Role of efflux pump activity in lapatinib/caelyx combination in breast cancer cell lines

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The aim of this study was to investigate, at preclinical level, efflux pump modulation induced by lapatinib, a small-molecule dual inhibitor of the epidermal growth factor receptor (EGFR), in HER2-negative or HER2-positive breast cancer cell lines (SkBr3 and BRC230). We also evaluated the cytotoxic activity and modulation of biomolecular cellular pathways regulated by caelyx and lapatinib, used singly or in combination, at concentrations corresponding to peak plasma level in the two cell lines. Lapatinib was active in the HER2-overexpressing cell line, SkBr3, but not in BRC230 cell line, which does not express HER2. Conversely, caelyx exerted a cytotoxic effect on both the cell lines. Simultaneous exposure to lapatinib and caelyx in SkBr3 cell line produced an additive cytotoxic effect with dephosphorylation of HER2 and EGFR, an upregulation of p21, and an induction of apoptosis through dephosphorylation of BAD^{Ser112} and caspase cleavage. In BRC230, simultaneous treatment induced a synergistic effect that was because of, at least in part, an upregulation of p21. Lapatinib also blocked efflux pumps, such as the breast cancer resistance protein I by increasing the length of time in which caelyx was present in tumor cell cytoplasm, which led to caspase cleavage, BAD^{Ser112} dephosphorylation, and apoptosis. Our data indicate that lapatinib used in combination with caelyx is active in HER2-expressing cells, probably because of lapatinib-induced dephosphorylation of the HER2-EGFR pathway, and also in non-HER2-expressing cells, possibly because lapatinib blocks efflux pump activity, increasing the length of time of intracellular exposure to caelyx and thereby increasing its cytotoxic effect. *Anti-Cancer Drugs* 20:918–925 © 2009 Wolters Kluwer Health | Lippincott Williams & Wilkins.

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Introduction

Members of the type I tyrosine receptor kinase family, including EGFR, HER2, HER3, and HER4, are activated in a large number of epithelial malignancies and play a role in regulating tumor growth, cell survival and differentiation, and in resistance to chemotherapy [1–4]. Family members have an extracellular ligand-binding domain and a cytoplasmic protein tyrosine kinase domain. However, unlike the rest of the EGFR family, HER3 lacks a cytoplasmic protein tyrosine kinase domain, and HER2 has no known ligand. Peptide growth factors bind the extracellular domain of ErbB/HER receptors, leading to the formation of homodimers and/or heterodimers that stimulate the tyrosine kinase activity of the receptors and trigger autophosphorylation of tyrosine residues within the cytoplasmic domain [5].

Overexpression of ErbB1/HER2 proteins is associated with poor survival in breast cancer patients. Synergistic inhibition of cancer cell growth has been shown by targeting both ErbB1 and HER2 in breast cancers, where the fraction of tumors overexpressing the two proteins varies from 27 to 30% and from 20 to 25%, respectively [6]. The inhibition of EGFR and HER2 has been shown to have important antitumorigenic effects, which has led to the development of several antibody and small-molecule treatments.

Lapatinib (GW572016; GlaxoSmithKline, Philadelphia, Pennsylvania, USA) is a small-molecule dual tyrosine kinase inhibitor that reversibly inhibits both ErbB1 (EGFR) and HER2 [7–10]. Lapatinib blocks downstream mitogen-activated protein kinase (MAPK) and phosphatidylinositol-3-kinase proliferation and survival signaling pathways in HER2-positive breast cancer cell lines, tumor xenografts, and patients with HER2-positive breast cancers. It is able to penetrate the blood–brain barrier and is consequently effective for the treatment of brain metastases. Moreover, oral lapatinib has been approved by the US Food and Drug Administration for use in combination with capecitabine in advanced breast cancers overexpressing HER2 and resistant to Herceptin, taxanes, and anthracyclines. Clinical studies have shown a significant antitumor activity of lapatinib both as a single agent and in combination with capecitabine in HER2-positive cancers, but not in EGFR-positive/HER2-negative tumors [11–13]. Furthermore, one study showed that lapatinib is a substrate for the efflux transporter

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P-glycoprotein (Pgp) and for the breast cancer resistance protein I (BCRP I), a member of the superfamily of ATP-binding cassette (ABC) transporters, which translocate unique compounds in addition to some overlapping substrates [14]. Drugs transported by Pgp include hydrophobic compounds, either uncharged or slightly positively charged, and chemotherapeutic agents, such as vinca alkaloids, anthracyclines, epipodophyllotoxins, and taxanes. BCRP I utilizes the energy of ATP hydrolysis to extrude metabolic products and drugs, such as anthracyclines, mitoxantrone, camptothecin-derived and indolocarbazole topoisomerase I inhibitors, methotrexate, and flavopiridol. The protein is found in various tissues involved in drug absorption, distribution, and elimination [15–17]. Other recent reports show that lapatinib may inhibit cellular Pgp and BCRP I functions at clinically relevant concentrations [18].

