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► **To cite this version:**

Amelia d'Alessio, Antonella Luca, Monica R. Maiello, Luana Lamura, Anna Maria Rachiglio, et al..
Effects of the combined blockade of EGFR and ErbB-2 on signal transduction and regulation of cell
cycle regulatory proteins in breast cancer cells. *Breast Cancer Research and Treatment*, 2009, 123 (2),
pp.387-396. 10.1007/s10549-009-0649-x . hal-00509362

HAL Id: hal-00509362

<https://hal.science/hal-00509362>

Submitted on 12 Aug 2010

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Effects of the combined blockade of EGFR and ErbB-2 on signal transduction and regulation of cell cycle regulatory proteins in breast cancer cells

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Running title: *EGFR and ErbB-2 blockade in breast cancer cells*

Abstract

Purpose: Treatment of breast cancer cells with a combination of the EGFR-tyrosine kinase inhibitor gefitinib and the anti-ErbB-2 monoclonal antibody trastuzumab results in a synergistic anti-tumor effect. In this study, we addressed the mechanisms involved in this phenomenon.

Methods: The activation of signalling pathways and the expression of cell cycle regulatory proteins were studied in SK-Br-3 and BT-474 breast cancer cells following treatment with EGFR and/or ErbB-2 inhibitors.

Results: Treatment with the gefitinib/trastuzumab combination produced, as compared with a single agent, a more prolonged blockade of AKT and MAPK activation, a more pronounced accumulation of cells in the G0/G1 phase of the cell cycle, a more significant increase in the levels of p27^{kip1} and of hypophosphorylated pRb2, and a decrease in the levels of Cyclin D1 and survivin. Similar findings were observed with the EGFR/ErbB-2 inhibitor lapatinib. Gefitinib, trastuzumab and their combination increased the stability of p27^{kip1} with the combination showing the highest effects. Blockade of both receptors with gefitinib/trastuzumab or lapatinib induced a significant increase in the levels of p27^{kip1} mRNA and in the nuclear levels of the p27^{kip1} transcription factor FKHRL-1. Inhibition of PI3K signalling also produced a significant raise in p27^{kip1} mRNA. Finally, down-modulation of FKHRL-1 with siRNAs prevented the lapatinib-induced increase of p27^{kip1} mRNA. **Conclusions:** The synergism deriving from EGFR and ErbB-2 blockade is mediated by several different alterations in the activation of signalling proteins and in the expression of cell cycle regulatory proteins, including transcriptional and post-transcriptional regulation of p27^{kip1} expression.

Key words: EGFR; ErbB-2; breast cancer; cell cycle

Introduction

It has long been established that the epidermal growth factor receptor (EGFR, ErbB-1, HER1) and ErbB-2 (HER2, neu) cooperate in inducing oncogenic transformation. For example, simultaneous overexpression of both p185c-neu and EGFR was required to induce transformation in NIH:3T3 cells [1]. In addition, mammary tumors derived from transgenic mice engineered to overexpress neu also exhibited co-overexpression of endogenous EGFR [2]. Similarly, a synergistic interaction of the *neu* proto-oncogene and the EGFR ligand transforming growth factor- α (TGF α) in inducing transformation in the mammary epithelium of transgenic mice has also been demonstrated [3]. Finally, treatment of ErbB-2 transgenic mice with an EGFR tyrosine kinase inhibitor such as gefitinib efficiently prevented the formation of mammary tumors [4].

Co-expression of EGFR and ErbB-2 has been observed in 10-36% primary human breast carcinomas, and it is generally associated with a poor prognosis as compared with expression of a single receptor [5-7]. DiGiovanna et al. [8] found EGFR expression in only 15% of 807 invasive breast cancers. However, the majority of EGFR-positive tumors (87%) were found to co-express ErbB-2. More importantly, almost all tumors that expressed the phosphorylated form of ErbB-2, co-expressed EGFR, and expression of phosphorylated ErbB-2 or co-expression of ErbB-2 and EGFR was associated with the shortest survival in cancer patients. These data clearly establish a link *in vivo* between expression of EGFR and activation of ErbB-2 in breast cancer patients. In agreement with these findings, co-expression of EGFR, ErbB-2 and ErbB-3 was also found to have a negative synergistic effect on patient outcome, independent of tumor size or lymph node status [9].

