The EGFR Pathway Regulates BCRP Expression in NSCLC Cells: Role of Erlotinib

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**Abstract:** While multidrug resistance (MDR) in cancer is well established, little is known about the cellular pathways regulating the expression and trafficking of the MDR eflux transporter like BCRP (ABCG2). Here we evaluated the role of signalling downstream of EGFR on BCRP expression and sub-cellular localization using lung cancer cells harboring BCRP but expressing various EGFR and Ras activating mutations; A549 (K-Ras-G12S), H292 wild-type EGFR and Ras, and H1650 (EGFR-Dele747-A750). Immunocytochemistry and immunofluorescence studies demonstrated that BCRP was predominantly intracellular but its expression was found also on the plasma membrane in A549 and H1650 cells with activated Ras and EGFR. Remarkably, EGFR inhibition by erlotinib at IC50 concentrations induced a differential time-dependent alteration in BCRP gene and protein expression. In H1650 cells, erlotinib enhanced both the total and plasma membrane degradation of BCRP by ubiquitination within 6-24 hours, whereas BCRP expression regained the original basal levels after 48 hours. In erlotinib treated H292 cells, BCRP levels decreased at 24 hours until 72 hours, whereas in A549 cells erlotinib initially reduced BCRP expression but then induced its accumulation on the plasma membrane at 72 hours. We further found that the PI3K/Akt inhibitor LY294002 down-regulated BCRP expression, hence showing that the Akt pathway is involved in the regulation of BCRP expression but not in its localization in these lung cancer cell lines. Finally, the selective BCRP transport inhibitor Ko143 did not increase erlotinib sensitivity, but did decrease the transport activity of BCRP in A549 and H1650 cells as it induced the accumulation of its transport substrate topotecan. In conclusion, our results suggest that the EGFR and Akt pathways are involved in regulation of BCRP expression, trafficking and drug transport activity. These findings warrant future studies on the pharmacologic modulation of these pathways to enhance the efficacy of anticancer combinations of erlotinib with drugs that are BCRP transport substrates.

**Keywords:** BCRP, chemotherapy, EGFR, erlotinib, mutations, Ras, ubiquitination.

**INTRODUCTION**

The ABC half-transporter breast cancer resistance protein (BCRP/ABCG2) extrudes a variety of therapeutic drugs in an ATP-dependent manner, and diminishes their pharmacological efficacy in tumor cells [1, 2]. BCRP has clinical relevance since it affects the pharmacokinetics of drugs, plays a role in stem cell regulation and protection against hypoxia [3-5]. This encouraged further investigation on the characterization of its role in optimization of cancer therapy. Several studies were performed to determine the relationship between the expression of BCRP in human cancers and the clinical efficacy of several drugs [6], as well as the role of BCRP on intracellular drug accumulation [7].

BCRP overexpression is associated with high levels of resistance to a variety of anticancer agents, including anthracyclines, mitoxantrone, antifolates and camptothecins in a large number of hematological malignancies and solid tumor cells [8]. Nevertheless the regulation of BCRP/ABCG2 expression is poorly understood [9]. Apart from increased expression in MDR cancer cells, little is known about the mechanisms affecting BCRP expression. A physiological induction in transporter gene expression has been observed in the mammary gland during lactation [10], while Imai and colleagues showed that estrogen down-regulates BCRP expression via a post-transcriptional mechanism [11]. Furthermore, Lemos et al. as well as Ifergan et al. demonstrated that folate status in cancer cell lines affects the expression of BCRP and subcellular localization of the transporter (plasma membrane or cytoplasmic organelles) [12-15].

The association between the expression of ABC transporters including BCRP, and cell growth signalling pathways has recently generated an increasing interest. It has been shown that EGF induces the expression of ABCG2 via the MAPK cascade [16], while we and other groups demonstrated that an active PI3K-Akt pathway is responsible, at least in part, for the maintenance of BCRP expression and subcellular localization [17-19].
The utilization of selective receptor tyrosine kinase inhibitors (TKIs) is an important therapeutic strategy in the treatment of NSCLC. Currently, gefitinib and erlotinib are approved TKIs for the treatment of patients with NSCLC, one of the leading causes of cancer-related deaths in the Western world. Erlotinib hydrochloride (Tarceva, OSI-774, CP-358774), and gefitinib (Iressa) are quinazoline derivatives orally active, selective, and reversible epidermal growth factor receptor 1 tyrosine kinase inhibitors that compete with the binding of ATP to the intracellular tyrosine kinase domain of epidermal growth factor receptor (EGFR), thereby inhibiting receptor auto-phosphorylation and blocking downstream signal transduction. Several pharmacologic interactions have been described between TKIs and BCRP [20, 21], while these drugs also affect BCRP expression inducing overexpression or inhibition of the transporter [22, 23].