Caelyx, a polyethylene glycol-coated (pegylated) liposomal doxorubicin, is a formulation of doxorubicin with a prolonged circulation half-life, specific pharmacodynamic properties, a favourable toxic profile, proven activity in solid tumors, and the ability to evade interception by the immune system. Pegylated drugs are also reported to partially overcome Pgp-mediated resistance, and encouraging results from clinical studies have highlighted the feasibility of using liposomal doxorubicin in combination with conventional drugs [19–21].

On the basis of the above findings, we evaluated the cytotoxic activity and the correlated molecular mechanisms of lapatinib and caelyx used singly or in combination in breast cancer cell lines characterized by different levels of ErbB1 and HER2 expression. Our results provide evidence that the activity of the lapatinib/caelyx combination in HER2-negative breast cancer cell line is dependent on BCRP I pump efflux activity.

Materials and methods

Cell lines

SkBr3 cell line was purchased from the American Type Culture Collection (ATCC, Rockville, Maryland, USA), whereas the BRC230 cell line was established and characterized in our laboratory [22]. Both the cell lines have a similar doubling time (32 and 30 h, respectively), are estrogen and progesterone receptor negative, and show p21 expression. Basal expression of MDR1 is not detectable in BRC230 cell line. Cell lines were maintained as a monolayer in a humidified atmosphere containing 5% CO_2 at 37°C and subcultured weekly. Culture medium was composed of DMEM/HAM'S F12 (1 : 1) supplemented with fetal calf serum (10%), glutamine (2 mmol/l), non-essential aminoacids (1%) (Mascia Brunelli s.p.a., Milan, Italy), and insulin (10 g/ml) (Sigma-Aldrich, St. Louis, Missouri, USA). Cells were used in the exponential growth phase in all the experiments.

Drugs and treatment schemes

Lapatinib (GlaxoSmithKline) and caelyx (Schering Plough, Bruxelles, Belgium) were diluted in 0.9% saline solution. Both drugs were stored at 4°C and freshly diluted in culture medium before each experiment. For single-drug exposure, cells were treated for 72 h, an exposure time that is compatible with the half-life of the drugs when administered in a clinical setting. Lapatinib was tested at 0.04, 0.4, and 4 μmol/l, whereas caelyx was studied at 0.5, 5, and 50 μmol/l, with concentrations not exceeding peak plasma levels (4 μmol/l for lapatinib and 50 μmol/l for caelyx). Three different schedules were tested for the drug combination treatments:

(1) caelyx 72 h → lapatinib 72 h,
(2) lapatinib 72 h → caelyx 72 h,
(3) lapatinib + caelyx 72 h.

Drugs were used at peak plasma level concentrations in all combinations. Control samples were processed in the same manner, but using drug-free medium.

In-vitro growth inhibition assay

Cell survival after drug exposure was measured by sulforhodamine B assay, as described earlier [23]. Samples were run in octuplet, and each experiment was repeated three times.

Data analysis

The method of Kern *et al.* [24], subsequently modified by Romanelli *et al.* [25], was used to construct a cell survival index (RI) for the evaluation of the interaction between the two drugs. RI values greater than 1.5 indicate synergy, values less than 0.5 show antagonism, and values between 0.5 and 1.5 indicate additivity. Standard deviations did not exceed 10% in any of the experiments, and only differences of ≥ 0.5 from unity in RI values were considered significant ($P < 0.05$). Experiments were run in octuplet, and each experiment was repeated three times.

Apoptosis

Apoptosis was investigated by TUNEL assay. Cells (2×10^6) were cultured in normal medium or in medium containing single drugs or a combination, used at peak plasma concentrations. At the end of drug exposure (72 h), cells were fixed in 1% paraformaldehyde in PBS on ice for 15 min, suspended into ice-cold ethanol (70%) and stored overnight at −20°C. Cells were then washed twice in PBS and incubated with 50 μl of solution containing TdT and FITC-conjugated dUTP deoxynucleotides (1 : 1; Roche Diagnostic GmbH, Mannheim, Germany) in a humidified atmosphere for 60 min at 37°C in the dark. Samples were then washed with PBS containing 0.1% Triton X-100, counterstained with 3 μg/ml of propidium iodide (Sigma Aldrich) and 10 Kunits/ml of RNase (Sigma-Aldrich) for 30 min at 4°C in the dark, and finally analyzed by flow cytometry using a FACS Vantage

flow cytometer (Becton Dickinson, San Jose, California, USA). Data acquisition and analysis were performed using CELLQuest software (Becton Dickinson). For each sample, 10 000 events were recorded.

