Contribution of the drug transporter ABCG2 (breast cancer resistance protein) to resistance against anticancer nucleosides

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Abstract

We have studied the potential contribution of ABCG2 (breast cancer resistance protein) to resistance to nucleoside analogues. In cells transfected with DNA constructs resulting in overexpression of human or mouse ABCG2, we found resistance against cladribine, clofarabine, fludarabine, 6-mercaptopurine, and 6-mercaptopurine riboside in both MDCKII and HEK293 cells and against gemcitabine only in HEK293 cells. With Transwell studies in MDCK cells and transport experiments with vesicles from SF9 and HEK293 cells, we show that ABCG2 is able to transport not only the nucleotide CdAMP, like several other ATP-binding cassette transporters of the ABC (multidrug resistance protein) family, but also the nucleoside cladribine itself. Expression of ABCG2 in cells results in a substantial decrease of intracellular CdATP, explaining the resistance against cladribine. The high transport rate of cladribine and clofarabine by ABCG2 deduced from Transwell experiments raises the possibility that this transporter could affect the disposition of nucleoside analogues in patients or cause resistance in tumors. [Mol Cancer Ther 2008;7(9):3092–102]

Introduction

Nucleoside, nucleotide, and nucleobase analogue drugs are commonly used in the treatment of various types of cancer as well as in antiretroviral (HIV) therapy. Uptake and efflux of these drugs is mediated by equilibrative (uptake and efflux) transporters (SLC29 family) and concentrative (uptake) nucleoside transporters (SLC28 family) located in the plasma membrane (1, 2). Their cytotoxic action requires conversion into nucleotides and often incorporation into DNA. Whereas resistance of retroviruses against these analogues is usually due to modification of the substrate specificity of the viral reverse transcriptase, resistance of cancer cells is mainly due to interference with nucleoside uptake (1) or decreased conversion of nucleoside into nucleoside monophosphate by nucleoside kinases (3, 4).

A new potential mechanism for nucleoside analogue resistance was discovered by Schuetz et al. (5), who showed that a lymphoid cell line selected for zidovudine resistance highly overexpressed the gene for multidrug resistance protein 4 (ABCC4). Experiments with transfected cells overexpressing transporter gene constructs subsequently showed that both ABCC4 and ABCC5 (multidrug resistance protein 5) can cause resistance to a range of nucleoside analogues and to the thiopurines 6-mercaptopurine (6-MP) and thioguanine (5–15). Invariably, resistance was due to the ability of ABCC4 and ABCC5 to extrude the nucleoside monophosphates from the resistant cells rather than the nucleoside or nucleobase analogues themselves or the corresponding nucleoside diphosphates and triphosphates. Whether ABCC4 and ABCC5 contribute to resistance in human patients remains to be seen. The low affinity of these transporters for nucleoside monophosphate analogues makes this doubtful (10, 11), but more studies are required for a definitive conclusion (12).

ABCC4 and ABCC5 belong to the C subfamily of the ATP-binding cassette (ABC) transporters. One other member of this large subfamily of organic anion transporters, ABCC11, is also able to mediate resistance to some nucleobase and nucleoside analogues (16). In addition, several groups recently reported that an ABC transporter from the G family, the ABCG2 (breast cancer resistance protein), is able to cause cellular resistance to various nucleoside analogues, such as the antiretroviral compounds zidovudine, lamivudine (17, 18), and PMEA (19) and the antileukemic purine analogue [8-3H]2-chloro-2-deoxycadenosine (cladribine, 2-CdA; ref. 19). ABCG2 transports a wide range of xenotoxins (reviewed in refs. 20–22) and endogenous metabolites, such as riboflavin (23). It is present in the apical membrane of many tissues, such as...
the bile canalicular membrane of hepatocytes in the liver, the syncytiotrophoblasts in the placenta, the microvessel endothelium at the blood brain barrier, and the villi in the intestine (20–23). This location makes ABCG2 an important determinant of the pharmacodynamics and thus efficacy of administered drugs that are a substrate of ABCG2 (20–23).

In the work of Wang et al. (17, 18), resistance to zidovudine caused by ABCG2 was shown to be due to reduced drug accumulation in the cells, but the nature of the metabolite exported was not defined. As we have shown that ABCG2, like ABCC4 and ABCC5 (6, 24–27), is able to transport cyclic GMP (28), this raised the possibility that ABCG2 is also able to export nucleotide analogues, like ABCC4 and ABCC5 do. To characterize the transport specificity of ABCG2 for nucleoside and nucleobase analogue drugs, we determined ABCG2-mediated resistance to a panel of clinically relevant drugs. In two independent cell lines, we found that ABCG2 mediated high-level resistance against cladribine, clofarabine, and mercaptopurine (6-MP) among several other drugs that were tested. Of these drugs, we chose cladribine as a model substrate for further analysis of the transported substrate(s). We show here that ABCG2 transports the intact nucleoside, cladribine, as well as the monophosphate metabolite.