Despite the positive outcome obtained with EGFR tyrosine kinase inhibitors, application of these agents may also be limited by the acquisition of drug resistance. Furthermore the combined treatment of patients with advanced NSCLC with either gefitinib or erlotinib and standard two-drug chemotheraphy regimens failed to improve survival [24-28]. The lack of the expected positive outcome may have been caused by several factors as reported by Giovannetti et al. [29].

One interesting approach to improve antitumor treatment efficacy is to identify and manipulate signalling mechanisms that control drug transporter expression, intracellular localization and thereby drug extrusion function. Towards this end, we investigated here the impact of inhibiting the EGFR pathway while focusing on the role of a TKI, erlotinib, on BCRP expression, stability and sub-cellular localization in human NSCLC cell lines. Thus, we investigated the implications of erlotinib-induced BCRP inhibition on the rational design of combination therapy involving EGFR inhibitors and chemotherapy, by evaluating the modulation of topotecan accumulation in these tumor cells. For this purpose the expression and genetic status of BCRP were characterized in a panel of NSCLC cell lines, with a high expression of the transporter while harboring the wild type BCRP but different K-Ras and EGFR activating mutations; namely A549 (K-Ras Mut G12S), H292 (K-Ras wild type, EGFR wild type), and H1650 (EGFR Mut DelE747-A750), enabled us to identify novel mechanisms underlying the regulation of BCRP expression and subcellular localization.

MATERIALS AND METHODS

Cells and Culture Conditions

RPMI 1640 (containing 2 mM L-glutamine) and DMEM were used as culture media and were supplemented with 10% heat-inactivated fetal calf serum (Gibco BRL, Life Technology, Breda, The Netherlands), 50 μg/ml penicillin and 50 μg/ml streptomycin. The human NSCLC cell lines NCI-H292 (H292) and A549 were from American Type Culture Collection (ATCC) (Manassas, VA, USA) and were cultured in RPMI 1640. The human NSCLC NCI-H1650 (H1650) was cultured in DMEM. Cells were grown at 37°C in a humidified atmosphere with 5% CO₂. Cells from exponentially growing cultures were used in all experiments.

Western Blot Analysis of BCRP Expression

BCRP expression was determined by Western blotting, essentially as previously described [4, 7]. “Total lysates were prepared in a buffer containing 50 mM Tris (pH 7.6), 20% (v/v) glycerol, 5 mM DTT, 0.5% (v/v) NP-40, and 4% (v/v) of a protease inhibitor cocktail. Lysates were sonicated for 3 x 5 s with 30 s intervals (MSE Soniprep 150, 4°C, amplitude 6-7) and centrifuged at 13,000 rpm for 10 min at 4°C. The protein-containing supernatant was collected and protein concentration was determined using the Bio-Rad protein assay. In each lane of a Bio-Rad minigel system (Bio-Rad), 40 μg protein were loaded, resolved by electrophoresis, and transferred to a PVDF membrane (Millipore). PVDF membranes were blocked for 1 h at room temperature in blocking buffer [5% bovine serum albumin in PBS 0.1% (v/v) Tween] and incubated overnight at 4°C with the primary antibody. For detection of BCRP, an anti-BCRP monoclonal antibody was used (BXP-53; 1:200; 1.25 μg/mL) [30]. As secondary antibody, horseradish peroxidase-conjugated rabbit anti-rat (Amersham Biosciences) was used. As a loading control, expression of β-actin was determined using an antibody against β-actin (clone C4 from Chemicon International; 1:10,000; 0.01 μg/mL). Bound antibody was detected by incubating PVDF membranes with enhanced chemiluminescence plus (GE Healthcare). Expression level was evaluated by scanning densitometric analysis using Quantity One software (Bio-Rad, Hercules, CA, USA).”

