Mitochondrial Expression and Functional Activity of Breast Cancer Resistance Protein in Different Multiple Drug-Resistant Cell Lines

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Abstract
The multidrug resistance (MDR) phenotype is characterized by the overexpression of a few transport proteins at the plasma membrane level, one of which is the breast cancer resistance protein (BCRP). These proteins are expressed in excretory organs, in the placenta and blood-brain barrier, and are involved in the transport of drugs and endogenous compounds. Because some of these proteins are expressed in the mitochondria, this study was designed to determine whether BCRP is expressed at a mitochondrial level and to investigate its function in various MDR and parental drug–sensitive cell lines. By using Western blot analysis, immunofluorescence confocal and electron microscopy, flow cytometry analysis, and the BCRP (ABCG-2) small interfering RNA, these experiments showed that BCRP is expressed in the mitochondrial cristae, in which it is functionally active. Mitoxantrone accumulation was significantly reduced in mitochondria and in cells that overexpress BCRP, in comparison to parental drug–sensitive cells. The specific inhibitor of BCRP, fumitremorgin c, increased the accumulation of mitoxantrone significantly in comparison with basal conditions in both whole cells and in mitochondria of BCRP-overexpressing cell lines. In conclusion, this study shows that BCRP is overexpressed and functionally active in the mitochondria of MDR-positive cancer cell lines. However, its presence in the mitochondria of parental drug–sensitive cells suggests that BCRP can be involved in the physiology of cancer cells. [Cancer Res 2009;69(18):7235–42]

Introduction
Multidrug resistance (MDR) in human cancer is the major obstacle to the successful treatment of cancer by chemotherapy. The MDR phenotype is characterized by the overexpression of the so-called ATP-binding cassette (ABC) transporters (1). It is known that the MDR phenotype may be due to the overexpression of ABC transporters such as P-glycoprotein (P-gp), MDR-associated protein 1 (MRP1), and/or breast cancer resistance protein (BCRP; refs. 2, 3). BCRP is a protein involved in the transport of several cytotoxic drugs and some endogenous substrates and fluorescent dyes (4–10). BCRP has been detected in many types of solid and hematologic tumors and a potential link between its expression and clinical drug resistance has been proposed in several recent clinical studies (10–13). However, BCRP, like P-gp, is also expressed in many normal tissues and could play an important role in the absorption, distribution, and elimination of drugs (14–19).

MDR transporters are mainly expressed at cell plasma membrane level to mediate drug transport reducing the intracellular accumulation of toxins and drugs (20). Nevertheless it was shown that these proteins could be expressed in subcellular compartments in which they actively sequester drugs away from their cellular targets (21–26). On the basis of these observations, we hypothesized that BCRP could be expressed in the mitochondria in which it could be involved in maintaining low concentrations of anticancer drugs. The aim of this study was to elucidate the protein expression, localization, and efflux function of BCRP in the mitochondria of different MDR cell lines and their parental drug–sensitive clones, by using immunofluorescence confocal microscopy, flow cytometry analysis, immunoblotting, electron microscopy, and BCRP (ABCG2) small interfering RNA (siRNA). The efflux activity of BCRP in the model we used was measured by using two fluorescent BCRP substrates (mitoxantrone and rhodamine 123) and a specific inhibitor for BCRP (fumitremorgin C, FTC; ref. 27).

Materials and Methods
Cell culture. Experiments were done using the following cell lines: MDR-positive P1(0.5) and drug-sensitive P5 HCC cell line, two cellular clones of a human HCC cell line (PLC/PBF/5; ref. 28); the human IGROV1 ovarian carcinoma cell line and their derivate topotecan-selected subline, T8; the Madin-Darby canine kidney cells (MDCKII) and the MDCKII transduced with human BCRP. P1(0.5) cells, grown in 0.5 μg/mL of doxorubicin, exhibit an MDR phenotype and are 100 times more resistant to doxorubicin than P5 (29). Cell lines were cultured in DMEM (Celbio) supplemented with 10% fetal bovine serum, 100 units/mL of penicillin, and 10 μg/mL of streptomycin (Celbio) at 37°C in 5% CO2. The human IGROV1 ovarian carcinoma cell line, their derivate topotecan-selected subline, T8, the MDCKII and the MDCKII transduced with human BCRP were kindly donated by Dr. J.H.M. Schellens (The Netherlands Cancer Institute, Amsterdam, the Netherlands).

