Mouse Breast Cancer Resistance Protein (Bcrp1/Abcg2) Mediates Etoposide Resistance and Transport, but Etoposide Oral Availability Is Limited Primarily by P-glycoprotein

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INTRODUCTION

Epipodophyllotoxins, including etoposide and teniposide, are known to be substrates of several drug transporters, notably P-gp and the multidrug resistance-associated protein MRPI, and also MRP2 and MRP3 (1-3). Elevated activity of these transporters can mediate resistance to the drugs, at least in specific instances in vitro. Various levels of cross resistance to etoposide, ranging from negligible to substantial, have also been observed in a number of drug-selected cell lines overexpressing BCRP/Bcrp1 (4-7). However, it has been unclear to what extent this cross resistance is due to BCRP/Bcrp1 activity versus other mechanisms of resistance, such as changes in the behavior of the etoposide target, topoisomerase II, because nearly all of these cell lines were heavily selected with other drugs targeting topoisomerase II, such as mitoxantrone and doxorubicin. Specific and effective inhibitors of BCRP, which could be used to address its contribution to drug resistance in such cases, have not been readily available until recently. Moreover, although it is now clear that a number of the BCRP/Bcrp1-expressing cell lines carry a mutation at codon 482 that substantially alters the substrate specificity of the transporter (8-10), it is unclear to what extent, if any, this mutation might contribute to cross resistance to etoposide.

These issues have potential consequences for the clinical use of epipodophyllotoxins as anticancer agents. One obvious question is whether elevated BCRP constitutes a potential resistance mechanism in tumors treated with these drugs, particularly if chemosensitizers that inhibit P-gp and/or MRPI function (such as PSC833/valspodar or VX-710/biricodar) are ultimately used in chemotherapy. A related question is the likelihood of outgrowth of drug-resistant tumor cells carrying BCRP mutations that enhance the efflux of epipodophyllotoxins. Another matter, of equal importance to chemotherapy and possibly greater immediacy, is that BCRP might influence the pharmacokinetics of epipodophyllotoxins. BCRP/Bcrp1 is expressed in locations that place it in a position to do so, including the intestinal epithelium, hepatic canalicular membranes, and placental trophoblasts (11, 12). We have already shown that murine Bcrp1 has a marked effect on the oral uptake, tissue distribution, and elimination of the substrate drug topotecan (11, 13), and results from a clinical trial indicate that inhibition of intestinal BCRP also improves the oral availability of topotecan in humans (14). Etoposide can be administered p.o. with a bioavailability averaging 50%, but there is great interpatient and intrapatient variability in this parameter (e.g., Ref. 15 or 25-80%, according to the manufacturer’s data sheet). This variability is a hindrance to optimal dosing. In principle, increasing the oral availability to nearer 100% might reduce that variability, as was found for topotecan.

To address these questions and also to identify new potential mechanisms of resistance to epipodophyllotoxins, we have used cell lines lacking functional P-gp and Mrp1, derived from mice in which both gene loci were inactivated by gene targeting (4). Up-regulation of these transporters as a resistance mechanism is thus precluded, and the lines exhibit very low innate resistance to many P-gp and Mrp1 substrate drugs, including epipodophyllotoxins (16). We show here that when such cell lines were selected for resistance to etoposide, consistent amplification and overexpression of the Bcrp1 gene were observed. Bcrp1 activity accounted for much of the drug resistance because it was markedly reduced by the specific inhibitor Ko143 (13) or by GF120918 (17). The latter compound also markedly improved the oral availability of etoposide in mice, but it did so as a result of inhibition of P-gp rather than Bcrp1.

MATERIALS AND METHODS

Cell Lines. The origins and drug sensitivities of the 4B.65, MEF3.8, and KOT52 lines have been described elsewhere (16). Briefly, they were obtained by spontaneous immortalization of fibroblasts from Mdr1a/1b-/- Mrp1-/- mice or embryos. The K114 line was obtained from the kidney of a Mdr1a/1b-/- Mrp1-/- mouse that also carried a SV40 transgene (18), in the manner described previously (19). The basal drug sensitivity of the K114 line is very similar to that of the fibroblast lines. Etoposide-resistant sublines were obtained by continuous exposure to increasing concentrations of the drug, usually in 2-fold steps. The production of the Bcrp1-transduced MDCK-II cells was described previously (11). All cell lines were grown in complete medium.
Bcrp1-MEDiated Etoposide Resistance