Membrane Fraction Preparation

Membrane fraction preparation was performed as described in Cardone et al. [31] with minor modifications. “Briefly, after drug treatment, monolayers were washed...
twice with ice-cold PBS and then lysed in ice-cold buffer A (10 mM HEPES pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol, and 0.5 mM phenylmethylsulfonyl fluoride) and homogenized by five passes through a 20-gauge needle to obtain a total cell homogenate. An aliquot was removed for the determination for total cellular protein determination. After separation of nuclear and endosomal fractions, the supernatant was centrifuged again at 100,000 x g for 1 h to obtain a plasma membrane rich pellet. Thirty-five micrograms of membrane fraction proteins were suspended in SDS sample buffer, resolved by SDS-PAGE and analyzed by Western blotting as described above.

Immunoprecipitation

This procedure was performed essentially as described [4, 23, 29]. “Cells were lysed in a buffer (20 mM Tris/HCl pH 8, 140 mM NaCl, 10 mM EDTA, 10% glycerol, 1% NP40, 1 mM Na-deoxycholic acid, and 1 mM PMSF) passed through a 22 gauge needle and cleared by centrifugation at 10,000 x g at 4°C for 10 min. Proteins were immunoprecipitated by incubating 35 μg of total cell lysate protein with 0.2 μg of ubiquitin-specific monoclonal antibody (Santa Cruz Biotechnology, USA) for 1 h at 4°C, then 2-5 μl of protein A/G agarose (Santa Cruz Biotechnology USA) were added and incubated over-night at 4°C. The cell suspension was centrifuged at 2,600 rpm and the pellet was washed three times with PBS and then resuspended in Læmli buffer. Each sample was separated on 10% polyacrylamide gels and Western blot analysis was performed as described above. The immunoprecipitates were analyzed by immunoblotting using anti-BCRP (BXP-53) antibody [23].”

Immunofluorescence Microscopy Studies

This procedure was performed essentially as described [32]. “H1650, H292 and A549 cells were seeded onto glass Lab-Tek Chamber Slides (8 wells; 0.8 cm2/well) at a density of 20 x 10^4 cells per well (400 μl/well medium) and incubated for 1 to 2 days at 37°C. Then, the growth medium was removed, and monolayers were washed twice with HBSS solution and fixed with 3.7% formaldehyde in PBS for 15 min at room temperature. Cells were washed twice with PBS and permeabilized by Triton X-100 [0.1% (w/v)] in PBS for 5 min at room temperature. Non-specific binding sites were blocked for 30 min at room temperature with PBS containing 5% rabbit serum (DakoCytomation). After serum removal, and without further washing, cells were incubated with a mouse anti-BCRP monoclonal antibody BXP-21, diluted 1:50 in PBS containing 4% bovine serum albumin, for 60 min at room temperature. BCRP staining was followed by incubation with FITC-conjugated rabbit anti-mouse antibody (1:30; DakoCytomation) for 60 min at room temperature. After a final wash with water instead of PBS, the slides were mounted on Vectashield (Vector Laboratories) and examined using a Leica TCS SP confocal laser scanning microscope equipped with X20 objective with numerical aperture of 0.70. FITC was excited using the 488 laserline. The same setting was used to take all pictures for allowing the comparison between the different cell lines. Control for non-specific staining was the replacement of BXP-21 [32] with a non-specific antibody from the same class, mouse IgG2a (DakoCytomation)”.

Analysis of BCRP mRNA Expression

Analysis of BCRP mRNA expression was performed by real-time PCR with the Applied Biosystems 7500HT sequence detection system (Applied Biosystems, Foster City, CA), essentially as described earlier [4]. “Primers and probes for ABCG2 were obtained from Applied Biosystems Assay-on-Demand Gene expression products (Hs01053790_m1). PCR reactions were performed in triplicates using 5 μl of cDNA, 12.5 μl of TaqMan Universal PCR Master Mix, and 2.5 μl of forward and reverse primers and probes in a final volume of 25 μl. Samples were amplified using the following thermal profile: an initial incubation at 50°C for 5 min, to prevent the reamplification of carryover-PCR products by AmpEraser uracil-N-glycosylase (UNG), followed by incubation at 95°C for 10 min, to suppress AmpEraser UNG activity and denature the DNA, 40 cycles of denaturation at 95°C for 15 sec followed by annealing and extension at 60°C for 1 min. Amplification data were normalized to β-actin, and quantification of gene expression was performed using the ΔΔCT calculation, where Ct is the threshold cycle; the amount of target gene, normalized to β-actin and relative to the calibrator (control cells), is given as 2−ΔΔCT. Preliminary experiments were carried out with dilutions of cDNA obtained from Quantitative PCR Human Reference Total RNA (Stratagene, La Jolla, CA, USA) to determine the primer concentrations that yield the minimum standard deviation between Ct values and to demonstrate that the efficiencies of amplification of all targets and reference (β-actin) genes are approximately equal. Experiments were done two independent times in duplicate”.

