Expression and activity of breast cancer ~~-related~~ resistance protein (BCRP/ABCG2) in human distal lung epithelial cells *in vitro*

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Abstract

Purpose: Breast cancer resistance protein (BCRP/ABCG2) has previously been identified with high expression levels in human lung. The subcellular localisation and functional activity of the transporter in lung epithelia, however, remains poorly investigated. The aim of this project was to study BCRP expression and activity in freshly isolated human alveolar epithelial type 2 (AT2) and type 1-like (AT1-like) cells in primary culture, and to compare these findings with data obtained from the NCI-H441 cell line.

Methods: BCRP expression levels in AT2 and AT1-like cells and in different passages of NCI-H441 cells were determined using q-PCR and immunoblot. Transporter localisation was confirmed by confocal laser scanning microscopy (CLSM). Efflux and transport studies using the BCRP substrate BODIPY FL prazosin and the inhibitor Ko143 were carried out to assess BCRP activity in the different cell models.

Results: BCRP expression decreased during transdifferentiation from AT2 to AT1-like phenotype. Culturing NCI-H441 cells at an air-liquid interface or submersed did not change BCRP abundance, but BCRP levels increased with passage number. BCRP was localised to the apical membrane and cytosol in NCI-H441 cells. In primary cells, the protein was found in the nucleus. Functional studies were consistent with expression data.

Conclusions: BCRP is differently expressed in AT2 and AT1-like cells with lower abundance and activity in the latter ones. Nuclear BCRP might play a transcriptional role in distal lung epithelium. In NCI-H441 cells, BCRP is expressed in apical cell membranes and its activity is consistent with the localisation.

Keywords

ABC transporter; NCI-H441 cells; Inhalation biopharmaceutics; Pulmonary drug disposition; Drug absorption

Abbreviations

AIC	Air-interface culture
BBB	Blood-brain barrier
BCRP	Breast cancer resistance protein
CLSM	Confocal laser scanning microscopy
Ko143	[(3S,6S,12aS)-1,2,3,4,6,7,12,12a-octahydro-9-methoxy-6-(2-methylpropyl)-1,4-dioxopyrazino[1',2':1,6]pyrido[3,4- b]indole-3-propanoic acid 1,1-dimethylethyl ester;
LCC	Liquid-covered culture
TEER	Transepithelial electrical resistance

Introduction

Breast cancer resistance protein (BCRP)/ATP-binding cassette subfamily G member 2 (ABCG2) is a 72 kDa half transporter encoded by the *ABCG2* gene (1). BCRP consists of six transmembrane domains and functions as a homodimer or homotetramer (2). BCRP effluxes and thus limits systemic and organ exposure of endogenous and exogenous substrates with diverse physicochemical properties (3). BCRP substrates often have a positive and/or negative charge but otherwise span a large range of molecular weight, lipophilicity and permeability; examples include oestrone 3-sulphate, rosuvastatin, sulfasalazine, furosemide and prazosin (3). BCRP is also an important player in cancer drug resistance as many of its substrates are chemotherapeutics such as doxorubicin, topotecan and imatinib (4). Consequently, the US Food and Drug Administration and European Medicines Agency ~~(EMA)~~ included BCRP in the panel of transporters recommend for preclinical evaluation and, when appropriate, clinical assessment (3).

Whilst the role of BCRP in intestinal absorption, hepatic excretion and limitation of endothelial barriers such as blood-brain barrier (BBB), retina and placenta has been intensely studied, BCRP expression and function in lung has received considerably less attention (5). According to Sakamoto and colleagues, who measured transporter expression in lung tissue by liquid chromatography-tandem mass spectrometry, BCRP together with MRP1 showed the highest abundance (6). In the same study, BCRP was also found at high levels in commercially obtained bronchial epithelial cells in primary culture, whilst signals in alveolar and tracheal epithelial cells were below the detection limit. A follow-up publication reported that BCRP had the highest expression of all ABC transporters investigated in a

number of continuously growing cell lines of human lung origin, i.e. Calu-3, BEAS-2B, NCI-H292, NCI-H441 and A549 (7).

