

The Mouse *Bcrp1/Mxr/Abcp* Gene: Amplification and Overexpression in Cell Lines Selected for Resistance to Topotecan, Mitoxantrone, or Doxorubicin¹

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Abstract

Mouse fibroblast cell lines lacking functional *Mdr1a*, *Mdr1b*, and *Mrp1* genes were selected for resistance to topotecan, mitoxantrone, or doxorubicin. Each of the resulting drug-resistant lines showed marked gene amplification of *Bcrp1*, the mouse homologue of the human ATP-binding cassette transporter gene *BCRP/MXR/ABCP*, and greatly elevated expression of *Bcrp1* mRNA. All three of the resistant cell lines were highly cross-resistant to topotecan and mitoxantrone and, to a variable extent, doxorubicin. All showed greatly reduced cellular accumulation and greatly increased efflux of mitoxantrone that was dependent on cellular ATP and efficiently reversed by the compound GF120918. The mouse *Bcrp1* cDNA encodes a 657-amino-acid protein with 81% identity (86% similarity) to the human breast cancer resistance protein (BCRP) and a virtually superimposable hydrophobicity profile. Our data argue strongly that mouse *Bcrp1* is functionally comparable with human BCRP, conferring multidrug resistance to topotecan, mitoxantrone, doxorubicin, and related compounds. Mouse models and cell lines should, therefore, be highly informative in understanding the clinical, pharmacological, and physiological roles of BCRP.

Introduction

Several sources of MDR³ have been identified and well studied. They include elevated levels of P-gp or the MDR protein MRP1, both members of the ABC transmembrane protein family (1–4). The proteins are efflux pumps situated in the plasma membrane, with broad substrate specificities including several clinically important antineoplastic drugs. Despite extensive knowledge of their activity *in vitro* and evidence of their involvement in MDR in certain types of tumors, it is still unclear what mechanisms contribute most to MDR in many clinical tumors. It is very possible that as yet unidentified or poorly understood mechanisms will turn out to be as important in clinical practice as P-gp and MRP1. The recently identified ABC gene *BCRP/MXR/ABCP* is a candidate for such a mechanism, inasmuch as its expression in transfected cell lines confers resistance to doxorubicin, mitoxantrone, and related drugs (5–7). Cross-resistance to mitoxantrone and doxorubicin that is associated with reduced drug accumulation but not elevated P-gp or MRP1 has been reported previously for a number of drug-selected cell lines (8–11). In some of these lines cross-resistance to topotecan was also noted (12, 13). Drug resistance in several of these lines has now been attributed to elevated expression of *BCRP* (5, 7, 14, 15).

Mouse cell lines lacking functional *Mdr1a* and *Mdr1b* (encoding mouse P-gps), and *Mrp1* genes constitute a potentially fertile resource

for identifying new mechanisms of drug resistance. Such lines are markedly more sensitive than equivalent wild-type lines are to P-gp and MRP1 substrate drugs, including doxorubicin, paclitaxel, topotecan, and vincristine.⁴ Selection of these lines for resistance to antineoplastic drugs may, therefore, invoke resistance mechanisms normally masked or overshadowed by the presence of P-gp or MRP1. Indeed, we report here that selection with topotecan, mitoxantrone, or doxorubicin readily resulted in overexpression of the mouse *Bcrp1* gene.

Materials and Methods

Cell Lines. Adherent, spontaneously immortalized embryo (MEF3.8) and ear (KOT52) fibroblast cell lines were derived by 3T3-like procedures (16) from *Mdr1a/b*^{-/-}*Mrp1*^{-/-} mice, obtained by crossing *Mdr1a*^{-/-}*Mdr1b*^{-/-} (17) and *Mrp1*^{-/-} (18) knockout mice. Cells were grown in complete medium, *i.e.*, DMEM supplemented with 10% FCS, and passaged by trypsinization. Drug-resistant sublines were selected by continuous exposure to topotecan, mitoxantrone, or doxorubicin, with repeated two-fold increments in drug concentration over a period of 4–8 months, corresponding to 20–40 passages.