Immunocytochemistry Studies

Cells were treated with erlotinib at the IC50 concentrations. At indicated time points, cells were harvested and cytocentrifuged preparations were made. Alternatively, the cells were cultured for 48 h on coverslips and then treated with erlotinib. In both preparations, the specimens were subsequently air-dried overnight and fixed for 7 min in acetone at room temperature. The slides were incubated with diluted primary antibody BXP-21 (or a negative control) in 1% PBS/BSA solution for 1 h at room temperature (RT). After 3 wash steps with PBS, biotinylated rabbit-antimouse serum (1:150, Zymed, San Francisco, CA) in 1% PBS/BSA solution for 1 h at RT and HRP-labelled streptavidin (1:500, Zymed) in 1% PBS/BSA solution for 45 minutes were used as secondary antibodies. Color development was with 3-amino-9-ethylcarbazole (AEC) ready to use solution (Zymed, San Francisco) and nuclei stained with hematoxylin.

Cellular Topotecan Accumulation

The effects of erlotinib or Ko143 on the cellular accumulation of topotecan were determined by flow cytometry. Cells were cultured in medium with indicated concentrations of erlotinib for 1 day in H1650 and H292 cells and for 1 and 3 days in A549 cells. Alternatively, cells were treated with Ko143 for 45 minutes. After trypsinization, cells were incubated with 20 μM topotecan for 45 min at 37°C, washed in ice-cold PBS, and subjected to fluorescence analysis using FACSCalibur (Becton Dickinson, CA) using an excitation wavelength of 488nm and an emission wavelength of 585 as previously described by Hendricks et al. [33].
RESULTS

Analysis of BCRP Expression after Erlotinib Treatment

Since we aimed at investigating the impact of erlotinib in each lung tumor cell line, we first determined the IC_{50} concentrations; these were found to be 2.8 μM in H292, A549, and H1650 cells, respectively, as reported earlier [29].

The effect of erlotinib at their IC_{50} concentrations on BCRP protein expression varied considerably between H292, A549, and H1650 cells as revealed by Western blot analysis (Fig. 2). In H292 cells the levels of BCRP remained relatively stable, but decreased at 24 h through 72 h. Conversely, a significant decrease (50%) in BCRP protein levels was found as early as 6 h through 24 h after erlotinib treatment and returned to the basal level after 48 h and 72 h of treatment in H1650 cells. Similarly, in A549 cells, BCRP levels were markedly decreased (down to 30%) at 6 h of erlotinib treatment and a 2.0-fold (P<0.05) increased expression compared to control cells (untreated) was found at 48 and 72 h of treatment.

Erlotinib-Dependent BCRP Down-Regulation is due to Transcriptional Repression

To determine whether erlotinib decreased BCRP levels via transcriptional repression, we used quantitative real-time PCR to determine BCRP mRNA levels after erlotinib treatment (Fig. 3). In general, gene expression data were consistent with the protein expression profiles obtained after treatment with erlotinib. In particular in H292 cells, BCRP mRNA levels were significantly reduced (p<0.005) to about 50% between 24 h and 72 h of treatment with erlotinib, hence being consistent with the protein levels. In A549 cells the reduction in BCRP mRNA levels reflected the down-regulation observed at protein level after 6 h of erlotinib treatment; however, after 24 h BCRP mRNA expression remained diminished in contrast to the protein levels. After 6 h of erlotinib, a slightly up-regulated BCRP mRNA expression, and about 1.5-fold decrease after 24 h in H1650 cells (p<0.005).

