Multidrug resistance mediated by the breast cancer resistance protein BCRP (ABCG2)

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Observations of functional adenosine triphosphate (ATP)-dependent drug efflux in certain multidrug-resistant cancer cell lines without overexpression of P-glycoprotein or multidrug resistance protein (MRP) family members suggested the existence of another ATP-binding cassette (ABC) transporter capable of causing cancer drug resistance. In one such cell line (MCF-7/AdrVp), the overexpression of a novel member of the G subfamily of ABC transporters was found. The new transporter was termed the breast cancer resistance protein (BCRP), because of its identification in MCF-7 human breast carcinoma cells. BCRP is a 655 amino-acid polypeptide, formally designated as ABCG2. Like all members of the ABC G (white) subfamily, BCRP is a half transporter. Transfection and enforced overexpression of BCRP in drug-sensitive MCF-7 or MDA-MB-231 cells recapitulates the drug-resistance phenotype of MCF-7/AdrVp cells, consistent with current evidence suggesting that functional BCRP is a homodimer. BCRP maps to chromosome 4q22, downstream from a TATA-less promoter. The spectrum of anticancer drugs effluxed by BCRP includes mitoxantrone, camptothecin-derived and indolocarbazole topoisomerase I inhibitors, methotrexate, flavopiridol, and quinazoline ErbB1 inhibitors. Transport of anthracyclines is variable and appears to depend on the presence of a BCRP mutation at codon 482. Potent and specific inhibitors of BCRP are now being developed, opening the door to clinical applications of BCRP inhibition. Owing to tissue localization in the placenta, bile canaliculi, colon, small bowel, and brain microvessel endothelium, BCRP may play a role in protecting the organism from potentially harmful xenobiotics. BCRP expression has also been demonstrated in pluripotential 'side population' stem cells, responsible for the characteristic ability of these cells to exclude Hoechst 33342 dye, and possibly for the maintenance of the stem cell phenotype. Studies are emerging on the role of BCRP expression in drug resistance in clinical cancers. More prospective studies are needed, preferably combining BCRP protein or mRNA quantification with functional

assays, in order to determine the contribution of BCRP to drug resistance in human cancers.

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Multidrug resistance (MDR) in cancer cells is the simultaneous development of resistance to a variety of anticancer drugs that appear to be structurally and mechanistically unrelated. One type of MDR is characterized by the decreased accumulation of hydrophobic natural product drugs. In some multidrug-resistant cells, drug efflux is mediated by an adenosine triphosphate (ATP)-dependent membrane transporter termed P-glycoprotein (Pgp), the product of the MDR1 gene (Juliano and Ling, 1976). Pgp functions as an active outward transport mechanism for a variety of molecules, including certain chemotherapeutic drugs. As data accumulated regarding the role of Pgp in drug resistance, it became clear that other transporters could confer resistance to cytotoxic agents. The MDR protein 1 (MRP1) gene was cloned from a multidrug-resistant lung cancer cell line and, like Pgp, was found to be a member of the ATP-binding cassette (ABC) superfamily of transporter genes (Cole *et al.*, 1992). Transfection studies indicated that, similar to MDR1, MRP1 overexpression is sufficient to confer resistance to a broad profile of lipophilic, natural product antineoplastics. MRP1 was the first identified member of a family of genes encoding multispecific organic anion transporter (MOAT) proteins (Borst *et al.*, 1999). Two other homologues of MRP1, the cMOAT/MRP2 and MRP3 genes encode proteins that mediate MDR when transfected into drug-sensitive cell (Borst *et al.*, 1999). All of these membrane-embedded proteins act as drug efflux pumps, preventing cytotoxic agents from reaching lethal levels within cells.

A number of cell lines selected for resistance to the anthracenedione mitoxantrone were discovered to have a transporter activity without overexpression of MDR1 or MRP1 (Dietel *et al.*, 1990; Taylor *et al.*, 1991; Nakagawa *et al.*, 1992; Futscher *et al.*, 1994; Hazlehurst *et al.*, 1999). These cell lines were highly resistant

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to mitoxantrone, with cross resistance to doxorubicin and daunorubicin, but were not resistant to vinblastine. The mitoxantrone-resistant breast cancer cell line MCF-7/MX was demonstrated to have markedly decreased accumulation of radiolabeled mitoxantrone, which was not reversed by verapamil (Nakagawa *et al.*, 1992). The enhanced efflux of mitoxantrone in these cells was blocked by sodium azide or 2,4-dinitrophenol, suggesting an ATP-dependent transporter activity. The MCF-7/MX cell line was also found to be highly resistant to the camptothecin analogs topotecan, 9-aminocamptothecin, CPT-11 and SN-38, but was only mildly crossresistant to the parent compound camptothecin (Nakagawa *et al.*, 1992).

A pattern of MDR similar to that found with mitoxantrone selection was found in another breast cancer subline made resistant to doxorubicin in the presence of the Pgp inhibitor verapamil (Chen *et al.*, 1990). The resulting doxorubicin-resistant subline, MCF-7/AdrVp, did not overexpress Pgp or MRP1 and exhibited a cross resistance pattern of high resistance to daunorubicin, mitoxantrone, rubidazole, VM-26, and melphalan, but only minimal resistance to vinca alkaloids, actinomycin D, cisplatin, or paclitaxel. The relative resistance of the MCF-7/AdrVp subline to mitoxantrone was strikingly higher than the resistance to the selecting agent doxorubicin (Lee *et al.*, 1997). The MCF-7/AdrVp subline did not display the altered subcellular distribution of drug seen in many cell lines that express MRP1 (Lee *et al.*, 1997). Decreased accumulation of daunorubicin and the fluorescent dye rhodamine 123 throughout the cytoplasm was seen in MCF-7/AdrVp cells (Doyle *et al.*, 1995; Lee *et al.*, 1997). Although this decreased accumulation was not reversed by cyclosporin A, depletion of ATP resulted in complete abrogation of the enhanced efflux of both daunorubicin and rhodamine (Lee *et al.*, 1997). These findings led to the hypothesis that an ATP-dependent xenobiotic transporter contributed to the MDR phenotype of MCF-7/AdrVp cells.

Identification of the BCRP gene

Our laboratory was able to identify a novel ABC transporter in MCF-7/AdrVp cells, and to demonstrate that the enforced expression of the transporter in drug-sensitive MCF-7 cells recapitulated the drug-resistant phenotype of the MCF-7/AdrVp cells (Doyle *et al.*, 1998). We named the new transporter breast cancer resistance protein (BCRP), because it was derived from a resistant breast cancer cell line. To isolate BCRP, we examined RNA transcripts overexpressed in the MCF-7/AdrVp subline relative to parental MCF-7 cells by RNA fingerprinting, a variation of differential display (Doyle *et al.*, 1998). Gel bands that represented differentially expressed cDNAs were isolated, amplified by PCR, ligated into a TA cloning vector and transfected into *Escherichia coli* cells. One differentially expressed PCR product insert identified a 2.4 kb mRNA

species that was confirmed by Northern analysis to be markedly overexpressed in MCF-7/AdrVp cells, compared with MCF-7 (Doyle *et al.*, 1998). Protein database searches of the deduced amino-acid sequence revealed a high degree of homology to members of the G or white subfamily of ATP-binding cassette (ABC) transport proteins. The PCR product was radiolabeled and used as a probe to isolate a full-length cDNA from a lambda phage library derived from MCF-7/AdrVp. The full-length cDNA coded for a novel 655 amino-acid protein with properties of a half-transporter, which is characteristic for members of the ABCG subfamily. A BLAST-N search of the nonredundant database of GenBank human expressed sequence tag (EST) entries for homology to the BCRP cDNA sequences revealed that a portion of the 3' end of BCRP cDNA had near identity to clone EST157481, one of 21 new genes predicted by Allikmets *et al.* (1996) to be in the human ABC transporter family on the basis of EST database analysis.

To determine whether BCRP was the putative mitoxantrone transporter described in many atypical multidrug-resistant cell lines, we examined a number of these cancer cell lines selected for BCRP expression (Ross *et al.*, 1999). A variety of mitoxantrone-resistant cell lines were found to overexpress BCRP mRNA markedly, including sublines of human breast carcinoma (MCF-7), colon carcinoma (S1 and HT29), gastric carcinoma (EPG85-257), fibrosarcoma (EPF86-079), and myeloma (8226). These results suggested that BCRP overexpression was likely to be a major cellular defense mechanism elicited in response to exposure to mitoxantrone.

Subsequent to our initial report of BCRP, a cDNA essentially identical to BCRP was cloned by Allikmets *et al.* (1998) by extending EST 157481, and given the name ABCP, relating to its expression in the placenta. A cDNA essentially the same as BCRP was also cloned from the mitoxantrone-resistant colon cancer cell line S1-M1-80 by Miyake *et al.* (1999), and given the name MXR, relating to the mitoxantrone resistance phenotype conferred by the expression of the gene. The Human Gene Nomenclature Committee formally designated BCRP as ABCG2, or the second member of the G (white) subfamily of ABC transporters. The gene nomenclature can be found on the ABC transporter web page (URL: <http://www.med.rug.nl/mdl/humanabc.htm>). Since most papers in the literature use the original name BCRP, we will employ it for this review.

ABC genes comprise a large superfamily of transporters characterized by extensive conservation of the ATP-binding domains throughout evolution (Higgins, 1995). ABC family members function as ATP-dependent active membrane transporters, translocating molecules across a cell membrane against a concentration gradient. Most functional ABC transporters consist of two ATP-binding domains and two sets of transmembrane domains. In eucaryotes, most ABC genes have all four domains in a single open reading frame. A smaller group of ABC transporters, termed half-transporters, have single transmembrane and ATP-binding domains. Half

transporters typically form heterodimers to produce a functional protein, such as the TAP1/TAP2 heterodimers involved in peptide transport into the endoplasmic reticulum (Abele and Tampe, 1999). The first genetic marker described in *Drosophila*, the white protein, is an ABC half-transporter closely related to BCRP. The *Drosophila* white protein forms complexes with two other white-related half transporters: brown to transport guanine or scarlet to transport tryptophan, the precursors to eye pigments (Ewart and Howells, 1998). White/white homodimers are not functional for transport of either tryptophan or guanine (Higgins, 1995).