Drug Resistance Assays. Growth inhibition (IC₅₀) assays were performed by seeding 250 or 500 cells per well in 96-well plates in complete medium and applying drugs in a dilution series, each concentration in quadruplicate wells. After 4–4.5 days, when unselected wells were still subconfluent, cells were lysed *in situ*, nucleic acids were stained with a proprietary dye (Cyquant, Molecular Probes, Eugene, OR), and quantified by UV fluorescence (485 nm excitation, 530 nm emission). All such assays were performed three times.

Mitoxantrone Accumulation and Efflux Assays. Relative cellular accumulation of mitoxantrone was determined by flow cytometry using excitation at 633 nm and a 661 nm band-pass filter to detect emission. All of the assays were conducted at 37°C with 10⁵ (subconfluent) cells per well in 12-well plates, seeded in complete medium without drug the night before. Mitoxantrone was added for timed intervals in fresh, prewarmed, complete medium containing 5% FCS. Accumulation or efflux was arrested by prompt cooling on ice, and the cells were maintained at 0°C during all of the subsequent steps, including trypsinizing. Where indicated (see “Results”), mitoxantrone accumulations were done in the presence of 2 μM GF120918; pilot experiments indicated that this concentration gave >95% maximal effect. Cells were preincubated with GF120918 for 30–60 min before adding mitoxantrone. Accumulation under ATP-depleting conditions was performed for 2 h in glucose-free, pyruvate-free DMEM containing 5% dialyzed FCS, plus 10 mM sodium azide to inhibit oxidative phosphorylation, as described previously (19). Mitoxantrone efflux was assayed after accumulation under ATP-depleting conditions: the medium and drug were removed by aspiration, wells were washed quickly with complete medium at room temperature, and the cells were then incubated with prewarmed complete medium at 37°C for timed intervals prior to harvesting. Assays were performed at least twice, each time with triplicate wells.

***Bcrp* Cloning and Sequencing.** Mouse *Bcrp1* cDNAs were amplified by PCR with primers based on mouse EST sequences homologous to the 5' and 3' ends of human *BCRP*, with *Xba*I linkers added: 5'-GAG TGA GAT CTA GAA GGC ATA AAT CCT AAA GAT GTC TTC C and 3'-AAG GTA AGT

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³ The abbreviations used are: MDR, multidrug resistance; MRP, MDR-associated protein; ABC, ATP-binding cassette; BCRP, breast cancer resistance protein; P-gp, P-glycoprotein; EST, expressed sequence tag.

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Table 1 Cross-resistance profile of *Mdr1a/b*^{-/-} *Mrp1*^{-/-} fibroblast lines selected for resistance to mitoxantrone, topotecan, or doxorubicin

The IC₅₀s shown (nM) are the means ± SD of results from three experiments, each performed in quadruplicate. Resistance factors (RF) in parentheses are the ratios of the mean IC₅₀s for the resistant sublines and their sensitive parental lines. Differences in those means were tested with one-tailed (resistant > sensitive) heteroscedastic *t* tests; they were statistically significant (*P* < 0.05) except where indicated by "ns" (not significant).

