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A Mutation Hot Spot in the Bcrp1 (Abcg2) Multidrug Transporter in Mouse Cell Lines Selected for Doxorubicin Resistance[1]

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ABSTRACT

The recent identification of mutations at arginine 482 (R482) in human Breast Cancer Resistance Protein (BCRP) in two drug-selected cell lines largely explains some discrepancies observed in the cross-resistance profiles of human cell lines overexpressing this multidrug transporter. We find that each of three mouse cell lines independently selected for resistance to the anthracycline doxorubicin also acquired mutations in the cognate mouse transporter Bcrp1 exclusively at R482. Although the mouse Bcrp1 amino acid substitutions (M or S) are distinct from those seen in the human cell lines (G or T), they all have similar consequences: (*a*) greater resistance to anthracyclines (and bisantrene); (*b*) relatively lower resistance to topotecan; (*c*) greatly enhanced efflux of the dye rhodamine 123. The ready selection of R482X mutations seen *in vitro* might also occur in tumors treated with anthracyclines. Thus, it is noteworthy that the efficacy of Bcrp1 inhibitors applicable *in vivo* was not markedly affected by the presence of the mutations. We found that the Bcrp1 mutations all occurred after previous amplification and overexpression of the wild-type gene under doxorubicin selection; wild-type Bcrp1 is evidently able to mediate substantial resistance to anthracyclines, and this was confirmed in *Bcrp1*-transduced cell lines. These observations emphasize the general importance of the arginine at amino acid 482 for substrate specificity of the transporter, while reminding us that unmutated Bcrp1 remains a potential source of resistance to anthracyclines and a potential factor in anthracycline pharmacokinetics. The same is most likely true of human BCRP, given its profound similarities to mouse Bcrp1.

INTRODUCTION

The ABC[3] multidrug transporter protein BCRP (ABCG2/MXR/ABCP) mediates resistance to a number of cytotoxic drugs in drug-selected cell line models, including mitoxantrone, topotecan, bisantrene, and doxorubicin (1–7). Human BCRP and its mouse cognate Bcrp1 also affect the pharmacokinetics of at least one substrate drug, topotecan, in that their presence in the intestinal epithelium limits the oral bioavailability of this drug (8, 9), and Bcrp1 in mouse placental trophoblasts limits penetration of systemic topotecan to the fetal compartment (8). *BCRP* cDNA was first cloned (1) from the MCF-7/AdrVp cell line, which had been selected for high resistance to doxorubicin in the presence of the P-gp inhibitor verapamil (10). Transfection of this cDNA into MCF-7 cells conferred relatively high resistance to anthracyclines, approaching the level of resistance conferred to mitoxantrone. However, the data from mitoxantrone- or topotecan-selected cell lines that overexpress *BCRP* indicated variable but usually modest resistance to anthracyclines compared with mitoxantrone resistance (1, 5, 6, 11). This situation was clarified recently when it was shown that the human BCRP overexpressed in the MCF-7/AdrVp cell line and also in the mitoxantrone-selected S1-M1–80 line (2) is altered at amino acid 482 (12, 13), with the parental arginine replaced by threonine or glycine, respectively. These changes resulted in greater resistance to anthracyclines and also enabled transport of the dye rhodamine 123.

We showed previously that mouse fibroblast lines lacking functional P-gp and Mrp1 also overexpress mouse *Bcrp1* when selected for resistance to topotecan, mitoxantrone, or doxorubicin (3). Of these, the doxorubicin-selected line KOT52/D320 showed much greater resistance to anthracyclines and bisantrene than the mitoxantrone- or topotecan-selected lines, although the levels of *Bcrp1* mRNA were comparable. We now show that this subline and two other fibroblast cell lines independently selected for doxorubicin resistance all harbor mutations altering amino acid 482. Although these mutations result in amino acid substitutions different from those seen in the human cell lines, their effects on drug resistance are remarkably similar.

MATERIALS AND METHODS

Cell Lines. All cell lines were maintained in complete medium: DMEM supplemented with 10% fetal bovine serum, penicillin, and streptomycin. Drug-resistant lines were adapted to continuous exposure to the selecting drug at a concentration indicated by the name of the subline. For example, the KOT52/D320 subline was adapted to 320 nM doxorubicin. Before cytotoxicity assays, drug concentration was reduced to 10% of the usual level for three days, to allow recovery from drug-selection damage.

RNA and DNA Analysis. Total RNAs were isolated from cell lines and tissues with phenol/guanidine isothiocyanate (Trizol; Life Technologies, Inc., Paisley, Scotland). DNAs were isolated by proteinase K/SDS digestion and phenol-chloroform extraction. RNA and DNA blots on Hybond-N (Amersham Pharmacia Biotech, Little Chalfont, England) were probed with ^{32}P-labeled antisense RNA probes for *Bcrp1* or *Mrp1,* or random-primed cDNA probes for the 18S rRNA or *Pim-1*, in Ultrahyb buffer (Ambion, Austin, TX) according to the manufacturer's recommendations. Signal levels were quantified on a phosphorimager (BAS Reader 2000; Fuji, Saitama City, Japan).