Hoechst test

Cell lines in exponential growth were trypsinized and resuspended at $0.5–1.0 \times 10^6$ cells/ml in complete RPMI (Sigma-Aldrich). Hoechst (Hoechst Invitrogen, Carlsbad, California, USA) was added to a final concentration of 5 mg/ml and cells were incubated for 45 min in a 37°C water bath. Cells were then washed in cold Hanks balanced saline solution/1 mmol/l HEPES/2% FCS (HBSS1; Euroclone, Milan, Italy), resuspended in warm (37°C) medium and incubated for a 45-min efflux period, which served to remove membrane-associated fluorescence and to reduce background staining. The samples were finally analyzed by flow cytometry.

siRNA knockdown

Three different double-strand siRNA oligonucleotides (Invitrogen) directed against BCRP I mRNA were used alone or pooled together. A validated medium GC scramble (SCR) double-strand siRNA oligonucleotide (Invitrogen) was used as control for transfection. The pooled siRNA oligonucleotides, used at a concentration of 30 nmol/l, showed the highest BCRP I mRNA knockdown in the BCR230 cell line. Transfections were carried out using lipofectamine RNAiMAX (Invitrogen) and Opti-MEM I (Invitrogen) medium without antibiotics in 25-cm^2 flasks and then supplemented with 18×10^5 cells (60% confluence) diluted in complete medium without antibiotics, as described for reverse transfections of RNAiMax (Invitrogen). Cells were incubated with oligonucleotide/lipofectamine RNAiMAX complexes for 72 h at 37°C.

RNA extraction and amplification

Total RNA was extracted from cell lines by RNA easy mini kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. One microgram of RNA was reverse transcribed using the iScript cDNA Synthesis Kit (BioRad, Hercules, California, USA) in a final volume of 20 µl. The reaction was carried out at 42°C for 30 min and was stopped by heating to 85°C for 5 min. Real-time PCR was performed with MyiQ Single Color Real Time PCR Detection System (BioRad) using SYBR Green I dye chemistry. β2-microglobulin mRNA was amplified as control to verify the quality and quantity of input RNA.

Primers used for β2-microglobulin and BCRP I amplification were designed using Beacon Designer Software (version 4, BioRad). Primer sequences were: forward 5′-CGC TAC TCT CTC TTT CTG GC-3′ and reverse 5′-AGA CAC ATA GCA ATT CAG GAA AT-3′ for β2-microglobulin; and forward 5′-AGA TGCATA TTC GGA CCC ACA-3′ and reverse 5′-CCT CAT GTT TGT GCA GGA GAG-3′ for BCRP I. Real-time PCR reactions were carried out in triplicate in a volume of 25 µl containing 50 ng of cDNA template, 1 × SYBR Green Mix, and 200 or 400 nmol/l of forward and reverse primers (for β2-microglobulin and BCRP I, respectively). Samples were heated to 95°C for one-and-a-half minute, followed by 30 amplification cycles for 15 s at 95°C and for 30 s at 60°C (for β2-microglobulin) or 61°C (for BCRP I) for annealing and amplification. Product purity was controlled by melting point analysis. The amount of BCRP I mRNA was normalized to the endogenous reference β2-microglobulin and expressed as *n*-fold BCRP I mRNA levels relative to SCR of each experiment using Gene Expression Macro Software (Version 1.1, BioRad).