Drug	IC ₅₀ ± SD, nM (resistance factor)							
	Unselected parent line MEF3.8	Mitoxantrone-selected M32	RF	Topotecan-selected T6400	RF	Unselected parent line KOT52	Doxorubicin-selected D320	RF
Mitoxantrone	0.81 ± 0.25	142 ± 8	(174)	53 ± 6	(66)	1.35 ± 0.15	403 ± 42	(299)
Bisantrene	12.5 ± 3.0	215 ± 22	(17)	110 ± 10	(8.8)	10.6 ± 2.1	1,420 ± 770	(134)
Doxorubicin	6.4 ± 1.0	56 ± 8	(8.7)	33 ± 9	(5.2)	3.4 ± 0.5	1,180 ± 300	(350)
Daunorubicin	7.4 ± 1.5	14.5 ± 0.5	(2.0)	12.3 ± 1.2	(1.7)	4.1 ± 0.3	318 ± 19	(78)
Etoposide	53 ± 6	593 ± 64	(11)	307 ± 123	(5.8)	28 ± 8	3,970 ± 1,110	(142)
Topotecan	49 ± 3	6,400 ± 2,000	(132)	16,000 ± 5,300	(329)	183 ± 74	28,700 ± 3,100	(156)
Vincristine	0.38 ± 0.03	0.64 ± 0.07	(1.7)ns	0.63 ± 0.23	(1.6)ns	0.66 ± 0.37	1.01 ± 0.17	(1.5)ns
Paclitaxel	4.5 ± 0.3	8.4 ± 1.9	(1.9)	9.1 ± 3.0	(2.0)ns	2.2 ± 0.5	4.9 ± 0.2	(2.2)
Cisplatin	1,500 ± 780	2,630 ± 250	(1.8)ns	3,270 ± 570	(2.2)	2,670 ± 380	2,050 ± 560	(0.77)ns

CTA GAG GAG TAC AAT TAA TAG TCC GTT AAA GG. The PCR was performed with *Pfu* polymerase, Stratagene, La Jolla, CA) on oligo-dT primed first-strand cDNA from liver, yielding a product of 2.0 kb, as expected by analogy with the human BCRP sequence. cDNA clones from two independent amplifications were completely sequenced on both strands.⁵ Minor discrepancies between the two clones and with existing mouse ESTs were resolved by sequencing relevant portions of a *Bcrp1* genomic clone derived from the 129/Ola mouse strain. A slightly shorter PCR product covering only the *Bcrp1* coding sequence was used for probing blots (nucleotides 19 through 1977 in the GenBank sequence⁵). A short probe for *Bcrp2* covering the 3' coding region was obtained similarly by PCR based on the sequence of mouse EST AA277174.

Results

Selection of Resistant Lines and Analysis of Cross-Resistance.

Cell lines nullizygous for *Mdr1a*, *Mdr1b* and *Mrp1* were selected for resistance to topotecan, mitoxantrone or doxorubicin (see "Materials and Methods" for details). The embryo fibroblast line MEF3.8 yielded the topotecan-resistant subline T6400 and the mitoxantrone-resistant subline M32. The ear fibroblast line KOT52 yielded a doxorubicin-resistant subline, D320. Compared with their parental lines, the sublines were each more than 100-fold resistant to the selecting drug but remained sensitive to paclitaxel, vincristine and cisplatin (Table 1). The T6400, M32 and D320 lines were all highly cross-resistant to topotecan and mitoxantrone. The T6400 and M32 lines also showed some cross-resistance to anthracyclines and bisantrene (a compound structurally related to mitoxantrone) but at a much lower level than the doxorubicin-selected D320 line. The D320 line was also much more resistant to etoposide.

Reduced Drug Accumulation and Increased Efflux. Doxorubicin and mitoxantrone interfere with topoisomerase II activity, whereas topotecan acts on a different target, topoisomerase I. Cross-resistance to both types of drugs could be explained most easily by reduced cellular drug accumulation, which can be readily measured for mitoxantrone by flow cytometry. Indeed, as a function of either time (Fig. 1A) or drug concentration (Fig. 1B), mitoxantrone accumulation was greatly reduced in all three of the resistant sublines compared with the sensitive parental lines. The accumulation deficits could be largely reversed by performing the assays under conditions that deplete cellular ATP (Fig. 1C), which indicates that they are mediated by an ATP-dependent mechanism. The accumulation deficits were similarly reversed in the presence of GF120918, a known P-gp inhibitor (19) also reported to inhibit human BCRP (20). Confocal scanning microscopy revealed no qualitative differences in the subcellular localization of mitoxantrone in resistant *versus* sensitive cells (data

not shown), implying that the mechanism responsible for reduced accumulation operates at the plasma membrane.