Cytotoxicity Assays. Cells were seeded in 96-well plates at a density (400 or 1000 cells/well, depending on the cell line) that allowed unimpeded growth for at least 4 days. After attachment (4 h), the cells were exposed to a concentration series of drug along the long plate axis, each concentration in quadruplicate, with or without a Bcrp1 inhibitor. After 4 days, while cells were still subconfluent in the untreated wells, proliferation was quantified by fluorescence of Sybr Green I nucleic acid stain (Molecular Probes, Eugene, OR) using a plate reader (Cytofluor 4000; PerSeptive Biosystems, Framingham, MA) with 485 nm excitation and 530 nm emission filters. The Bcrp1 inhibitors included in some assays had no effect alone on cell proliferation at the concentrations used: 200 nM Ko143[4] or 400 nM GF120918. These concentrations are adequate to inhibit nearly all of the transport activity of wild-type Bcrp1 for the drugs mitoxantrone and topotecan, representing ~8 times the respective effective concentration for 90% reversal of drug resistance concentrations.[4]

Drug Accumulation Assays. Cells were seeded in 24-well plates at 25,000/well. The following day, drug (20 μM daunorubicin or mitoxantrone) or dye (1 μM rhodamine 123) was added in complete medium, and plates were incubated for 1 h at 37°C. All of the subsequent operations were performed on

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[3] The abbreviations used are: ABC, ATP-binding cassette; BCRP, breast cancer resistance protein; Bcrp1, mouse cognate of breast cancer resistance protein; MRP, multidrug resistance protein; P-gp, P-glycoprotein; GFP, green fluorescent protein.

[4] J. D. Allen, A. van Loevezijn, J. M. Lakhai, M. van der Valk, O. van Tellingen, G. Reid, J. H. M. Schellens, G-J. Koomen, and A. H. Schinkel. Potent and specific inhibition of the breast cancer resistance protein multidrug transporter *in vitro* and in mouse intestine by a novel analogue of fumitremorgin C, Mol. Cancer Ther., in press.

ice to minimize transporter activity, including washing, trypsinization, and centrifugation. Relative intracellular drug or dye levels were determined by flow cytometry (rhodamine 123: 488 nm excitation, 530 nm emission; daunorubicin: 488 nm excitation, 670 nm emission; and mitoxantrone: 633 nm excitation, 661 nm emission).

Sequence Analysis. *Bcrp1* cDNA was synthesized by reverse transcription from cell line total RNAs using specific antisense primer Bcrp1-z: AAGGTAAGTCTAGACAAAGTGCCCATATTTAATTGGAGTAC. Complete coding sequences were amplified with *Pfx* high fidelity polymerase (Life Technologies, Inc.) using primers Bcrp1-z and Bcrp1-x: GAGTGAGATCTAGAAGGCATAAATCCTAAAGATGTCTTCC. PCR products were sequenced completely on both strands using BigDye Terminator chemistry (ABI-Prism) and additional specific primers. To confirm the presence of the mutations in codon 482, a ~420-bp segment was amplified from the cell line cDNAs using primers M13-Bcrp1-S3: TGTAAAACGACGGCCAGTACTTGCTCGGGAACCCTCAAGC and M13R-Bcrp1-AS6: CAGGAAACAGCTATGACCCTATGGCCAGTGCCATGGAACTG. The resulting PCR products were sequenced on both strands using BigDye primer chemistry (ABI-Prism) with M13 and M13-reverse primers.

Transduction of MEF3.8 Cells with Wild-type Bcrp1. The LZRS-Bcrp1-IRES-GFP construct has been described (8). Ecotropic recombinant retrovirus supernatants were produced by calcium-phosphate transfection of Phoenix packaging cells (14) with the pLZRS-Bcrp1-IRES-GFP construct. MEF3.8 cells were transduced with the supernatants by coincubation for 4 h in the presence of 4 μg/ml Polybrene. After 24 h, ~70% of the cells were positive for GFP is GFP$^+$. Single GFP$^+$ cells were sorted into 96-well plates containing MEF3.8-conditioned medium. After expansion, clones were screened for expression of functional Bcrp1 on the basis of reduced mitoxantrone accumulation.

RESULTS

Doxorubicin-resistant Cell Lines with Elevated Expression of Bcrp1. The 88.6 fibroblast line (15), derived from *Mdr1a/1b*$^{-/-}$ mice (16), completely lacks functional P-gp. Two independently selected doxorubicin-resistant sublines, 88.6/D800-A and 88.6/D800-B, were obtained by gradual adaptation to increasing concentrations of doxorubicin, up to 800 nM (~100 times the starting IC_{50}), over a cumulative period of 8 months or ~50 passages. The derivation of the doxorubicin-resistant line KOT52/D320, the topotecan-selected line MEF3.8/T6400, and the mitoxantrone-selected line MEF3.8/M32 has been described (3). The latter fibroblast cell lines lack both P-gp and Mrp1, their drug-sensitive parent lines (KOT52 and MEF3.8) having been derived from *Mdr1a/1b*$^{-/-}$*Mrp1*$^{-/-}$ mice. Note that the naming convention for all of these drug-resistant sublines includes the initial of the selecting drug and the concentration, in nM, to which the cells were adapted.