Analysis of the phylogenetic relationship of BCRP to other members of the ABC transporter superfamily (Figure 1) reveals that BCRP is only distantly related to

Pgp or MRP1, but is closely related to ABCG1 (Doyle *et al.*, 1998), the human homologue of the *Drosophila* white (w) gene (Chen *et al.*, 1996; Croop *et al.*, 1997). ABCG1 has 638 amino acids, which are 29.3% identical to BCRP. In humans, ABCG1 has been recently found to be a regulator of macrophage cholesterol and phospholipid transport (Klucken *et al.*, 2000). Members of the human ABCG subfamily are summarized in Table 1.

Analysis of the BCRP peptide sequence (Figure 2) reveals a single ATP/GTP-binding region and ABC signature motif within a relatively hydrophilic amino-terminal domain (amino acids 1–400), and a relatively hydrophobic carboxyl-terminal domain (amino acids 401–663) containing six putative transmembrane domains and four potential N-glycosylation sites (Doyle *et al.*, 1998). The BCRP or white sequences are similar to one-half of the duplicated Pgp or MRP1 molecule, except that Pgp or MRP1 have the configuration NH₂–(transmembrane domains)–(ATP binding 1)–(transmembrane domains)–(ATP binding 2)–COOH, whereas BCRP or white have NH₂–(ATP binding)–(transmembrane domains)–COOH. Members of the ABCG subfamily are the only known human ABC transporters with a domain arrangement in which the ABC fold precedes the transmembrane region.

Figure 1 Relationship of BCRP (ABCG2) amongst ABC transporters. This dendrogram was produced using multiple sequence alignments with Clustal_X 1.8 (Thompson *et al.*, 1997), with graphical representation in a tree diagram using TreeView 1.6.6

Figure 2 Physical structure of BCRP (ABCG2) compared to ABCB and ABCC family members (used with permission from Macmillan)

Table 1 Members of the human ABC transporter subfamily G (white)^a

Name ^b	Chromosome	Amino acids	Tissue	Substrates
ABCG1 (white)	21q22.3	638	Brain, spleen, lung	Sterols, lipids
ABCG2 (BCRP, MXR, ABCP)	4q22	655	Placenta, liver, colon, small bowel, venules – see Table 3	See Table 2
ABCG3	8p12	? Has unusual ATP-binding domain – may not be transcribed	—	—
ABCG4 (white 2)	11q23	646	—	—
ABCG5 (white 3)	2p21	651	Liver, small bowel	Sterols?
ABCG8 (white 4)	2p21	673	Liver, small bowel	Sterols

^aAdapted from Muller, M: <http://humanabc.4t.com/humanabc.htm>.

^bHuman Gene Nomenclature Committee designation (common name)

Functional configuration of BCRP

Recent evidence suggests that BCRP functions as a homodimer bridged by disulfide bonds. The initial demonstration that BCRP transfection directly confers MDR supports evidence that BCRP might be able to function by homodimerization (Doyle *et al.*, 1998). Kage *et al.* (2002) established BCRP transfectants with either Myc- or HA-tagged BCRP. The exogenous BCRP proteins migrated as 70 kDa bands in SDS-PAGE under reducing conditions, but as a 140 kDa complex in the absence of reducing agents. The 140 kDa BCRP complex dissociated into 70 kDa polypeptides with the addition of 2-mercaptoethanol. The 140 kDa BCRP complex was immunoprecipitated with anti-Myc antibody from lysates of cells cotransfected with Myc- and HA-tagged BCRP constructs. The 140 kDa complex reacted with anti-HA and anti-BCRP antibodies. After the addition of reducing agents, a 70 kDa BCRP band was seen, reactive with both anti-HA and anti-Myc antibodies. Furthermore, a dominant-negative mutant of BCRP, with an L554P alteration in the fifth transmembrane domain, was found to inhibit BCRP function partially when cotransfected with BCRP. These results elegantly demonstrate that BCRP forms a homodimer bridged by disulfide bonds. A molecular mass shift from a 72 kDa band under denaturing conditions to a 180 kDa band after treatment with crosslinking agents was also noted in Western blots by Litman *et al.* (2002), using polyclonal antibodies directed against peptide epitopes of BCRP.

The functional activity of BCRP in transfected insect cells, using a baculovirus-Sf9 cell system, also argues strongly for the activity of the protein as a homodimer (Ozvegy *et al.*, 2001, 2002). In this heterologous system, it is unlikely that any possible heterodimeric partners of BCRP are expressed. These investigators demonstrated that a mutation of BCRP (K86M) in the Walker A nucleotide-binding domain lacked transporter activity, and served as an effective dominant-negative inhibitor of BCRP function when cotransfected with nonmutated BCRP (Ozvegy *et al.*, 2002).

Chromosomal localization studies

The BCRP gene was mapped using a radiation hybrid panel to human chromosome 4q22, between the markers D4S2462 and D4S1557 (Knutsen *et al.*, 2000). No other ABC transporter gene had previously been assigned to human chromosome 4. These investigators performed cytogenetic studies on multidrug-resistant MCF-7/AdrVp 3000, MCF-7 MX, and S1-M1-80 cells, all of which overexpress BCRP relative to the parental cell lines. The MCF-7/AdrVp 3000 and MCF-7 MX cells demonstrated an amplification peak at 4q21–4q22 by comparative genomic hybridization, while S1-M1-80 cells did not. Fluorescent *in situ* hybridization (FISH) studies, using chromosome 4 paint markers, demonstrated multiple rearrangements of chromosome 4 in

MCF-7/AdrVp 3000 and MCF-7/MX cells, while S1-M1-80 cells had a simple reciprocal translocation between chromosomes 4 and 17. The balanced translocation without amplification of BCRP in S1-M1-80 cells suggests that rearrangement of BCRP in this line results in overexpression through the loss of an inhibitory region or that BCRP comes under the control of an active promoter in this subline. Spectral karyotyping revealed a balanced translocation, *t*(4;17)9q21–q22:p13 in S1-M1-80 and multiple clonal translocations of chromosome 4 in MCF-7/AdrVp3000 and MCF-7/MX. FISH studies with a bacterial artificial chromosome probe containing BCRP confirmed localization of the gene at 4q21–q22 in the normal chromosome 4 and showed amplification of BCRP in one translocation juncture in MCF-7/AdrVp 3000 and MCF-7 MX cells. The absence of cytogenetic evidence of coamplification of other regions was interpreted as being consistent with the absence of a half-transporter partner and the sufficiency of BCRP homodimerization to create an active transporter.

Murine and porcine orthologues of BCRP

The murine orthologue of BCRP was found to be located on chromosome 6, at a distance of 2829 cM from the centromere (Allen *et al.*, 1999). The BCRP gene demonstrates a new syntenic segment between human chromosome 4 and mouse chromosome 6. Sequencing of mouse cDNA clones revealed two transcripts differing by approximately 200 bp at the 3' end. Both transcripts contain the same open reading frame of 658 amino acids, corresponding closely in sequence to human BCRP.

Mouse Bcrp1 cDNAs were amplified by high-fidelity PCR with primers based on mouse EST sequences homologous to the 5' and 3' ends of human BCRP (Allen *et al.*, 1999). The mouse Bcrp1 and human BCRP amino-acid sequences are 81% identical and 86% homologous, with very high homology in the ATP-binding region. Hydrophobicity plots of mouse Bcrp1 and human BCRP are almost identical, with six putative transmembrane domains. Four potential sites for N-linked glycosylation were observed. While the first two are in a likely cytosolic part of the protein, the latter two are closely spaced in the loop between the fifth and sixth transmembrane domains and are likely extracellular. Only one of these latter two glycosylation sites is conserved in the human BCRP sequence.

A 656 amino-acid ABC transporter that is 85% identical and 90% homologous to human BCRP was recently isolated from porcine brain capillary endothelial cells (Eisenblatter and Galla, 2002). Termed the brain multidrug resistance protein (BMDP), the protein is expressed in brain tissue *in vivo*, predominantly within the brain capillary endothelial cells. As a potential xenobiotic transporter, BMDP was postulated to play a role in the blood–brain barrier. In contrast to porcine brain capillary endothelium, low

expression of BCRP was found in porcine oocytes (Takebayashi *et al.*, 2001).

Organization of the BCRP gene and promoter

We identified the genomic sequence of BCRP by screening human genomic lambda phage and BAC libraries and by searching the human genome database. The BCRP gene spans over 66 kb and consists of 16 exons and 15 introns (Bailey-Dell *et al.*, 2001). The exons are small in size, ranging from 60 to 532 bp, with the translational start site found in the second exon. The majority of the 5' untranslated region is in exon 1. The BCRP promoter is TATA-less, contains a CAAT box, and several putative Sp1 sites downstream from a putative CpG island. This is similar to the promoters for the MDR1 (ABCB1), MRP1 (ABCC1) and ABCG1 genes, which lack TATA boxes, and have multiple Sp1 sites (Ueda *et al.*, 1987; Zhu and Center, 1994; Langmann *et al.*, 2000). Unlike the ABCG1 (human white homologue) promoter, the BCRP promoter does not contain a sterol response element, strengthening the argument that BCRP is not involved with lipid transport, as is ABCG1 (Klucken *et al.*, 2000; Langmann *et al.*, 2000). We employed a luciferase reporter assay and transient transfection of human breast cancer MCF-7 cells and the human choriocarcinoma cell lines BeWo, JAR, and JEG-3 to investigate promoter activity in deletion constructs made from the BCRP 5' upstream region. These cell lines were used because we found that they have high endogenous expression of BCRP. The reporter analyses indicated that a 312 bp sequence directly upstream from the transcriptional start site conferred basal promoter activity, with positive and negative *cis*-regulatory elements identified in the region

between -1285 and +362 relative to the transcriptional start site (Figure 3) (Bailey-Dell *et al.*, 2001).