Comparisons of mitoxantrone efflux rates under normal cell culture conditions were forestalled by the large differences in drug accumulation; it was not feasible to load the drug-resistant sublines to intracellular mitoxantrone levels similar to those obtained in the drug-sensitive parental lines, even using 200-fold greater concentrations of applied mitoxantrone. Nevertheless, similar mitoxantrone loadings were possible under ATP-depleting conditions, and efflux could be monitored after returning the loaded cells to normal conditions. The results (Fig. 1D) show dramatically increased mitoxantrone efflux from the resistant sublines compared with the sensitive parental lines. Because recovery from ATP-depleted conditions is probably not immediate, the efflux curves likely underestimate the normal mitoxantrone efflux activity in these lines.

Cloning and Sequencing of Mouse *Bcrp1*. The cross-resistance patterns and the ATP-dependent changes in mitoxantrone accumulation and efflux and their inhibition by GF120918 suggested the possibility of up-regulation of a mouse homologue of BCRP in one or more of the resistant lines. We, therefore, cloned and analyzed a mouse cDNA, *Bcrp1*, closely homologous to human BCRP. cDNAs containing the full *Bcrp1* coding sequence were obtained by high-fidelity PCR based on existing mouse EST sequences homologous to the 5' and 3' ends of human BCRP. The cDNA sequence⁵ contains an extended open reading frame starting four codons downstream of an in-frame stop codon. This encodes an ABC transporter "half molecule" of 657 amino acids corresponding closely in sequence and structure to human BCRP (Fig. 2). The mouse *Bcrp1* and human BCRP amino acid sequences are 81% identical and 86% homologous. Conservation is, as expected, very high in the ATP-binding cassette. The level of conservation between the mouse and human polypeptides is comparable with that between human MDR1 and mouse *Mdr1a* (87% identity) or *Mdr1b* (81% identity). Hydrophobicity plots of mouse *Bcrp1* and human BCRP are almost identical (Fig. 2), increasing confidence in the assignment of six putative transmembrane domains (5, 6). However, the locations of charged amino acids in the mouse sequence merited small shifts in the positions assigned to some of the transmembrane domains relative to those proposed for human BCRP. Four potential sites for N-linked glycosylation are apparent; the first two lie in what is likely to be the cytosolic part of the protein, whereas the latter two are closely spaced in the loop between the fifth and sixth putative transmembrane domains and are, thus, probably extracellular (only one is conserved in human BCRP).

***Bcrp1* Overexpression and Amplification in Resistant Cell Lines.** On Northern blots (Fig. 3A), the *Bcrp1* coding sequence probe identified either one or two bands at about 2.6 kb in the resistant

⁵ The *Bcrp1* sequence was deposited in GenBank under accession number AF140218.