Materials and Methods

Materials

2-CdA, [8-3H]2-chloro-2'-dAMP (2-CdAmp), and [8-14C]6-MP were purchased from Moravek Biochemicals. [14C]inulin and [3',5',7-3H]methotrexate were purchased from GE Healthcare. 2-Chloro-2'-deoxy-2'-fluoro-β-D-adenosine (clofarabine) and [8-14C]clofarabine were a gift from Genzyme. Ko143 was a gift from Dr. A. Schinkel. The Netherlands Cancer Institute. Rat anti-mouse ABCG2 antibody, BXP-53, was a gift from Dr. G.L. Scheffer (Free University Amsterdam). Rabbit anti-rat IgG horseradish peroxidase was obtained from DAKO. All culture reagents were obtained from Invitrogen. All other chemicals and reagents were purchased from Sigma-Aldrich.

Synthesis of 2-CdAMP

2-CdAMP was synthesized using a previously described phosphorylation method with modifications (29, 30). In brief, 0.1 mmol dried 2-CdA (Sequova Research Products) was dissolved in 2.5 mL trimethylphosphate together with 0.15 mmol 1,8-bis(dimethylamino) naphthalene (Proton Sponge). The reaction was carried out on ice water. Phosphorous oxychloride (0.4 mmol) was added dropwise to the stirred solution. After 2 h, the reaction mixture was poured into 20 mL of 1 mol/L triethylammonium bicarbonate solution (pH 8.5). The reaction yield was 69%. The solvents were evaporated under reduced pressure and the remaining semisolid was washed twice with diethylther. After reconstitution in water, 100 μL volumes of the product were injected onto a Biosep DEAE-PEI column (75 × 7.8 mm, 7 μm; Phenomenex) and chro-matographed using an ammonium bicarbonate gradient (0-360 mmol/L) at 0.5 mL/min. The UV absorption of the eluate was monitored at 264 nm and the fractions containing 2-CdAMP were collected and lyophilized twice. High-performance liquid chromatography (HPLC)-mass spectrometry analysis of the product on a weak anion exchange column coupled to a Quantum Ultra mass spectrometer (Thermo Fisher Scientific) operated in the positive ion mode (31) confirmed the identity of the product [m/z 366 (M + H)+]. The purity of the product was 96% as determined by HPLC-UV (31). Solutions of the final product in water were quantitated using HPLC-UV (31) and stored at −70°C.

Cell Culture and Transduction

Wild-type (WT) Madin-Darby canine kidney (MDCKII) cells and MDCKII cells stably transduced with human ABCG2 (32) or mouse Abcg2 (33) were described before and were kindly provided by Dr. A. Schinkel. Additionally, human embryonic kidney (HEK293) cells were stably transduced with the retroviral LZR5-ires-GFP expression vector (34) containing the cDNA for either human ABCG2 or mouse Abcg2 as described previously (32). After expansion, clones were screened for expression of (functional) ABCG2/Abcg2 by determination of increased resistance to mitoxantrone in a cytotoxicity assay and by immunoblot analysis. Membrane localization was determined by immunocytochemistry. All cell lines were cultured in DMEM, which contained 10% FCS and was supplemented with 100 units/mL penicillin/streptomycin.

Immunoblot Analysis

Membrane vesicles (20 μg protein) were fractionated on a denaturing 7.5% polyacrylamide slab gel and transferred onto a nitrocellulose membrane. After blocking for 1 h in PBS containing 1% nonfat dry milk, 1% bovine serum albumin, and 0.05% Tween 20, the membrane was incubated at 1 h at room temperature with the specific antibody. ABCG2 and Abcg2 were detected with the monoclonal antibody BXP-53 (1:400; ref. 35). As secondary antibody, horseradish peroxidase-conjugated rabbit anti-rat IgG was used at a dilution of 1:1,000. Enhanced chemiluminescence was used for detection by incubating the membrane for 1 min with freshly mixed 1.25 mmol/L 3-aminophtalhydrazide, 0.2 mmol/L p-coumaric acid, and 0.01% (v/v) H2O2 in 0.1 mol/L Tris (pH 8.5).

Semi quantitative PCR

Total RNA was isolated from cell cultures using Trizol reagent (Invitrogen) according to the manufacturer’s recommendations. Total RNA (2 μg) was reverse transcribed using SuperScript II reverse transcriptase and random hexamers (Invitrogen). One-sixtieth of the cDNA was used in the PCR analysis. ABCG2/Abcg2 transcript levels were determined by densitometric measurement of PCR product using Syngene densitometric software and normalized to the internal standard, β-actin. Degenerate ABCG2/Abcg2 primers, 5'-CTTACAGTTTC-AGCAGCTC-3' and 5'-ATGGAGAAGATGATTTCG-3', were used to generate a PCR product of 309 bp spanning exons 4 to 7. For canine β-actin (MDCKII cells), the
following primers were used, 5'-GGGCAGGCAGGCGGT-GATG-3' and 5'-GCCCTGGATGCACATACATGC-3', to generate a PCR product of 299 bp. For human β-actin (HEK293 cells), the following primers were used, 5'-AG-GCACCAGGGCGTGATG-3' and 5'-GCCCTGGATAGCAGTGACATG-3', to generate a PCR product of 299 bp. Using the method, the monophosphate, diphosphate, and triphosphate could not be determined due to the low levels present and interference of growth medium. This method was therefore used only to determine cladribine levels. Briefly, samples (10 μL) were injected onto a Gemini C18 column (150 × 4.6 mm, 3 μm; Phenomenex) thermostatted at 45°C. The sample was eluted using a 0.2 mL/min isocratic flow of a triethylamine phosphate [pH 6.75; prepared by mixing 0.1 mol/L triethylamine and 0.09 mol/L phosphoric acid (pH 6.75)] containing 10% methanol. UV absorption was measured at 265 nm. The lower limit of quantitation was 20 nmol/L 2-CdA.