Little is known about the regulation of BCRP transcription. Classic enzyme inducers, such as valproic acid, phenytoin, and carbamazepine, induced the tissue expressions of bcrp1 in mouse kidney 1.5–2-fold (Schellens *et al.*, 2002). Phenytoin and carbamazepine also induced Bcrp1 levels significantly in the liver. While dexamethasone was not found to induce Bcrp1, a second study, using a cDNA subtraction technique to compare gene expression in untreated versus hydrocortisone-treated porcine endothelial cells, demonstrated over-expression of porcine BCRP after hydrocortisone (Eisenblatter and Galla, 2002). Elevated basal expression of BCRP, Pgp, and MRP1 were noted in thermoresistant human gastric carcinoma EPG85-257 cells, suggesting the possibility that cellular responses to heat may induce the expression of these transporters (Stein *et al.*, 2002).

Normal allelic variants of BCRP

Partial sequencing of BCRP exons from multiple genomic DNAs demonstrates that the genetic sequence is highly conserved, although single-nucleotide polymorphisms (SNPs) are found. Allelic variation as a result of SNPs results in alterations of the BCRP protein at amino acids 12 (V12M), 141 (Q141K), 206 (I206L), and 590 (N590Y), with the most frequent polymorphisms being the exon 2 SNP (G34A) and the exon 5 SNP (C421A), which produce changes in amino acids 12 and 141 (Honjo *et al.*, 2002; Imai *et al.*, 2002a; Zamber *et al.*, 2003). Additionally, a splice variant was found that resulted in deletions of A315 and T316 (Imai *et al.*, 2002a). The effect of these polymorphisms on BCRP function remains to be determined, although one study suggested that the C421A/Q141K variant may result in

Figure 3 Structure of BCRP gene and promoter. Adapted from (Bailey-Dell *et al.*, 2001), with permission from Elsevier

very low levels of BCRP protein expression (Imai *et al.*, 2002a). These investigators found that in a normal Japanese population, 39% were heterozygous and 7% were homozygous for the variant C412A allele.

Resistance phenotype conferred by BCRP expression: substrate specificity

Our laboratory inserted the full-length cDNA of BCRP into the expression vector pcDNA3, and transfected MCF-7 (Doyle *et al.*, 1998) or MDAMB231 cells with this vector, then isolated clones with high BCRP expression. These clones displayed decreased daunorubicin accumulation and retention by flow cytometry. The BCRP-expressing MCF-7 transfectants were also shown to have decreased accumulation of rhodamine 123 relative to cells transfected with empty vector, and this decreased accumulation was reversed by depletion of ATP (Doyle *et al.*, 1998). The sensitivities of the BCRP-transfected sublines to chemotherapeutic agents were tested by the sulforhodamine-B cytotoxicity assay. To date, the BCRP-overexpressing clones displayed resistance to mitoxantrone, daunorubicin, doxorubicin, and topotecan, but not to cisplatin, paclitaxel, etoposide, or vincristine, compared to control transfectants and parental MCF-7 or MDA MB231 breast cancer cells. The pattern of crossresistance in the transfectants was very similar to that of the original MCF-7/AdrVp-resistant cell line, although the degree of resistance to cytotoxic agents in the MCF-7/AdrVp subline was greater than that of transfected cells with comparable levels of BCRP by Northern blotting.

Resistance to mitoxantrone: efflux of fluorescent dyes

Strong resistance to mitoxantrone characterizes most drug-selected cell lines that overexpress BCRP, even if the selecting agent is not mitoxantrone. For example, MCF-7/AdrVp cells are over 1000-fold resistant to mitoxantrone but only 150-fold resistant to doxorubicin (Doyle *et al.*, 1998), despite being selected by doxorubicin in the presence of verapamil (Chen *et al.*, 1990). Litman *et al.* (2000) examined the pharmacodynamics of mitoxantrone and other fluorescent drugs by confocal microscopy in multidrug cell lines overexpressing BCRP. The expression of BCRP correlated with marked decreases in the accumulation of mitoxantrone, daunorubicin, bisantrene, topotecan, prazosin, lysotracker, and rhodamine 123. The Pgp substrates vinblastine, paclitaxel, and verapamil, and the MRP1 substrate calcein were not extruded from BCRP-overexpressing cells, distinguishing BCRP resistance from that of both Pgp and MRP1. BCRP-expressing cells were also found to be resistant to epirubicin (Brangi *et al.*, 1999). MCF-7 and K562 cells made resistant to doxorubicin encapsulated in polyisohexylcyanoacrylate nanospheres demonstrated BCRP rather than Pgp overexpression (Laurand *et al.*, 2001).

BCRP was found to be an efficient efflux transporter of the fluorescent dye Hoechst 33342 (Scharenberg *et al.*,

2002). This property is particularly germane to primitive stem cells termed 'side population' (SP) stem cells, which strongly efflux Hoechst 33342, and which recently were found to express BCRP (discussed subsequently in this paper).

The aza-anthrapyrazole BBR 3390 (Boeringer Mannheim), which is structurally similar to the anthracenedione structure of mitoxantrone, was demonstrated to have increased efflux in mitoxantrone-resistant 8226 multiple myeloma cells, which overexpress BCRP relative to parental cells (Hazlehurst *et al.*, 1999). The 8226/MR4 subline is 34-fold resistant to BBR 3390. BBR 3390 is a highly fluorescent drug, making it ideal for investigational drug accumulation studies. Decreased accumulation of BBR 3390 in BCRP-transfected cell lines has been demonstrated in our laboratory (Rabindran *et al.*, 2000). BODIPY prazosin, a fluorescent conjugate of the quinazoline α blocker prazosin, can also be used to monitor BCRP-mediated efflux (Honjo *et al.*, 2001b).

Efflux of anthracyclines, methotrexate, and rhodamine 123: influence of a mutational 'hot-spot' at codon 482

Anthracycline resistance and rhodamine efflux were noted in the BCRP-overexpressing MCF-7/AdrVp and S1-M1-80 cell lines but not in 10 other sublines that overexpress BCRP (Honjo *et al.*, 2001a). Sequencing of BCRP in these 12 lines demonstrated that MCF-7/AdrVp and S1-M1-80 contain a threonine and glycine, respectively, at codon 482, while their parental cell lines, as well as the other 10 resistant cell lines, have an arginine residue at that position. Sequencing of BCRP derived from normal tissues such as placenta reveal arginine at amino acid position 482, indicating that R482 is the 'wild-type' configuration, and that the threonine or glycine substitutions are mutations (R482T or R482G). These results suggest that a mutation at position 482 of BCRP, in the third transmembrane domain, may alter substrate specificity of the transporter to include rhodamine efflux. Transfection studies with a vaccinia virus expression system confirm that rhodamine efflux is observed when BCRP has a glycine or threonine at 482, but does not occur with arginine at 482 (Honjo *et al.*, 2001a). Three mouse cell lines independently selected for doxorubicin resistance were found to acquire mutations of murine Bcrp1 at arginine 482, leading to greater resistance to anthracyclines and bisantrene and to increased efflux of rhodamine 123 (Allen *et al.*, 2002a). The Bcrp1 mutations occurred after previous amplification and overexpression of the wild-type gene under doxorubicin selection. The findings demonstrate the crucial role of arginine 482 for BCRP substrate specificity, while suggesting that even unmutated BCRP has a role in anthracycline resistance. A Japanese study also suggested that crossresistance to mitoxantrone was relatively high in cell lines transfected with BCRP R482T as compared with those transfected with BCRP R482 (Komatani *et al.*, 2001). This conclusion was refuted by another study demonstrating high levels of mitoxantrone resistance in cell lines

expressing wild-type R482 as well as mutated R482G and R482T (Volk *et al.*, 2002a).

As with other anthracyclines, wild-type BCRP does not confer resistance to idarubicin (Abbott *et al.*, 2002). However, MCF-7/AdrVp cells, which overexpress the R482T mutation of BCRP and display considerable resistance to daunorubicin (25–40-fold) (Ross *et al.*, 1995; Doyle *et al.*, 1998), accumulate and retain idarubicin well, and are only minimally resistant to idarubicin (1.8-fold) (Ross *et al.*, 1995).

A high level of ATP-dependent cross resistance to methotrexate was noted in mitoxantrone-selected MCF-7/MX cells, which display considerable overexpression of BCRP (Volk *et al.*, 2000). Furthermore, this methotrexate resistance was reversible with the BCRP inhibitor GF120918. However, in this same work, investigations of the BCRP-transfected MCF-7 or MDAMB-231 cells developed in our laboratory did not find resistance or low accumulation of methotrexate in the transfected cells. Since these MCF-7 or MDAMB-231 cells were transfected with BCRP cDNA derived from MCF-7/AdrVp cells, the transfected cells consequently overexpressed the R482T mutation of BCRP. This realization led to further studies of methotrexate transport, which revealed that methotrexate resistance, reversible with GF120918, correlated with BCRP expression in cell lines that overexpressed wild-type BCRP, but not the mutant forms (R482T or R482G) (Volk *et al.*, 2002b).

Mutations of BCRP at position 482 have been investigated using the Baculovirus-Sf9 insect system (Ozvegy *et al.*, 2001, 2002). BCRP transporters having different amino acids (Arg, Gly or Thr) at position 482 were expressed in Sf9 cells and membranes from these cells were studied for ATPase and transport characteristics. All three BCRP proteins had high basal, vanadate-sensitive ATPase activity. However, only the mutant 482G and 482T transporters showed any drug-stimulated ATPase activity, when measured in the presence of known BCRP substrates. Fumitremorgin C (FTC), a specific inhibitor of BCRP (described subsequently), decreased the ATPase activity for both wild-type and 482 mutant variants of BCRP.