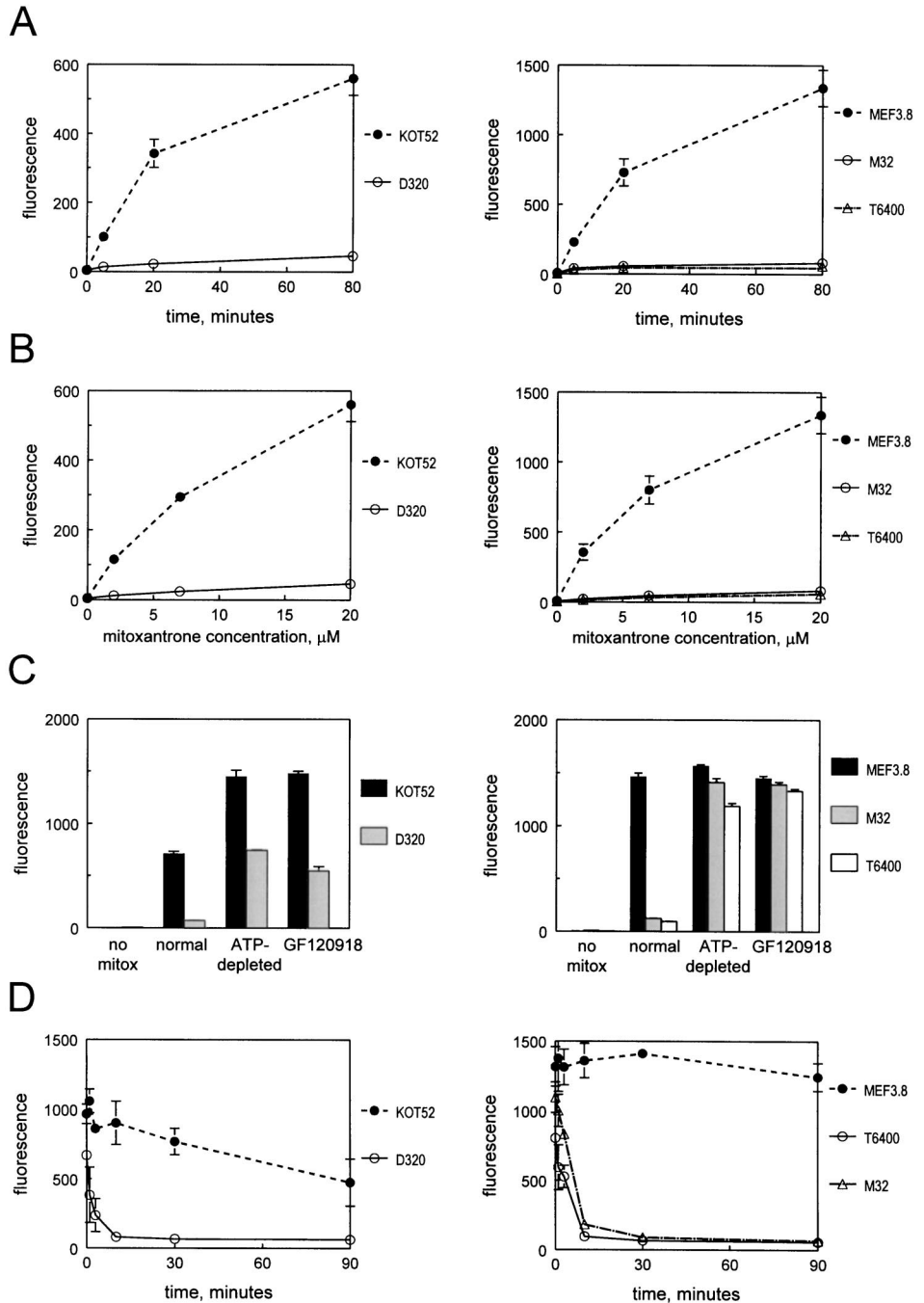


Fig. 1. Mitoxantrone accumulation as measured by flow cytometry in resistant cell lines *versus* sensitive parental lines: *A*, as a function of time; *B*, as a function of mitoxantrone concentration (accumulation for 80 min); and *C*, under ATP-depleting conditions (glucose-free medium plus sodium azide; see "Materials and Methods" for details) or in the presence of 2 μM GF120918 for 2 h. *D*, mitoxantrone efflux from resistant *versus* sensitive lines under normal conditions after loading under ATP-depleted conditions. Unless otherwise indicated, mitoxantrone was applied at a concentration of 20 μM . All of the assays were performed in triplicate, at least twice. *Error bars*, the SDs of mean values from individual experiments.