Western blot

Cell proteins were isolated by lysing cells in 50 mmol/l Tris–HCl (pH 8.0), 150 mmol/l NaCl, 1% Triton X-100 and 0.1% SDS supplemented with 1 mmol/l phenylmethylsulfonylfluoride/protease inhibitor mixture (Sigma-Aldrich). Cells were incubated in ice for 1 h and centrifuged at 9500*g* to obtain cell protein supernatant. Cellular proteins were denaturated, separated on 10% SDS–polyacrylamide gel and then electroblotted onto Hybond-C extra membrane (Amersham Biosciences, Piscataway, New Jersey, USA). The membrane was stained with Ponceau S (Sigma-Aldrich) to verify equal amounts of sample loading and then incubated for 2 h at room temperature with T-PBS 5% non-fat dry milk. After that, the membrane was probed overnight at 4°C with the primary antibody and then with a horseradish peroxidase-conjugated secondary antibody diluted 1 : 1000 (Dako Corporation, Glostrup, Denmark). The bound antibodies were detected by enhanced chemiluminescence using an ECL kit (Amersham Biosciences). The following primary antibodies were used: anti-caspase 3, rabbit polyclonal antibody (1 : 500), anti-caspase 7, rabbit polyclonal antibody (1 : 500), anti-caspase 9, rabbit polyclonal antibody (1 : 500), anti-phospho-Bad^{Ser112}, mouse monoclonal antibody (1 : 1000), anti-HER2, rabbit polyclonal antibody (1 : 1000), anti-MEK 1/2, rabbit polyclonal antibody (1 : 1000), anti-phospho-MEK 1/2 (Ser217/221), rabbit polyclonal antibody (1 : 1000), anti-p44/42MAP, rabbit polyclonal antibody (1 : 1000), anti-survivin, mouse monoclonal antibody (1 : 1000), anti-phospho-p44/42MAP, rabbit polyclonal antibody (1 : 1000) (Cell Signaling Technology, Danvers, Massachusetts, USA); anti-p21, mouse monoclonal antibody (1 : 100) (NeoMarkers, Fremont, California, USA); anti-caspase 8, mouse monoclonal antibody, 1 : 500 (Alexis Biochemicals, San Diego, California, USA); anti-phospho-HER2 (Tyr 1248), rabbit polyclonal antibody (1 : 1000), anti-EGFR, mouse monoclonal antibody, 1 : 500, anti-phospho-EGFR (Tyr 1173), rabbit monoclonal antibody, 1 : 1000 (Upstate Biotechnology, Charlottesville, Virginia, USA), anti-p-Tyr (PY99), mouse monoclonal antibody, dilution 2 µg of antibody per 500 µg of total protein rabbit polyclonal antibody (1 : 1000)

(Santa Cruz Biotechnology, Santa Cruz, California, USA); and anti-actin, rabbit polyclonal antibody, dilution 1 : 5000 (Sigma-Aldrich).

Immunoprecipitation

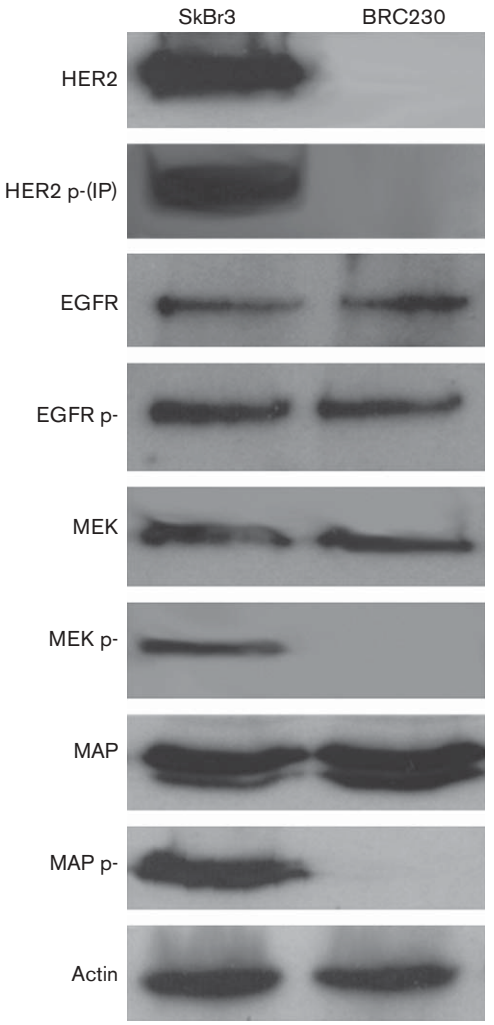
Cells (2×10^6) were lysed with RIPA lysis buffer (Santa Cruz Biotechnology) and by repeated aspiration through a 21-gauge needle. Supernatant was obtained from cells lysed after centrifugation at 10000*g* for 10 min at 4°C. Proteins (750 µg) were incubated for 2 h with 1.5 µg of anti-HER2 antibody (BD Pharmingen, San Diego, California, USA) followed by 30 µl of protein A/G agarose beads (Santa Cruz Biotechnology) for 2 h. The immunoprecipitates were pelleted and washed four times with RIPA lysis buffer. The captured immune complexes were subjected to immune-blot analysis, as described above.