For the determination of cladribine monophosphate, diphosphate, and triphosphate, a more sensitive and specific weak anion exchange HPLC method coupled to tandem mass spectrometry was developed and validated (31). Samples were spiked with a mixture of internal standards and a volume of 25 μL was injected onto a BioBasic AX column (50 × 2.1 mm, 5 μm; Thermo Fisher Scientific). A 0.25 mL/min binary gradient of 10 mmol/L ammonium acetate in water/acetonitrile (70:30, v/v; pH 6.0; solvent A) and 1 mmol/L ammonium acetate in water/acetonitrile (70:30, v/v; pH 10.5; solvent B) was used to separate the nucleotides. The following gradient was used: 0 to 0.5 min 10% solvent B, 0.51 to 1.75 min 50% solvent B, 1.76 to 6.5 100% solvent B, 6.5 to 6.6 min linear to 10% solvent B, 6.6 to 10 min 10% solvent B. A Quantum Ultra mass spectrometer (Thermo Fisher Scientific) operated in the positive ion mode was used to monitor specific mass transitions of each analyte (2-CdATP m/z 525.9→170, 2-CdADP m/z 445.9→170, and 2-CdAMP m/z 365.9→170). The validated range was 1.11 to 27.7, 0.550 to 55.0, and 1.31 to 52.3 nmol/L for 2-CdAMP, 2-CdADP, and 2-CdATP, respectively.

**Vesicular Uptake Assay**

Inside-out membrane vesicles were prepared from Spodoptera frugiperda insect cells (S9) and HEK293 cells overproducing human ABCG2 and mouse Abcg2 as described previously (38) with the following modification. The final homogenization and vesicular storage buffer for HEK293 cells was 10 mmol/L Tris-HCl (pH 7.4) instead of 50 mmol/L Tris-HCl/250 mmol/L sucrose (pH 7.4). ATP-dependent transport of radiolabeled compounds into inside-out membrane vesicles was measured using the rapid filtration technique as described previously (38) with the following modifications. Vesicular uptake buffer for incubations with S9 vesicles consisted of 50 mmol/L Tris-HCl/250 mmol/L sucrose (pH 7.4). ATP-dependent transport of radiolabeled compounds into inside-out membrane vesicles was measured using the rapid filtration technique as described previously (38) with the following modifications. Vesicular uptake buffer for incubations with S9 vesicles consisted of 50 mmol/L Tris-HCl/250 mmol/L sucrose (pH 7.4). For incubations with HEK293 vesicles, the vesicular uptake buffer was 10 mmol/L Tris-HCl (pH 7.4). High-throughput filtration assays were done in Multiscreen HTS FB 96-well filter plates on the Millipore Multiscreen HTS vacuum manifold (Millipore). Filters were washed four times with 200 μL PBS and the radioactivity retained on the filter was counted by liquid scintillation counting.

**Statistical Analysis**

A two-sided Student’s t test was used to determine the statistical significance between experimental groups.
ABCG2/Abcg2 RNA and Protein Levels in Transfected MDCKII and HEK293 Cells

In addition to the available MDCKII cells overexpressing human ABCG2 (32) and mouse Abcg2 (33), we constructed HEK293 cells overexpressing these transporter genes to extend the range of cells available for resistance studies. The continued expression of ABCG2/Abcg2 was routinely checked by Western protein blot analysis and was stable over at least 20 cell culture passages. ABCG2/Abcg2 was located at the apical membrane in a monolayer of polarized MDCKII-cells and at the membrane of HEK293 cells as determined by immunohistochemistry (results not shown). As shown in Supplementary Fig. S1A, cells making mouse Abcg2 produced a more prominent band on Western blots than cells making human ABCG2 and the mouse band also migrated more slowly through polyacrylamide gels. The difference in migration is due to a difference in N-linked glycosylation: after deglycosylation with endoglycosidase F, the two proteins comigrated as expected based on their estimated open reading frames, 656 (ABCG2; ref. 39) and 657 (Abcg2; ref. 40) amino acids, respectively (results not shown). The difference in the intensity of the ABCG2 and Abcg2 band could be due to a higher affinity of the antibody for the mouse protein, as this was used to raise the BXP-53 antibody (35). To check expression levels, transcript levels were analyzed by semiquantitative PCR with degenerate ABCG2/Abcg2 primers (Supplementary Fig. S1B). Approximately 2-fold more Abcg2 transcripts were calculated to be present in cDNA prepared from both a confluent and a log-phase cell culture from the MDCKII-mAbcg2 clone than in the MDCKII-hABCG2 clone, whereas similar transcript amounts were calculated for HEK293-mAbcg2 and HEK293-hABCG2.

Table 1.