Different anti-EGFR agents, including the EGFR tyrosine kinase inhibitor (EGFR-TKI) gefitinib (ZD1839, Iressa), have shown to inhibit the growth of human breast carcinoma cells [10-14]. We and other research groups have demonstrated that breast cancer cell lines with high levels of expression of ErbB-2 are more sensitive to gefitinib as compared with ErbB-2-negative cells [11-14]. However, clinical studies of gefitinib in breast cancer resulted in few clinical responses and in a disease control rate of approximately 10% [15-17]. ErbB-2-positive breast cancer patients enrolled in trials of gefitinib failed to respond, suggesting that ErbB-2-positive tumor cells might acquire resistance to anti-EGFR agents [17-19]. In this regard, Sergina et al. [20] have recently demonstrated that breast cancer cells that co-express EGFR, ErbB-2 and ErbB-3 escape the activity of EGFR-TKIs through an ErbB-3/PI3K/AKT pathway. Interestingly, they found that ErbB-3 activation was dependent on ErbB-2, whose residual kinase activity was able to drive ErbB-3 phosphorylation. Interestingly, acquired resistance to anti-ErbB-2 agents can occur through different mechanisms including increased activation of EGFR signalling [21], suggesting that a

cross-talk between these two receptors might limit the activity of target based agents directed against either the EGFR or ErbB-2 in breast cancer patients.

We and other groups have previously demonstrated that a synergistic anti-tumor effect occurs when breast cancer cells that co-express EGFR and ErbB-2 are treated with a combination of gefitinib and the anti-ErbB-2 monoclonal antibody trastuzumab [11-13]. **In particular, we demonstrated that the two drugs have a strong synergism in breast cancer cells by using a wide range of concentrations of both drugs and isobolographic analysis [13].** Such combination failed to show significant activity in a phase I/II clinical trial [22]. However, patients enrolled in this trial were not selected for expression of the EGFR, since the aim of this study was to evaluate the ability of gefitinib to prevent the occurrence of resistance to trastuzumab in ErbB-2 positive patients. Contemporary targeting of both EGFR and ErbB-2 can be also accomplished by using tyrosine kinase inhibitors that block the activity of different ErbB-receptors. In this regard, lapatinib is a dual EGFR/ErbB-2 tyrosine kinase inhibitor that has been recently approved for treatment of ErbB-2 expressing patients [21].

In this paper we investigated the molecular mechanisms involved in the synergism of combined blockade of EGFR and ErbB-2 by using either a combination of gefitinib and trastuzumab or the dual inhibitor EGFR/ErbB-2 inhibitor lapatinib. In particular, the effects of blockade of EGFR and/or ErbB-2 on the activation of signal transduction pathways and on the expression of cell cycle regulatory proteins were assessed.

Materials and methods

Materials

Gefitinib was kindly provided by AstraZeneca (Macclesfield, UK); trastuzumab (Herceptin) was purchased from Roche (Milan, Italy); lapatinib was obtained from Glaxo Smith Kline; cycloheximide was purchased from Sigma (Saint Louis, Missouri USA); U0126 and LY294002 were purchased from Promega (Madison, WI).

Cell Culture

The human breast cancer cell lines SK-Br-3 and BT-474 were obtained from the American Type Culture Collection (Rockville, MD, USA) and were cultured as previously described [13].

Small Interfering RNA

ON-TARGET plus SMARTpool against human FKHRL1 (containing four individual siRNA motifs: GCACAGAGUUGGAUGAAGU, GUACUCAACUAGUGCAAAC, CGAAUCAGCUGACGACAGU, UAACUUUGAUUCCCUCAUC) and control non targeting siRNA were purchased from Dharmacon Inc. (Lafayette, CO). Transfections were performed by using 50 nM siRNA and the INTERFERinTM siRNA transfection reagent (Polyplus transfection).

Protein extraction and western blot analysis

Cells were seeded in 100 mm dishes (1 x 10⁶ SK-Br-3 cells; 2 x 10⁶ BT-474 cells) and treated at time 0 with gefitinib (2 μM SK-Br-3 cells; 1 μM BT-474 cells), trastuzumab (20 μg/ml) or lapatinib (140 nM). For experiments of protein stability, cells were also treated with 10 μM cycloheximide for 2, 4 and 8 hours. Whole protein extraction and western blot analysis were performed as previously described [13]. Nuclear or cytoplasmic proteins were differentially extracted by lysing cells in ice cold hypotonic buffer (0,2% NP-40, 10 mM HEPES, pH 7.9, 1 mM EDTA, 60 mM KCl). Nuclei were separated through a 30% sucrose cushion and lysed in hypertonic buffer (250 mM Tris-HCl, pH 7.8, 60 mM KCl). Primary antibodies used for western blotting analysis were: rabbit polyclonal anti-p27^{kip-1}, anti cyclin D1 and anti-Sp1 (PEP 2) (Santa Cruz); **rabbit monoclonal anti-phospho-HER2/ErbB2 (Tyr1221/1222) (6B12)**, rabbit polyclonal **anti-phospho-EGF Receptor (Tyr 1068)**, anti-p42/44 and -phospho-p42/44 (Thr202/Tyr204) MAPK and anti-AKT and -phospho-Akt (Ser 473) (Cell Signaling); rabbit polyclonal anti-human survivin (R&D); mouse monoclonal anti-human Rb2 (BD Biosciences); rabbit polyclonal anti-FKHRL1 (U.B.I.); mouse monoclonal anti-