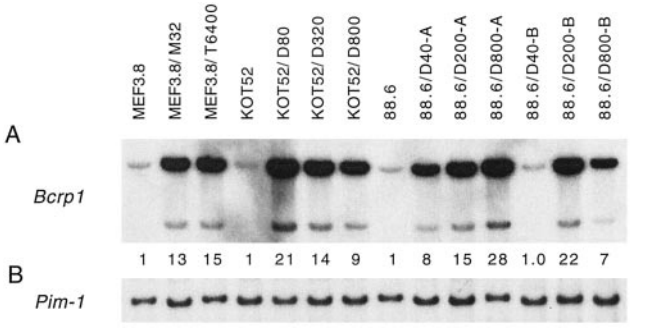

Fig. 2. Southern analysis of *Bcrp1* amplification in the doxorubicin-selected cell lines. Parallel blots of *Eco*RI-digested DNAs were hybridized to either (*A*) *Bcrp1* or (*B*) *Pim-1* probes. Numerical estimates of *Bcrp1* amplification relative to the parent cell lines, shown below (*A*), were obtained by quantifying the *Bcrp1* hybridization signals and correcting for loading on the basis of the *Pim-1* signals.

Each of the three doxorubicin-resistant cell lines had highly elevated levels of *Bcrp1* mRNA compared with the drug-sensitive parent cell lines, similar to mitoxantrone- or topotecan-selected cell lines (Fig. 1*A*), and each had markedly amplified the *Bcrp1* gene locus (Fig. 2*A*). Results are presented for both early and late passages during doxorubicin selection (see legend for Fig. 1). It is interesting to note that in the resistant lines, *Bcrp1* mRNA levels and the gene copy number were not closely correlated, and also did not increase proportionately with adaptation to higher concentrations of doxorubicin (see below for additional discussion).

Changes in the ratio of the two main *Bcrp1* transcripts are evident during the course of doxorubicin selection in at least one of the sublines (Fig. 1*A*). As documented previously (1–3), two such Bcrp1/BCRP transcripts are present in normal mouse and human tissues in ratios that vary with the source. Their physiological significance, if any, is not known, but neither transcript appears to be specifically associated with resistance to any particular drug in drug-selected cell lines, either in the present case or others known. Instability of transcript ratios is not surprising given the corresponding flux in *Bcrp1* gene amplification and deletion.

Functional Mrp1 is present in the 88.6 line and its drug-resistant sublines, and mouse Mrp1 can confer resistance to anthracyclines, albeit poorly (15, 17). However, the level of *Mrp1* mRNA was not

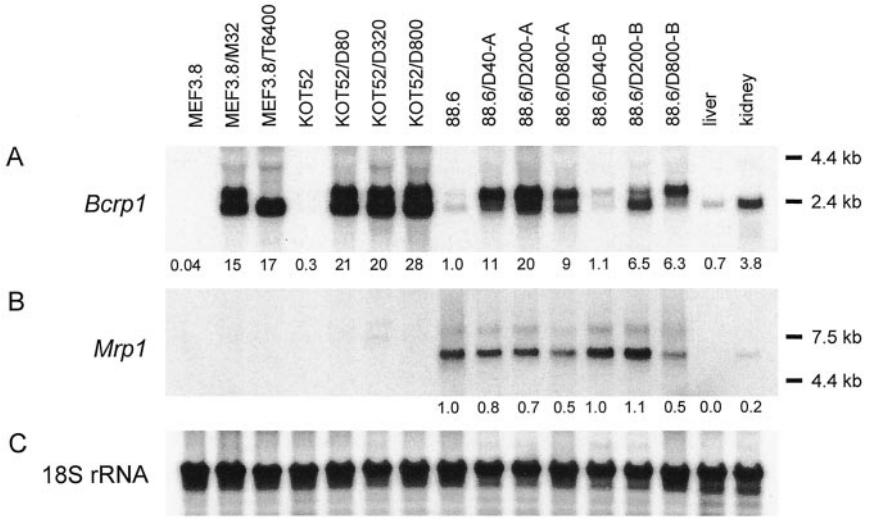

Fig. 1. Northern analysis of (*A*) *Bcrp1* and (*B*) *Mrp1* mRNA levels in doxorubicin-resistant fibroblast cell lines during the development of doxorubicin resistance, with mitoxantrone- or topotecan-resistant cell lines and normal mouse tissues for comparison. Names of drug-selected sublines reflect the drug (D, M, or T prefix for doxorubicin, mitoxantrone, or topotecan, respectively) and drug concentration, in nM, to which the cells were adapted. Duplicate blots were hybridized with antisense RNA probes for *Bcrp1* or *Mrp1*. Total RNA loading (*C*) was checked by rehybridizing one blot with a probe for the 18S rRNA. Signal levels were quantified, corrected for loading, and are presented below the panels in the figure relative to the level of *Bcrp1* (or *Mrp1*) mRNA in the 88.6 cell line.

elevated in the doxorubicin-selected sublines (Fig. 1*B*). The KOT52 line and its derivatives have no functional Mrp1 (see above). Neither of two other candidate anthracycline transporters, Mrp2 and Mrp3, could be detected in any of the fibroblast cell lines by Northern analysis, although both mRNAs were readily detected in tissue controls (not shown). Thus, of the known or suspected transporters of anthracyclines, only *Bcrp1* is likely to play a part in the observed drug resistance.