(A) Growth inhibition of MDCKII, MDCKII-hABCG2, and MDCKII-mAbcg2 cells by nucleoside, nucleotide, and nucleobase analogues

<table>
<thead>
<tr>
<th>Drug</th>
<th>IC50 (SE) WT</th>
<th>IC50 (SE) hABCG2</th>
<th>IC50 (SE) mAbcg2</th>
<th>RF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mitoxantrone</td>
<td>0.02 (0.01)</td>
<td>0.20 (0.06)</td>
<td>1.69 (0.25)</td>
<td>12.1*</td>
</tr>
<tr>
<td>CPT11</td>
<td>0.02 (0.00)</td>
<td>0.02 (0.00)</td>
<td>0.07 (0.01)</td>
<td>0.9</td>
</tr>
<tr>
<td>Cladribine</td>
<td>0.51 (0.03)</td>
<td>2.02 (0.32)</td>
<td>3.46 (2.06)</td>
<td>4</td>
</tr>
<tr>
<td>Clofarabine</td>
<td>0.45 (0.06)</td>
<td>2.79 (0.45)</td>
<td>85.75 (22.61)</td>
<td>6.2</td>
</tr>
<tr>
<td>Fludarabine</td>
<td>139.9 (18.1)</td>
<td>491.6 (142.2)</td>
<td>206.4 (15.3)</td>
<td>3.5*</td>
</tr>
<tr>
<td>Gemcitabine</td>
<td>0.19 (0.02)</td>
<td>0.16 (0.02)</td>
<td>0.12 (0.01)</td>
<td>0.8</td>
</tr>
<tr>
<td>6-MP riboside</td>
<td>13.9 (0.8)</td>
<td>139.2 (0.9)</td>
<td>5.4 (0.5)</td>
<td>10</td>
</tr>
<tr>
<td>Thioguanine riboside</td>
<td>10.0 (0.7)</td>
<td>21.4 (0.7)</td>
<td>5318.6 (543.5)</td>
<td>2.1*</td>
</tr>
<tr>
<td>6-MP</td>
<td>29.5 (1.6)</td>
<td>2938.7 (415.3)</td>
<td>180.1</td>
<td>99.5</td>
</tr>
<tr>
<td>Thioguanine</td>
<td>25.4 (1.1)</td>
<td>370 (3.7)</td>
<td>31.4 (3.4)</td>
<td>5.8</td>
</tr>
<tr>
<td>5-Fluorouracil</td>
<td>1.75 (0.38)</td>
<td>1.75 (0.06)</td>
<td>0.76 (0.10)</td>
<td>1.2</td>
</tr>
<tr>
<td>Bis-POM-PMEA</td>
<td>13.40 (1.49)</td>
<td>36.80 (5.01)</td>
<td>53.48 (1.62)</td>
<td>2.8</td>
</tr>
</tbody>
</table>

(B) Growth inhibition of HEK293, HEK293-hABCG2, and HEK293-mAbcg2 cells by nucleoside, nucleotide, and nucleobase analogues

<table>
<thead>
<tr>
<th>Drug</th>
<th>IC50 (SE) WT</th>
<th>IC50 (SE) hABCG2</th>
<th>IC50 (SE) mAbcg2</th>
<th>RF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mitoxantrone</td>
<td>0.06 (0.01)</td>
<td>0.11 (0.01)</td>
<td>0.38 (0.07)</td>
<td>1.8*</td>
</tr>
<tr>
<td>CPT11</td>
<td>0.01 (0.00)</td>
<td>0.02 (0.00)</td>
<td>0.02 (0.00)</td>
<td>1.8*</td>
</tr>
<tr>
<td>Cladribine</td>
<td>0.64 (0.21)</td>
<td>2.62 (0.46)</td>
<td>3.71 (0.49)</td>
<td>4</td>
</tr>
<tr>
<td>Clofarabine</td>
<td>0.37 (0.10)</td>
<td>0.88 (0.11)</td>
<td>1.78 (0.21)</td>
<td>2.4</td>
</tr>
<tr>
<td>Fludarabine</td>
<td>54.85 (11.6)</td>
<td>90.71 (2.7)</td>
<td>85.83 (2.8)</td>
<td>2</td>
</tr>
<tr>
<td>Gemcitabine</td>
<td>0.02 (0.01)</td>
<td>0.06 (0.01)</td>
<td>0.05 (0.00)</td>
<td>1.6*</td>
</tr>
<tr>
<td>6-MP riboside</td>
<td>0.74 (0.11)</td>
<td>2.86 (0.29)</td>
<td>2.11 (0.24)</td>
<td>3.9</td>
</tr>
<tr>
<td>Thioguanine riboside</td>
<td>0.42 (0.6)</td>
<td>0.83 (0.16)</td>
<td>0.66 (0.09)</td>
<td>2.2</td>
</tr>
<tr>
<td>6-MP</td>
<td>1.66 (0.19)</td>
<td>7.01 (1.87)</td>
<td>6.02 (1.03)</td>
<td>2.3</td>
</tr>
<tr>
<td>Thioguanine</td>
<td>1.03 (0.04)</td>
<td>2.23 (0.57)</td>
<td>4.43 (0.61)</td>
<td>2.2</td>
</tr>
<tr>
<td>5-Fluorouracil</td>
<td>4.97 (1.48)</td>
<td>4.66 (0.51)</td>
<td>8.20 (0.44)</td>
<td>1.0</td>
</tr>
<tr>
<td>Bis-POM-PMEA</td>
<td>0.81 (0.15)</td>
<td>1.85 (0.05)</td>
<td>1.85 (0.05)</td>
<td>2.8</td>
</tr>
</tbody>
</table>