tubulin- α Ab-2 clone DM1A (Lab Vision Co. USA). Densitometric analysis of the blots was performed by using the NIH Image 1.62f software.

Flow cytometric analysis

Cells were trypsinized, washed two times with ice-cold PBS, and then re-suspended on vortex in 1 ml PBS plus 3 ml ice-cold 95% EtOH. Then cells were incubated in 1 ml of propidium iodide staining solution (50 μ g/ml propidium iodide, 0,5 mg/ml RNase, 0,2% NP-40). After 30 min at room temperature in the dark, the DNA content was analyzed using a FACScan flow cytometer (Becton Dickinson, San Jose, CA). Cell cycle data analysis was performed by the CellFIT program (Becton Dickinson).

Real-time PCR

Total cellular RNA extraction and reverse transcriptase reaction were performed as previously described [23]. Real Time PCR for p27^{kip1} was carried out with a 7900HT ABI PRISM Instrument (Applied Biosystems) using the SybrGreen PCR Master Mix (Applied Biosystems) and the RT2 qPCR Primer Assays SybrGreen Human CDKN1B (p27) and Human GAPDH (Superarray, Frederick, MD, USA). Relative quantification of p27 transcript was performed by using the $\Delta\Delta$ Ct method as previously described [23].

Results

We explored the molecular mechanisms involved in the synergism of the EGFR tyrosine kinase inhibitor gefitinib and the anti-ErbB-2 humanized antibody trastuzumab in the breast cancer cell lines SK-Br-3 and BT-474, which overexpress both EGFR and ErbB-2. In these experiments we used concentrations of gefitinib (1 or 2 μM) and trastuzumab (20 $\mu\text{g}/\text{ml}$) that have shown to produce a significant synergism in both breast cancer cell lines [13]. We and other groups have previously demonstrated that at the above mentioned concentrations gefitinib does not directly affect the kinase activity of ErbB-2 in live cells [11, 13].

Effects of treatment with gefitinib and trastuzumab on the activation of ErbB receptors and MAPK and AKT signalling

In preliminary experiments, we assessed the effects of treatment with gefitinib, trastuzumab and the combination of the two drugs on the activation of both EGFR and ErbB-2. In agreement with previous findings, we found that treatment with gefitinib reduced the activation of both EGFR and ErbB-2 in the SK-Br-3 and BT-474 breast cancer cell lines [10, 11, 13] (Supplementary Figure 1). As previously reported, trastuzumab treatment produced a reduction in the levels of activation of ErbB-2 that was more pronounced in BT-474 cells as compared with SK-Br-3 cells [24]. The effects of the combination of the two agents on the activation of the EGFR were comparable to gefitinib, whereas a more pronounced reduction in the levels of activation of ErbB-2 was found as compared with each single agent.

We have previously demonstrated that treatment with gefitinib and trastuzumab, alone or in combination, reduces the activation of intracellular transducers of growth factor signalling, such as p42/p44 MAP kinases (MAPK) and AKT in both breast carcinoma cell lines [13]. We hypothesized that the combination of the two drugs might lead to a more prolonged reduction of the activation of both signalling pathways. Therefore, we performed a time course experiment in order to evaluate differences between treatment with a single agent and the combination of gefitinib and trastuzumab on the activation of these pathways. In these experiments, cells were treated once and proteins were extracted at the indicated times.