Drug Resistance Phenotype of the Doxorubicin-selected Cell Lines. The cross-resistance properties of early and late passages of the three doxorubicin-resistant sublines are presented in Table 1. Data published previously for the mitoxantrone- and topotecan-resistant MEF3.8 lines (3) are reproduced for comparison. The data for the parent lines are in agreement with values we have determined previously (3, 15). It should be noted that the 88.6 cell line expresses some Mrp1 and is consequently somewhat more resistant to various drugs than the KOT52 or MEF3.8 lines. Therefore, relative resistance factors of the 88.6-derived sublines are somewhat lower in these cases.

As observed previously for the KOT52/D320 line (3), the 88.6/D800-A and 88.6/D800-B lines exhibited high resistance to doxorubicin and bisantrene, with resistance factors of the same order as those for mitoxantrone (Table 1). This is in sharp contrast to the mitoxantrone-selected MEF3.8/M32 and topotecan-selected MEF3.8/T6400 lines, where resistance factors for doxorubicin and bisantrene were 8–20-fold lower than for mitoxantrone.

The differences in anthracycline resistance between these two groups of cell lines were reflected in their relative cellular accumulation of daunorubicin (Fig. 3). All of the drug-resistant lines showed low accumulation of mitoxantrone (Fig. 3*A*) consistent with high Bcrp1 levels. However, the three doxorubicin-selected cell lines exhibited much lower relative accumulation of daunorubicin than the mitoxantrone- or topotecan-selected lines, or the Bcrp1-transduced MEF3.8 clone A2 (Fig. 3*B*). Note the extensive reversal of this low daunorubicin accumulation, shown in Fig. 3*B*, by the highly potent and specific Bcrp1/BCRP inhibitor Ko143, a fumitremorgin C analogue that we developed recently.[4] Similar reversals were obtained with the structurally unrelated Bcrp1/BCRP inhibitor GF120918 (Ref. 18; not shown). Therefore, we conclude that differences in drug resistance between the doxorubicin-selected sublines and the mitoxantrone- or topotecan-selected sublines are mostly the result of differences in the behavior of Bcrp1.

Bcrp1 Mutations in the Doxorubicin-selected Mouse Cell Lines. The identification of R482T and R482G mutations in human BCRP in highly drug-selected human cell lines (12, 13), which increase resistance to anthracyclines, suggested that the doxorubicin-selected mouse lines might harbor similar mutations. Indeed, sequencing of full-length *Bcrp1* cDNAs from the 88.6/D800-A, 88.6/D800-B, and KOT52/D320 lines revealed mutations in codon 482 in all three of the lines (Fig. 4; Table 2). However, the resulting amino acid changes differed from those seen in the human lines, arginine being replaced by methionine in the 88.6/D800-A and KOT52/D320 lines and by serine in the 88.6/D800-B line. The mutations were not evident in the sequence electropherograms of cDNAs from earlier passages of these cell lines, *i.e.*, KOT52/D80, 88.6/D200-A, and 88.6/D200-B (Table 2). In contrast, the late passage 88.6/D800-A and 88.6/D800-B lines retained little or no wild-type *Bcrp1* mRNA. The KOT52/D320 line contained mutant *Bcrp1* mRNA as a minority species (~30% of the