A number of inhaled drugs have shown to be BCRP substrates, for example ciprofloxacin, beclomethasone diproprionate, budesonide, ciclesonide and mometasone furoate (9-11). Moreover, BCRP has been suggested to be involved in detoxification of xenobiotics by way of extrusion of (toxic) metabolites, such as sulphate or glucuronide conjugates (12-14). Alfaras *et al.* reported that that Bcrp is crucial in the pulmonary accumulation of resveratrol conjugates resulting from phase II metabolism. Ten minutes after oral administration of trans-resveratrol in *Bcrp*$^{-/-}$ mice and wildtype controls, trans-resveratrol glucuronide was 2.1-fold higher and the sulphate conjugate could only be detected in lungs of the knockout mice (15). It is therefore conceivable that BCRP plays a pivotal role in the absorption, disposition and clearance of inhaled pharmaceuticals and/or in the lung accumulation of drugs administered by other routes.

The aims of this study were to determine the expression and activity of BCRP in freshly isolated human alveolar epithelial cells in primary culture and to compare those to the human cell line NCI-H441.~~, which has previously been put forward as an *in vitro* model for biopharmaceutical studies in the distal lung~~ We have focused our efforts on the NCI-H441 cell line, because data from our laboratory and others' recent studies, confirmed the cells as probably the most promising continuously growing *in vitro* surrogate of human distal lung epithelium (16, 29, 35, 36). Calu-3 and BEAS-2B are other widely used models, but they rather resemble the proximal airways, whereas A549 cells might bear some similarity to AT2 cells, however, they are lacking the ability to form electrically tight monolayers of polarised cells (37). ~~(16).~~

Materials and Methods

Cell culture

NCI-H441 (ATCC HTB-174) cells were purchased from LGC Promochem (Teddington, UK). Cells of passage numbers 55 – 90 were cultured at 75,000 cells/cm^2 in flasks using RPMI-1640 medium supplemented with 5% FBS, 1 mM sodium pyruvate, 100 U/ml penicillin and 100 µg/ml streptomycin. Twenty-four hours post-seeding in Transwell Clear inserts, this medium was further supplemented with dexamethasone (1 µM) and 1% of insulin-transferrin-selenious acid-bovine serum albumin-linoleic acid (ITS premix universal culture supplement (Corning, Bedford, MA)).

Human alveolar type 2 epithelial (AT2) cells were isolated according to a protocol modified from Demling *et al.* from non-tumour lung tissue obtained from patients undergoing lung surgery (17; 18). Briefly, purified AT2 cells were either used directly for protein and RNA isolation or seeded at a density of 200,000 cells/cm^2 on collagen/fibronectin-coated surfaces using small airways growth medium (Lonza, Verviers, Belgium) supplemented with 1% FBS, 100 U/ml penicillin and 100 µg/ml streptomycin. Cells were kept in culture for at least one week, so that transdifferentiation into an alveolar type 1-like (AT1-like) phenotype could occur, before being used. The use of human tissue specimens was approved by Saarland State Medical Board (Saarbrücken, Germany).

All cell types were grown in a humidified atmosphere at 37°C in 5% CO_2. Cell culture media were changed every other day. The transepithelial electrical resistance (TEER) of cell monolayers grown on Transwell Clear inserts was measured using a Millicell ERS volt-ohm meter equipped with STX-2 electrodes (Millipore, Carrigtwohill, Ireland) and corrected for the background value contributed by the Transwell inserts and medium.