We found that treatment of SK-Br-3 and BT-474 cells with gefitinib at concentrations of 2 μM and 1 μM , respectively, produced a significant reduction in the levels of activation of MAPK and AKT at 6 hours from treatment. After 24 and 48 hours, the levels of phosphorylation of both kinase gradually raised. In contrast, treatment with 20 $\mu\text{g}/\text{ml}$ trastuzumab had little effect on MAPK activation in SK-Br-3 cells, whereas it led to a gradual reduction of MAPK phosphorylation in the

BT-474 cell line. In both cell lines, trastuzumab produced a significant reduction of AKT activation that reached a maximum at 6 hours or 24 hours from treatment in BT-474 and SK-Br-3 cells, respectively. Combined treatment with gefitinib and trastuzumab produced a more significant and prolonged reduction of MAPK and AKT signaling in both breast cancer cell lines (Figure 1). Treatment with gefitinib, trastuzumab or the combination had no effects on endogenous levels of p42/p44 MAPK and AKT proteins in both cell lines (Figure 1).

Cell cycle distribution of breast cancer cells following treatment with gefitinib and trastuzumab

We analyzed the cell cycle distribution of the SK-Br-3 and BT-474 cell lines following treatment with gefitinib and trastuzumab, alone or in combination, at the above mentioned concentrations for 24 and 48 hours. The combination of the two drugs produced a more significant accumulation of cells in the G0/G1 phase of the cell cycle and reduction of cells in the S-phase, as compared with treatment with a single drug. Although such difference was evident after 24 hours, it became more significant at 48 hours of treatment (Figure 2 and data not shown). In agreement with previous findings, a sub-G0/G1 peak, suggestive of apoptosis, was evident in cells treated with the combination but not with gefitinib or trastuzumab alone (Figure 2).

Effects of treatment with gefitinib and trastuzumab on cell cycle regulatory proteins in breast cancer cells

We next determined by western blot analysis the expression of p27^{kip1} in whole protein lysates, and in cytosolic and nuclear fractions from untreated and treated breast cancer cells. The combination of gefitinib plus trastuzumab produced a bigger increase in the levels of p27^{kip1} in all protein extracts, as compared with treatment with a single drug (Figure 3 and data not shown). No significant effects were observed on p21^{cip1} expression (data not shown). Treatment of SK-Br-3 and BT-474 cells with gefitinib and trastuzumab also affected the expression of other proteins involved in cell cycle progression and apoptosis. In particular, a significant reduction of cyclin D1 and survivin levels and an increase of hypo-phosphorylated forms of p130Rb2 (pRb2) were observed in treated cells as compared with untreated cells, with the combination of drugs having a more pronounced effect at 48 h of treatment (Figure 3). **These experiments were repeated three times and we always observed a more pronounced effect of the combination as compared with each single agent.**

Molecular mechanisms of p27^{kip1} induction: protein stabilization and increase of p27^{kip1} mRNA

In order to investigate the mechanisms by which treatment with gefitinib and trastuzumab leads to the increase of p27^{kip1} expression, we evaluated the effects of these drugs on p27^{kip1} protein stability.

We exposed SK-Br-3 cells to cycloheximide, an inhibitor of protein synthesis, following 48 hours of treatment with the different drugs. All treatments increased the stability of p27^{kip1} protein. However the combination showed higher effects as compared to treatments with a single drugs, and gefitinib had higher effects than trastuzumab (gefitinib plus trastuzumab>gefitinib>trastuzumab) (Figure 4). **Similar findings were obtained in BT-474 cells in which trastuzumab and gefitinib produced analogous effects on p27^{kip1} protein stability, whereas the combination showed higher effects as compared with each drug alone (Supplementary Figure 2).**

We next analysed the effects of EGFR and/or ErbB-2 blockade on the expression of p27^{Kip1} mRNA, by using quantitative REAL TIME PCR. A significant increase in the levels of p27^{kip1} mRNA was found in SK-Br-3 cells treated with gefitinib plus trastuzumab for 48 hours as compared with untreated cells or cells treated with a single drug (Figure 5b). Similar findings were obtained in BT-474 cells (Figure 5c).

Analysis of the nuclear fraction from SK-Br-3 and BT-474 cells revealed that treatment with the combination produced a more pronounced increase in the nuclear levels of the p27^{kip1} transcription factor FKHRL1 (forkhead transcription factor in rhabdomyosarcoma-like 1, FOXO3a), as compared with gefitinib or trastuzumab alone (Figure 5a). AKT phosphorylates FKHRL-1 thus preventing its migration to the nucleus [25]. In agreement with these findings, blockade of AKT signalling with the PI3K inhibitor LY294002 produced a significant increase in the levels of p27^{kip1} mRNA in SK-Br-3 cells (Figure 5d). In contrast, treatment with the MEK inhibitor U0126 had no significant effects on p27^{kip1} mRNA levels (Figure 5d).