Results

Biomolecular cell line profiles

The presence of lapatinib-specific targets, HER2 and EGFR, and their phosphorylated forms were detected in SkBr3 cells, whereas only EGFR expression and its phosphorylated form were present in BRC-230 cell line. Moreover, while both cell lines were characterized by MAP and MEK expression, the phosphorylated forms of the two proteins were only present in SkBr3 (Fig. 1).

Fig. 1



Basal biomolecular profiles of BRC230 and SkBr3 cell lines. IP, immunoprecipitation; p-, phosphorylation.

Cytotoxic activity of single drugs and drug combinations

Lapatinib did not exhibit a cytostatic or cytotoxic effect on BRC230 cells at any of the tested concentrations, whereas a cell-killing effect was observed in SkBr3 starting from a 0.3-µmol/l drug concentration (Fig. 2). Caelyx activity was modulated as a function of the cell line and drug concentration used (Fig. 2). Specifically, the drug produced a cytostatic, dose-dependent effect in BRC230 cell line, which became cytocidal at the peak plasma level (50 µmol/l) concentration. In SkBR3 cell line, caelyx was more cytotoxic, inducing a cytocidal effect starting at the concentration corresponding to one-tenth of the peak plasma level (5 µmol/l). Drug interaction analysis showed an antagonistic effect following the sequence caelyx→lapatinib (RI = 0.1) and also the inverse sequence, lapatinib→caelyx (RI = 0.3) in SkBr3. In BRC230, both sequences produced an additive effect [caelyx→lapatinib (RI = 0.8), lapatinib→caelyx (RI = 0.8)]. Simultaneous exposure to the two drugs showed an additive effect (RI = 0.7) in SkBr3, whereas a synergistic interaction was observed in BRC230 cell line (RI = 1.6). On the basis of these results, we investigated only the effects induced by simultaneous exposure in both cell lines.

Apoptosis

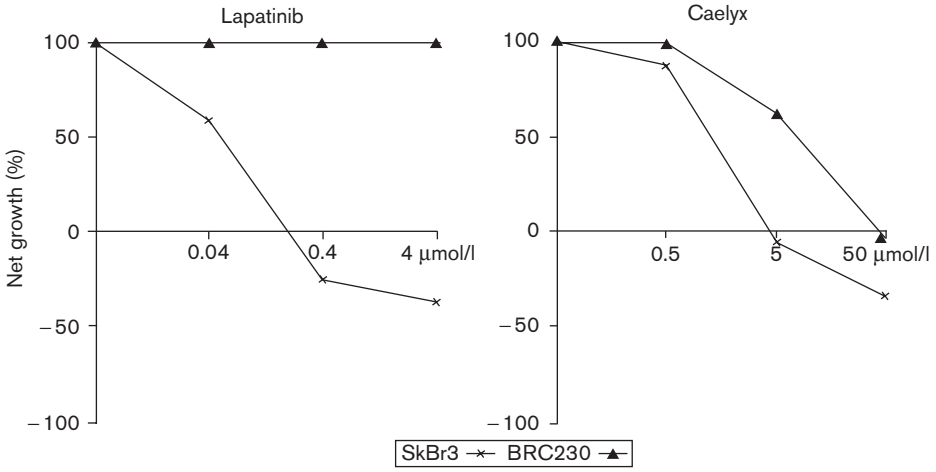
Apoptosis, evaluated by TUNEL assay, showed a higher percentage of apoptotic cells in SkBr3 than in BRC-230 after lapatinib treatment (15 vs. 5%) and a similar rate (18 vs. 15%) after exposure to caelyx. Simultaneous treatment with the two drugs increased the percentage of apoptotic cells in both the lines (SkBr3, 35% and BRC230, 30%; Fig. 3).

Modulation of apoptosis markers

In SkBr3 cell line, lapatinib induced the activation of caspase 3, 7, and 8 (the last to a lower degree), suppressed survivin expression, and had no effect on p21 expression. Exposure to caelyx resulted in the upregulation of p21 and caspase 3, 7, and 8 activation. Simultaneous treatment produced p21 upregulation, caspase 3, 7, and 8 activation, increased BAD^{Ser112} dephosphorylation and decreased survivin expression (Fig. 4).