NOTE: IC50 values are mean ± SE (μmol/L) drug of three to six experiments in triplicate. Cell number was stained and quantified by the sulforhodamine B colorimetric assay as described in Materials and Methods, except for mitoxantrone and CPT11. Cytotoxicity of these drugs was quantified by cell counting and trypan blue exclusion. Statistical significance was calculated with an unpaired two-tailed Student’s t test.

* P < 0.05.
† P < 0.01.
‡ P < 0.001.

Results

ABCG2/Abcg2 RNA and Protein Levels in Transfected MDCKII and HEK293 Cells

In addition to the available MDCKII cells overexpressing human ABCG2 (32) and mouse Abcg2 (33), we constructed HEK293 cells overexpressing these transporter genes to extend the range of cells available for resistance studies. The continued expression of ABCG2/Abcg2 was routinely checked by Western protein blot analysis and was stable over at least 20 cell culture passages. ABCG2/Abcg2 was located at the apical membrane in a monolayer of polarized MDCKII-cells and at the membrane of HEK293 cells as determined by immunohistochemistry (results not shown).

As shown in Supplementary Fig. S1A, cells making mouse Abcg2 produced a more prominent band on Western blots than cells making human ABCG2 and the mouse band also migrated more slowly through polyacrylamide gels. The difference in migration is due to a difference in N-linked glycosylation: after deglycosylation with endoglycosidase F, the two proteins comigrated as expected based on their estimated open reading frames, 656 (ABCG2; ref. 39) and 657 (Abcg2; ref. 40) amino acids, respectively (results not shown). The difference in the intensity of the ABCG2 and Abcg2 band could be due to a higher affinity of the antibody for the mouse protein, as this was used to raise the BXP-53 antibody (35). To check expression levels, transcript levels were analyzed by semiquantitative PCR with degenerate ABCG2/Abcg2 primers (Supplementary Fig. S1B). Approximately 2-fold more Abcg2 transcripts were calculated to be present in cDNA prepared from both a confluent and a log-phase cell culture from the MDCKII-mAbcg2 clone than in the MDCKII-hABCG2 clone, whereas similar transcript amounts were calculated for HEK293-mAbcg2 and HEK293-hABCG2.

4 Supplementary material for this article is available at Molecular Cancer Therapeutics Online (http://mct.aacrjournals.org/).
cells. Hence, the apparent difference in protein levels in Supplementary Fig. S1A is not reflected in transcript levels in Supplementary Fig. S1B.

**Resistance to Nucleoside and Nucleobase Analogues Mediated by Human and Mouse ABCG2/Abcg2**

Table 1 summarizes our results of the effects of ABCG2 and Abcg2 on cellular resistance to nucleoside and nucleobase analogues used in treatment of cancer. For comparison, resistance to the classic ABCG2 substrates mitoxantrone and CPT11 and to the antiviral nucleotide analogue bis-POM-PMEA was determined. In general, resistance levels found were higher for Abcg2-transfected cells than for ABCG2-transfected cells and for MDCKII cells than for HEK293 cells. Notable exceptions were found, however. Whereas we did not find any resistance to gemcitabine in MDCKII cells, substantial resistance to this drug was found in the transfected HEK293 cells.

High resistance was found against the purine analogues cladribine, clofarabine, and 6-MP. Resistance against 6-MP was especially high in MDCKII cells, with 100-fold (ABCG2) and 180-fold (Abcg2) resistance ($P < 0.001$), respectively. Resistance was much less in the transfected...
HEK293 cells, 4.2-fold ($P < 0.05$) and 3.6-fold ($P < 0.01$), respectively (Table 1B). In both cell lines, increased resistance mediated by ABCG2/Abcg2 was also found for 6-MP riboside.

**Transport of Cladribine and Clofarabine by ABCG2/Abcg2 through MDCKII Monolayers**

Figure 1A shows the effect of the presence of human or mouse ABCG2 on the transport of radioactive cladribine and clofarabine through monolayers of MDCKII cells. Nearly 30% of the radioactivity associated with [3H]cladribine goes through the monolayer in WT cells and the rate of transport is the same in both directions. Uptake of both cladribine and clofarabine into cells is known to be mediated mainly by the equilibrative nucleoside transporter ENT1 and the concentrative nucleoside transporter CNT3 and to a lesser extent also by ENT2 and CNT2 (41, 42). Diffusion through the membrane is probably minimal, as transport of [3H]cladribine through the monolayer was inhibited >90% by excess cold clofarabine (results not shown). The presence of ABCG2/Abcg2 in the apical membrane increased transport in the apical direction and decreased transport in the basal direction. These effects were completely blocked by addition of 1 μmol/L of the specific ABCG2 inhibitor Ko143 (43) to the cell culture medium (Fig. 1B). We have shown previously that resistance to nucleoside or nucleobase analogues caused by the ABC transporters ABCC4 and ABCC5 is due to transport of the corresponding nucleoside monophosphates formed in the cell (10). To determine which form of cladribine is transported by ABCG/Abcg2, we used a recently developed HPLC-tandem mass spectrometry method that can separate cladribine and cladribine monophosphate, diphosphate, and triphosphate (31).