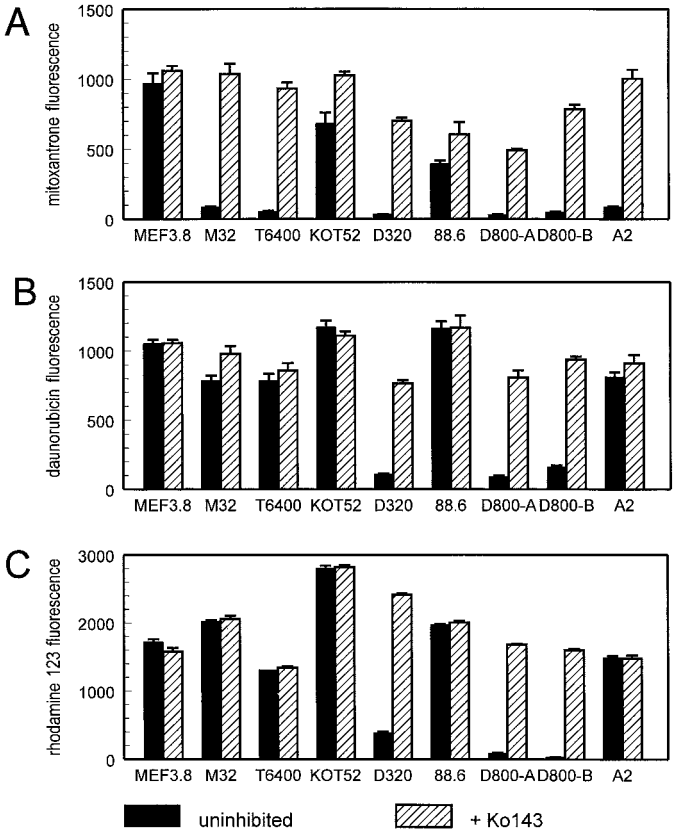

Fig. 3. Accumulation of (*A*) mitoxantrone, (*B*) daunorubicin, and (*C*) rhodamine 123 in drug-selected mouse cell lines and in *Bcrp1*-transduced MEF3.8 clone A2, in the absence or presence of 200 nM Ko143, a Bcrp1 inhibitor. Accumulation is shown in terms of relative arbitrary units of drug or dye fluorescence. Experiments were performed in triplicate; *bars*, ±SD.

Table 1 *Cross-resistance profiles of doxorubicin-resistant fibroblast cell lines*

Shown for each cell line and drug combination: IC_{50} ± SD (nM) with resistance factors in bold below the IC_{50}s.

Drug	MEF3.8[a] $n = 3$	MEF3.8/M32[a] $n = 3$	MEF3.8/T6400[a] $n = 3$	KOT52 $n = 4$	KOT52/D80 $n = 2$	KOT52/D320 $n = 5$	88.6 $n = 3$	88.6/D200-A $n = 2$	88.6/D800-A $n = 3$	88.6/D200-B $n = 2$	88.6/D800-B $n = 3$
Mitoxantrone	0.81 ± 0.25	142 ± 8	53 ± 6	1.33 ± 0.13	210 ± 70	420 ± 60	2.4 ± 0.3	295 ± 30	600 ± 90	275 ± 20	650 ± 150
		174	**66**		**158**	**318**		**123**	**250**	**115**	**269**
Bisantrene	12.5 ± 3.0	215 ± 22	110 ± 10	9.7 ± 2.5	385 ± 64	1850 ± 400	11 ± 1.1	540 ± 85	2500 ± 560	450 ± 90	4400 ± 600
		17	**8.8**		**40**	**191**		**48**	**222**	**40**	**394**
Doxorubicin	6.4 ± 1.0	56 ± 8	33 ± 9	3.5 ± 0.5	140 ± 32	1100 ± 300	8.3 ± 1.6	260 ± 50	1400 ± 180	230 ± 15	1300 ± 200
		8.7	**5.2**		**39**	**312**		**31**	**172**	**28**	**156**
Daunorubicin	7.4 ± 1.5	14.5 ± 0.5	12.3 ± 1.2	4.0 ± 0.3	34 ± 6	330 ± 20	6.5 ± 0.2	67 ± 4	500 ± 140	51 ± 9	390 ± 60
		2.0	**1.7**		**8.6**	**82**		**10**	**77**	**7.8**	**60**
Topotecan	49 ± 3	6400 ± 2000	16000 ± 5300	166 ± 69	17500 ± 700	23000 ± 5000	98 ± 11	11000 ± 2000	4300 ± 1750	6700 ± 600	3200 ± 1200
		132	**329**		**105**	**137**		**109**	**44**	**69**	**32**
Vincristine	0.38 ± 0.03	0.64 ± 0.07	0.63 ± 0.23	0.82 ± 0.09	0.72 ± 0.18	0.98 ± 0.22	4.1 ± 0.4	6.1 ± 0.4	4.1 ± 0.7	6.2 ± 0.4	7.3 ± 0.7
		1.7	**1.6**		**0.87**	**1.2**		**1.5**	**1.0**	**1.5**	**1.8**
Paclitaxel	4.5 ± 0.3	8.4 ± 1.9	9.1 ± 3.0	2.2 ± 0.4	2.7 ± 0.1	3.4 ± 1.4	5.8 ± 0.4	8.0 ± 0.1	5.9 ± 1.5	3.8 ± 0.6	4.1 ± 0.8
		1.9	**2.0**		**1.2**	**1.7**		**1.4**	**1.0**	**0.7**	**0.7**

[a] These data are taken for comparison from Ref. 3.