Resistance to topoisomerase I inhibitors

Mitoxantrone-resistant cell lines had frequently been demonstrated to have crossresistance to camptothecin-derived topoisomerase I poisons (Nakagawa *et al.*, 1992). Experiments with BCRP-transfected cells also demonstrated low-level resistance to the planar aromatic topoisomerase I inhibitors topotecan (Rabindran *et al.*, 2000) and irinotecan, as well as to SN-38, the active metabolite of irinotecan, reversible with FTC, a specific inhibitor of BCRP. This information was used as the basis for investigating the role of BCRP in cancer cells made resistant to topoisomerase I inhibitors *in vitro* (Maliepaard *et al.*, 1999). Resistant ovarian cell lines T8 and MX3 were developed by exposure of IGROV-1 tumor cells to topotecan or mitoxantrone, respectively. Both lines are resistant to topotecan, SN-38, 9-amino-

camptothecin, and mitoxantrone, but are not resistant to camptothecin, doxorubicin, paclitaxel, 5-FU, or cisplatin. Topotecan and mitoxantrone levels are markedly decreased in the resistant cell lines by a rapid, energy-dependent efflux mechanism, in the absence of overexpression of MRP1 or Pgp. Northern blotting revealed that BCRP mRNA was undetectable in the IGROV1 cells, while abundant expression was observed in the T8 and MX3 cells. The expression levels of BCRP in various revertant T8 sublines were qualitatively correlated to the level of resistance. Another topotecan-resistant breast cancer cell line, MCF-7/TPT300, with increased topotecan efflux, was also derived from MCF-7 parent cells (Yang *et al.*, 2000). MCF-7/TPT300 cells did not overexpress Pgp or MRP1, but had overexpression of BCRP, demonstrated by Northern and Western blotting. There was a lack of resistance to doxorubicin in topotecan-selected resistant cancer cells expressing BCRP, in contrast to the doxorubicin resistance demonstrated in mitoxantrone-resistant or BCRP-transfected cells.

Irinotecan is transformed to SN-38, an active metabolite that is further glucuronidated by uridine diphosphate glucuronosyltransferases (UGTs) in cells. An SN-38-resistant PC-6/SN2-5 small-cell lung cancer cell line was derived from parental PC-6 cells (Kawabata *et al.*, 2001b). A more highly resistant PC-6/SN2-5H cell line was also derived. These cell lines are 20- and 50-fold resistant to SN-38, respectively, and have reduced intracellular accumulation of the drug with crossresistance to mitoxantrone and to other topoisomerase I inhibitors (Kawabata *et al.*, 2001b). No alterations in topoisomerase I gene sequence or activity, and no differences in UGT1A1 enzyme content or total UGT enzyme activity were seen in the resistant cells. The level of BCRP expression in these sublines correlated with resistance to SN-38. BCRP-antisense oligonucleotides significantly enhanced the SN-38 sensitivity of the resistant cells with reduction of BCRP mRNA levels and increased intracellular accumulation of SN-38 (Kawabata *et al.*, 2001b).

Resistance to two potent indolocarbazole topoisomerase I inhibitors, NB-506 and J-107088, was found to be mediated by BCRP (Komatani *et al.*, 2001). An NB-506-resistant mouse fibroblast cell line and its parental cell line were analysed for gene expression by oligonucleotide microarray. Of 34,020 gene sequences analysed, the BCRP gene showed the highest relative increase in expression (31-fold).

Differences in structure of the topoisomerase I inhibitors appear to determine the specificity of the drugs as BCRP substrates. Cancer cells that express BCRP are not resistant to camptothecin itself, or to the camptothecin analogs BNP1350, DX8951f, or NX211 (Ishii *et al.*, 2000; Maliepaard *et al.*, 2001b; Van Hattum *et al.*, 2001; Van Hattum *et al.*, 2002). Studies of SN-38- and irinotecan-resistant PC-6 sublines, which overexpress BCRP, also demonstrated that these cells were not resistant to the clinically promising water-soluble camptothecin analog DX-8951f (Ishii *et al.*, 2000). However, ovarian cancer cells made resistant to

DX-8951f itself overexpressed BCRP but not Pgp, MRP1, or LRP (Van Hattum *et al.*, 2001). The DX-8951f-resistant ovarian cancer cell line was much more resistant to topotecan, SN-38, and mitoxantrone than it was to the selecting agent. Experimental data suggest that the accumulation of camptothecin analogs in BCRP-expressing cells is related to the polarity of the molecule (Yoshikawa *et al.*, 2002). Nucleophilic groups at the ninth or tenth position of the camptothecin A ring facilitate interaction with BCRP. For example, BCRP expression confers resistance to 9-aminocamptothecin but not 9-nitrocamptothecin, associated with decreased intracellular concentrations of the former but not the latter (Rajendra *et al.*, 2002). ST1481, a lipophilic 7-modified camptothecin analog with good oral bioavailability, was reported to be a poor substrate for BCRP (Perego *et al.*, 2001).

Flavopiridol resistance

Flavopiridol is an *N*-methylpiperidiny chlorophenyl flavone, which was the first cyclin-dependent kinase inhibitor used in human clinical trials (Senderowicz, 1999). Flavopiridol arrests cell growth at both G1 and G2 junctures and induces apoptosis as demonstrated by Annexin-V labeling and cytologic studies. Previously reported mechanisms of resistance to flavopiridol involve alterations in apoptosis due to overexpression of bcl-2, cyclin-D1, and p53 inactivation. Resistance to flavopiridol has not been seen in MDR cells overexpressing Pgp, MRP1, or MRP2, but was observed in the mitoxantrone-resistant cell lines 8226/MR20 and MCF-7/MR, which overexpress BCRP (Schlegel *et al.*, 1999). The myeloma cell line 8226/MR20 and the breast cancer subline MCF-7/MR have 4.8- and 3.2-fold resistance to flavopiridol, respectively. MCF-7 cells made 30-fold resistant to flavopiridol are also highly crossresistant to mitoxantrone, topotecan, and SN-38, but not to 5-FU or paclitaxel (Robey *et al.*, 2001b). Northern analysis of this MCF-7/FLV1000 subline revealed overexpression of the BCRP gene. Furthermore, flavopiridol, at a concentration of 100 μ M, was able to antagonize mitoxantrone transport from both mitoxantrone-resistant S1-M1-80 cells and the flavopiridol-resistant MCF-7/FLV1000 subline. In BCRP-transfected MCF-7 cells, flavopiridol was also effective in blocking mitoxantrone efflux (Robey *et al.*, 2001b).

BCRP transport of drug conjugates

Preliminary investigations have examined whether BCRP may transport drug conjugates analogous to the glutathione conjugates transported by MRP1. Many of the cytotoxic drugs extruded by BCRP, such as irinotecan, SN-38, and epirubicin, are detoxified by glucuronidation with UDP-glucuronyltransferases. Brangi *et al.* (1999) compared glucuronidation of 4-methylumbelliferone (4-MU), SN-38, mitoxantrone, and epirubicin in the mitoxantrone-resistant colon cell line S1-M1-80 and the doxorubicin-resistant MCF-7/AdrVp

3000 breast cancer cell lines to that of the parental cell lines. The MCF-7/AdrVp 3000 cells demonstrated increased glucuronide formation for 4-MU and epirubicin relative to the parental cells. The extent of glucuronidation with each compound was similar between the sensitive and resistant colon cancer cell lines, which have increased UDP-glucuronyltransferase activity relative to the breast cancer sublines, suggesting that glucuronidation is not rate limiting for the resistance mechanism in the S1-M1-80 cells.

To determine whether BCRP transports SN-38 and/or SN-38 glucuronide, Nakatomi *et al.* (2001) isolated membrane vesicles from SN-38-resistant PC-6/SN2-5H cells, which overexpress BCRP, and from the parental PC-6 small-cell lung cancer line (Kawabata *et al.*, 2001b). The concentrations of free SN-38 and SN-38-glucuronide within the membrane vesicles were measured by HPLC. Both SN-38 and SN-38 glucuronide were transported into the vesicles of PC-6/SN2-5 cells, in an ATP-dependent fashion, but not into the vesicles of PC-6 cells. These findings suggest that BCRP transports SN-38 glucuronide and confers resistance to SN-38. However, the $V_{\max}/K_m$ ratio for SN-38 in PC-6/SN2-5H membrane proteins was substantially higher than the ratio of those kinetic parameters for SN-38-glucuronide, suggesting that BCRP interacts directly with SN-38 and preferentially transports SN-38 rather than SN-38-glucuronide (Nakatomi *et al.*, 2001).

Modulation of BCRP transport

Fumitremorgin C

Most inhibitors of Pgp, such as verapamil, cyclosporin A, PSC-833, and the new Pgp antagonists XR9576 and LY335979, do not increase drug accumulation or cytotoxicity in cells expressing BCRP (Litman *et al.*, 2000). However, a number of potent inhibitors of BCRP transport have been described. Even before the discovery of the BCRP transporter, the drug FTC had been described as reversing mitoxantrone resistance and transport (Rabindran *et al.*, 1998). Rabindran and Greenberger used the mitoxantrone-resistant colon cancer cell line S1-M1-3.2 in a cell-based screen with a library of extracts derived from a variety of microorganisms. Extracts that had little or no toxicity by themselves but that, when combined with mitoxantrone, showed substantially increased cell death were analysed further. One extract from a solid medium fermentation broth of *Aspergillus fumigatus* was found to be highly active. The pharmacologically active ingredient in this broth was purified by chromatographic methods. The active ingredient was shown to be FTC, belonging to a class of diketopiperazines that are potent mycotoxins. FTC caused tremors in cockerels at 25 mg/kg orally (Cole and Cox, 1981). FTC, at a 5 μ M concentration, significantly increased the toxicity of mitoxantrone (93-fold), doxorubicin (26-fold), topotecan (24-fold), and bisantrene (25-fold). At 1 μ M, FTC was also

demonstrated to increase the amounts of mitoxantrone and doxorubicin retained by S1-M1-3.2 cells.