Isolation of RNA

RNA was isolated from NCI-H441, AT2 and AT1-like cells using Tri-Reagent (Sigma-Aldrich, Dublin, Ireland) according to manufacturer's instructions. Cells grown in 6-well plates were used for isolation. After the medium was removed, 1 ml of Tri Reagent was applied onto the monolayer and incubated for 5 min at RT. Lysate was homogenized by pipetting up and down and was mixed with 0.2 ml chloroform. Samples were shaken for 15 s followed by another incubation for 15 min at RT. Subsequently, samples were centrifuged at 4 °C and 12,000x*g* for 15 min. The resulting upper phase containing the RNA was used for further isolation. One point six microliters of oyster-glycogen and 500 µl isopropanol were added and samples were incubated for another 15 min at RT, followed by 10 min centrifugation at 12,000x*g* and 4°C. The supernatant was discarded and the RNA pellet was washed with 750 µl ethanol (75% w/v), followed by a 5 min centrifugation step at 7,500x*g* and 4°C. Supernatant was discarded and the pellet air dried for 5 min at RT. Pellet was dissolved for 2 min at 55°C in 50 µl nuclease-free water.

RNA concentration was determined using the NanoDrop 2000 UV-Vis spectrophotometer (Thermo fisher, Waltham, MA) according to manufacturer's instructions. For semi-quantitative, one-step real time PCRs the QuantiFast SYBR® Green RT-PCR Kit (Qiagen, Hilden, Germany) was used. Forty nanograms of RNA were added to the master mix containing the SYBR green dye, dNTPs and the fast reverse transcriptase. Primers were added to yield a final concentration of 1 µM for each primer. Nuclease free water was added to a final amount of 10 µl per reaction. PCR was carried out on a 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA) under the following conditions: 50°C, 5 min hold stage

for reverse transcription, initial PCR activation step at 95°C for 10 min and 35 amplification cycles consisting of a denaturation step at 95°C for 10 s and a combined annealing-elongation step at 60°C for 30 s. A melting curve protocol was included to check for specific amplification. Primers used were KiCqStart predesigned Primers (Sigma-Aldrich, St. Louis, MO) for *ACTB* (forward GACGACATGGAGAAAATCTG; reverse ATGATCTGGGTCATCTTCTC) and *ABCG2* (forward AAAGCCACAGAGATCATAGAG; reverse GATCTTCTTCTTCTTCTCACC).

Immunoblot

Lysis of all cells was performed on ice in Invitrogen cell extraction buffer (Thermo Fisher Scientific, Waltham, MA) containing protease inhibitor cocktail (Roche)). Cell samples were sonicated twice for 10 s and then the lysate was centrifuged (10,000x*g* at 4°C) for 20 min. The total protein amount was determined by Pierce BCA protein assay kit (Thermo Fisher) using bovine serum albumin (BSA) as standard. Samples were standardised to equal protein concentrations, loading buffer was added and the mixture was heated up to 95°C for 5 min, before samples were loaded onto SDS gels. Polyacrylamide gel electrophoresis was performed at 120 V, followed by transfer onto immunoblot polyvinylidine fluoride membranes (Bio-Rad, Hercules, CA) at 22 V for 40 min. Blots were blocked in washing buffer (PBS containing 0.05% Tween 20 (PBST)) containing 1% (w/v) BSA and 1% (w/v) milk for at least one hour at room temperature. Membranes were then incubated with rat monoclonal anti-BCRP antibody (BXP-53, sc-58224, Santa Cruz ~~b~~Biotechnology, 1:100 dilutions) in PBST containing 1% (w/v) BSA. After washing with PBST, the secondary anti-rat antibody (1:5000; Santa Cruz Biotechnology) was added for 1 h at room temperature. Peroxidase activity was

detected with Immobilon Western Chemiluminescent HRP substrate (Millipore, Carrigtwohill, Ireland). Signals were documented using a ChemiDoc system (Bio-Rad).