We analyzed the medium and cell lysates from 4 h Transwell transport assays in which 10 μmol/L cladribine was added to either the medium at the basolateral or the apical side of the MDCKII monolayer. The results of these experiments are summarized in Fig. 2 and Table 2. The striking result is that more than 99% of the transported compounds were identified as cladribine under all conditions. Whereas parental cells effluxed similar amounts of cladribine apically and basolaterally, the presence of ABCG2 or Abcg2 in the cells increased efflux at the apical side and reduced basolateral efflux to minimal values. Only low levels of 2-CdAMP were formed intracellularly, even in parental MDCKII cells, suggesting that deoxycytidine kinase levels are low in these cells. Nevertheless, the increased appearance of 2-CdAMP in the apical medium from ABCG2/Abcg2-overexpressing cells compared with WT MDCKII cells (Table 2A) indicated that 2-CdAMP is a substrate of...
Table 2. Levels of cladribine and metabolites in medium and cell lysates of MDCKII, MDCKII-ABCG2, and MDCKII-Abcg2 cells after a 4 h transepithelial transport assay

<table>
<thead>
<tr>
<th></th>
<th>MDCKII WT</th>
<th>ABCG2</th>
<th>Abcg2</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Apical medium</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cladribine</td>
<td>1,348.4 ± 108.7</td>
<td>2,794.7 ± 108.8</td>
<td>4,480.2 ± 753.8</td>
</tr>
<tr>
<td>CdAMP</td>
<td>3.4 ± 1.1</td>
<td>22.4 ± 6.6</td>
<td>12.1 ± 3.3</td>
</tr>
<tr>
<td><strong>Basolateral medium</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cladribine</td>
<td>5,963.2 ± 166.9</td>
<td>4,484.6 ± 167.1</td>
<td>3,533.1 ± 655.8</td>
</tr>
<tr>
<td>CdAMP</td>
<td>19.7 ± 6.3</td>
<td>23.5 ± 6.9</td>
<td>3.4 ± 0.4</td>
</tr>
<tr>
<td>Lysate</td>
<td>6.6 ± 0.7</td>
<td>2.6 ± 1.0</td>
<td>0.4 ± 0.1</td>
</tr>
<tr>
<td>CdADP</td>
<td>1.5 ± 0.2</td>
<td>0.5 ± 0.2</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>CdATP</td>
<td>4.2 ± 0.5</td>
<td>1.3 ± 0.5</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td><strong>Basolateral medium</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cladribine</td>
<td>1,270.2 ± 37.4</td>
<td>357.9 ± 78.8</td>
<td>56.1 ± 11.0</td>
</tr>
<tr>
<td>CdAMP</td>
<td>11.1 ± 2.8</td>
<td>8.8 ± 3.4</td>
<td>0.7 ± 0.0</td>
</tr>
<tr>
<td>Apical medium</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cladribine</td>
<td>5,966.4 ± 60.5</td>
<td>6,874.3 ± 237.7</td>
<td>7,269.6 ± 176.8</td>
</tr>
<tr>
<td>CdAMP</td>
<td>7.2 ± 2.2</td>
<td>21.6 ± 10.2</td>
<td>9.3 ± 1.4</td>
</tr>
<tr>
<td>Lysate</td>
<td>5.6 ± 0.4</td>
<td>1.6 ± 0.5</td>
<td>0.3 ± 0.1</td>
</tr>
<tr>
<td>CdADP</td>
<td>1.1 ± 0.1</td>
<td>0.3 ± 0.1</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>CdATP</td>
<td>3.4 ± 0.3</td>
<td>0.8 ± 0.3</td>
<td>0.1 ± 0.1</td>
</tr>
</tbody>
</table>

NOTE: (A) Cladribine (750 µL, 10 µmol/L) was added to the basolateral compartment at t = 0. (B) Cladribine (750 µL, 10 µmol/L) was added to the apical compartment at t = 0. Cladribine and metabolites were measured and quantified as described in Materials and Methods. Values are mean ± SE (in pmol) of three independent experiments in duplicate.

ABCG2/Abcg2. The possibility that 2-CdAMP levels were underestimated due to 2-CdAMP dephosphorylation by extracellular 5'-nucleotidases after extrusion from the cell was excluded by addition of 5'-nucleotidase inhibitors to the medium: Addition of high concentrations (100 µmol/L) of the inhibitors AMP-PCP and ARL67156 had no effect on 2-CdAMP levels (results not shown). No CdADP or CdATP was detectable in the medium and these compounds are presumably not transported by ABCG2/Abcg2. In WT cells, 2-CdAMP was effluxed more into the basolateral than into the apical medium, suggesting the presence of an endogenous transporter at the basolateral membrane, which is capable of 2-CdAMP transport. This could be a member of the ABCC family, as efflux was inhibited >90% by 100 µmol/L indomethacin, a general inhibitor of ABCC-type transporters (results not shown; ref. 44).