sublines and mouse liver, similar to observations for the human *BCRP* mRNA (5–7). *Bcrp1* mRNA was expressed in each of the three resistant cell lines at roughly similar levels. The mRNA was not detectable in the parent cell lines at this sensitivity; this implies an increase in expression of more than 10-fold. The significance of the two transcripts is not known, but in humans, they may stem from alternate polyadenylation sites (7) or differences at the 5' end of the mRNA (6). The elevated *Bcrp1* expression was associated with heavy amplification of the gene locus, in each case greater than 20-fold compared with the drug-sensitive parent lines (Fig. 3B), as has been observed in several drug-resistant human cell lines overexpressing *BCRP* (7, 15).

Two mouse ESTs for a sequence related to human *BCRP* but distinct from *Bcrp1* were also identified (AA277174 and AI463023).

We tentatively denote this sequence *Bcrp2*. One might speculate that the molecule encoded by *Bcrp2* represents a partner for *Bcrp1* in heterodimers. However, its expression, already very low in the MEF3.8 and KOT52 parental lines as determined by RNase protection, was not elevated in the drug-resistant sublines (data not shown).

Discussion

Our data show that the mouse *Bcrp1* gene is readily amplified and overexpressed in cell lines made resistant to three different drugs—topotecan, mitoxantrone, and doxorubicin. The resistant sublines display greatly reduced mitoxantrone accumulation via an ATP-dependent efflux mechanism. All of the lines are characterized by cross-resistance to topotecan and mitoxantrone, and also to

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1  MSSSSNDHVLV PMSQRNNNGL PRMNSRAVRT LAEGDVLSEH HITYRVKVKV
1  VE F I V G T F ATA ND I KA FT A N C I L
51  GFL-VRKTVE KEILSDINGI MKPGLNAILG PTGGGKSSLL DVLAARKDPK
51  PC P N S
100 GLSGDVLING APQPAHFKCC SGYVVQDDVV MGLTLVRENL QFSAALRLPT
101 R N N A
150 TMKNHEKNER INTI I KELGL EKVADSKVGT QFIRGISGGE RKRTSIGMEL
151 T R V Q D V
200 ITDPSILFLD EPTTGLDSSST ANAVLLLLKR MSKQGRITIF SIHQPRYSIF
201
250 KLFDSLTLA SGKLVFHGPA QKALEYFASA GYHCEPYNP ADFFLDVI
251 R M E G E A I
300 DSSAVMLNRE EQDNEANKTE EPSKGEKPI HNLSEFYINS AIYGETKAEL
301 T A - FK TEII QD L K A I V SF K
350 DQLPGAQEKK GTSAFKEPVY VTSFCHQLRW IARRSFKNLL GNPQASVAQL
350 H S GEK KITV IS T VSK I I
400 IVTVILGLII GAIYFDLKYD AAGMQNRAGV LFFLTNNQCF SSVSAVELFV
400 V V G N ST I
450 VEKKLFIHEY ISGYRVSYY FFGKVMDDL PMRFLPSVIF TCILYFMLGL
450 L LL TM I V
500 KKTVDAFFIM MFTLIMVAYT ASSMALATAT GQSVVSVATL LMTIAFVFM
500 PKA V M S A C
550 LFSGLLVNLR TIGPWLWLQ YFSIPRYGFT ALQYNEFLGQ EFCPGENVTD
550 I T AS H N L A G
600 NSTCVNSYAI CTGNEYLINQ GIELSPWGLW KNHVALACMI IIFLTIAYLK
600 NP --N T E V K D V
650 LLFLKKYS 657
648 655
    
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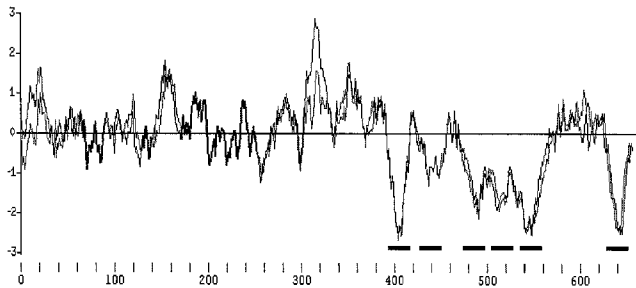