The TPT-resistant T8 cells were developed from the IGROV1 human ovarian carcinoma cell line, as explained elsewhere (30, 31). These cell lines were cultured in RPMI 1640 supplemented with 25 mmol/L of HEPES, 10% fetal bovine serum, 100 units/mL of penicillin, and 100 μg/mL of streptomycin (Celbio) at 37°C in 5% CO2.

Western blot analysis. Preparation of total protein lysates and Western blot analysis were performed as previously described (32). Monoclonal anti-BCRP (BXP-21) was purchased from Chemicon International. Rat monoclonal anti-BCRP (BXP-53) was purchased from Santa Cruz Biotechnology. Polyclonal anti-integrin β3 was kindly donated by Dr. E. Rovida and polyclonal anti-AIF was from Dr. A. Chiarugi (University of Florence, Florence, Italy).

Preparation of mitochondrial fractions. Two methodologies were used to prepare and purify mitochondria. In the first (standard method; ref. 33) cells were washed twice with ice-cold PBS, collected, and the pellet was suspended in 500 μL of ice-cold buffer A (20 mmol/L, HEPES (pH 7.5), 1.5 mmol/L MgCl2, 10 mmol/L KCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 1 mmol/L DTT, 0.1 mmol/L phenylmethylsulfonyl fluoride, and 10 μg/mL
each leupepin, aprotinin, and pepstatin A containing 250 mmol/L of sucrose. Afterwards, cell suspension was passed five times through a 26-gauge needle fitted to a syringe to lyse the cells. Unbroken cells, large plasma membrane pieces, and nuclei were removed by centrifuging the homogenates at 1,000 × g at 4°C for 10 min. The resulting supernatant was subjected to 10,000 × g centrifugation at 4°C for 20 min. The pellet fraction (i.e., mitochondria) was first washed with the above buffer containing sucrose and then solubilized in 50 μL of TNC buffer [10 mmol/L Tris-acetate (pH 8.0), 0.5% Igepal CA-630, and 5 mmol/L CaCl₂]. HSP-60 and cytochrome c proteins were used as markers for the purity of mitochondrial preparations. The supernatant was recentrifuged (“low” speed, 1,000 × g, 1 h) to generate cytosol, that was used to detect BCRP also in this fraction (33).

By using another methodology, as recently proposed (34), cells were washed (three times in PBS), resuspended in homogenization buffer [150 mmol/L MgCl₂, 10 mmol/L KC1, 10 mmol/L Tris (pH 6.7), protease inhibitors; 1 mL/1 × 10⁶ cells], incubated on ice (15 min), and Dounce homogenized (35 strokes). Homogenization buffer sucrose (34.2%, 1/3 vol) was added and centrifuged (“low” speed, 1,000 × g, 5 min, 4°C) to remove nuclei and unlysed cells. The supernatant was centrifuged (“medium” speed, 5,000 × g, 10 min, 4°C), the pellet (“crude mitochondria”) was resuspended in homogenization buffer + sucrose (20 mL), and centrifuged (5,000 × g, 10 min, 4°C). The resultant pellet was resuspended in solution A [3 mL; 20 mL 150 mmol/L HEPES, 1 mmol/L EDTA, and 250 mmol/L sucrose (pH 7.4)], Iodixanol solution [50% iodixanol, 120 mmol/L HEPES, 6 mmol/L EDTA, and 250 mmol/L sucrose (pH 7.4)] was added (final concentration of 36%), placed in a centrifuge tube, overlaid with solution B [10 mL, 30% iodixanol, 80 mmol/L HEPES, 4 mL EDTA, and 250 mmol/L sucrose (pH 7.4)], then solution C [to top, 10% iodixanol], 80 mmol/L HEPES, 4 mL EDTA, and 250 mmol/L sucrose (pH 7.4)], and centrifuged (50,000 × g, 4 h, 4°C, swinging bucket rotor). Protein was collected at the 30%/10% iodixanol interface, an equal volume of solution A (10 mL) was added, followed by centrifugation (30,000 × g, 10 min, 4°C). The resulting pellet was resuspended in mitochondrial suspension buffer [250 mmol/L sucrose, 10 mmol/L Tris (pH 7.0) with protease inhibitors; ref. (34)].