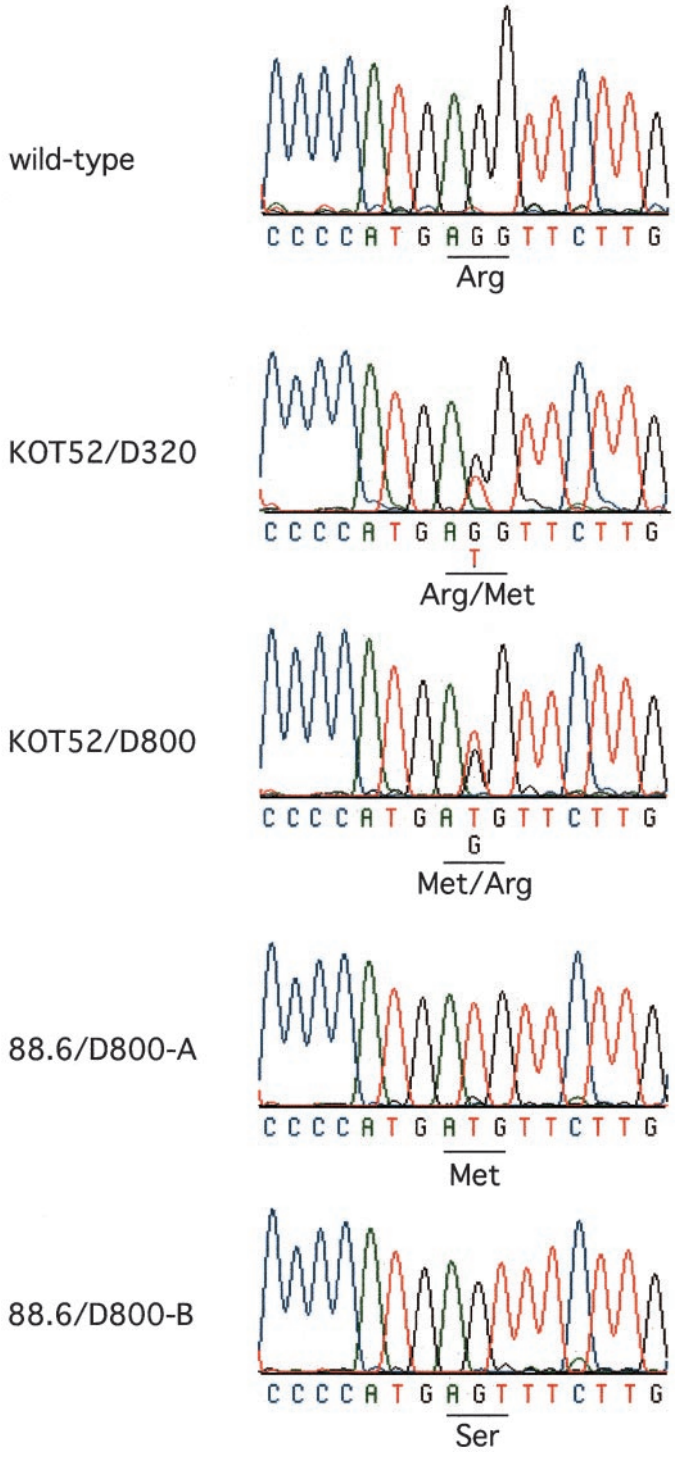

Fig. 4. Mutations in codon 482 of *Bcrp1* from the doxorubicin-selected cell lines. Sequence electropherograms for the vicinity of the mutations are shown for each of the late-passage doxorubicin-selected cell lines. The wild-type example is taken from the KOT52/D80 line but is the same as found in earlier passages of all of the doxorubicin-selected lines, the mitoxantrone- or topotecan-selected lines, the parent cell lines, and mouse liver (Table 2 and "Results").

total *Bcrp1* mRNA), of which the relative abundance increased after additional selection for greater resistance to doxorubicin (Fig. 4). cDNAs from the topotecan- or mitoxantrone-selected cell lines and all of the drug-sensitive parent cell lines coded for arginine at position 482. No other differences with the sequence of mouse liver *Bcrp1* cDNA (3) were detected in any of the cell lines.

Effect of the Mutations on Bcrp1 Properties. It is clear that early passages of each of the doxorubicin-selected cell lines (KOT52/D80, 88.6/D200-A, and 88.6/D200-B) had acquired substantial amplification and overexpression of *Bcrp1* (Figs. 1 and 2), and accompanying doxorubicin resistance (Table 1), well before the mutations in codon 482 became apparent (Fig. 4; Table 2). This must mean that wild-type Bcrp1 can also mediate significant resistance to doxorubicin, a point that was confirmed using MEF3.8 cells transduced with a retroviral *Bcrp1* expression construct. The latter cell line allows the contribution of Bcrp1 to resistance to various drugs to be assessed in the absence of the confounding influences of P-gp and Mrp1, and against a very low background of endogenous Bcrp1. Ectopic *Bcrp1* expression in this system conferred modest but significant (4-fold) resistance to doxorubicin (Table 3). In comparison, resistance conferred to mitoxantrone or topotecan was roughly 10-fold higher. The same cells showed corresponding differences in accumulation of mitoxantrone and daunorubicin. Comparable drug resistance and drug accumulation results were obtained for *Bcrp1*-transduced KOT52 cells (not shown).

Amplification of the wild-type *Bcrp1* gene locus during the early development of resistance in the drug-selected cell lines increases the probability of obtaining favorable mutations in *Bcrp1* that improve the efficiency of doxorubicin efflux. Subsequently, with additional doxorubicin selection, increases in the proportion of mutant *versus* wild-type Bcrp1 mRNA can occur through preferential amplification of the mutated gene locus. Unmutated *Bcrp1* gene copies can then be lost with relatively little impact on doxorubicin resistance. Such processes can readily explain the absence of a simple correlation between total *Bcrp1* mRNA levels, total gene copy number, and drug resistance (Figs. 1 and 2; Table 1).