FTC does not reverse resistance to cells that over-express Pgp (S1-B1-20, 8226/DOX6) or MRP1 (HL-60/AR) (Rabindran *et al.*, 1998). FTC was found, however, to reverse resistance to mitoxantrone and doxorubicin in a breast cancer cell line (MCF-7/mtxR) selected in mitoxantrone and expressing BCRP, as well as with the MCF-7/AdrVp cell line. We evaluated the activity of FTC in cells transfected with BCRP to confirm that FTC could reverse resistance mediated by this transporter (Rabindran *et al.*, 2000). Cells transfected with the pcDNA3 vector (MCF-7/pcDNA3) or the BCRP expression construct (MCF-7/BCRP) were exposed to cytotoxic drugs in sulforhodamine blue cytotoxicity assays, with or without the addition of FTC. FTC, at 5 μ M, almost completely reversed resistance mediated by BCRP *in vitro* to mitoxantrone, doxorubicin, and topotecan. FTC also enhanced the toxicity of topotecan but not mitoxantrone in vector-transfected MCF-7 cells. The enhanced cytotoxicity of topotecan by FTC may be related to the known low-level expression of BCRP in parental MCF-7 cells, but a second, FTC-sensitive, mechanism of drug resistance cannot be completely discounted (Rabindran *et al.*, 2000). Flow cytometric measurement of the inhibition of mitoxantrone or prazosin efflux by FTC correlates with levels of BCRP mRNA by Northern analysis, and as such, constitutes a sensitive and specific functional assay for BCRP (de Bruin *et al.*, 1999; Robey *et al.*, 2001a).

Resistance to flavopiridol is reversible by FTC, and FTC is also able to enhance the accumulation and inhibit the efflux of the fluorescent aza-anthrapyrazole drug BBR 3390 (Rabindran *et al.*, 2000; Robey *et al.*, 2001b). Sensitivity to flavopiridol has also been restored by concurrent exposure to BIB-E, a dipyridamole analog that inhibits BCRP-mediated drug export (Schlegel *et al.*, 1999).

BCRP-mediated drug efflux can also be inhibited by other fumitremorgin-type indolyl diketopiperazines (van Loevezijn *et al.*, 2001). A library of diastereoisomeric mixtures of these compounds, synthesized by solid phase techniques, were screened for BCRP inhibitory properties in both the Igrov1/T8 human ovarian carcinoma cell line and the mouse T6400 cell line. The T6400 cell line is a topotecan-selected subline of the embryo fibroblast line MEF3.8, lacking functional genes encoding mouse Pgp and Mrp1. This subline is highly resistant to topotecan and mitoxantrone and expresses the murine homologue Bcrp1 (Allen *et al.*, 1999). Structure-activity relationship studies of the fumitremorgin-related compounds demonstrated that several analogs had potent effects on mitoxantrone accumulation and MDR in both cell systems. The tetracyclic FTC analog Ko143 was found to be a highly potent and specific inhibitor of BCRP, without the neurotoxicity that is associated with FTC, as assessed in murine systems (Allen *et al.*, 2002b). Ko143, at 100 nM concentration, modulated BCRP-mediated transport of mitoxantrone, but did not modulate P-gp or MRP1-mediated efflux (Minderman *et al.*, 2002a).

Novobiocin

We have demonstrated that partial reversal of BCRP-mediated drug resistance and transport can be achieved by coincubation of resistant cells with novobiocin, a coumermycin antibiotic (Doyle *et al.*, 1996). Modulation of BCRP-mediated resistance by novobiocin and FTC was studied using a novel coculture comparative growth assay (Hausner *et al.*, 1999). MCF-7 cells, stably transfected with pcDNA-3 BCRP, were compared with MCF-7 cells transfected with a control plasmid. One cell line of each coculture was permanently labeled by stably expressing the yellow fluorescent protein. The coculture was exposed to vincristine, mitoxantrone, topotecan, daunorubicin, and SN-38, with or without the addition of putative inhibitors FTC or novobiocin. The coculture was exposed to drugs for 4 days, evaluated by flow cytometry and the average daily selection rate was calculated from the ratio of fluorescent and nonfluorescent cells in exposed cocultures compared to baseline cocultures. BCRP-overexpressing MCF-7 cells were selected by mitoxantrone, topotecan, SN-38, and daunorubicin. Novobiocin decreased the selection rate of daunorubicin and FTC completely reverted the selective advantage of BCRP-expressing cells exposed to topotecan or mitoxantrone.

GF120918

GF120918, an acridone carboxamide, is a second-generation Pgp inhibitor developed by GlaxoSmith-Kline, which increases drug accumulation and cytotoxicity in cells expressing BCRP (de Bruin *et al.*, 1999). Higher concentrations of GF120918 are required to reverse resistance in BCRP-expressing cells than in Pgp-expressing cells. GF120918 does not sensitize MRP1-overexpressing MCF-7/VP-16 cells to etoposide (de Bruin *et al.*, 1999). GF120918 has the advantage over FTC of synthetic production, making large-scale production of this agent more feasible. GF120918 also does not have neurotoxicity, the principal side effect of FTC. The disadvantage of GF120918, as a laboratory or clinical tool, is that it is a less specific inhibitor of BCRP than is FTC.

GF120918 increases the cytotoxicity and accumulation of mitoxantrone and topotecan in BCRP-expressing cells. In the drug-resistant human ovarian cancer lines T8 and MX3, developed by exposure of Igrv1 cells to topotecan and mitoxantrone, respectively, resistance associated with overexpression of BCRP was completely abrogated by GF120918 at a nontoxic dose of 100 nM (Maliepaard *et al.*, 2000). Reversal of resistance to the promising hydrophilic topoisomerase I inhibitor DX9581f by GF120918, in cells overexpressing BCRP, is variable and may depend on the cell line (Ishii *et al.*, 2000; Van Hattum *et al.*, 2001).

Other BCRP substrates/inhibitors

The ErbB1 tyrosine kinase inhibitor CI1033 inhibits BCRP-mediated efflux of topotecan and SN-38, leading

to synergistic cytotoxicity with these agents in glioblastoma and colon cancer cells (Erlichman *et al.*, 2001). CI1033 enhanced the uptake and cytotoxicity of SN-38 and topotecan in cells transfected with BCRP but not empty vector, suggesting that CI1033 is a substrate for this efflux pump. Erlichman *et al.* (2001) demonstrated protein expression of BCRP in unselected HCT8 colorectal carcinoma cells as well as T98G glioblastoma cells by immunoblotting studies with a monoclonal antibody reactive to BCRP. The expression of BCRP in these cell lines correlated with the ability of CI1033 to modulate the cytotoxicity of topotecan and SN-38 in the cells. In contrast, BCRP levels were much lower in PC-3 or Ovarcar-3, two cell lines in which CI1033 failed to modulate SN-38 cytotoxicity.

The accumulation of CI1033 in MDA-MB-231 cells transfected with empty vector or BCRP was measured by HPLC (Erlichman *et al.*, 2001). This analysis demonstrated that CI1033 accumulation was 4.9-fold lower in cells transfected with BCRP, suggesting that CI1033 is itself a substrate for BCRP, and providing an explanation for the drug's ability to inhibit BCRP-mediated efflux of cytotoxic agents.

Another quinazoline ErbB1 inhibitor, Iressa (ZD1839), has also been shown to cause potent BCRP inhibition and to enhance the oral bioavailability of topotecan and irinotecan in mice (Schuetz *et al.*, 2002). In Saos cells transfected with BCRP, Iressa at a concentration less than 1 μ M reversed topotecan, irinotecan and SN-38 resistance. In mice, oral Iressa (100 mg/kg) markedly increased the oral bioavailability of topotecan and irinotecan. In contrast to structurally related CI1033, Iressa is apparently not a substrate for BCRP transport.

The rauwolfia alkaloid reserpine, a prototypical inhibitor of Pgp, was also found to be an effective inhibitor of BCRP function in stem cell populations (Zhou *et al.*, 2001). The pipercolinate derivatives VX-710 (biricodar, Incel) and VX-853 were found to be effective inhibitors of drug efflux mediated by Pgp, MRP1, and BCRP in acute myeloid leukemia cells (Baer *et al.*, 2002).

Estrogen agonists and antiestrogens were found recently to have potent BCRP antagonistic activity (Sugimoto *et al.*, 2003). Using BCRP-transfected K562 cells, strong BCRP-reversing activity was found for diethylstilbesterol, and estrone. BCRP antagonist activity was also found for the estrogen antagonists tamoxifen and toremifene, and for the tamoxifen derivatives TAG-11 and TAG-139.

Molecular imaging of the functional transport activity of ABC transporters *in vivo* with radiopharmaceuticals may allow noninvasive monitoring of chemotherapy and MDR gene therapy agents. Chen *et al.* (2000) investigated the transport of ^{99m}Tc-tetrofosmin, a candidate radiopharmaceutical substrate of ABC transporters in cells overexpressing BCRP. Neither ^{99m}Tc-Sestimibi, a previously validated MDR imaging agent, nor ^{99m}Tc-Tetrofosmin was a substrate for BCRP, although both agents were transported by cells overexpressing Pgp.

Table 2 Substrate dyes, drugs, and inhibitors of BCRP

Drugs	Dyes/natural substrates	Inhibitors
Mitoxantrone	Rhodamine 123 ^a	Fumitremorgin C (FTC)
Topotecan	Lysotracker	FTC analogues (Ko143)
Irinotecan, SN-38	Prazocin-BODIPY	GF120918
9-Aminocamptothecin	Hoechst 33342	BIB-E
Doxorubicin ^a	Pheophorbide a	Flavopiridol
Daunorubicin ^a	Protoporphyrin IX	CI1033
Epirubicin ^a	Hydrocortisone?	Iressa
Idarubicin ^a	Estradiol 17- β ?	Novobiocin
Flavopiridol	Estrone?	Reserpine
CI1033		VX-710 (biricodar)
BBR3390		VX-853
Methotrexate ^a		Diethylstilbesterol (DES)
Prazocin		Estrone
Indolocarbazole topoisomerase I inhibitors: NB-506, J-107088		Antiestrogens
		Tamoxifen, and derivatives TAG-11, TAG-139
		Toremifene
Zidovudine (AZT), lamivudine		

^aEffect varies, depending on the amino acid at codon 482 (see text)

ATP-dependent efflux of active phosphorylated metabolites of zidovudine (AZT) in a BCRP-overexpressing human CD4⁺ T-cell line (MT-4) was recently described. The activity of AZT and lamivudine against HIV-1 viral replication in these BCRP-overexpressing T cells was considerably reduced compared to parental MT-4 cells (Wang *et al.*, 2003).