Erlotinib Promotes BCRP Degradation

To explore the mechanism underlying erlotinib-induced BCRP down-regulation in H1650 cells despite the fact that transcript levels were increased, we assessed BCRP ubiquitination at 6 h and 24 h after erlotinib treatment. BCRP was found to be ubiquitinated at 6 h and 24 h (Fig. 4a). Hence, since BCRP transcript levels increased, the decrease in BCRP occurred at the protein level. Therefore, we examined the biosynthesis of BCRP by blocking protein synthesis using cycloheximide (50μg/ml) alone or in combination with erlotinib. BCRP degradation occurred already after 6 h in cells treated with this drug combination (Fig. 4b) but not with cycloheximide alone (Fig. 4c). This experiment demonstrated that erlotinib caused BCRP protein degradation in H1650 and suggests that the increase in BCRP levels is apparently aimed at compensating for the loss of BCRP protein. Thus, erlotinib decreased BCRP expression at the post-translational level in H1650 cells and at the transcriptional level in A549 and H292 cells.
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**Fig. (3).** Determination of BCRP mRNA levels after 6, 24 and 48 h of erlotinib treatment. Analysis was performed by real-time PCR with the Applied Biosystems 7500HT sequence detection system. Shown are the ratios between BCRP and the housekeeping gene, $\beta$-actin. Data are presented as % of untreated cells. Erlotinib induced a tumor-model dependent modulation of BCRP expression.

**Fig. (4).** (a) BCRP ubiquitination after treatment with erlotinib. BCRP was found ubiquitinated after 6 and 24 h in H1650 cells. Proteins immunoprecipitated with ubiquitin or BCRP-specific antibodies were loaded onto SDS-PAGE gels and Western blotting was performed. Blots for the detection of BCRP were subsequently stripped and re-probed with an antibody to Ubiquitin, and vice versa. Experiments were repeated twice and similar results were obtained. (b) Cells were treated with either erlotinib (IC$_{50}$ concentration) in combination with cycloheximide (50 $\mu$g/ml) or (c) cycloheximide alone. Cells were harvested before the initiation of cycloheximide treatment (0 hr) as well as at 3, 6 and 24 h post-treatment. Immunoblotting was performed for BCRP and $\beta$-actin. The BCRP protein was stable for up 1 h. In combination with erlotinib, the down-regulation of BCRP started as early as 6 h after treatment.

Evaluation of BCRP expression on the plasma membrane fraction after treatment with the TKI, revealed that erlotinib induced elimination of BCRP from the plasma membrane of H1650 cells with the same kinetics of total BCRP in these tumor cells; conversely, a strong accumulation of BCRP was found in the membrane of A549 cells after 72 h of continuous exposure to erlotinib (Fig. 5a). Immunocytochemistry.

**Fig. (5).** Plasma membrane localization of BCRP after erlotinib treatment. (a) BCRP expression on the plasma membrane fraction of H1650 and A549 cells was determined by Western blotting. As a loading control, $\beta$-actin levels are also shown. Early reduction in BCRP on cell surface was evident in H1650 cells; while late accumulation on the plasma membrane was found in A549 cells. (b) Staining of cytospins from A549 and H1650 cell specimens with the BCRP specific monoclonal antibody BXP-21. Biotinylated rabbit-anti-mouse serum and HRP-labeled streptavidin were used as secondary antibodies. Nuclei were stained with hematoxylin. Color development was performed with AEC. (The color version of the figure is available in the electronic copy of the article).
studies confirmed that after 6 h of erlotinib treatment, BCRP was no longer intensely localized in the vesicles-like structures that are part of the plasma membrane in H1650 cells. Instead BCRP appeared diffusely distributed in the cytosolic region close to the plasma membrane and returned to the cell surface after 24 h of erlotinib treatment (Fig. 5b). Consistent with the Western blot analysis were the results of the immunocytochemistry in A549 cells, which confirmed the increase of BCRP expression on plasma membrane in these cells after 72 h of erlotinib treatment (Fig. 5b).

**Phosphatidylinositol 3-Kinase Inhibitor Down-Regulates BCRP Expression in a Time-Dependent Manner Without Affecting Transporter Localization**

To test the hypothesis that the PI3K-Akt signalling pathway regulates BCRP expression in these lung tumor cells, we studied the effects of LY294002, an inhibitor of the Akt effector protein. Towards this end, H292, A549 and H1650 cells were treated with 20 µM LY294002 or erlotinib for 3 h and 6 h, and BCRP expression levels were determined by Western blot analysis (Fig. 6). LY294002 neither reduced BCRP levels nor those of phosphorylated Akt (Ser473) in H1650 cells. In contrast, LY294002 decreased the levels of phosphorylated Akt (Ser473) and BCRP in A549 and H292 cells (Fig. 6).