The consequences of such changes for drug resistance can be seen in a comparison of late *versus* early passages of the 88.6-derived

Table 2 *Mutations in BCRP/Bcrp1 codon 482 in drug-resistant cell lines*

Altered nucleotides are underlined.

cDNA source	Selecting drug	Codon 482	Encodes
Human			
placenta[a]	–	AGG	R
MCF-7/AdrVp[a]	doxorubicin, 3 μM	ACG	T
S1-M1-80[a]	mitoxantrone, 80 μM	GGG	G
Mouse			
liver[a]	–	AGG	R
MEF3.8		AGG	R
MEF3.8/M32	mitoxantrone, 32 nM	AGG	R
MEF3.8/T6400	topotecan, 6400 nM	AGG	R
KOT52	–	AGG	R
KOT52/D80	doxorubicin, 80 nM	AGG	R
KOT52/D320	doxorubicin, 320 nM	AGG/ATG	R/M: 70/30%
KOT52/D800	doxorubicin, 800 nM	AGG/ATG	R/M: 40/60%
88.6	–	AGG	R
88.6/D200-A	doxorubicin, 200 nM	AGG	R
88.6/D800-A	doxorubicin, 800 nM	ATG	M
88.6/D200-B	doxorubicin, 200 nM	AGG	R
88.6/D800-B	doxorubicin, 800 nM	AGT	S

[a] From Refs. (1–3, 12, 13, 19) and GenBank records.

Table 3 *Drug resistance conferred by ectopic expression of Bcrp1 in MEF3.8 cells*

Shown are the IC_{50}s (in nM), determined by *n* assays and the resistance factors for a representative *Bcrp1*-transduced clone. Values shown here for the MEF3.8 parent line may differ somewhat from the earlier determinations shown in Table 1 because of variations in drug batches and details of the assay procedure. Results of two-tailed heteroscedastic *t* tests comparing the IC_{50}s of the parent and transduced lines are shown.

Drug	Mean IC_{50} ± SD, nM, and (*n*)		RF	*t* test
	MEF3.8	MEF3.8/Bcrp1 clone A2		
Mitoxantrone	0.40 ± 0.10 (6)	19 ± 2 (5)	48	$P < 0.01$
Doxorubicin	3.6 ± 0.3 (3)	13 ± 1.2 (3)	3.6	$P < 0.01$
Topotecan	45 ± 7 (4)	1800 ± 100 (3)	40	$P < 0.01$
Vincristine	0.25 ± 0.10 (4)	0.26 ± 0.01 (3)	1.0	ns[a]

[a] ns, not significant.

Table 4 *Reversal of cross-resistance in doxorubicin-selected cell lines by the BCRP/Bcrp1 inhibitor Ko143*

Values shown are the resistance factors in the absence (as per Table 1) or presence of 200 nM Ko143. Some assays in the presence of Ko143 were performed on two occasions; in these cases both results are shown.

		Cell line				
Drug	Ko143	KOT52	KOT52/D320	88.6	88.6/D800-A	88.6/D800-B
Mitoxantrone	−	1	318	1	250	269
	+	0.53	2.1	0.40	1.6	3.3
Bisantrene	−	1	191	1	222	394
	+	0.48	2.3, 2.4	0.96, 1.1	2.1, 2.3	4.8, 7.6
Doxorubicin	−	1	312	1	172	156
	+	0.69	6.6, 7.1	0.82, 1.35	2.9, 3.6	3.7, 5.9
Topotecan	−	1	137	1	44	32
	+	0.30	1.0	0.68	0.60	0.76

sublines (Table 1). During adaptation to a 4-fold increase in doxorubicin concentration (from 200 nM to 800 nM), both sublines became at least 5-fold more resistant to anthracyclines, as expected, and resistance to bisantrene also increased by at least this much. However, resistance to mitoxantrone increased only 2-fold over the same period, whereas resistance to topotecan was substantially decreased, by at least 2-fold. Some of these trends can also be discerned in the history of the KOT52/D320 line although they are obscured to a considerable extent by the presence of a majority of residual wild-type *Bcrp1* mRNA in this line. Note that in all of the cases the level of total *Bcrp1* mRNA either remained the same or even decreased (Fig. 1). Finally, each of the doxorubicin-selected cell lines remained sensitive to the hydrophobic drugs vincristine and paclitaxel (Table 1).

Clearly, the mouse Bcrp1 R482M and R482S mutants confer enhanced resistance to anthracyclines compared with the wild-type transporter. The same is true for bisantrene, a structurally related drug. Comparison of the changes in *Bcrp1* mRNA levels (Fig. 1) and the corresponding changes in mitoxantrone resistance suggests that the mutations also enhance resistance to this drug. Resistance to topotecan was at least 10-fold lower, relative to the anthracyclines and bisantrene, in the 88.6-derived R482M and R482S mutant lines, as was found for the human R482G mutant (13). In this context it is also noteworthy that cell lines carrying either the R482M or R482S Bcrp1 mutants showed greatly reduced (and Ko143-reversible) accumulation of the dye rhodamine 123 (Fig. 3*C*), as was observed previously for the R482G and R482T mutants of human BCRP (13). In contrast, wild-type Bcrp1 did not appreciably lower cellular rhodamine 123 accumulation (Fig. 3*C*).