Biotinylation of cell surface proteins

Cell surface proteins were labelled with biotin, isolated and analysed according to a previously published protocol (34). Briefly, Transwell-grown cell monolayers were washed with ice-cold PBS and incubated for 20 min at 4°C with Pierce sulfo-NHS-biotin (Thermo Fisher Scientific). BSA-supplemented PBS was added to the respective opposite compartments. To stop the biotinylation reaction, BSA-supplemented PBS was added to both compartments for another 20 min, before cell monolayers were washed and lysed. Protein concentrations of the whole cell lysates were determined by Pierce BCA protein assay kit and equal amounts of protein were loaded onto streptavidin-agarose beads (500 µg whole cell protein/200 µl streptavidin-agarose beads) for overnight incubation at 4°C. The following day, biotinylated membrane proteins, were separated from the non-biotinylated proteins by centrifugation at 12000-x*g* for 20 min, washed with cell extraction buffer, resuspended in protein loading buffer and used for immunoblot as described above.

Isolation of nuclear proteins

For isolation of nuclear protein, NCI-H441 and human alveolar epithelial cells were grown to confluency in 25 cm^2 flasks. Confluent monolayers were washed twice with ice cold PBS, scraped off and collected into 15 ml tubes, centrifuged at 1000x*g* for 5 min at 4°C to obtain cell pellets. Cell pellets were then resuspended in 300 µl of cytoplasmic lysis buffer composed of 10 mM HEPES (pH 7.9), 1.5 mM $MgCl_2$, 10mM KCl, 0.5mM dithiothreitol (DTT),

0.2 mM phenylmethylsulfonyl fluoride (PMSF), and 0.6% Nonidet P-40 along with the protease inhibitor cocktail. The cell suspensions were incubated on ice for 15 min, with intermittent shaking, vortexed and centrifuged for 15 min at 3000x*g*. The supernatants were collected as cytoplasmic extracts.

The remaining pellets were washed three times with the same buffer as above, but without NP-40, centrifuged after each washing step for 15 min at 3000x*g* and the supernatants were discarded. The remaining nuclear pellet was suspended in 300 µl of nuclear lysis buffer composed of 20 mM HEPES (pH 7.9), 1.5 mM $MgCl_2$, 2mM KCl, 25% glycerol, 400 mM NaCl, 0.2 mM EDTA, 0.5 mM DTT, 0.2 mM PMSF and protease inhibitor cocktail. The mixture was incubated on ice for 30 min with shaking and sonicated for 10 s. The nuclear proteins in the supernatants were collected by centrifugation at 4°C to and 16000x*g* for 20 min.

Nuclear and cytoplasmic protein concentrations were determined by Pierce BCA protein assay kit and equal amounts of proteins were used for immunoblotting as described above. A rabbit polyclonal anti-lamin B1 antibody (GTX103292S, Gene Tex, 1:500 dilution) was used as a nuclear loading control.

Confocal laser scanning microscopy (CLSM)

Cells grown on filters were fixed with 2% paraformaldehyde in PBS for 7 min followed by 7 min incubation with NH_4Cl in PBS (50 mM). Monolayers were washed twice with PBS and subsequently permeabilised with 0.1% Triton-X-100 in PBS for 8 min. Monolayers were blocked with 2% BSA in PBS for 30 min and cells were incubated over night at 4°C with the BXP-53 antibody at a 1:50 dilution. After two 10 min washing steps, Alexa Fluor 488-

conjugated goat anti-rat secondary antibody (ab150165, abcam, Cambridge, UK) at a 1:100 dilution was applied at RT for 1 h, followed by 5 min incubation with a Hoechst 33342 solution (1 µg/ml) to counterstain nuclei. After two additional 10 min washing steps, Transwell inserts were cut out using a scalpel blade and transferred to a microscopic slide. FluorSafe mounting medium (EMD Millipore, Billerica, MA) was applied and monolayers were covered with a coverslip. Samples were allowed to dry in a cool, dark place for at least one hour before analysis. Samples were visualised on a Leica SP8 confocal laser scanning microscope with a 63x oil immersion objective, a 488 nm diode laser and 520 nm Alexa Fluor 488 detection filter (antibody signal) and a UV laser in combination with a Hoechst 33342 filter (nucleic signal).