Effects of treatment with lapatinib on cell cycle regulatory proteins in SK-Br-3 cells

We next evaluated the effects of lapatinib on the above described mechanisms. Treatment of SK-Br-3 cells with lapatinib produced a significant increase in the levels of p27^{kip1} and of hypophosphorylated pRb2, and a significant decrease in the levels of cyclin D1 and survivin, similar to the effects of the gefitinib/trastuzumab combination (Figure 6).

More importantly, an increase in p27^{kip1} mRNA (Figure 7a) and in the nuclear levels of FKHRL-1 (Figure 7b) were also observed following treatment of SK-Br-3 cells with lapatinib. Therefore, we examined the effect of siRNA-mediated FKHRL-1 silencing on p27^{Kip1} mRNA induction by lapatinib in SK-Br-3 cells. As shown in figure 7c, transfection of SK-Br-3 cells with 50 nM of specific FKHRL-1 siRNAs produced a significant reduction of FKHRL-1 protein expression. The FKHRL-1 siRNAs, but not control non targeting siRNAs, significantly reduced the ability of lapatinib to induce an increase of p27^{kip1} mRNA in SK-Br-3 cells (Figure 7d). A slight effect of the

FKHRL-1 siRNAs on the basal levels of p27^{Kip1} mRNA in untreated cells was also observed. These results demonstrate that lapatinib-induced p27^{Kip1} mRNA expression is mediated by FKHRL-1.

Discussion

Breast cancer cells that overexpress ErbB-2 are dependent on the activation of this oncogene for their proliferation and survival and are significantly inhibited by agents directed against ErbB-2. Although spontaneous activation of ErbB-2 might occur in cells that overexpress the receptor, transactivation by other receptors of the same family such as EGFR and ErbB-3 might lead to its phosphorylation [26]. Therefore, it is conceivable that this phenomenon might play a role in determining the sensitivity/resistance to anti-ErbB-2 agents. In this regard, it has been shown that resistance to trastuzumab might involve the activation of a TGF α /EGFR autocrine loop [27]. We and others have demonstrated that gefitinib and trastuzumab have a synergistic anti-tumor effect in breast cancer cells that express both receptors. Synergism of anti-EGFR and anti-ErbB-2 agents has also been demonstrated in cells derived from tumors of different histological type, such as ovarian, non-small-cell lung and pancreatic carcinomas [28-30].

Our study is the first to address the mechanisms involved in the synergism of contemporary blockade of EGFR and ErbB-2 in breast cancer cells. In particular, we have shown that blockade of both tyrosine kinases causes a prolonged inhibition of MAPK and AKT activation as compared with inhibition of a single receptor. Both these pathways are involved in the intrinsic and acquired resistance of breast cancer cells to anti-EGFR and anti-ErbB-2 drugs [26, 31, 32]. **Interestingly, our findings show that following treatment with trastuzumab an increase in the levels of activation of MAPK is observed (Figure 1). This phenomenon might represent an attempt of tumor cells to escape the activity of the drug that mainly blocks the ErbB-2/AKT pathway. The use of a combination of drugs targeting both the EGFR and ErbB-2 prevented trastuzumab-induced MAPK activation, which might therefore involve the EGFR.** In addition, combined blockade had a synergistic effect on the expression of different proteins that regulate cell cycle progression, such as p27^{kip1}, cyclin D1 and pRb2, and survival (survivin) and resulted in a more pronounced G0/G1 block and induction of apoptosis as compared with inhibition of a single pathway.

Regulation of p27^{kip1} expression and/or intracellular localization is one of the principal mechanisms through which anti-EGFR and anti-ErbB-2 drugs block cell cycle progression in cancer cells [33-37]. Several studies have demonstrated that p27^{kip1} expression is mainly regulated at post-transcriptional level by protein stabilization [38-42]. However, we found that when both EGFR and ErbB-2 are blocked by using either a combination of trastuzumab plus gefitinib or the dual inhibitor lapatinib, a significant increase in the levels of p27^{kip1} mRNA is also observed. This phenomenon was found to be mediated by FKHRL-1, a member of the Forkhead family of transcription factor [43] that is one of the principal regulators of p27^{kip1} mRNA expression. FKHRL-1 is also a AKT substrate: AKT inactivates FKHRL1 by phosphorylation that produces a retention of the transcription factor into the

cytoplasm [25]. The finding that FKHRL1 nuclear levels increased only in cells treated with the combination of the two drugs suggests that a near full inhibition of AKT activation is required to cause an increase of p27^{kip1} gene transcription. These results also highlight that the mechanism of action of combinations of biological agents might significantly differ from the activity of a single drug, by inducing profound biochemical modifications in the target cells.