Confocal immunofluorescence. Living cells grown on glass coverslips were incubated for 30 min at 37°C with 100 mmol/L of MitoTracker red CMXRos (Molecular Probes), a cell-permeable mitochondria-selective dye, fixed in 0.5% buffered paraformaldehyde for 10 min at room temperature and then processed by confocal immunofluorescence to reveal BCRP protein. After permeabilization with cold acetone for 3 min, washed cells were incubated in blocking solution [0.5% bovine serum albumin (Sigma) and 3% glycerol in PBS] for 30 min and then with mouse monoclonal anti-BCRP (BXP-21) antibody (1:50; Chemicon) for 1 h at room temperature. The immunoreactions were revealed by incubation of the cells with goat anti-mouse Alexa 488-conjugated IgG (Molecular Probes, Inc.). The MDCKII and MDCK-BCRP cells were also treated with rat monoclonal anti-BCRP (BXP-53) antibody (1:10; Santa Cruz Biotechnology). In this case, the immunoreactions were revealed by incubation of the cells with goat anti-rat IgG-FITC (Santa Cruz Biotechnology). Negative controls were carried out. Ultra-thin sections counterstained with uranyl acetate, were analyzed using a Jeol 1010 transmission electron microscope (Jeol) at 80 kV.

Cellular uptake experiments. Experiments of uptake of fluorescent dyes were performed by using a previously reported method (27).

Briefly, the cell culture medium was removed and each well was washed twice with PBS. Because it was shown that BCRP-mediated transport of rhodamine 123 (Rho 123; Sigma) depends on acquired mutations in the BCRP gene that result in changes in amino acid 482 that alter the substrate specificity of the protein (36–38), whereas mitoxantrone (MXR; Sigma) is the BCRP substrate transported in all cell lines, cellular uptake experiments were performed by using both substrates. However, because MXR is also a substrate for P-gp and MRP1, to study the specific BCRP-mediated MXR transport, we used a BCRP-specific modulator such as FTC (39).

The fluorescent substrates MXR or Rho 123 were added at a final concentration of 20 μmol/L and 5 μg/mL, respectively; FTC was added at a final concentration of 10 μmol/L. MXR or Rho 123–related fluorescence in the cells was detected by flow cytometry using a FACScanto cytometer (Becton Dickinson) equipped with a 633 nm red diode laser and 660/20 nm band-pass filter for MXR and FACScan equipped with a 488 nm argon laser and a 530 nm band-pass filter to detect Rho 123. The mean channel number for each histogram (mean fluorescence intensity) was used as the measure of fluorescence for the calculation of uptake values.

For cellular uptake experiments, fluorescence was determined in each sample with a FACScanto flow cytometer and measured at a flow rate of 2,000 events/s. At least three independent experiments were carried out for each assay.

siRNA transfection. Positive control glyceraldehyde-3-phosphate dehydrogenase siRNA, siCONTROL non-targeting siRNA, and a pool of four different double-stranded RNA oligonucleotides directed against BCRP (siGENOME SMARTpool, human ABCG2) were purchased from Dharmacon. The IGROV1 and T8 cells were seeded in 60 mm dishes to reach 30% to 50% confluency after 24 h of incubation, and transfected with a total of 100 nmol/L of siRNA using Lipofectamine 2000 (Invitrogen Life Technologies).
in antibiotic-free medium, according to the instructions of the manufacturer. Seventy-two hours after transfection, mitochondrial fractions and total proteins were extracted for functional assay with MXR and Western blot analysis.