**Fig. (6).** Involvement of PI3K/Akt pathway in BCRP expression. Erlotinib was applied at the IC₅₀ concentrations and LY294002 at 20 µM for 3 h and 6 h. Protein expression was determined by Western blotting using a primary BCRP-specific antibody (BXP-53) followed by a secondary HRP-conjugated antibody. The blots were then stripped and reacted with an antibody against phosphorylated Akt (Ser473). To correct for loading differences, the blots were stripped and reacted with an antibody against β-actin. The expression of both phosphorylated Akt (Ser473) and BCRP was decreased in H292 and A549 cells.

Although, erlotinib at IC₅₀ concentration slightly affected the phosphorylation of Akt in our lung cancer cell models, it induced a reduction in BCRP levels, suggesting that the down-regulation effect of BCRP-lowering induced by erlotinib is not only the result of the inhibition of the PI3K/Akt. In contrast, LY294002 down-regulated BCRP protein levels in A549 and H292 cells, thus the PI3K/Akt pathway is important for maintaining BCRP expression in these two lung tumor cell lines. Based on these results, we further determined whether or not an inhibitor of the MEK-ERK pathway UO126 suppressed BCRP expression in H1650 cells. Hence, cells were treated for 3 h and 6 h with 10 µM UO126; however, no reduction in BCRP expression was observed (data not shown) suggesting that the activation of the ubiquitination pathway by erlotinib, is important for maintaining BCRP expression in this cell line. Regarding the localization of BCRP, after inhibition of the PI3K/Akt pathway we found no specific re-localization of BCRP in these lung tumor cell lines (data not shown).

**Erlotinib Affects Topotecan Uptake into H1650 and A549 Cells**

In order to determine whether or not BCRP was functionally active on the plasma membrane of H1650 and A549 cells after erlotinib treatment, topotecan was used as a fluorescent probe to measure cellular drug accumulation by flow cytometry. Twenty-four h of exposure to erlotinib led to an increase in the intracellular accumulation of topotecan in H1650 cells compared to untreated cells (Fig. 7a), suggesting the loss of BCRP transport activity or inhibition of BCRP activity after treatment with erlotinib. In A549 cells, erlotinib induced a lower topotecan accumulation suggesting that this membrane transporter is functionally active in extruding topotecan (Fig. 7c). In H292 cells, consistent with the absence of BCRP on plasma membrane after erlotinib treatment, we failed to observe any variation in topotecan accumulation (data not shown). The selective BCRP transport inhibitor, Ko143 [34], induced the accumulation of topotecan to the same extent observed with erlotinib (Fig. 7b) in H1650 cells, hence demonstrating that BCRP is still active on the plasma membrane, whereas erlotinib affected its drug efflux activity. In A549 cells, Ko143 induced a slight but significantly higher increase in topotecan accumulation (Fig. 7d) than observed with erlotinib, suggesting that in this tumor cell line, BCRP remains active on plasma membrane after erlotinib treatment.

**Erlotinib is not a Substrate of BCRP in our Study Models**

To determine whether or not erlotinib could be a transport substrate of BCRP in H1650 and A549 cells we determined whether Ko143, as shown in Fig. (8A, B) would affect the sensitivity of cells to erlotinib; the IC₅₀ value of erlotinib was evaluated in the presence of Ko143 and no difference in erlotinib sensitivity was observed.