Cytotoxicity, Drug Accumulation, and Transport Assays. Cytotoxicity assays and mitoxantrone accumulation assays were performed as described previously (4, 16). Etoposide accumulation was performed by first plating 2 \times 10^5 cells/well in 12-well plates, 12–16 h beforehand. Wells were then refreshed with 1 ml of complete medium containing 1 \mu M etoposide spiked with 0.2 \mu Ci/ml \[^{[3]H}\]etoposide (Moravek, Brea, CA), with or without 0.2 \mu M Ko143 or 0.4 \mu M GFI29018 to inhibit Bcrp1 activity. After incubation for 60 min at 37°C in a humidified CO\(_2\) incubator, plates were then placed on ice, the medium was removed, and cells were washed once with ice-cold PBS and then solubilized with 0.2 ml of Solvable (Packard, Meridian, CT). Etoposide content was determined by scintillation counting. All accumulations were performed in triplicate wells.

Transport assays were performed in Costar Trans-well plates with 3-\mu m-pore membranes. MDCK-II and MDCK-II-Bcrp1 cells were seeded at a density of 2 \times 10^5 cells/well and allowed to form tight monolayers over 3 days, with a medium change at the end of the second day. Ninety min before the experiment, medium on both sides of the monolayer was replaced with 2 ml of serum-free Optimem (Life Technologies, Inc.) containing 2 mM t-glutamine, 10 \mu M PSC833 to inhibit confounding P-gp activity, and antibiotics as described above. The experiment was started by replacing the Optimem in either the basal or the apical compartment with 2 ml of Optimem containing the same additives listed above plus 2 \mu M \[^{[3]H}\]etoposide (\sim 0.25 \mu Ci/ml) and 4 \mu M [\(^{[3]}\)H]inulin (\sim 0.025 \mu Ci/ml; included to check the integrity of the cell layer). The plates were then maintained at 37°C in a humidified CO\(_2\) incubator. Samples of 50 \mu l were removed from the basal and apical compartments at 0.5, 2, 4, 6, and 24 h time points. The samples were monitored for etoposide and inulin content by scintillation counting. Trans-epithelial transport of etoposide and paracellular leakage of inulin through the monolayers were expressed as a percentage of total radioactivity added.

Statistical Analysis. As distributions of drug resistance or accumulation data are by nature positively skewed, values were transformed to logarithms, which are more normally distributed, for the purpose of statistical comparisons. All tests were two-tailed tests assuming unequal variance.

DNA and RNA Analysis and Sequence Analysis. DNA was isolated from cells by digestion with proteinase K/SDS and extraction with phenol/chloroform. RNA was isolated with phenol/guanidine isothiocyanate (Trizol; Life Technologies, Inc.). RNAs or EcoRI-digested DNA fragments were fractionated by electrophoresis and transferred to Hybond-N membrane (Amersham-Phar- macia Biotech) by capillary blotting. Blots were probed with 32P-labeled antisense RNA probes for Bcrp1, MRP2, or MRP3 or random-prime DNA probes for Pim-1 or the 18S RNA.

Full-length Bcrp1 cDNAs were synthesized by reverse transcription from cell line total RNAs, and 420-bp segments spanning the R482 codon were amplified by PCR and then sequenced, as described previously (10).

Oral Etoposide Availability. Mice were housed and handled and experiments were conducted according to institutional ethical guidelines complying with Dutch legislation. The mice used were FVB strain wild type or Mdr1a/1b knockout males (20), of 7–12 weeks of age. Litters were allocated evenly across comparison groups. GF120918 was suspended at a concentration of 5 mg/ml in aqueous hydroxypropylmethyl cellulose (10 mg/ml + 5% (v/v) Tween 80, as described previously (11), and administered by oral gavage, so that 10 \mu l/g body weight was equivalent to 50 mg/kg of the suspended compound. Control mice were given vehicle only. After 15 min, etoposide was administered p.o. at a dose of 30 mg/kg, as 5 \mu l/g body weight of Vesepsid formulation (20 mg/ml) diluted to 6 mg/ml in 5% glucose. At the assigned time point, i.e., 1 or 2 h after etoposide administration, mice were anesthetized with methoxyflurane, heparinized blood was collected by cardiac puncture, and the mice were then killed by cervical dislocation. Blood cells and particulates were removed by centrifugation, and plasma samples were stored at \sim 70°C. Plasma etoposide concentrations were determined blind by high-performance liquid chromatography, as described previously (21).