Results

Western blot analysis of BCRP in total cell lysates and in mitochondrial and cytosolic fractions of different cell lines. The expression of BCRP protein was analyzed by immunoblot, using BXP-21 as the primary antibody (Fig. 1). Western blot analysis clearly shows that the expression of BCRP is higher in T8 and in MDCK-BCRP cells than in parental IGROV1 and MDCKII cells. BCRP is also weakly expressed in P5 and P1(0.5) human HCC cell lines (Fig. 1A).

To study the localization of BCRP, mitochondrial, and cytosolic fractions were isolated from P5, P1(0.5), IGROV1, T8, MDCKII, and MDCK-BCRP cells, and these fractions were used to determine BCRP, cytochrome c, and HSP-60 protein expression by Western blot analysis (Fig. 1B, i and ii). Cytochrome c and HSP-60 were used as markers for the purity of mitochondrial preparations. Results clearly show that BCRP is expressed in the mitochondrial fraction of all cell lines we studied (Fig. 1B, i and ii). BCRP expression in mitochondria is higher in T8 and in MDCK-BCRP cells in comparison to parental drug–sensitive clones. HSP-60 was more expressed in the mitochondrial than in the cytosolic fraction of all cell lines studied. HeLa whole cell lysate was the positive control for HSP-60 detection. The integrity of mitochondria during the process of purification was tested by measuring the expression of cytochrome c both in the mitochondrial as well as in the cytosolic fraction. Western blot analysis showed that cytochrome c was only expressed in the mitochondrial fraction of all cells we studied, confirming that during the process of purification of mitochondria no leaking of cytochrome c into the cytosol occurred (Fig. 1B, i and ii).

Mitochondrial localization of BCRP in different cell lines by confocal and electron microscopy. Both confocal immunofluorescence and immunoelectron microscopy confirmed the results obtained by Western blot analysis, showing the presence of BCRP in mitochondria of intact P5, P1(0.5), IGROV1, T8, MDCKII, and MDCK-BCRP cells (Figs. 2 and 3). In particular, confocal microscopy shows the expression of BCRP in the mitochondria of intact cells, using either the BXP-21 or the BXP-53 antibodies (Fig. 2A, B, C, and D, respectively).

By using two different specific antibodies, we ruled out the possibility of a cross-reaction with one of the other proteins. BCRP was also abundantly expressed at the plasma membrane level, as shown in Fig. 2.

These results were confirmed by the immunoelectron microscopy studies. As shown in Fig. 3, BCRP immunogold labeling of Epon 812-embedded MDCK-BCRP cells with anti-BCRP antibodies showed that both plasma membrane (Fig. 3B) and mitochondria (cristae; Fig. 3C) were decorated with gold particles (arrows).

Cellular uptake of Rho 123 and MXR in different cell lines. To investigate whether BCRP is active and works like a pump, the efflux function of BCRP in MDCKII, MDCK-BCRP, IGROV1, and T8 cell lines was studied. Experiments to study the uptake of Rho 123 and MXR, in the presence or absence of 10 μmol/L of the BCRP-specific inhibitor FTC were performed. Rho 123 uptake was only observed in the presence of FTC, in contrast to the results obtained in parental IGROV1 cells.
accumulated in great amounts in all cell lines tested, and there was no effect with 10 μmol/L of FTC (data not shown). Experiments done by using MXR showed that cell accumulation of the drug was significantly increased in parental drug–sensitive MDCKII and in IGROV1 cells compared with cell autofluorescence, and no effect of the BCRP inhibitor, FTC (10 μmol/L), was observed in both cell lines (Fig. 4). On the contrary, MXR accumulation was significantly reduced in MDCK-BCRP and in T8 cells that overexpress BCRP as compared with parental drug–sensitive cell lines. The reduced accumulation of MXR was probably due to BCRP-mediated efflux in these cells as 10 μmol/L of FTC significantly increased cell fluorescence, compared with basal conditions, in the transfected MDCK-BCRP and in T8 cells.