Transport studies

Transport experiments using a solution of BODIPY FL prazosin (BP) (Life Technologies, Eugene, OR) in KRB (3 µM) were performed as described by Salomon *et al.* (16). Transwell-grown NCl-H441 and human alveolar epithelial cell monolayers were only used when TEER values exceeded 500 Ohm·cm^2. TEER values were recorded before and after the transport studies, in order to verify the cell layer integrity. In BCRP inhibition experiments, [(3S,6S,12aS)-1,2,3,4,6,7,12,12a-octahydro-9-methoxy-6-(2-methylpropyl)-1,4-dioxopyrazino[1',2':1,6]pyrido[3,4- b]indole-3-propanoic acid 1,1-dimethylethyl ester (Ko143; 10 µM), a non-toxic analogue of fungal toxin fumitremorgin C was added to donor and receiver fluids.

The fluorescence activity of samples was assessed in 96-well plates using an automated plate reader (FLUOstar Optima, BMG Labtech, Offenburg, Germany) at excitation and

emission wavelengths of 485 nm and 520 nm, respectively. Samples were diluted with KRB solution, where appropriate.

The apparent permeability coefficient (P_{app}) was calculated using the following equation:

$P_{app}= (\Delta Q/\Delta t)/ (A*C_0)$ Eq. 1

where ΔQ was the change in concentration of BODIPY FL prazosin over the designated period of time (Δt), A was the nominal surface area of the growth supports (1.13 cm^2) and C_0 was the initial concentration of BP in the donor fluids.

BODIPY FL prazosin release studies

Release studies of the fluorescent BCRP probe, BP, were performed using NCI-H441 and AT 1-like cells cultured in 24-well plates. All experiments were carried out in KRB at 37°C. Cell monolayers were initially loaded with BP solution (3 µM) for 60 min, which was then replaced by fresh KRB or KRB containing either Ko143 (5 or 10 µM), and BP release was followed for 1 hour. At 0, 15, 30, 45 and 60 min, 200 µl samples were taken from the cell supernatant. Immediately afterwards, all remaining supernatant was removed from the relevant cell layers and they were washed twice with ice cold KRB, before being left for lysis in KRB containing 1% (w/v) Triton X-100. BP activity was measured in the cell lysate using an automated plate reader (FLUOstar Optima) at excitation and emission wavelengths of 485 and 520 nm, respectively. The samples were diluted with KRB, where appropriate. For standardisation, the total protein amount of cell layers was determined by Pierce BCA assay (Thermo Fisher Scientific) according to the manufacturer's instructions.

Data analysis

Results were expressed as means ± SD. The significance of differences between the mean values was determined by unpaired, two-tailed Student's *t*-test. $P < 0.05$ was considered significant. All experiments were carried out at least in triplicate.

Results

Expression analysis in human alveolar epithelial cells

The expression of *ABCG2* mRNA and BCRP abundance in human alveolar epithelial cells was measured by semi-quantitative real-time-PCR and immunoblot, respectively. Moreover, BCRP localisation was studied by CLSM, surface protein biotinylation and nuclear protein isolation.

When analysed by q-PCR, *ABCG2* mRNA in freshly isolated AT2 cells was significantly ($P < 0.05$) higher than in the corresponding AT1-like cells in primary culture for 7-9 days in all three studied patient samples (Fig. 1B). However, when data was pooled, the trend was maintained, but differences were no longer significant due to inter-specimen variability (Fig. 1A).

Immunoblots from protein isolated from the same ~~primary cells~~patients confirmed a decrease of BCRP abundance during transdifferentiation from the AT2 to an AT1-like phenotype (Fig. 1C). Densitometric analysis of immunoblot data demonstrated that BCRP levels were significantly ($P < 0.05$) lower in AT1-like cells than in AT2 and NCI-H441 cells.

CLSM analysis of BCRP revealed distinct BCRP signals along the cell membranes and in nuclei of AT2 cells (Fig. 1E - G). In the corresponding AT1-like cells, membraneous and nuclear signals were noticeably weaker (Fig. 1H - J).