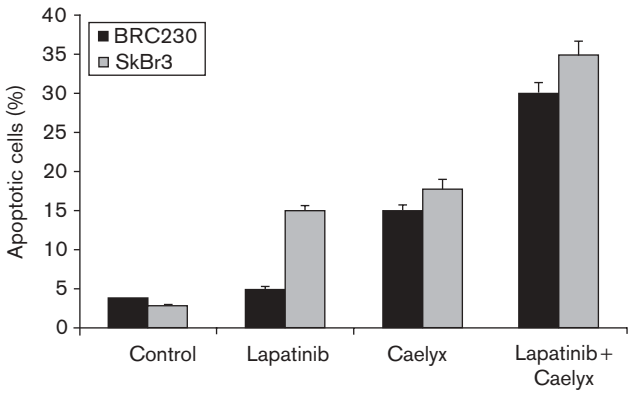
In BRC-230 cell line, lapatinib caused only a slight upregulation of p21 and had no effect on survivin, whereas caelyx resulted in the upregulation of p21 and

Fig. 2



Dose–response curves following a 72 h exposure to single-agent lapatinib and caelyx.

Fig. 3



Apoptosis evaluated by TUNEL assay after a 72 h treatment.

caspase 3, 7, 8, and 9 activation. Similar marker modulation and decreased BAD^{Ser112} dephosphorylation were observed after the drug combination schedule (Fig. 4).

Modulation of proliferation markers

In the SkBr3 HER2-overexpressing cell line, lapatinib caused the dephosphorylation of human HER2 and a reduction in EGFR, MEK, and MAP phosphorylation levels, whereas caelyx did not modify marker phosphorylation. Simultaneous treatment with the two drugs reproduced the pattern observed after lapatinib-only exposure (Fig. 5). In the nonexpressing-HER2 cell line, BRC230, single-drug or combination treatment did not induce any modulations in EGFR, MAP, or MEK phosphorylation (data not shown).

Pump efflux functionality and siBCRP I transfection

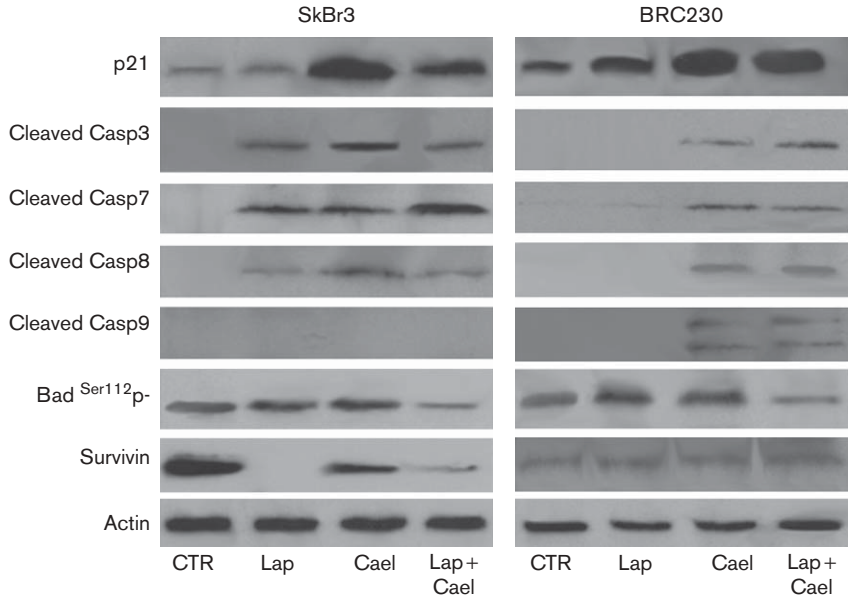
The flow cytometric Hoechst test showed that the efflux pumps are basally active in BRC230 and not in SkBr3 cell line. Treatment of the BRC230 cell line with lapatinib (4 µmol/l) for 72 h induced a blockage of efflux pumps (Fig. 6). To better evaluate the role of the BCRP I efflux pump, BCR230 cells were exposed to three siRNA double-strand BCRP I-specific short-interfering oligonucleotides (siBCRP I). A marked downregulation of BCRP I mRNA was observed after a 72 h exposure to siBCRP I with respect to that observed after exposure to an appropriate oligonucleotide control (scramble, siCTR) (Fig. 7a). A similar number of Hoechst-negative cells were observed when lapatinib was administered at a concentration of 4 µmol/l for 72 h (Fig. 6) and when siBCRPI was transfected for 72 h (Fig. 7b) (6.43% lapatinib vs. 6.77% siBCRP I).