**BCRP-mediated efflux in mitochondrial fraction of different cell lines.** To show that BCRP is functionally active in the mitochondrion, mitochondrial localization of BCRP protein in different cell lines was examined by confocal and immunoelectron microscopy. Confocal immunofluorescence analysis showed that BCRP protein is localized in the mitochondrial fraction of all cell lines tested. Immunoelectron microscopy also confirmed BCRP localization in the mitochondrial fraction, with BCRP-gold particles observed at the plasma membrane and in the mitochondrial cristae (Fig. 3). These results indicate that BCRP is functionally active in the mitochondrial fraction of these cell lines.

**Figure 2.** Confocal immunofluorescence analysis of mitochondrial localization of BCRP protein in different cell lines. PS and P1(0.5) (A, I and III), IGROV1 and T8 (B, I and III), and MDCKII, and MDCK-BCRP living cells (C, I and III; D, I and III) cultured on coverslips were incubated with 100 nM MitoTracker red CMXRs to label mitochondria (red), fixed and processed for BCRP protein immunostaining (green). Confocal micrographs in A, B, and C are representative of cells immunostained with mouse monoclonal anti-BCRP antibody (BXP-21; Chemicon). Immunostaining in cells (D) was performed with rat monoclonal anti-ABCG2 (BXP-53) antibody (Santa Cruz Biotechnology). A (II and IV), B (II and IV), C (II and IV), and D (II and IV), represent images of colocalized points (white) between red (mitochondria) and green (BCRP) fluorescence signals obtained using ImageJ colocalization plugin software (NIH). Note the mitochondrial localization of BCRP protein in all cell lines examined (overlap coefficients between green and red signals: 0.823 ± 0.01, 0.654 ± 0.02, 0.44 ± 0.01, 0.489 ± 0.05, 0.551 ± 0.03, 0.892 ± 0.05, 0.404 ± 0.01, 0.492 ± 0.05 in PS, P1(0.5), IGROV1, T8, MDCKII (BXP-21), MDCK-BCRP (BXP-21), MDCKII (BXP-53), and MDCK-BCRP (BXP-53), respectively). Bar, 12 μm. The images are representative of at least three independent experiments with similar results.

**Figure 3.** Immunoelectron cytochemistry analysis of BCRP protein. A to C, TEM images of Epon 812–embedded samples of MDCK-BCRP cells processed for immunogold labeling of anti-BCRP monoclonal antibody. Arrows, BCRP-gold particles at the plasma membrane level and in the mitochondria cristae, respectively (B and C). The photographs are representative of at least three independent experiments with similar results.
mitochondria, efflux experiments were performed in isolated mitochondria of MDCKII, MDCK-BCRP, IGROV1, and T8 cells using only MXR.

As shown in Fig. 5, a high level of fluorescence due to MXR was detected in the presence of 10 μmol/L of FTC in the mitochondria of MDCK-BCRP and T8. This level of fluorescence was more than 2-fold higher as compared with mitochondria of the FTC untreated cells. Mitochondria of parental drug–sensitive MDCKII and IGROV1 cells showed less efflux of fluorescence compared with BCRP-overexpressing cell lines.

**Effect of ABCG-2 siRNA transfection on BCRP-mediated transport.** Efflux experiments were also performed, by using flow cytometry, in isolated mitochondria from IGROV1 and T8 cells which were previously transfected with ABCG-2 siRNA. As shown in Fig. 6A, fluorescence due to MXR accumulation was higher in the mitochondria of T8 cells transfected with ABCG-2 siRNA than in the mitochondria of the untreated cells. On the other hand, in the mitochondria of parental IGROV1 cells transfected with ABCG-2 siRNA, which showed low levels of BCRP, the MXR efflux was much less than that observed in BCRP-overexpressing cell lines.

Western blot analysis, performed to estimate the expression of BCRP both in whole cell lysates and in isolated mitochondria from IGROV1 and T8 cells which were previously transfected with ABCG-2 siRNA showed that the expression of BCRP was reduced by ABCG-2–targeted siRNA both in whole cell lysates and in the mitochondria of T8-transfected cells (Fig. 6B). The expression of BCRP was also reduced by ABCG-2–targeted siRNA in the whole cell lysate of IGROV1, whereas no effect was found in the mitochondria of parental drug–sensitive cells (data not shown). The inhibition of BCRP expression was not observed using the nonspecific control siRNA.