The observation of a nuclear localisation of BCRP was further confirmed by isolating nuclear protein and comparing it to cytosolic protein abundance (Fig. 3). Equal amounts of BCRP were present in the cytosolic and nuclear fractions of AT2 cells, whereas the protein is virtually absent in the corresponding AT1-like cells. Of note, the band for nuclear BCRP

appears at a higher molecular weight suggesting a different isoform or post-translational modification pattern.

Biotinylation of cell surface proteins and subsequent analysis by Western blot confirmed that BCRP was predominantly localised to the apical cell membranes in human AT1-like cell monolayers (Fig. 4A and B).

Expression analysis in NCI-H441 cells

BCRP was clearly expressed in the NCI-H441 cell line (Fig. 2). BCRP was studied in cells of passage numbers 59, 71 and 89 by q-PCR and Western blot. There was a significant increase of BCRP protein abundance with higher passage number detected by immunoblot (Fig. 2B and C). This was consistent with a trend to higher *ABCG2* mRNA expression levels (in AIC grown cells) between passage numbers 59 and 71 (Fig. 2A) (mRNA from passage number 89 was not included due to contamination). Culturing NCI-H441 monolayers at an air-liquid interface or under submersed culture conditions did not influence BCRP protein abundance (Fig. 2B and C).

CLSM analysis of BCRP in NCI-H441 cells grown under LCC (Fig. 2D - F) and AIC (Fig. 2G - I) conditions revealed localisation along the apical cell membranes as well as throughout the cytosol. The data were confirmed by nuclear protein analysis (Fig. 3) and cell surface protein analysis (Fig. 4A and C).

~~***Cell surface protein analysis***~~

~~Biotinylation of cell surface proteins and subsequent analysis by Western blot confirmed that human AT1-like cell monolayers and NCI-H441 cell monolayers had BCRP predominantly localised to their apical cell membranes (Fig. 3).~~

***BCRP activity in distal lung epithelial cells* in vitro**

The functional activity of BCRP in human alveolar epithelial cells and the NCI-H441 cell line was determined by efflux and bi-directional transport experiments using the BCRP substrate BODIPY FL prazosin (BP) and the inhibitor Ko143. In AT1-like cell monolayers grown in 24-well plates, the efflux assay resulted in relatively low amounts (i.e. 1.88 ± 1.16 pmol/µg protein after 60 min) of released BP. Moreover, the efflux was insensitive to inhibition with Ko143 (Fig. 5~~4~~A). When BP efflux was studied in NCI-H441 cell monolayers, efflux after 60 min reached 28.11 ± 2.10 pmol/µg protein (Fig. 5~~4~~B). Ko143 at 5 and 10 µM dose-dependently inhibited BP efflux from NCI-H441 cells.

Bi-directional transport studies of BP across Transwell-grown monolayers of AT1-like cells showed a significant net absorption of BP (Fig. 5~~4~~C). The presence of Ko143 increased flux rates in both transport directions. BP transport across NCI-H441 monolayers resulted in net-secretion, consistent with an apical localisation of BCRP, which was attenuated in the presence of Ko143 (Fig. 5~~4~~D).

Discussion

To our knowledge, this is the first time BCRP expression and activity has been comprehensively studied in freshly isolated human alveolar epithelial cells in primary culture. Previously, low but distinct amounts of BCRP were reported in the epithelial layer, in seromucinous glands and in small endothelial capillaries of human lungs by immunohistochemistry (19). Expression of the *ABCG2* gene in lung tissue was published by Bleasby and colleagues to be in the 50-75 percentile (20). By liquid chromatography-tandem mass spectrometry Sakamoto *et al.* found BCRP at high levels in lung tissue and bronchial epithelial cells in primary culture, whereas the protein was absent from alveolar and tracheal epithelial cells (6). No abnormalities of lungs in *Bcrp*$^{(-/-)}$ mice have been reported thus far (5). BCRP was also identified in a number of cell lines of pulmonary origin that are frequently used in biopharmaceutical studies (7; 8). Paturi *et al.* have previously determined BCRP expression and activity in Calu-3 cells, however, in their study neither the influence of AIC *vs.* LCC conditions or different passage levels were investigated, nor were efflux or transmonolayer transport studies conducted (21). Topotecan efflux from lung cancer cells, including NCI-H441 cells, measured by FACS analysis correlated well with *ABCG2* mRNA expression levels (22) and Ohbayashi *et al.* observed that the transport activity of the BCRP substrate, ^{3}H-methotrexate was significantly higher in primary mouse alveolar epithelial cell, when compared to primary mouse lung fibroblasts (23).