The 2-CdAMP efflux observed is a complex function of the rate of cladribine transport and the side of the monolayer to which cladribine is added. In the ABCG2-transfected cells, apical efflux of cladribine is moderately increased, but there is still sufficient 2-CdAMP formed in the cell (Fig. 2C and D; Table 2) to allow ABCG2 to substantially increase 2-CdAMP efflux relative to WT cells at the apical side, without influencing efflux at the basolateral side (Fig. 2B). In the Abcg2-transfected cells, the apical efflux of cladribine is strongly increased. This appears to result in such a reduced level of intracellular cladribine—intracellular cladribine concentrations were consistently below the limit of detection and could therefore not be measured—that transport of cladribine from apical to basolateral virtually ceases (Fig. 2A; Table 2). As a consequence, also 2-CdAMP synthesis is reduced, explaining why the apical efflux of 2-CdAMP relative to ABCG2-transfected cells is reduced and why basolateral efflux is nearly abolished.

The side at which cladribine is added to the monolayer affects the amount of 2-CdAMP effluxed into the basolateral medium. In both ABCG2- and Abcg2-transfected cells, more 2-CdAMP tends to come out basolaterally when cladribine is added basolaterally than when it is added apically (ABCG2 cells: 23.5 versus 8.8 pmol, P = 0.1; Abcg2 cells: 3.4 versus 0.7 pmol, P = 0.002). This indicates that the presence of ABCG2/Abcg2 in the apical membrane results in a locally low intracellular cladribine concentration near the apical membrane. If cladribine is added at the apical side, drug entering the cell is immediately pumped out. If it is added at the basolateral side, it has to diffuse through the cell to the apical membrane before it can be pumped out, thereby setting up a diffusion gradient within the cell with higher cladribine concentrations near the basolateral membrane and hence more 2-CdAMP synthesis than near the apical membrane.

Because the cytotoxic effect of cladribine mainly results from its conversion into 2-CdATP and its subsequent incorporation into DNA, it is important to note that the
2-CdATP concentrations in the ABCG2/Abcg2-transfected cells are much lower than in the WT cells explaining the substantial resistance to cladribine observed in these cells (Fig. 2C and D; Table 1).

Because over 99% of total transported substrate was cladribine, we determined the apparent affinity (K_m) of ABCG2 for cladribine from net transport curves with radiolabeled substrate (Fig. 4); transport in MDCKII-ABCG2/Abcg2 monolayers minus that in MDCKII parental monolayers. This affinity is only very approximate, as the cladribine concentration in the medium is only distantly related to the intracellular cladribine concentration seen by the transporter. ABCG2 had a higher affinity for cladribine than the murine orthologue (80 ± 7 versus 248 ± 26 μmol/L). On the other hand, the velocity of cladribine transport by Abcg2 was much higher than that of ABCG2 with V_{max} values of 121 ± 4 versus 17 ± 0.5 nmol (monolayer) h^{-1}. We think that the possible difference in the amount of ABCG2/Abcg2 in the cell membrane (Supplementary Fig. S1) is not enough to explain this difference in velocity and that the Abcg2 present may be more active.

To test whether other ABC transporters can transport cladribine as well, additional Transwell assays were conducted with MDCKII monolayers overexpressing the genes for ABCC transporters 1 to 5 as well as ABCB1. We did not find an increased efflux of [3H]cladribine to the apical (ABC1, ABCC2) or basolateral (ABCC1, ABCC3, ABCC4, and ABCC5) medium in any of these cell lines (result not shown). From these experiments, we infer that none of these ABC-transporters transport cladribine.

Vesicular Transport of Cladribine and 2-CdAMP by ABCG2/Abcg2

To determine kinetic variables for the transport of cladribine and 2-CdAMP by ABCG2/Abcg2, we tested both substrates in vesicular transport by vesicles from Sf9 or HEK293 cells overexpressing ABCG2 or Abcg2. No transport was observed with [3H]cladribine. Presumably, cladribine is transported into the vesicles but leaks out through equilibrative nucleoside transporters or by passive diffusion. We therefore tested cladribine transport in an indirect fashion by determining its ability to competitively inhibit uptake of [3H]methotrexate into Sf9 vesicles. The experiments were done at pH 7.4 rather than at pH 5.5, where transport rates are higher (45), to make the results obtained comparable with those of the Transwell experiments done at pH 7.4. The affinity of Abcg2 for methotrexate, not determined previously, proved to be similar to that of ABCG2 at about 2 mmol/L, in line with published data (45–47). Similar values were obtained with Sf9 (Fig. 4A) and HEK293 (Fig. 4B) vesicles. Cladribine inhibited methotrexate transport in a competitive fashion, inhibition being higher at 0.5 than at 4 mmol/L methotrexate. Human ABCG2 was much more inhibited than the murine Abcg2, the apparent inhibitory constants K_i being about 50 and 300 to 400 μmol/L (Fig. 4). This is in agreement with the Transwell experiments in which the apparent K_m of ABCG2 for cladribine was much lower than of Abcg2 (Fig. 3).