Fig. 2. The mouse *Bcrp1* polypeptide sequence and superimposed hydrophobicity plots of the mouse *Bcrp1* and human BCRP polypeptides. Amino acids that differ in the human BCRP sequence (taken from GenBank, accession AF098951) are shown below their mouse counterparts. Colons indicate conservative substitutions, and dashes are alignment gaps. Putative features are marked: *overscore*, transmembrane segments; *double overscore*, Walker A and B motifs; *box*, potential N-linked glycosylation sites, although the first two of these are presumed to lie within the cytosolic portion of the protein. The hydrophobicity plot was determined by the Kyte-Doolittle method using a window-length of 17 amino acids; *bars*, putative transmembrane regions.

doxorubicin, albeit to a lesser extent in the M32 and T6400 lines than in the doxorubicin-selected D320 line. The evidence at hand strongly favors the interpretation that *Bcrp1* overexpression is responsible for much of this pattern of resistance. Cross-resistance to mitoxantrone and doxorubicin in drug-selected human cell lines has, in several cases, now been attributed to *BCRP* overexpression (5, 7, 15). Although concomitant cross-resistance to topotecan has been observed in a few cases (12, 13), we have now shown that *Bcrp1* expression is also readily elevated by topotecan selection *per se*. The same could occur in human tumors, some of which are extensively treated with topotecan in the clinic.

Mouse *Bcrp1*, thus, appears functionally comparable with the human BCRP as a multidrug transporter. Functional homology is also suggested by the close structural similarities between the mouse and human polypeptides. Mouse models, therefore, will likely be appropriate and valuable for investigating the biochemistry and physiological functions of the BCRP/*Bcrp1* protein, and its significance for drug pharmacokinetics and drug-resistance in tumors. We are currently developing such models.

The availability of effective inhibitors of *Bcrp1* will be invaluable to such studies. It is of great interest that GF120918 turns out to be an effective inhibitor of both human BCRP (20) and mouse *Bcrp1*.