BCRP substrates and inhibitors are summarized in Table 2.

Gene therapy approaches to modulating BCRP

Modulation of the BCRP multidrug-resistant phenotype by a hammerhead ribozyme directed against the transporter has recently been reported (Kowalski *et al.*, 2002, 2001). When the anti-BCRP hammerhead ribozyme was introduced into the human gastric carcinoma cell line EPG85-257RNOV, the levels of BCRP mRNA and protein were greatly reduced and cellular accumulation of BCRP substrate drugs was increased.

BCRP transport function can be inhibited by dominant-negative BCRP mutants, strongly suggesting that homodimer formation is essential for BCRP function (Kage *et al.*, 2002). BCRP mutants, generated by PCR mutagenesis, were transfected into PA317 cells and tested for the loss of BCRP function. A mutant with an amino-acid change from Leu to Pro at residue 554 in the fifth transmembrane domain of BCRP resulted in the loss of transporter function, and was able to reverse partially the drug resistance of cotransfected wild-type BCRP. The results suggest dominant-negative inhibition as a possible new strategy to circumvent clinical drug resistance.

Antibodies directed at BCRP

Litman *et al.* (2000, 2002) developed polyclonal antibodies against several epitopes on BCRP. Rabbit anti-BCRP antisera 84705 was raised against an 18-mer peptide of the ATP-binding region of the protein and could detect BCRP polypeptide at a dilution of 1:2000. The antisera detected a well-defined 72 kDa band corresponding to the predicted MW of the protein. Abundant expression of BCRP could be detected in MCF-7 AdVp3000 and S1-M1-80 cells by immunoblot assays and immunohistochemistry. Further Western analysis by Yang *et al.* (2000) demonstrated relative overexpression of BCRP in a topotecan-resistant subline MCF-7/TPT300.

Scheffer *et al.* (2000) developed a monoclonal antibody, BXP-34, by immunizing mice with MCF-7 Igrov1 MR cells and using a cytospin-based screening method with mitoxantrone-resistant and -sensitive cell lines. The BXP-34 monoclonal antibody, thus derived, was found to detect BCRP in the plasma membranes of the mitoxantrone-resistant cell lines MCF-7 Igrov1 MR, 8226 MR20, Igrov1 MX3, and the topotecan-resistant line Igrov1 T8. BXP-34 was able to detect a 72 kD BCRP band in overexpressing cells by immunoprecipitation but not by Western blotting. A second monoclonal antibody, BXP-21, was subsequently developed which, unlike BXP-34, can detect BCRP by immunoblotting and in formalin-fixed paraffin-embedded tissues (Maliepaard *et al.*, 2001a). These antibodies are now available commercially. BXP-34 and BXP-21 also allow detection of BCRP expression by flow cytometry in multiple BCRP-expressing cell lines (Minderman *et al.*, 2002b). Flow cytometric analysis of FTC modulation of mitoxantrone retention has also been found to be a sensitive functional assay for BCRP transport suitable for analysis of clinical drug resistance (Minderman *et al.*, 2002b). A new anti-human BCRP monoclonal antibody (clone 5D3; MAB4155, Chemicon), which recognizes an external epitope of BCRP on living cells, has also been developed for flow cytometry (Zhou *et al.*, 2001).

Cellular localization studies

Immunohistochemical studies with monoclonal and polyclonal antibody probes confirm the predominant plasma membrane localization of the BCRP transporter. Initial studies with the 87405 antisera suggested cytoplasmic staining as well, but subsequent immunofluorescence studies with confocal microscopy and careful preadsorption of nonspecific binding antibodies demonstrated the predominance of plasma membrane immunostaining (Rocchi *et al.*, 2000; Scheffer *et al.*, 2000).

The plasma membrane location of BCRP differentiates this transporter from almost all other known half-transporters. Most half-transporters are predominantly localized to intracellular membranes, such as the mitochondria (M-ABC1 and ABC-7), the endoplasmic reticulum (TAP 1 and 2), or the peroxisomes (ALDP/

ABCD1). The only other half-transporter with evidence suggesting partial cell surface localization is the human white homologue ABCG1 (Ewart and Howells, 1998). Models for the *Drosophila* white protein also predict it to be localized in the plasma membrane of eye pigment cells (Ewart *et al.*, 1994).

The results from immunohistochemical studies with BCRP antibodies have been corroborated by confocal microscopy drug localization assays performed in MCF-7 and S1 parental cells and resistant sublines (Litman *et al.*, 2000). Reduced cytoplasmic accumulation of mitoxantrone, daunorubicin, and topotecan was observed in the BCRP-expressing MCF-7 Adr-Vp3000 and S1-M1-80 sublines. In the S1-M1-80 cells, hot-spots consistent with accumulation in acidic vesicles were observed. Colocalization studies with lysotracker demonstrated sequestration of mitoxantrone in acidic vesicles in both parental S1 and resistant sublines (Litman *et al.*, 2000).

Kinetics of drug transport by BCRP

Ozvegy *et al.* (2001) characterized the ATPase activity of BCRP in a variety of BCRP-overexpressing cell types, including Baculovirus-transfected Sf9 insect cells. BCRP demonstrates a baseline level of ATPase activity, which is 2–3-fold higher than that of Pgp. The ATPase activity in plasma membranes from these cells was vanadate sensitive, with a K_i of 8 μ M, and was directly proportional to the amount of BCRP detected. The ATPase activity of BCRP demonstrated classical Michaelis-Menten kinetics. The transporter showed specific ATP binding, but adenine nucleotide trapping could not be detected using 8-azido-ATP. The ATPase activity was stimulated by transport substrates of BCRP, such as mitoxantrone, prazosin, doxorubicin, daunorubicin, and rhodamine 123, but was inhibited by FTC. This ATPase assay could potentially be used as a screen to detect BCRP–drug interactions.

Physiologic function of BCRP

Expression of BCRP in normal tissues

We used BCRP cDNA as a probe in Northern blots to examine the expression of BCRP mRNA in selected normal human tissues (Doyle *et al.*, 1998). The greatest expression was seen in placental tissue, which had approximately 100 times greater expression than brain, prostate, small intestine, testis, ovary, colon, or liver. BCRP transcripts were undetectable by Northern analysis in heart, lung, skeletal muscle, kidney, pancreas, spleen, thymus, and peripheral blood leukocytes. In subsequent studies, we used a commercially available dot blot that contained RNA from 50 human tissues (Human RNA Master Blot, Clontech). This blot was probed with a 795-bp cDNA probe corresponding to the 3' portion of BCRP cDNA; quantification of probe

Figure 4 Multiple tissue dot blots using radiolabeled BCRP cDNA as probe. Blots were performed using Human RNA Master Blot (Clontech)

binding was carried out by Phosphorimager determination of the intensity of autoradiographic image of each dot in the blot. As illustrated in Figure 4, placenta was again found to have the highest expression of BCRP mRNA. High expression was also seen in the liver and small intestine. Interestingly, certain areas of the brain (particularly the midbrain: putamen, substantia nigra, pituitary gland, thalamus, amygdala, caudate, and subthalamic nuclei) had relatively high expression of BCRP mRNA. In contrast to our earlier Northern blot studies, the dot blots showed moderate expression of BCRP in lung tissue, and low expression in testis, ovary, and colon.

Immunohistochemical studies with BXP-21 demonstrated moderate to strong BCRP expression in placenta, liver canaliculi, colon, small intestine, cardiac muscle, endocrine pancreas, adrenal cortex, thyroid, and parathyroid (Izquierdo *et al.*, 2002). A weaker expression of BCRP was observed in other tissues such as hypophysis, prostate, bronchial epithelium, and peribronchial glands. In the kidneys, glomeruli were negative but faint BCRP expression was detected in certain tubules. While mouse kidney expresses high levels of BCRP expression by immunohistochemistry, human kidney does not (Schuetz *et al.*, 2002). Normal tissues without detectable BCRP expression included endometrial cells and squamous epithelial cells in the skin or esophagus. BCRP is variably expressed by immunohistochemistry in normal breast epithelium and blood vessels (Faneyte *et al.*, 2002). Low or undetectable BCRP expression in human and murine lung were also observed by immunohistochemical studies (Scheffer *et al.*, 2002). The expression of BCRP in normal tissues as detected by immunohistochemistry is summarized in Table 3.

Immunohistochemical studies of the placenta, using the monoclonal antibody BXP-34, suggest that BCRP

Table 3 Expression of BCRP in normal tissues, detected by immunohistochemistry

High	Intermediate	Low
Placental syncytiotrophoblast	Hypophysis	Heart
Brain microvessel	Prostate	Lung
Endothelium (porcine human)		Squamous epithelium – skin and esophagus
Venules		Skeletal muscle
Kidney (mouse)		Kidney (human)
Small intestine		Pancreas
Liver		Spleen
Testis		Thymus
Ovary		Peripheral blood leukocytes
Colon		

expression is highest in the placental syncytiotrophoblast, at the apical surface of the chorionic villus (Maliepaard *et al.*, 2001a). This localization suggests that BCRP plays a protective role for the fetus by effluxing drugs that enter the placenta back into the maternal circulation. Evidence for this hypothesis was provided by Jonker *et al.* (2000), who demonstrated that in pregnant, Pgp-deficient mice, fetal penetration of topotecan is increased at least twofold by the BCRP inhibitor GF120918. A recent review of efflux transporters in the human placenta is available (Young *et al.*, 2003).