The main question that needs to be addressed is whether contemporary blockade of EGFR and ErbB-2 might result in a significant activity in breast cancer patients. Contrasting results have been obtained in clinical trials of combinations of anti-ErbB-2 and anti-EGFR agents. The combination of gefitinib and trastuzumab failed to show significant activity in a phase I/II clinical trial in which patients were not selected for the expression of the EGFR [22]. In fact, this study aimed to address the ability of gefitinib to prevent the occurrence of resistance to trastuzumab, following pre-clinical and clinical observations suggesting a potential role of TGF α signalling in the acquired resistance to trastuzumab. Post-study evaluation of EGFR expression revealed that this receptor was expressed only in 6/28 patients. In addition, a low dose of gefitinib (250 mg/die corresponding to one third of the maximum tolerated dose) was used in this trial, due to the significant toxicity that was observed in patients treated with full dose trastuzumab and 500 mg/die gefitinib in the Phase I part of the study.

Overall, a number of studies have clearly demonstrated that lapatinib is active only in patients that overexpress ErbB-2. A recent report confirmed that the response to lapatinib in breast cancer patients correlates with ErbB-2 status (amplified vs non-amplified) but not with EGFR expression [44]. Patients with ErbB-2 amplification benefited of lapatinib treatment regardless of EGFR expression. However, the levels of activation of the EGFR were not assessed in this study. Therefore, the potential role of EGFR in increasing ErbB-2 activation in selected patients was not explored.

Interestingly, the combination of erlotinib and trastuzumab showed preliminary clinical activity in ErbB-2 overexpressing breast cancer patients that were not previously treated with trastuzumab [45]. In this study, the dose of erlotinib (100 or 150 mg/die, corresponding to two-thirds or full maximum tolerated dose, respectively) was adjusted on individual patient tolerability. However, very few patients were enrolled in this study that was closed early due to the difficulty to enrol patients following the availability of lapatinib.

Taken together, these data suggest that the specific drugs used in the different trials might account in part for the diverse clinical efficacy of such combinations. More importantly, the levels of activation rather than the expression of the target receptors might result more informative for an appropriate selection of patients that might benefit of this approach. Indeed, no correlation between

the levels of expression of EGFR and activity of anti-EGFR agents has been found in different tumor types, including lung and colon carcinoma [26]. Interestingly, increasing evidence suggests that the levels of expression of EGFR ligands correlates with activity of the anti-EGFR monoclonal antibody cetuximab in metastatic colorectal cancer patients [46]. High levels of ligands might represent an indirect marker of the levels of activation of EGFR or other ErbB receptors.

In conclusion, contemporary blockade of EGFR and ErbB-2 receptors in breast cancer cells is able to produce a significant arrest of cell proliferation together with changes in the expression of factors that regulate cell cycle progression such as p27^{kip1}. Although preliminary studies failed to demonstrate the clinical efficacy of this strategy in women affected by breast carcinoma, these results suggest that blockade of EGFR and ErbB2 might represent a novel and effective therapeutic approach in selected patients.

Acknowledgements

This work was partially supported, through research grants, by the non-profit “Associazione Italiana per la Ricerca sul Cancro” (AIRC) and Ministero della Salute to N.N.

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Figure legends:

Figure 1. Effect of treatment with gefitinib and trastuzumab on the activation of MAPK and AKT. Western blot analysis for phosphorylated or total p42/p44 MAPK and for phosphorylated or total AKT expression in SK-Br-3 and BT-474 cells following treatment for the indicated time. C: control, untreated cells; G: cells treated with gefitinib; T: cells treated with trastuzumab; G+T: cells treated with gefitinib plus trastuzumab.

Figure 2. Effect of gefitinib and trastuzumab on cell cycle distribution in breast cancer cells. Cell cycle distribution of SK-Br-3 and BT-474 cells untreated, or treated for 48 hours with gefitinib and trastuzumab, alone or in combination.

Figure 3. Effects of treatment with gefitinib and trastuzumab on cell cycle regulatory proteins in breast cancer cells. Western blot analysis for the expression of p27^{kip1}, pRb2, cyclin D1, and survivin in SK-Br-3 and BT-474 cells whole lysates untreated or treated with gefitinib and trastuzumab, alone or in combination. The blots were normalized with α -tubulin antibody.