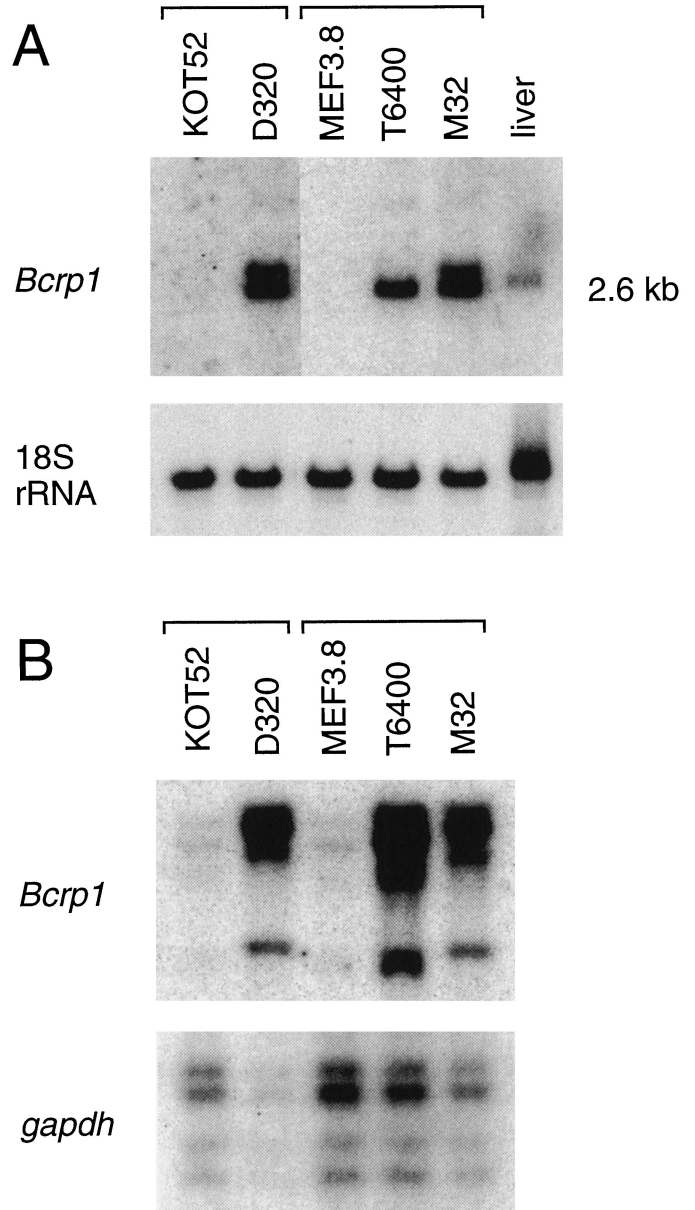


Fig. 3. A, Northern analysis of *Bcrp1* mRNA expression in the drug-resistant cell lines versus their sensitive parental lines. A 1.95-kb *Bcrp1* coding sequence probe was used. A mouse liver sample was included for comparison of expression level, but its migration in the gel was slightly retarded by salt in the sample. Each lane on the blot contained 5 μ g total RNA. Subsequent hybridization of the blot with an 18S-rRNA probe provided a loading control. B, Southern analysis of *Bcrp1* copy number in the same panel of cell lines, using 5 μ g of *KpnI*-digested genomic DNA per lane. Hybridization of the blot with a glyceraldehyde phosphate dehydrogenase (*gapdh*) probe provided an independent loading control, indicating that the D320 lane was substantially underloaded. The quantitation of *Bcrp1* amplification reported in the text included normalization for the *gapdh* signal.

GF120918 has very low toxicity, and it has already been administered at considerable doses to both animals (19) and patients⁶ to inhibit P-gp activity. Pilot experiments indicate that GF120918 is nearly as efficient at inhibiting murine *Bcrp1*. Thus, if BCRP contributes to clinical drug resistance, GF120918 may well be attractive as a dual-action reversal/sensitizing agent, coadministered to enhance the response to chemotherapy.

The doxorubicin-selected D320 subline showed considerably greater resistance to anthracyclines bisantrene and etoposide than the other two resistant sublines (Table 1), whereas the *Bcrp1* expression level did not differ markedly (Fig. 3). Evidently, additional changes occurred in this subline. Each of the above drugs affects topoisomerase II activity; therefore, it may be that topoisomerase II function is altered in the D320 line. Alternatively, the function of *Bcrp1* may be changed, for instance, by a mutation that affects substrate specificity. Because *Bcrp1* is an ABC transporter half molecule, it may form homodimers or heterodimers. In the latter case, although speculative, different partner molecules could confer different substrate specificities on the dimer. These possibilities are now under investigation.

Finally, we note that the ranges of drugs transported by BCRP and P-gp overlap. If BCRP does prove significant in clinical drug resistance, this significance can only increase when drug-resistance reversal agents that inhibit P-gp are used in chemotherapy. Indeed, clinical use of inhibitors for any of the drug transporters will likely bring new drug resistance mechanisms to the fore. The value of anticipating such clinical developments in the laboratory is obvious. Our results illustrate the utility of cell lines nullizygous for known MDR genes for identification and characterization of such potential new resistance mechanisms.

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⁶ J. H. M. Schellens, personal communication.

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The Mouse *Bcrp1/Mxr/Abcp* Gene: Amplification and Overexpression in Cell Lines Selected for Resistance to Topotecan, Mitoxantrone, or Doxorubicin

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