV infection, especially because there is a strong link between liver cancer and HBV infection—the lifetime risk for developing liver cancer in a chronically infected person is 10% to 25% (41). Thus, it is a formal possibility that Mrp4 and Abcg2, by decreasing hepatic purine nucleotide concentrations, impair the effectiveness of PMEA against HBV, which leads to chronic HBV infection.

A major dose-limiting toxicity of PMEA (also known as adefovir) is renal toxicity (37, 42). Adefovir nephrotoxocity would require renal tubular uptake by the organic anion transporter OAT1 (43); however, renal excretion has been proposed as rate limiting to nephrotoxicity (44). Our studies show that both Mrp4 and Abcg2 reduce renal PMEA concentration and support the idea that excretion is rate limiting as no difference is observed in the expression of the PMEA uptake carrier OAT1 (data not shown). Furthermore, as both ABCG2 and Mrp4 protect against PMEA cytotoxicity (this article and refs. 5, 28, 45), their location on the apical side of the proximal tubules can facilitate excretion of PMEA into the urine. However, it remains to be tested how Mrp4 and Bcrp individually contribute *in vivo* to protect against renal toxicity of PMEA derivatives. Nevertheless, it is notable that another apical renal transporter, Mrp2, does not affect kidney elimination of PMEA (46).

2-CdA is a purine nucleoside analogue that is converted intracellularly to nucleotide analogues and has been successfully used for the treatment of myeloid malignancies (12). Nitrobenzylthioinosine is a compound that can increase the intracellular concentration of 2-CdA nucleotide metabolites (47). Wright et al. (48) reported enhanced intracellular retention of 2-CdA and its metabolites in an erythroleukemia cell line by 0.5 μmol/L nitrobenzylthioinosine. Our study provides the first evidence that ABCG2 reduces intracellular 2-CdA accumulation and confers 2-CdA resistance. Moreover, preliminary studies indicate that nitrobenzylthioinosine inhibits ABCG2 (data not shown). Thus, leukemia cells expressing ABCG2 might be less sensitive to 2-CdA and we speculate that leukemias expressing high levels of ABCG2 as well as MRP4 (45) may be refractory to 2-CdA due to extrusion of metabolites.

An understanding of the substrate binding sites of ABC transporters is essential for identifying mechanisms of multidrug resistance, drug-drug interactions, and the design of specific inhibitors. As reported previously, some ABC transporters, such as P-glycoprotein, Abcg2, Mrp2, and Mrp4, have more than one substrate binding site (26, 49–51). Shapiro et al. (52) showed three distinct substrate binding sites for P-glycoprotein. In addition, the binding site of methotrexate for Mrp4 is different from that of urate, which provides a convenient explanation for the inhibition of methotrexate transport by urate (51). In our studies, we evaluated if PMEA and puromycin interact with the prazosin binding site on ABCG2 by testing how these compounds reduced [^{125}I]IAAP labeling. Neither puromycin nor PMEA [and Bis (POM)PMEA; data not shown] up to 25 μmol/L reduced [^{125}I]IAAP labeling of Abcg2, thus suggesting that both PMEA and puromycin do not compete with the [^{125}I]IAAP binding site on ABCG2. This is analogous to daunomycin, mitoxantrone, rhodamine 123, and methotrexate, all of which are ABCG2 substrates but are ineffective in competing with [^{125}I]IAAP labeling (17, 26). These findings lend further support to the concept that Abcg2 has more than one substrate binding site. Determining the identity of this binding site may provide insight into the development of ABCG2 inhibitors that might selectively affect substrates.

In conclusion, our results not only extend the list of ABC transporters that efflux purine analogues to include Abcg2 but also reveal how Mrp4 and Abcg2, in combination and separately, affect tissue concentrations of purine analogues *in vivo*. Although our studies are important for hematologic malignancies (Abcg2 is highly expressed in hematopoietic progenitors; refs. 11, 29) and especially for the chemotherapy of leukemias in which 2-CdA is used, these findings have important implications for antiviral therapy because not only can MRP4 and ABCG2 potentially affect renal toxicity of PMEA derivatives but both MRP4 and ABCG2 may affect the concentration of PMEA in liver. Currently, PMEA derivatives are highly effective against HBV; however, our studies imply that these two transporters may combine to reduce the accumulation of PMEA analogues, thus contributing to the reduced antiviral efficacy and increasing the possibility that persistent HBV infections may progress to HBV-initiated liver cancer.

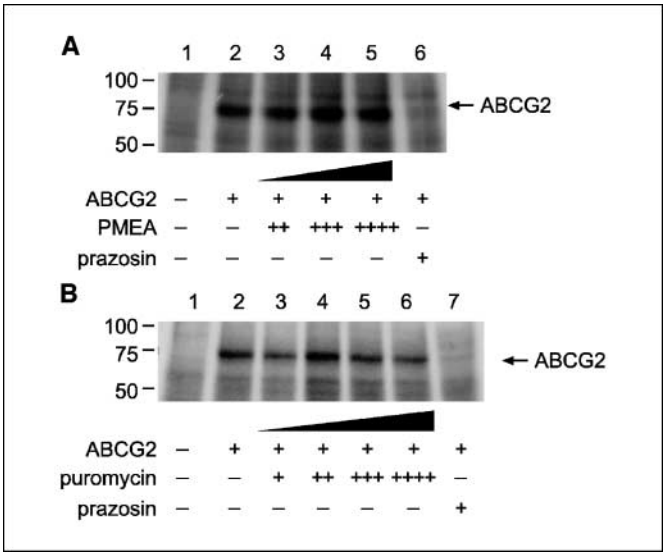

Figure 6. Neither PMEA nor puromycin competed with [^{125}I]IAAP for binding to ABCG2. [^{125}I]IAAP photoaffinity labeling of ABCG2 in membranes derived from cells expressing ABCG2 [MCF-7/FLV1000 cells (these cells express the WT R482 allele)] and the effect of the addition of various concentrations of PMEA [1(++), 5 (+++), and 10 (++++) μmol/L; *A*] and puromycin [1 (+), 5 (++), 10 (+++), and 25 (++++) μmol/L; *B*] in the presence of prazosin (10 μmol/L) or DMSO as controls on [^{125}I]IAAP labeling. *Dark triangle,* increasing concentration.

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