Figure 4. Effects of cycloheximide treatment (10 μ M at 2, 4 and 8 hours) on the levels of expression of p27^{kip1} in SK-Br-3 cells. **a)** western blot analysis for p27^{kip1} expression in cells untreated or treated for 48 hours with gefitinib and trastuzumab, alone or in combination. **b)** Densitometric analysis of the blots shown in a.

Figure 5. Effects of treatment for 48 hours with gefitinib or trastuzumab, or with the combination, on the nuclear levels of FKHRL1 protein in SK-Br-3 and BT-474 cells (**a**) and on p27^{kip1} mRNA expression in SK-Br-3 (**b**) and BT-474 (**c**) cells. Effects of treatment with U0126 and LY294002 for 48 hours on p27^{kip1} mRNA expression in SK-Br-3 cells (**d**). Nuclear proteins were normalized with Sp1 antibody in a.

Figure 6. Effects of treatment with lapatinib on cell cycle regulatory proteins in SK-Br-3 cells. Western blot analysis for the expression of p27^{kip1}, pRb2, cyclin D1, and survivin in SK-Br-3 cells whole lysates untreated (C) or treated with the combination of gefitinib and trastuzumab (G+T), or with lapatinib (L). The blot was normalized with α -tubulin antibody.

Figure 7 Effects of treatment with gefitinib plus trastuzumab, or with lapatinib, for 48 hours on p27^{kip1} mRNA expression (**a**) and on the nuclear levels of FKHRL1 protein (**b**) in SK-Br-3 cells. Nuclear proteins were normalized with Sp1 antibody. (**c**) Western blot analysis of total FKHRL-1 expression in SK-Br-3 cells: untreated; transfected with interferin alone; transfected with increasing concentrations of control non targeting siRNA; transfected with increasing concentrations of specific FKHRL-1 siRNA. The blots were normalized with α -tubulin antibody. (**d**) Effects of transfection with specific FKHRL1 siRNA (50 nM) on the levels of p27^{kip1} mRNA induced by treatment with lapatinib in SK-Br-3 cells.

Supplementary Figure 1. Western blot analysis for phosphorylated EGFR and ErbB-2 in SK-Br-3 and BT-474 cells following treatment with gefitinib (2 μ M SK-Br-3 and 1 μ M BT-474) and/or trastuzumab (20 μ g/ml) for 2 hours. C: control, untreated cells; G: gefitinib; T: trastuzumab; G+T: gefitinib plus trastuzumab.

Supplementary Figure 2. Effects of cycloheximide treatment (10 μ M at 2, 4 and 8 hours) on the levels of expression of p27^{kip1} in BT-474 cells. **a)** western blot analysis for p27^{kip1} expression in

cells untreated or treated for 48 hours with gefitinib and trastuzumab, alone or in combination. **b)** Densitometric analysis of the blots shown in a.