Our data suggest that BCRP is predominantly expressed in AT2 cells and expression decreases upon transdifferentiation to a type 1-like phenotype. While this trend was observed in samples from all patients, significant variation in BCRP abundance was noted between the patient specimens. In type 1-like primary cells and polarised monolayers of

NCI-H441 cells, BCRP protein was localised to the apical cell membrane, which is consistent with the protein's role as an apical effluxer in other epithelial barriers (3; 4). In the primary alveolar epithelial cells, BCRP was also present in cell nuclei. Several other localisation studies have also demonstrated the possible presence of BCRP in the nucleus in head and neck squamous cell carcinoma cells, chorion trophoblasts, and glioblastoma multiforme cells (24-26). Liang *et al.* recently suggested a dual role for BCRP: as a transporter on the plasma membrane and as a transcriptional factor in the nucleus regulating gene expressions (27). They reported that BCRP protein binds to the E-box of *CDH1* (E-cadherin) promoter and positively regulates transcription of this gene in A549 cells (27). This implies a role of BCRP in cell migration and possibly tumour colonisation. BCRP overexpression has also shown to modulate the expression and activity of other transporters involved in the uptake of different substrates into the cells (28). Whilst the molecular mechanism underlying the interaction between the overexpressed BCRP transporter and other influx transporters has not been elucidated yet, it is conceivable that overexpression increases nuclear BCRP levels and leads to subsequent transcriptional activity.

On functional level, AT1-like cell monolayers and NCI-H441 monolayers behaved quite differently. The cell line has previously been proposed to have similar transporter expression and activity compared to human alveolar epithelial cells *in vitro* in the case of P-glycoprotein, organic cation transporters and peptide transporters (16; 29; 30). BODIPY FL prazosin efflux, however, showed very different extend and sensitivity to inhibition, when data from AT1-like monolayers was compared with NCI-H441 cells. Similarly, bi-directional BP transport data were markedly different in the two cell types. NCI-H441 monolayers displayed a Ko143 sensitive net-secretion, consistent with apical localisation of BCRP transporter protein, whereas in AT1-like cell monolayers net absorption was observed. The

expression analysis found relatively low levels of BCRP in AT1-like cells. This alone, however, is insufficient to explain the observed effect. Al-Damluji *et al.* have reported a desipramin-sensitive uptake pathway for prazosin in neurons, which was not identified, but could be a monoamine transporter or a novel system similar to the one recently identified for the transport of verapamil or nicotine in human lung cell lines (31-33). If this hypothetical uptake system was active in AT1-like cells, it could explain the observed prazosin net absorption.

Conclusions

BCRP is expressed in human alveolar epithelial type 2 pneumocytes, but its expression decreases when the cells transdifferentiate into a type 1-like phenotype. In addition to the cell membranes, BCRP protein is also expressed in the nucleus and here it might play a transcriptional role in distal lung epithelium. In the NCI-H441 cell line, BCRP is abundantly expressed in apical cell membranes and its activity, as determined by BP efflux, is consistent with its localisation. Due to the observed differences, NCI-H441 are not an ideal model for the study of BCRP effects in human distal lung *in vitro*.