Efficient Inhibition of Mutant Bcrp1 by Ko143 or GF120918. Despite these differences in substrate specificity, the drug resistance mediated by mutant Bcrp1 in the doxorubicin-selected cell lines was still effectively reversed (Table 4) by application of the potent Bcrp1 inhibitor Ko143. This compound had relatively little effect on the drug sensitivity of the parent cell lines KOT52 and 88.6, where Bcrp1 levels are low, but in almost all of the cases it dramatically decreased (or eliminated) the resistance of the three doxorubicin-selected sublines to four drugs, to levels near those of the parent cell lines. Similar results were obtained with the structurally unrelated BCRP inhibitor GF120918 (not shown). As already noted above, these inhibitors also effectively reversed the low cellular accumulation of mitoxantrone, daunorubicin, and rhodamine 123 in the mutant cell lines (Fig. 3).

DISCUSSION

In three mouse cell lines, each selected independently for resistance to doxorubicin, Bcrp1 was mutated at R482, resulting in higher resistance to anthracyclines and bisantrene, possibly also higher resistance to mitoxantrone, and lower resistance to the topoisomerase I poison topotecan. The mutations also enabled efflux of the dye rhodamine 123, which was absent with wild-type Bcrp1. This apparently wider substrate specificity of the mutant Bcrp1 transporters did not extend to the hydrophobic drugs vincristine and paclitaxel.

Honjo *et al.* (13) found previously that human BCRP was mutated at R482 to glycine or threonine in, respectively, mitoxantrone-selected and doxorubicin-selected cell lines. Likewise, these mutations resulted in higher resistance to doxorubicin, possibly lower resistance to topotecan in the R482G mutant, and the capacity to efflux rhodamine 123. The independent mutation of R482 in five different human and mouse cell lines selected for resistance to doxorubicin or mitoxantrone clearly cannot be a coincidence. In all likelihood, substitution of R482 is one of the few, if not the only, readily obtainable mutation in Bcrp1/BCRP that enhances transport of these drugs. The results underscore the conclusion that this amino acid has a key role in determining the substrate specificity in BCRP from both species, possibly by virtue of its tentative strategic location at the cytoplasmic end of transmembrane domain 3 (13).

It is interesting that the amino acid changes observed thus far [threonine and glycine in human BCRP, methionine (twice), and serine in mouse Bcrp1] represent four of the six amino acid alternatives possible by single-base mutations in codon 482 (AGG) and that all four are uncharged. The other possibilities are tryptophan and lysine. Tryptophan is also uncharged but perhaps too bulky to be accommodated. Lysine, like arginine, is basic and may represent too conservative a change to affect BCRP function noticeably. Of course, it may also be a matter of chance that these two substitutions did not occur yet. In any case, the results suggest that it may be the loss of basic arginine at position 482 that is critical for altering the substrate specificity of Bcrp1/BCRP and not the exact identity of the replacing amino acid. We note in passing that in a zebra fish (*Danio rerio*) BCRP homologue, arginine is retained at the position equivalent to R482 although the surrounding amino acids are not so well conserved (see contig formed by GenBank expressed sequence tags BF156737, AI497110, and AW018623). This suggests that R482 may also be important for the normal physiological function of BCRP in vertebrates.

Amplification and overexpression of the *Bcrp1* gene preceded appearance of the mutations in all three of the doxorubicin-selected cell lines examined herein. Thus, although mutations at the R482 hot spot are evidently highly advantageous for resistance to anthracyclines, wild-type Bcrp1 can also contribute significantly to resistance to these drugs *in vitro*, and this was confirmed in transduced cells expressing ectopic *Bcrp1*. To date, no obvious differences have been seen in the substrate specificity or drug resistance conferred by mouse Bcrp1 and human BCRP, if the effects of mutations at R482 are taken into account. Indeed, all of our findings thus far reinforce the similarities of the mouse and human transporters. Hence, although the question remains open as to whether wild-type (or perhaps even R482 mutant) BCRP is a significant source of clinical resistance to anthracyclines in tumors, it is a possibility that cannot be ignored. It is equally possible that wild-type BCRP has a significant impact on the pharmacokinetics of anthracyclines, at least in certain tissues, as is the case for topotecan (8, 9). Anthracyclines are good substrates of P-gp, so it is noteworthy that at least one good inhibitor of both P-gp and BCRP (GF120918) is potentially available for reversal of resistance to anthracyclines or modulation of their pharmacokinetics. In this regard, it is significant that the anthracycline resistance of the Bcrp1 mutants considered here was still effectively inhibited by GF120918 and the specific BCRP inhibitor Ko143, although we have not investigated in great detail whether the efficiency of inhibition might have been reduced for some drugs. Both GF120918 and Ko143[4] can be used p.o. *in vivo* at dosages high enough to inhibit BCRP/Bcrp1 function (8, 9).

Thus, it is clear that potential clinical use of these BCRP inhibitors is unlikely to be seriously compromised by the presence of mutations at R482, should they occur in doxorubicin-treated tumors.

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A Mutation Hot Spot in the Bcrp1 (Abcg2) Multidrug Transporter in Mouse Cell Lines Selected for Doxorubicin Resistance

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