Discussion

Given the role of ErbB family tyrosine kinases, EGFR and HER2, in breast cancer biology, treatments that inhibit both kinases are widely considered to be more effective than those directed at either kinase [1–5]. As lapatinib is a dual inhibitor of EGFR and HER2, it is considered a strong inhibitor of cell growth and an inducer of cell death, as shown *in vitro* and *in vivo* in several histotypes, including human breast cancers [6–8].

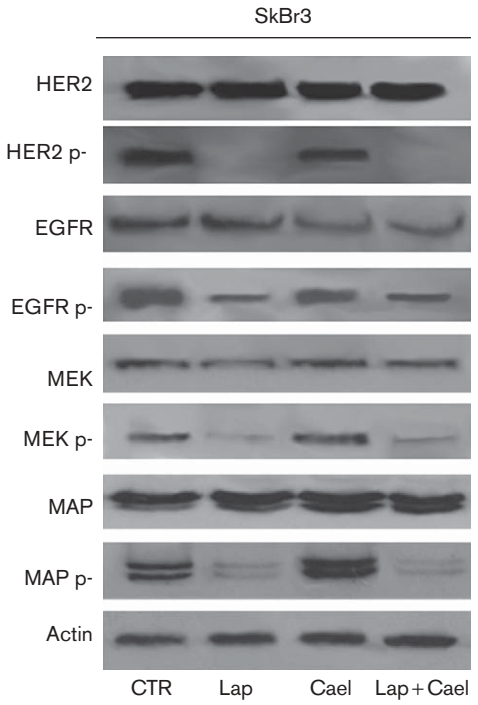
Results from a recent study [26] reported a direct relationship between the antiproliferative activity of lapatinib and HER2 or EGFR expression in breast cancer cell lines. Moreover, the study showed that lapatinib was more effective in inhibiting cell growth and inducing cell

Fig. 4



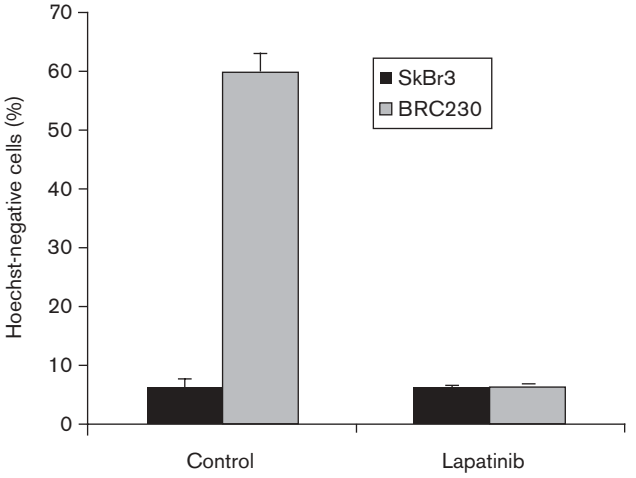
Modulation of apoptotic markers after a 72 h exposure to drugs. Casp, caspase; Cael, caelyx; Lap, lapatinib; p-, phosphorylation.

Fig. 5



Modulation of proliferation markers in SkBr3 cell line. Cael, caelyx; Lap, lapatinib; p-, phosphorylation.

Fig. 6

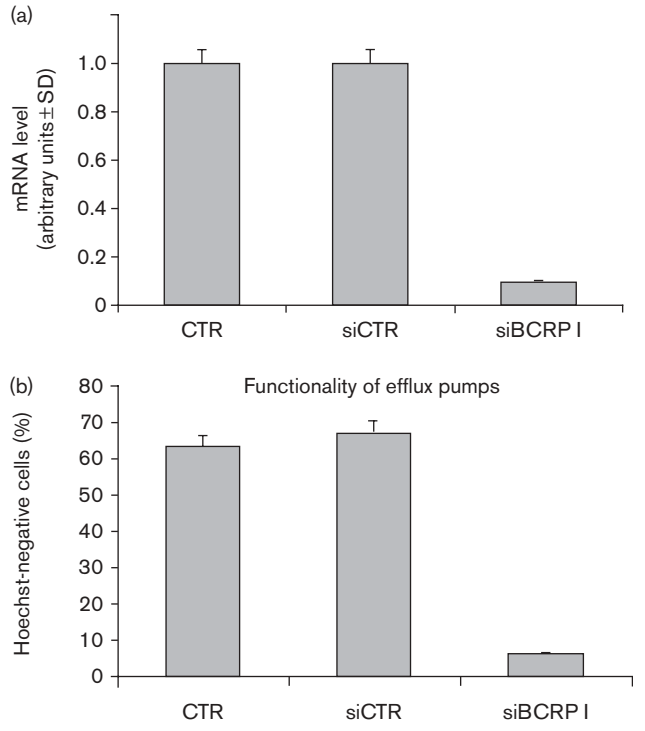


Pump activity in control and tumor cells treated for 72 h with lapatinib (4 μmol/l).

death in breast cancer cell lines overexpressing HER2. These results were recently confirmed at clinical level in a number of trials [11,27,28].