Obtaining kinetic variables for CdAMP uptake was unexpectedly complicated. At low CdAMP concentrations, vesicles from WT Sf9 cells bound significant amounts of [3H]CdAMP and this apparent accumulation was not prevented by addition of nonhydrolyzable ATP analogues (not shown). The nature of this association of [3H]CdAMP with the vesicles is not known. To assess transport by ABCG2, we corrected all values for CdAMP accumulation by ABCG2-Sf9 and Abcg2-Sf9 vesicles with the values obtained with WT vesicles. With these corrections, CdAMP uptake by ABCG2/Abcg2 was linear for about 10 min (not shown) and transport was saturable as shown in Supplementary Fig. S2. At the high CdAMP concentrations required for saturation, the values are inaccurate because of the relatively low specific radioactivity of the [3H]CdAMP available. Hence, only approximate values for the kinetic variables can be deduced. For ABCG2, we find in Sf9 cells in three experiments a K_m of about 148 μmol/L (range, 126-165) and a V_{max} of about 775 pmol/mg protein/min (range, 578-984). For the Abcg2, data are even less accurate, and in two experiments, we find...
an average $K_m$ of about 0.5 mmol/L and a $V_{max}$ of 0.4 nmol/mg protein/min. Compared with other ABCG2 substrates, these rates of transport are substantial, but the affinity for this substrate is relatively low.

Transport of 6-MP by Human and Mouse ABCG2/Abcg2

ABCG2/Abcg2 conferred increased resistance in cytotoxicity assays to both 6-MP and 6-MP riboside in both cell lines tested, MDCKII and HEK293 (Table 1). Also, the directional transport of $[^{14}C]$6-MP in epithelial transport assays with monolayers of MDCKII cells was higher in the basal to apical direction in ABCG2/Abcg2-overexpressing cells than in WT cells and this increased rate was reduced to WT levels on addition of the ABCG2-specific inhibitor Ko143 (results not shown). Preliminary HPLC data indicate that 6-MP riboside as well as the monophosphate metabolite, tIMP, is transported by ABCG2/Abcg2.$^5$

Discussion

We have found that ABCG2 can cause resistance to several nucleobase and nucleoside analogues used in cancer chemotherapy in addition to its effect on the antiretroviral nucleosides zidovudine and lamivudine (17, 18). Remarkably, ABCG2 transports not only the nucleoside monophosphate metabolite of cladribine—in analogy with other ABC transporters that can cause resistance to nucleobase and nucleoside analogues (6, 11, 12, 14)—but also cladribine itself. Presumably, this holds also for (some of) the other nucleoside analogues affected by ABCG2, such as clofarabine, fludarabine, and 6-MP riboside. Our preliminary results for 6-MP confirm this assumption.$^5$ Moreover, while our work was being finished, Pan et al. (48) reported evidence that the antiretroviral nucleosides zidovudine and abacavir are transported as nucleosides as well by Abcg2.

In the cell clones used here, transport of cladribine by Abcg2 was more effective than by ABCG2. Presumably, this is due to a higher catalytic activity of Abcg2 than ABCG2, as the affinity of Abcg2 for cladribine seems to be lower rather than higher than that of ABCG2 (Figs. 3 and 4). The results in Table 2 indicate that, at high transport rates of ABCG2/Abcg2, the effects on nucleoside transport contribute more to resistance than the effects on transport of 2-CdAMP: in the Abcg2 transfectant, intracellular 2-CdAMP is reduced 16-fold relative to WT cells. This cannot be due to increased 2-CdAMP transport, because both

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$^5$ Unpublished data.
apical and basolateral efflux of 2-CdAMP are actually decreased relative to the ABCG2 transfectant. This implies that high concentrations of Abcg2 can reduce intracellular cladribine concentrations to such an extent that synthesis of 2-CdAMP is lowered. This is quite an accomplishment of Abcg2 given the high rate at which cladribine enters the cells via nucleoside transporters. Obviously, the potential effect of the presence of ABCG2/Abcg2 on toxicity of cladribine to cells must depend on the ability of several metabolic processes to compete with export of cladribine/2-CdAMP by the transporter: the rate of influx of cladribine via nucleoside transporters, the rate of conversion of cladribine into 2-CdAMP by cytosolic deoxyctydine kinase and mitochondrial deoxyguanosine kinase (4), and the conversion into 2-CdADP and 2-CdATP, which can be the rate-limiting step in 2-CdATP synthesis (49). It is of interest that ABCG2/Abcg2 appears to transport clofarabine at least as efficiently as cladribine (Fig. 1; Table 1). Clofarabine has clinical advantages over cladribine and fluorarabine, because it can be administered orally (reviewed in ref. 50). ABCG2 is prominently present in the apical membrane of the intestinal epithelium and is known to interfere with drug uptake (21–23). It will be of interest to test in Abcg2−/− mice (20) whether Abcg2 affects oral availability of clofarabine.

Disclosure of Potential Conflicts of Interest

The authors reported no potential conflicts of interest.

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Transport of anti-cancer nucleosides by ABCG2


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Contribution of the drug transporter ABCG2 (breast cancer resistance protein) to resistance against anticancer nucleosides

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