**Discussion**

Because the overexpression of ABC transporters in tumors plays an important role in resistance to chemotherapeutic agents, a better understanding of molecular mechanisms of MDR phenotype occurrence could be useful to bypass drug resistance (2, 40). It is known that cancer cells which express the MDR phenotype are characterized by several biological changes that make cells more resistant to various mechanisms of cell damage (41–43). Recently, it was proposed that P-gp could be expressed in the mitochondrial membrane of MDR–positive cells, but this observation is still controversial (32, 34, 44).

We studied the localization and activity of BCRP in the mitochondria of different MDR and their parental drug–sensitive cell lines. Using Western blot analysis and confocal microscopy, we show that BCRP is highly expressed in T8 and MDCK-BCRP, and weakly in P5, P1(0.5), IGROV1, and MDCK-II cells, both in whole cell lysates and in mitochondria. In addition, because the mitochondrial fractions obtained by using the standard methodology (33) for
separation of these organelles from the other cell parts could be contaminated by plasma and other cell membranes, as recently suggested, mitochondria were purified using the iodixanol-based separation methodology (34). Also by using this method, the expression of BCRP in the mitochondria of drug-resistant MDCK-BCRP cells was confirmed. By using two specific antibodies against different epitopes of BCRP, confocal microscopy immunofluorescence showed that BCRP is expressed in the mitochondria of parental and drug-resistant MDCK-BCRP intact cells.

Accordingly, the presence of BCRP at a mitochondrial level was also shown by using immunoelectron microscopy that showed the expression of BCRP in the mitochondrial cristae (inner membrane; Fig. 3). Uptake and efflux experiments using MXR and Rho 123 as fluorescent probes, in the presence or absence of FTC, a specific and potent inhibitor of BCRP activity, show that FTC inhibits BCRP-mediated transport of MXR either from the cell or from the mitochondria. The molecular mechanisms by which FTC inhibits BCRP are unknown, but possibly work through direct inhibition of ATPase activity (45).

To confirm the results obtained by exploiting the inhibitory effect of FTC, we studied the activity of BCRP in isolated mitochondria from IGROV1 and T8 cells, previously transfected with ABCG-2 siRNA. Altogether, our findings suggest that BCRP activity is responsible for MXR efflux in cells overexpressing BCRP.

The observation that BCRP is expressed in cells in which P-gp is not expressed, such as MDCKII cells, suggests that the two proteins have different mechanisms to regulate their expression. BCRP, like P-gp, is quite an unspecific ABC transporter, although its expression both at the plasma membrane level and in several cell organelles suggests roles for this protein that have not yet been clarified (46). The notion that BCRP protects cells from hypoxia-related cell damage (47), together with the demonstration in the present work of its expression on the inner membrane (cristae) of the mitochondrion, suggest that BCRP could be involved in maintaining the electrochemical gradient between matrix and intermembrane space of mitochondria but more studies are necessary to elucidate this hypothesis.

Cells expressing the MDR phenotype are usually more resistant to several agents that trigger apoptosis compared with parental drug-sensitive cells. Recently, it was shown that inhibitors of cyclooxygenase are involved in the activation of both the extrinsic and intrinsic mediated-apoptosis pathway in HCC cells (48). We have recently shown that apoptosis can be restored by low concentrations of celecoxib in a P-gp–dependent mechanism in MDR-positive cell lines (49). Celecoxib exerts a direct effect on the expression of P-gp and therefore on apoptotic pathways, however, it is unknown whether this drug and other coxibs could exert some effect on BCRP expression and/or activity.

To conclude, in the present study, we bring evidence that BCRP is functionally expressed in the mitochondria of MDR cell lines. The most probable hypothesis is that BCRP could be involved in protecting mitochondrial DNA from damage due to anticancer drugs in MDR-positive cells. However, its expression, although to a
lesser extent, in parental drug–sensitive cancer cells and its localization in the inner membrane of mitochondrion suggests that BCRP could be involved in still unknown physiologic pathways of cell metabolism.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

References


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