In this study, we used two breast cancer cell lines in parallel: SkBr3, which expresses EGFR receptor and overexpresses HER2 receptor, and BRC230, which expresses EGFR, but not HER2. As expected, lapatinib induced cytotoxicity in SkBr3, but not in BRC230 at peak plasma level, confirming the primary importance of HER2 receptor for lapatinib activity. In fact, we observed a dramatic reduction in HER2 phosphorylation, a modulation of EGFR/MEK/MAPK pathways, and also the complete disappearance of survivin, a member of the

Fig. 7



(a) mRNA levels by real-time PCR in control, siCTR-treated and siBCRP I-treated BRC230 cell line. (b) Functionality of efflux pump evaluated by Hoechst test in control, siCTR-treated and siBCRP I-treated BRC230 cell line. The figure shows the functionality of the efflux pump using the Hoechst test where cells without Hoechst coloring (negative) have efflux pump activity, whereas cells with Hoechst coloring (positive) indicate a block of the efflux caused by siRNA knockdown of BCRP I.

antiapoptotic protein family, which is widely expressed in a variety of human cancers and inhibits caspase activation. It has been suggested that survivin positivity, detected by immunohistochemical staining, may be correlated with HER2 and EGFR expression through ERK activation and phosphatidylinositol-3-kinase signaling [29]. Our data support this hypothesis in that we detected high basal levels of survivin and its subsequent modulation only in HER2-overexpressing Skbr3 cells. Conversely, in the HER2-negative cell line BRC230, lapatinib did not cause caspase pathway activation or modify survivin expression.

Caelyx treatment was cytotoxic in both cell lines, inducing a similar percentage of apoptotic cells. The drug determined caspase activation and p21 upregulation in both cell lines, and survivin downregulation in SkBr3 cells.

p21 (WAF1/Cip1), initially identified as a cell cycle regulatory protein that can cause cell cycle arrest, is induced by both p53-dependent and p53-independent mechanisms. As an inhibitor of cell proliferation, p21 plays an important role in drug-induced tumor suppression. However, a number of recent studies have shown that p21 can take up both proapoptotic and antiapoptotic functions in response to antitumor agents, depending on the cell type and cellular context [30]. In our study, the protein seems to have been modulated in different ways as a function of cell line and drug treatment. Its level of expression in SkBr3 was unmodified after exposure to lapatinib and upregulated after caelyx and combination treatment. In BRC230, p21 was upregulated after both caelyx and combination treatment.

We also investigated the molecular mechanisms underlying the activity of simultaneous drug treatment, which was additive in SkBr3 and synergistic in BRC230. As the percentage of apoptotic cells induced by simultaneous treatment was similar in the two cell lines (35% in SkBr3 and 30% in BRC230), treatment resulted additive in HER2-positive cells and synergistic in the HER2-negative cell line. We can speculate that the apoptotic SkBr3 cells observed after combination treatment derived from a modulation of markers similar to that observed after exposure to single-agent lapatinib, but with p21 upregulation and decreased BAD^{Ser112} dephosphorylation. Conversely, in BRC230 cells, as combination treatment resulted in apoptotic marker modulation similar to that observed after caelyx treatment, with a further decrease in BAD^{Ser112} dephosphorylation, we also detected a second apoptotic mechanism involved in the synergistic activity of the drug combination. In fact, as efflux pumps were not functional in the HER2-positive cell line, we observed that lapatinib induced BCRP I efflux pump blockage in Pgp-negative and HER2-negative BRC230 cells, preventing the efflux of caelyx from cells and resulting in a higher cytotoxic effect. This hypothesis was supported by results recently obtained in in-vivo experimental models [14].

In conclusion, the lapatinib/caelyx combination in our study produced, *in vitro*, an additive effect in HER2-overexpressing breast cancer cells and a synergistic effect in HER2-negative breast cancer cell lines. The activity of this treatment combination seems to be because of the capacity of lapatinib to overcome multidrug resistance through the targeting of the efflux pump BCRP I. These results suggest the potential clinical usefulness of the lapatinib/caelyx combination in patients with BRCP I-expressing HER2-negative breast cancer.

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