RESULTS

Etoposide-resistant Cell Lines. Three sublines of the 4B.65 cell line and two sublines of the K114 cell line (all lacking functional P-gp and MRP1) were independently selected for resistance to etoposide (see “Materials and Methods”). After 40–50 passages, averaging 9 months, all sublines grew continuously in 2 \mu M etoposide, equivalent to 50–70 times the starting IC\(_{50}\). Continuous drug selection was required to maintain this level of resistance. Three of the five sublines were tested for resistance to a panel of cytotoxic drugs (Table 1). Each was found to be more than 100-fold resistant to etoposide compared with the parent cell line and highly resistant to another epipodophyllotoxin, teniposide, as well as three other topoisomerase II inhibitors, mitoxantrone, bisantrene and doxorubicin. The etoposide-selected lines were also highly resistant to the topoisomerase I inhibitor topotecan, but not to the microtubule poisons vincristine or paclitaxel. Changes in the molecular target of etoposide, the topoisomerase II complex, might account for much of this pattern, but not for the resistance to topotecan. However, the cross-resistance profile strongly resembled that seen previously in mouse cell lines that overexpress Bcrp1 (4).

High Bcrp1 Activity in Etoposide-resistant Cell Lines. Bcrp1 mRNA was indeed highly elevated in all five of the etoposide-selected cell lines (Fig. 1A), and the Bcrp1 gene locus was highly amplified (Fig. 2). Both of the two sublines tested accumulated only low levels of etoposide (Fig. 3) and mitoxantrone or 4,4,4-difluoro-4-bora-3a,4a-dia-indene-prazosin (data not shown), and the accumulation of these compounds could be increased to levels comparable with those in the parent cell lines by either of the structurally unrelated Bcrp1

<table>
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<th>Drug</th>
<th>IC(_{50}) (nm)</th>
<th>IC(_{50}) (nm)</th>
<th>IC(_{50}) (nm)</th>
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<tbody>
<tr>
<td></td>
<td>Mean \pm SD</td>
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<td>415</td>
<td>5,200 \pm 500</td>
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<tr>
<td>+ 400 nm GFI29018</td>
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<td>1,220 \pm 100</td>
<td>42</td>
<td>290 \pm 90</td>
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<td></td>
<td></td>
<td>26 \pm 4</td>
<td>770 \pm 50</td>
<td>29</td>
</tr>
<tr>
<td>Teniposide</td>
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<td>660 \pm 30</td>
<td>190</td>
<td>390 \pm 11</td>
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<tr>
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<td>600 \pm 80</td>
<td>330</td>
<td>190 \pm 20</td>
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<tr>
<td>Bisantrene</td>
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<td>790 \pm 70</td>
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<tr>
<td>Doxorubicin</td>
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<td>92</td>
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<tr>
<td>Topotecan</td>
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<td>12,800 \pm 1,500</td>
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<td>4,800 \pm 70</td>
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<td>4.5 \pm 0.4</td>
<td>1.1 ns</td>
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Table 1 Cross-resistance profile of three Mdr1a1b/−/− MRP1−/− cell lines selected for resistance to etoposide, compared to their unselected parent cell lines and a Bcrp1-transduced line

Some of the data for MEF3.8 and its Bcrp1-transduced clone appeared previously (16).

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Bcrp1-MEDIATED ETOPOSIDE RESISTANCE

Fig. 1. Northern analysis of (A) Bcrp1, (B) Mrp2, and (C) Mrp3 mRNA levels in etoposide-selected cell lines, their unselected parent lines, and normal mouse tissues, using identical replicate blots. The membrane in a was stripped and rehybridized to a probe for the 18S rRNA as a loading control (D). Lanes were loaded with 5 μg of total RNA. The positions and sizes (kb) of markers are indicated at the right.

Fig. 2. Southern analysis of Bcrp1 amplification in the etoposide-resistant cell lines compared with their unselected parent cell lines. The topotecan-selected cell line MEF3.8/T6400 and its parent cell line, MEF3.8 (4), were included for comparison. The numbers below the Bcrp1 panel show the fold increases in hybridization signal compared with the relevant parent cell line, adjusted for loading according to the Pim1 control. DNAs were digested with EcoRI.

inhibitors, GF120918 (17) and Ko143 (Ref. 13; inhibition of P-gp by GF120918 does not occur in the present case because the lines are nullizygous for Mdr1α and Mdr1β). These inhibitors also substantially reversed resistance to etoposide in all of the three sublines tested, lowering the IC50 values 10–20-fold (Table 1). They were used at concentrations that fully reverse Bcrp1/BCRP-mediated drug resistance in other systems, including cell lines very similar to those studied herein (10, 13). Hence, the residual resistance in the present case suggests that reduced etoposide accumulation is not the only resistance mechanism operating but that it is a quantitatively important one.