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Figure Legends

Figure 1. Expression of *ABCG2* and BCRP in human alveolar epithelial cells. *ABCG2* mRNA was analysed by q-PCR in freshly isolated AT2 cells and AT1-like cells in primary culture for 7-9 days. A) During transdifferentiation from AT2 to AT1-like phenotype, expression of *ABCG2* mRNA decreased. Data shown are pooled from three patients and normalised to expression in AT2 cells. B) *ABCG2* mRNA data shown for the individual patients and normalised to expression in AT2 cells from patient 1. C) Representative Western blot of BCRP in AT2 and AT1-like cells isolated from three subjects. BCRP abundance in NCI-H441 cells (passage number 78) is show as comparison. D) Densitometric analysis of immunoblot data shows that BCRP levels are significantly ($P < 0.05$) lower in AT1-like cells than in AT2 cells. Confocal laser scanning microscopic analysis of BCRP in AT2 (E - G) and AT1-like (H - J) cells. BCRP signals can be observed along the cell membranes as well as in cell nuclei (arrows). Signals are considerable weaker in AT1-like cells.

Figure 2. Expression of *ABCG2* and BCRP in the NCI-H441 cell line. A) *ABCG2* mRNA was analysed by q-PCR in NCI-H441 cells from passages 59 and 71. Cells were grown under air-interfaced culture (AIC) or liquid-covered culture (LCC) conditions for at least 7 days. Data are represented as means ± S.D., $n = 3$. B) Representative Western blot of BCRP in NCI-H441 cells from passages 59, 71 and 89. Cells were grown under air-interfaced culture (AIC) or liquid-covered culture (LCC) conditions for at least 7 days. C) Densitometric analysis of immunoblot data shows that BCRP levels are significantly increased in cells of higher passage numbers, whilst there are no differences between AIC and corresponding LCC grown cells. Data are represented as means ± S.D., $n = 3$, $*P < 0.05$, $** P < 0.01$. Confocal laser scanning

microscopic analysis of BCRP in NCI-H441 cells grown under LCC (D - F) and AIC (G - I) conditions. BCRP signals can be observed along the apical cell membranes as well as throughout the cytosol.

Figure 3. Determination of nuclear BCRP. Human AT2 and AT1-like cells as well as NCI-H441 cells were grown in tissue culture flasks and nuclear proteins were isolated and detected by immunoblot. During transdifferentiation from AT2 to AT1-like (AT1) phenotype, expression of ABCG2 decreases. Equal amounts of BCRP are present in the cytosolic and nuclear fractions of AT2 cells. Of note, the band for nuclear BCRP appears at a higher molecular weight. BCRP is absent from nuclei of NCI-H441 cells.

Figure ~~3~~4. Biotinylation of cell surface proteins. Human AT1-like cells and NCI-H441 cells were grown to monolayers for >8 days under liquid-covered culture conditions on Transwell inserts and cell surface proteins were biotinylated, isolated and detected by immunoblot. A) Representative Western blots are presented. AT1-like monolayers (upper panel, 1 band) as well as NCI-H441 monolayers (lower panel, 2 bands) clearly show BCRP localisation to apical cell membranes. Beta actin was used as control. Densitometric analysis of immunoblot data shows that BCRP levels are significantly higher in apical membranes of AT1-like (B) and NCI-H441 (C) cells. Data are represented as means ± S.D., $n = 3$, ** $P < 0.01$.

Figure ~~4~~5. Activity of BCRP in human alveolar epithelial cells and the NCI-H441 cell line. Inhibitory effect of Ko143 on the efflux of BODIPY FL prazosin (BP) from monolayers of AT1-

like cells (A) and NCI-H441 cells (B) grown in 24-well plates. BP release from AT1-like cells was significantly lower than from NCI-H441 cells and was not affected by Ko143, whilst the inhibitor showed a dose-dependent effect on BP efflux from NCI-H441 cells. Bi-directional transport studies of BP across Transwell-grown monolayers of AT1-like cells (C) showed a significant net absorption of BP. The presence of Ko143 increased flux rates in both transport directions. BP transport across NCI-H441 monolayers resulted in net-secretion, consistent with an apical localisation of BCRP, which was attenuated in the presence of Ko143 (D). Data are represented as means ± S.D., $n \geq 3$, *$P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.