BCRP may also be an important component of the blood–brain barrier, excluding xenobiotics from the brain. The porcine homologue of BCRP was found in cultured brain capillary endothelial cells, which have high transendothelial electrical resistances and low cell monolayer permeabilities for small solutes very similar to the blood–brain barrier (Eisenblatter and Galla, 2002). Further analysis revealed that the porcine BCRP was expressed in brain tissue *in vivo* and was predominantly localized within the endothelial cells isolated from brain capillaries. The presence of BCRP in the luminal surface of the endothelium of human brain microvessels was recently demonstrated by immunofluorescence confocal microscopy (Cooray *et al.*, 2002). This suggests that BCRP, like Pgp and MRP1, contributes to the blood–brain barrier.

The expression of porcine BCRP was induced in brain capillary endothelial cells by hydrocortisone (Eisenblatter and Galla, 2002), suggesting that this steroid may be a substrate for the transporter. Estrone and 17β -estradiol have been found to potentiate the cytotoxicity of SN-38, mitoxantrone, and topotecan in BCRP-transfected cells but not in a parental K562 line (Imai *et al.*, 2002b). Estrone and 17β -estradiol also increased the cellular accumulation of topotecan in K562/BCRP cells. Since BCRP is expressed in the placenta, where these steroid hormones are produced, these findings suggest that BCRP may be important in the transport or secretion of these hormones or their precursors in the placenta.

BCRP effects on drug bioavailability, pharmacokinetics

Strong staining for BCRP was observed at the apical membrane of the small intestine and colon epithelium

and in the bile canalicular membrane (Jonker *et al.*, 2000). In fact, BCRP and MRP2 transcripts measured by quantitative PCR were more abundant in jejunum biopsies from healthy volunteers than were MDR1 transcripts (Taipalensuu *et al.*, 2001). The apical localization of BCRP in gut tissue suggests a role of BCRP in reducing the uptake of orally administered BCRP substrates. In mice, Bcrp1 mediates apically directed transport of topotecan in the polarized kidney epithelial cell line LLC-PK1 (Jonker *et al.*, 2000). The oral availability of topotecan is dramatically increased by oral coadministration of the Bcrp1 inhibitor GF120918 in both Pgp-deficient and wild-type mice. The plasma clearance and hepatobiliary excretion of topotecan were also decreased by GF120918. These findings suggest that BCRP plays an important role in the intestine and liver, limiting the oral availability of BCRP substrates by active backtransport of these molecules entering from the gut lumen. The role of transporters including BCRP in hepatobiliary transport is discussed in a recent review (Elferink and Groen, 2002).

Coadministration of GF120918 also resulted in a significant increase in the systemic exposure of oral topotecan in patients (Kruijtz *et al.*, 2002). The oral bioavailability of topotecan increased from 40% without to 97% with GF120918. Since topotecan has a much greater affinity for BCRP than Pgp, inhibition of BCRP by GF120918 is likely to be the major means by which the drug causes increased bioavailability of topotecan by increased intestinal absorption and reduced biliary excretion. Kruijtz *et al.* (2002) also demonstrated evidence for decreased biliary and renal excretion of topotecan by GF120918 by showing that the drug reduces plasma clearance of topotecan administered intravenously. Newer camptothecin derivatives such as BNP1350, likely owe their superior oral bioavailability to the fact that they are poor substrates for BCRP (Van Hattum *et al.*, 2002).

An important model for examining BCRP function has been developed by Allen *et al.* (1999), using mouse fibroblast cells lacking functional Mdr1a, Mdr1b, and Mrp1 genes. These cell lines were selected for resistance to topotecan, mitoxantrone, or doxorubicin. Each of the resultant drug-resistant cell lines demonstrated gene amplification of Bcrp1, the mouse homologue of the human gene, and elevated expression of Bcrp1 mRNA. Resistance to the three drugs in each cell line was completely reversed by coadministration of GF120918. These studies demonstrate that GF120918 reverses MDR in BCRP-overexpressing cells in which overexpression of Pgp species on Mrp1 cannot contribute to the resistant phenotype.

Expression and function of BCRP in stem cells

Evidence is recently evolving that ABC transporters play a crucial role in the regulation of stem cell biology (Bunting, 2002). Hematopoietic stem cells from a variety of sources can be recognized with flow cytometry by their appearance as a 'side population' (SP) of cells, manifested by the ability of the stem cells to exclude the dye Hoechst

33342, and by cell surface phenotypic characteristics, including low or no expression of the CD34 antigen, and no expression of lineage markers (Goodell *et al.*, 1997). Furthermore, SP cells are highly enriched in long-term culture-initiating cells (Goodell *et al.*, 1997). Initial attention was drawn to Pgp as the transporter responsible for Hoechst dye exclusion in the SP cells; however, studies in Mdr1 knockout mice found no difference in the numbers of SP cells in the Pgp-deficient and Pgp-replete animals (Zhou *et al.*, 2000, 2001; Uchida *et al.*, 2002). SP cells from mouse or rhesus monkey sources were found to express high levels of BCRP mRNA (Zhou *et al.*, 2001). Further, these investigators demonstrated that in primitive mouse hematopoietic stem cells (CD34⁻), BCRP mRNA was highly expressed; when the hematopoietic stem cells differentiated to a CD34⁺ phenotype, the expression of BCRP mRNA decreased markedly, with a concomitant increase in the expression of MRP1, -2, and -4. The enforced expression of BCRP in bone marrow cells expanded the SP population, and caused a reduction in mature cell numbers, suggesting that BCRP is both a stem cell marker and an important determinant of the SP phenotype. These investigators postulated that possibly BCRP was preventing the cellular accumulation of a substrate requisite for the differentiation of the stem cells.

The demonstration that BCRP is an efficient Hoechst dye efflux pump, with overexpression in hematopoietic stem cells has been observed by other investigators (Kim *et al.*, 2002; Scharenberg *et al.*, 2002). Transfection of BCRP into human embryonic kidney cells generated an SP cell phenotype (Scharenberg *et al.*, 2002). No Bcrp1 expression was seen in mature lymphoid or myeloid cells except for NK lymphocytes and Ter119⁺ erythroid precursor cells. Bcrp1 was sharply downregulated upon differentiation of murine hematopoietic stem cells into lineage-specific development (Scharenberg *et al.*, 2002).

Expression of Bcrp1 was noted in CD34⁺/CD45⁻ murine myogenic-endothelial progenitor cells, which are resident in the interstitial spaces of mammalian skeletal muscle (Tamaki *et al.*, 2002). BCRP expression has also been demonstrated in multipotential progenitor cells in islets isolated from adult human pancreas cells (Lechner *et al.*, 2002). These cells, which exclude Hoechst dyes, might be useful for the production of islet tissue, which could be transplanted into diabetic subjects. The isolation of such stem cells might be facilitated by the use of a new anti-human BCRP monoclonal antibody (clone 5D3), which recognizes an external epitope of BCRP on living cells, and which has successfully been used to confirm marrow SP cells by flow cytometry (Zhou *et al.*, 2001). Reviews concerning BCRP and the role of ABC transporters in stem cells are available (Bunting, 2002; Guo *et al.*, 2003).

Studies of BCRP-null mice

Recently, Bcrp1-null mice have been developed (Zhou *et al.*, 2002). These Bcrp1^{-/-} mice display a marked reduction in SP cell numbers in both bone marrow and skeletal muscle. Interestingly, these mice had normal hematopoietic. However, hematopoietic stem cells

from the Bcrp1-null mice were more sensitive to mitoxantrone, indicating a function of BCRP in protecting these cells from xenobiotic agents.

BCRP knockout mice were also produced independently by a group in the Netherlands (Jonker *et al.*, 2002). These investigators found that the Bcrp1^{-/-} mice displayed photosensitivity in light-exposed areas of their skin. This phenomenon was attributed in part to absorption in the Bcrp1-null animals of a diet-derived compound pheophorbide a. Pheophorbide a is a breakdown product of chlorophyll. In Bcrp1-replete animals, absorption of pheophorbide was minimized by Bcrp1 transport back into the gut lumen. These investigators also found that the Bcrp1-null mice developed a novel protoporphyria, characterized by accumulation of protoporphyrin IX in erythrocytes. These studies suggest that humans with low or absent BCRP expression may be at risk for developing photosensitivity dependent on diet and protoporphyria.

Expression of BCRP in human cancers

Leukemia studies (summarized in Table 4)

Correlative studies in adult leukemia suggest the presence of a novel transport mechanism operative in some leukemic blast cells. MDR1/Pgp expression, which is

highly correlated with cyclosporine-inhibited efflux by leukemic blasts, was noted in only 35% of younger acute myelogenous leukemia (AML) patients in a Southwest Oncology Group study, distinctly lower than the frequency of 71% previously reported in AML in the elderly (Leith *et al.*, 1999). A distinct and nonoverlapping phenotype was detected in 18% of young AML cases: cyclosporine-resistant efflux not associated with MDR1, MRP1, or LRP expression, implying the existence of other as yet undefined efflux mechanisms in this malignancy.

Leukemia patients may benefit from treatment with cytotoxic drugs in combination with inhibitors of Pgp, but these patients may also be at particular risk to develop non-Pgp mechanisms of MDR. List *et al.* (1999) used cyclosporine in combination with daunorubicin in patients with AML. They found that Pgp-positive leukemic blasts were eliminated and that patients relapsed with a non-Pgp mechanism of drug resistance.