The drug resistance and low etoposide accumulation could not be explained by the activity of any known or suspected transporter of etoposide other than Bcrp1 because the cell lines lack functional P-gp and Mrp1; Northern analysis failed to detect any transcripts of Mrp2 or Mrp3 in the cell lines, whereas these were readily detected in tissue controls (Fig. 1, B and C). Mrp4 and Mrp5 were also not up-regulated (data not shown). It may be added that at the concentration used, Ko143 has no noticeable inhibitory effect on any of these transporters other than Bcrp1/BCRP (13). Taken together, the data indicate that Bcrp1 is responsible for much of the etoposide resistance seen in the etoposide-selected cell lines.

Wild-type Bcrp1 Transports Etoposide and Confers Etoposide Resistance in Bcrp1-transduced Cells. We have previously observed mutations at codon 482 in doxorubicin-selected mouse cell lines (10), and at least one of those lines displayed high resistance to etoposide (4), raising the possibility that etoposide resistance is dependent on mutations at this site. Segments of cDNA were therefore amplified by PCR from each of the five etoposide-selected sublines and their parent cell lines. Sequence analysis indicated that they were all wild type over a 420-bp stretch spanning the R482 codon ("Materials and Methods"). Moreover, none of the etoposide-selected cell lines showed the reduced accumulation of rhodamine 123 (data not shown) that is characteristic of R482 mutations (9, 10).

Wild-type Bcrp1 cDNA also conferred modest resistance to both etoposide (4.8-fold) and teniposide (4.4-fold) when expressed ectopically in MEF3.8 cells (Table 1). The level of resistance to these drugs was comparable to that for doxorubicin (3.6-fold) but much less than the resistance to mitoxantrone (48-fold) or topotecan (41-fold). Qualitatively similar results were obtained with Bcrp1-transduced KOT52 cells (data not shown).

Transport of [3H]etoposide by wild-type Bcrp1 was confirmed in a Transwell system using MDCK-II cells (22) expressing wild-type mouse Bcrp1 cDNA (11). To suppress etoposide transport by endogenous canine P-gp, the P-gp inhibitor PSC833 was included in all assays (PSC833 has no activity against Bcrp1). In each of two MDCK-II/Bcrp1 clones tested, clearly enhanced transport in the basolateral-to-apical direction was observed, compared with untransduced cells (Fig. 4; additional data not shown). The ratio of apically directed transport versus basolaterally directed translocation was modest compared with results we obtained previously for topotecan in the same cells (11) or with results obtained by others for etoposide transport in MDR1-transfected MDCK-II cells (3), suggesting that etoposide is not a particularly good substrate of Bcrp1. This is in
keeping with the cross-resistance data (Table 1), although the rate of etoposide uptake at the basolateral surface may also limit the observed apically directed transport.

**P-gp, not Bcrp1, Is the Dominant Transporter Affecting Oral Availability of Etoposide.** We have shown previously that oral administration of the combined P-gp/BCRP inhibitor GF120918 (17, 23) alters the pharmacokinetics of several P-gp substrate drugs in mice (24) and that it also markedly increases the oral uptake of the BCRP substrate topotecan by inhibition of intestinal Bcrp1 (11). The potential of Bcrp1 to limit the oral availability of etoposide, compared with the effects of P-gp in this regard, was therefore tested in wild-type and Mdr1a/1b<sup>–/–</sup> mice pretreated with GF120918 or vehicle only. Plasma etoposide concentrations were quantified at two time points (1 and 2 h after administration) that span the peak of the plasma curve reported previously for oral etoposide in rats (25).