**DISCUSSION**

As recently reported, an intercalated regimen of chemotherapy and erlotinib resulted in improved efficacy for patients with advanced NSCLC [35]. In the current paper we provide novel information regarding the mechanisms underlying drug-drug interaction in NSCLC cells, by showing that treatment with erlotinib affects BCRP expression and
drug efflux activity. We found that erlotinib induced a stable decrease in BCRP expression only in a wild type NSCLC cell line model of H292, while an early and transient reduction of the MDR transporter BCRP was observed in A549 and H1650 cells harboring activated (i.e mutated) Ras and EGFR models. It is noteworthy that the decrease in BCRP levels in H292 was time-dependent and occurred at the mRNA level, while it is completely absent in A549 cells in which mutant Ras and in H1650 cells in which mutant EGFR was present, respectively. These findings suggested that the EGFR pathway exerts a transcriptional control on ABCG2 expression in the wild type model, perhaps via the PI3K-Akt signalling pathway, as both erlotinib and the inhibitor of PI3K-Akt signalling LY294002 strongly reduced the expression of BCRP in H292 cells. The PI3K-Akt signalling pathway is likely involved in both a transcriptional and post-transcriptional regulation of BCRP in mutant Ras-containing A549 cells, as LY294002 down-regulated BCRP levels in this tumor cell line. Conversely, PI3K-Akt was not involved in regulating BCRP expression in H1650 cells. Consistent with these findings, erlotinib did not decrease the phosphorylation of Akt in H1650 cells, hence the BCRP-lowering effect of erlotinib is neither the result of inhibition of the PI3K/Akt signalling pathway nor the MEK/ERK signalling, since the MEK inhibitor U0126 did not affect BCRP expression. This observation is in agreement with recent findings reported by Sos et al., regarding the uncoupling of mutant EGFR from receptor downstream signalling at the level of Akt [36]. Regulation of protein degradation plays a role in the stability of MDR efflux transporters of the ABC super-
family. The ubiquitination-proteasome system has been shown to play a role in the protein turnover and thereby in the functionality of P-gp, while ubiquitination mediated proteasomal-degradation of BCRP is a quality control system for misfolded proteins. Muller et al. demonstrated that P-gp is a relatively stable protein with a half-life of 14 h to 17 h. In H1650 cells, a post-translational mechanism that leads to degradation of BCRP, conceivably through the proteasome, may be involved as we found ubiquitination of BCRP early after treatment with the TKI erlotinib. However, we found that in H1650 cells the MDR transporter is a stable protein at least for 24 h, and that both total and membrane BCRP levels dropped soon after treatment and resumed the baseline expression already after 24 h of erlotinib treatment but not with LY294002. Thus, we conclude that the EGFR signalling pathway is required to maintain a marked expression of BCRP on the plasma membrane and that after erlotinib, plasma membrane BCRP undergoes internalization and subsequent degradation, through a mechanism that is not dependent on the PI3K/Akt pathway. Conversely, in A549 cells, the prolonged treatment with erlotinib induced a post-transcriptional increase in BCRP expression which strongly accumulated on the plasma membrane, through a mechanism that is not dependent on the PI3K/Akt pathway, indicating that in these NSCLC model cells, erlotinib that induces the up regulation of BCRP, may cause chemoresistance to drugs that are BCRP transport substrates. By testing this hypothesis, we found here that erlotinib completely inhibited the functionality of BCRP in H1650 cells and to a lesser extent in A549 cells, since it increased the intracellular level of topotecan. Similar results have been recently reported, demonstrating that several EGFR TKIs including erlotinib, decreased surface expression of BCRP via the PI3K/Akt signalling pathway and increased the intracellular level of chemotherapeutic agents by limiting drug efflux via ABC transporters. To the best of our knowledge, our current study provides the first evidence that erlotinib down-regulates BCRP expression in NSCLC cells at the transcriptional and post-translational level by the activation of PI3K/Akt pathway and protein ubiquitination pathway, modulates surface expression and inhibits activity of the BCRP. In addition, we evaluated the sensitivity of tumor cells to erlotinib in the presence of Ko143, at the same time point when BCRP was strongly expressed on the plasma membrane; however, we found no modification in cell sensitivity to erlotinib.

Interestingly, the current study pointed out that a transcriptional regulation of BCRP occurred in NSCLC cells with a wild type EGFR, whereas in cells with activating mutations of EGFR and of the downstream signalling molecule Ras, additional effectors are involved in determining BCRP trafficking, which may ultimately impact on the intrinsic resistance of NSCLC to chemotherapy and to combination schedules with TKI and other chemotherapeutics. Therefore, the current paper expands our knowledge on the influence of activating EGFR and Ras mutations on BCRP expression, subcellular localization and drug efflux which could predict the clinical treatment outcome and hence could pave the way towards the rational choice of the high priority anti-cancer therapies to be applied in NSCLC, especially through carefully designed clinical studies.

### Abbreviations

- **ABC** = ATP-binding cassette
- **AEC** = 3-amino-9-ethylcarbazole
- **BCRP** = Breast cancer resistance protein
- **CHX** = Cycloheximide
- **EGFR** = Epidermal growth factor receptor
- **HRP** = Horseradish peroxidise
- **MTT** = 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazoliumbromide
- **NSCLC** = Non-small-cell lung carcinoma
- **PCR** = Polymerase Chain Reaction
- **TIF** = Topotecan Intensity Fluorescence

### Conflict of Interest

The authors confirm this article content has no conflict of interest.

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