We have examined blast cells from 21 acute leukemia patients (one acute lymphoblastic leukemia (ALL), 20 AML) for BCRP expression with quantitative RT-PCR (Ross *et al.*, 2000). Blast cells in seven patients with clinical drug-resistant disease had relatively high expression of BCRP mRNA. BCRP expression was low or barely detectable in blast cells from the remaining 14 patients. Expression of BCRP did not correlate with the expression of Pgp. However, other studies have demonstrated that high expression of BCRP correlated with an

Table 4 BCRP Expression in human leukemias

Disease type	Number studied	Methods	Findings	Reference
AML, ALL	20 AML, one ALL. In all, 14 <i>de novo</i> , seven previously treated	RT-PCR	BCRP mRNA expression varies among samples. In all, 33% have expression more than fivefold that of the median value	Ross <i>et al.</i> (2000)
AML	20 paired samples	IHC, function	BCRP correlated with immature phenotype; no correlation in relapsed/refractory disease	van der Kolk <i>et al.</i> (2002b)
AML	20 paired samples	Real-time (Taqman) RT-PCR	BCRP expression increased in relapsed/refractory disease	van den Heuvel-Eibrink <i>et al.</i> (2002)
AML (newly diagnosed)	40	Real-time RT-PCR	BCRP mRNA comparable to expression in drug-resistant BCRP-transfected cells in 7% of patient samples	Abbott <i>et al.</i> (2002)
AML	20 (12 from previously treated patients)	Immunohistochemistry (BXP-34 monoclonal antibody)	No correlation of BCRP expression with <i>de novo</i> versus previously treated cases; patients with + BCRP expression had higher LC ₅₀ to daunorubicin <i>in vitro</i>	Sargent <i>et al.</i> (2001)
AML	21	Real-time (Taqman) RT-PCR	BCRP expression correlated with flavopiridol toxicity <i>in vitro</i> ; all samples expressed wild-type (arginine) at codon 482	Nakanishi <i>et al.</i> (2003)
ALL (childhood)	67	Real-time (Taqman) RT-PCR	BCRP of no prognostic significance	Sauerbrey <i>et al.</i> (2002)
AML (childhood)	59	Real-time (Taqman) RT-PCR	BCRP correlated with poor response to remission induction therapy	Steinbach <i>et al.</i> (2002)

immature phenotype, determined by expression of the surface marker CD34 ($R=0.54$, $P=0.001$) (van der Kolk *et al.*, 2002b). The association of BCRP expression with relapsed/refractory AML remains unclear. One study, using 20 paired clinical AML samples from diagnosis and relapse or refractory disease, demonstrated that BCRP was the only resistance protein that was expressed at a significantly higher RNA level (median 1.7-fold, $P=0.04$) in the relapsed/refractory state relative to that at diagnosis (van den Heuvel-Eibrink *et al.*, 2002). However, a similar sized study, using flow cytometry with BXP-21 and BXP-34 monoclonal antibodies, as well as FTC-inhibitable mitoxantrone accumulation, did not demonstrate a consistent upregulation of BCRP in relapsed or refractory disease (van der Kolk *et al.*, 2002b). A study of blast cell samples from 40 newly diagnosed AML patients revealed that 7% had BCRP expression comparable to BCRP-transfected cells with detectable functional activity; however, 78% of the samples expressed BCRP mRNA at levels higher than normal blood and bone marrow (Abbott *et al.*, 2002).

BCRP expression has also been examined in relation to *in vivo* chemoresistance of leukemia blast cells from AML patients (Sargent *et al.*, 2001). Six out of 22 AML samples had greater than 10% of cells staining positively for BCRP. Although there was no correlation between BCRP positivity and *in vivo* sensitivity to mitoxantrone, topotecan, or doxorubicin, the median daunorubicin LC₅₀ value of BCRP(+) cells was fourfold higher than that of BCRP(-) cells ($P<0.05$). The results are inconclusive but suggest that BCRP may be involved in resistance to one of the agents commonly used in front-line treatment of AML. These studies did not evaluate for mutations of the BCRP expressed at codon 482, the 'mutational hot-spot' found in drug-selected cells (Honjo *et al.*, 2001a). In studies of blast cells from acute leukemia patients at the University of Maryland, we were able to correlate high expression of BCRP with

in vitro resistance of the blast cells to flavopiridol (Nakanishi *et al.*, 2003). In the region of BCRP mRNA corresponding to codon 482, all of the blast cell samples tested expressed the wild-type sequence.

A study of 47 initial stage and 20 relapsed children with ALL indicated low expression of BCRP, particularly in T-lineage ALL, and no prognostic significance of BCRP expression was observed (Sauerbrey *et al.*, 2002). In contrast, this same group found that in childhood AML, BCRP expression was associated with a poor response to remission induction therapy (Steinbach *et al.*, 2002).

The prognostic significance of BCRP and other transporters in leukemias is the subject of some recent review papers (Ross, 2000; van den Heuvel-Eibrink *et al.*, 2000; van der Kolk *et al.*, 2002a).

Solid tumor studies (summarized in Table 5)

Initial immunohistochemical studies with monoclonal antibody BXP-34 using a panel of human tumors showed BCRP to be low or undetectable except for one case of small intestine adenocarcinoma (Scheffer *et al.*, 2000). However, subsequent investigation by the same investigators, using a second monoclonal antibody, BXP-21, in formalin-fixed paraffin-embedded specimens, demonstrated BCRP immunoreactivity in over 40% of solid tumors tested (Diestra *et al.*, 2002). These studies included 150 tumors from 21 different types of untreated cancer, and demonstrated weak to extremely strong BCRP immunoreactivity in some examples of most tumor types tested. BCRP reactivity was absent or low in bladder cancer, ovarian cancer, and small-cell carcinomas, but was detected in colon cancers, esophageal cancers, endometrial cancers, lung cancers, and melanoma, with staining of both cytoplasm and plasma membrane noted (Diestra *et al.*, 2002). These results, if confirmed, have important implications as to the role of BCRP in clinical oncology.

Table 5 BCRP Expression in Human Solid Tumors

Disease type	Number studied	Methods	Findings	Reference
Variety of solid tumors; 16 AML samples also	41 solid tumor samples, all but one pretreatment	IHC ^a BXP-34 monoclonal antibody	Low or undetectable BCRP expression	Scheffer <i>et al.</i> (2000)
Breast Cancer	25 primary, 27 pretreated with anthracyclines	IHC	BCRP not detected in tumor tissue	Faneyte <i>et al.</i> (2002)
21 types of solid tumors	150 pretreatment samples, formalin-fixed, paraffin-embedded	IHC ^a and Western blots, BXP-34, BXP-21 monoclonal antibodies	Frequent expression of BCRP staining with the BXP-21 antibody, confirmed by Western blot. Highest expression was in gastrointestinal adenocarcinomas, endometrial, and lung carcinomas and myeloma. Membrane and cytoplasmic staining was observed. No staining was observed with the BXP-34 antibody under the same conditions	Diestra <i>et al.</i> (2002)
Breast cancer	43 pretreatment samples	RT-PCR	Low BCRP mRNA expression	Kanzaki <i>et al.</i> (2001)

^aIHC = immunohistochemistry

The expression of BCRP in lung tumors was confirmed by Kawabata *et al.* (2001a), who used RT-PCR to detect low levels of BCRP in four of 12 lung cancer cell lines and approximately one-half of 44 lung tumor tissues tested. Topotecan efflux in the lung cancer cell lines correlated with the levels of BCRP mRNA expressed. The expression of BCRP in venules, as detected by immunohistochemistry, suggests that low levels of BCRP detected in tissue biopsies by PCR-based techniques must be interpreted with caution.

Ironically, despite its original isolation from multi-drug-resistant human breast cancer cells, the level of BCRP expression in clinical breast cancer cases appears low by RT-PCR (Kanzaki *et al.*, 2001). Immunohistochemical studies confirmed undetectable expression of BCRP in breast tumors, even in biopsies from women relapsing after doxorubicin-based chemotherapy (Faneyte *et al.*, 2002).

Reviews of the role of BCRP in drug resistance of clinical cancers are available (Bates *et al.*, 2001; Litman *et al.*, 2001; Ejendal and Hrycyna, 2002).

Unanswered questions and pending studies

Current investigations of the BCRP transporter are in progress using models developed to characterize Pgp and MRP family members. The potential role for homodimerization and heterodimerization of the BCRP half-transporter adds a level of complexity of the studies required and limits the firmness of conclusions drawn about BCRP function. Conclusions regarding the substrate specificity of BCRP transport may also be limited by a lack of understanding of other necessary cofactors for transport. The physiologic function of BCRP is incompletely understood, although the localization of the protein to the placenta, lining cells of the intestine, and small blood vessels is suggestive for some normal functions of the protein. The expression of BCRP in the pluripotent SP stem cells from many tissues may allow the transporter to be used as a marker to define and characterize these interesting cells. Transplant studies with Bcrp1-transduced bone marrow cells are ongoing and suggest that expression of Bcrp1 may have important functional effects in the stem cell compartment. The spectrum of drugs transported by BCRP and/or inhibiting BCRP function is evolving

rapidly. The inhibition of BCRP by the tyrosine kinase inhibitors CII033 and Iressa, for example, suggests the possibility that other clinically important quinazoline drugs may also be substrates and/or inhibitors of BCRP. Another way of revising BCRP-mediated resistance is to cleave the protein specifically using hammerhead ribozymes. A specific high-activity ribozyme directed at BCRP has recently been developed and found to cleave BCRP mRNA efficiently in a cell-free system (Kowalski *et al.*, 2001, 2002).

The role of BCRP in clinical cancer resistance is currently under investigation, now that immunologic and other molecular probes have become available. Furthermore, the sequence at codon 482 should be investigated, particularly in studies relating the clinical response to anthracyclines or methotrexate with BCRP expression. BCRP may have a role in recurrent tumors, especially in hematologic malignancies in which patients have been treated with chemotherapy concurrent with inhibitors of Pgp. Ribozyme-based gene therapy may become clinically applicable in preventing or reversing BCRP-mediated drug resistance (van der Kolk *et al.*, 2002b). Furthermore, inhibition of the BCRP transporter present at various tissue barriers may suggest ways to alter the pharmacokinetics of certain drugs. The oral coadministration of BCRP inhibitors could improve the intestinal availability of BCRP-substrate drugs such as topotecan and irinotecan. The use of new antibody probes, specific pharmacologic inhibitors, and animal models of BCRP function should allow rapid development of understanding of the physiologic and pathologic role of this interesting transporter.

Abbreviations

BCRP, breast cancer resistance protein; Pgp, P-glycoprotein; MRP1, multidrug resistance protein 1; MDR, multidrug resistance; FTC, fumitremorgin C; SP, side population.

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