Comparison of the vehicle-treated wild-type and Mdr1a<sup>–/–</sup> mice indicates that absence of P-gp increased etoposide plasma levels 4–5-fold. However, plasma etoposide levels did not increase further in Mdr1a<sup>–/–</sup> mice as a result of pretreatment with GF120918 (Fig. 5). Because the same dose of GF120918 previously proved highly effective for increasing the oral availability of topotecan (11), the data indicate that intestinal Bcrp1 activity has at best a minor role in limiting the oral availability of etoposide, whereas P-gp has a major role in this process. Pretreatment of wild-type mice with GF120918 increased the plasma etoposide concentrations 4–5-fold at both time points ($P < 0.0001$ and $P = 0.0017$, respectively). The plasma concentrations obtained in the GF120918-treated wild-type mice did not differ significantly from those in vehicle- or GF120918-treated Mdr1a<sup>–/–</sup> mice, suggesting that the inhibition of P-gp by GF120918 was effectively complete.

**DISCUSSION**

This study demonstrates that wild-type Bcrp1 can confer significant etoposide resistance and transport. When mouse cell lines lacking functional P-gp and Mrp1 were selected for etoposide resistance, amplification and concomitant overexpression of Bcrp1 emerged as a potent resistance mechanism in five of five cases. The resistance to etoposide was accompanied by reduced intracellular etoposide accumulation, and both processes were reversed by the structurally dissimilar Bcrp1 inhibitors Ko143 and GF120918. Substantial residual resistance to etoposide remained in the presence of the Bcrp1 inhibitors, indicating that other resistance mechanisms are also at work in the etoposide-selected lines, but several arguments weigh against involvement of other known drug transporters. First, etoposide accumulation was returned to the levels of the unselected parent lines by the Bcrp1 inhibitors. Second, the cells lack functional P-gp and Mrp1, the most obvious candidates. Finally, mRNAs of Mrp2 and Mrp3 were not expressed at significant levels in any of the resistant sublines.

Mutations at R482 are not necessary for Bcrp1-mediated resistance to etoposide because the Bcrp1 expressed in the etoposide-resistant lines was wild type at this position, and ectopic expression of a liver-derived Bcrp1 cDNA in fibroblasts conferred substantial etoposide resistance. Indeed, analysis of doxorubicin-selected cell lines that are mutated at R482 (10) suggests that the mutations decrease rather than increase resistance to etoposide.5

Elevated BCRP has not been described previously in etoposide-selected cell lines. The comparative cross-resistance data and drug transport rates suggest that etoposide is only a moderately good substrate for Bcrp1 compared with mitoxantrone, bisantrene, or topotecan. Thus, it is interesting that up-regulation of Bcrp1 should arise as the dominant transporter-mediated resistance mechanism in five of five cases, rather than Mrp2 or Mrp3, which can also transport etoposide, or its glucuronides or other conjugates (1–3, 26). This might be attributed to higher initial expression of Bcrp1 in the unselected parent lines (Fig. 1). Even so, it is remarkable that Mrp2- or Mrp3-expressing variants did not arise in any of the five sublines during the 50 passages of selection. This indicates that Bcrp1 is the most effective transporter under the conditions of selection (continuous exposure) or in the cell types used. For example, MRP2 does not route properly to the plasma membrane in many nonpolarized cultured

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5 John D. Allen, unpublished data.
cells (1) and therefore might not be a good candidate for up-regulation.

Although the data suggest that etoposide is not a particularly good substrate of Bcrp1, the transporter is a dominant mechanism of resistance in cell lines where P-gp and Mrp1 are not expressed. We thus speculate that in tumors that do not express P-gp or Mrp1, BCRP is potentially the dominant source of transporter-mediated resistance to epipodophyllotoxins. A similar situation could arise if efficacious inhibitors of P-gp and/or Mrp1, but not BCRP (such as PSC833, VX-710, or LY335979), are used as chemosensitizers in therapy. Finally, given previous evidence that even low levels of drug transporters may have a substantial effect on the basal resistance to substrates drugs (16), BCRP should at least be taken into account as a potential contributor to resistance to epipodophyllotoxins in tumors.

Like P-gp, BCRP is present in the gut mucosa (12, 27), where it could decrease the oral availability of some substrate drugs, as is clearly the case for topotecan (11, 14). However, the lack of a significant effect of GF120918 on the oral uptake of etoposide in the same Mdr1a/1b-deficient mice indicates that Bcrp1 activity is unlikely to have much impact on the oral availability of etoposide in humans. The most likely explanation for this lack of effect is that Bcrp1 is relatively inefficient in transporting etoposide compared with other transporters that occur in the intestinal epithelium, such as P-gp and MRP2 (3, 27), given that BCRP appears to be at least as abundant as in intestinal cells compared with the breast cancer resistance protein. Cancer Res., 60: 47–50, 2000.


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