Fig. 1

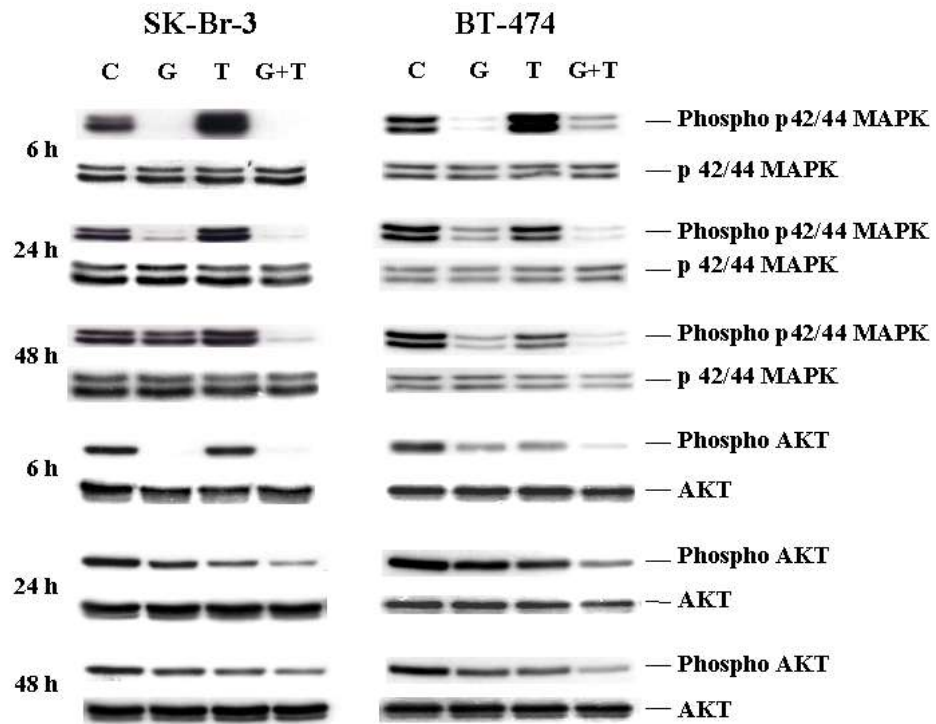


Fig. 2

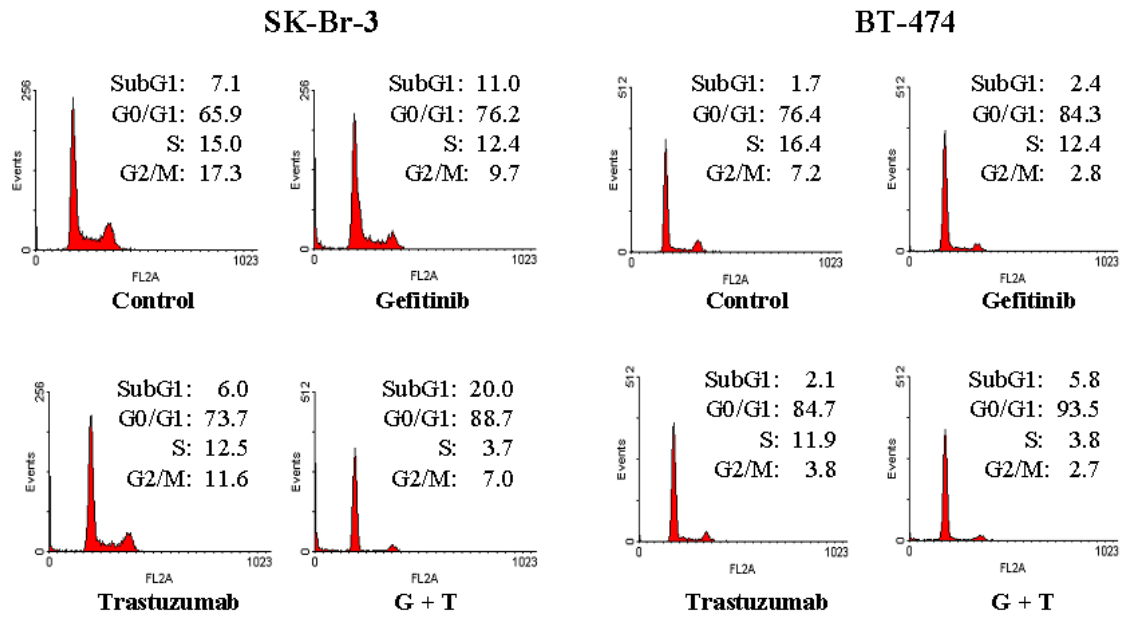


Fig. 3

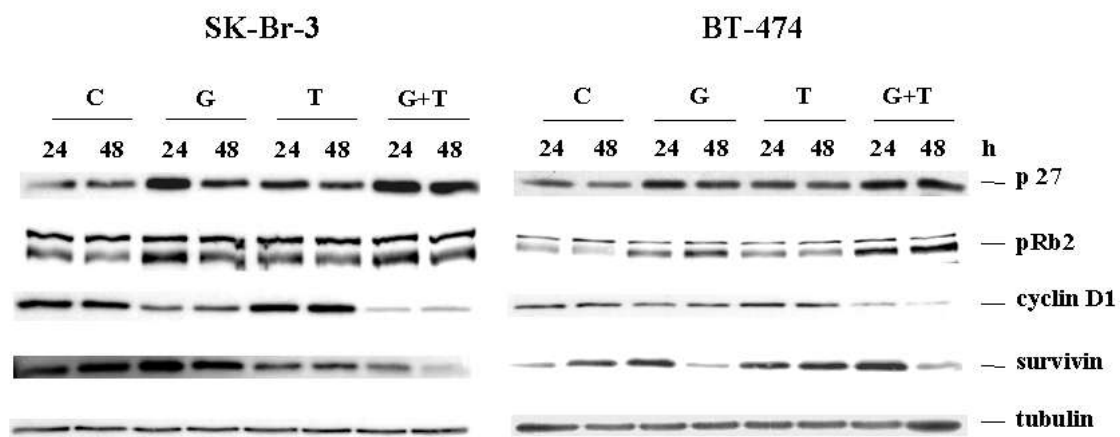


Fig. 4

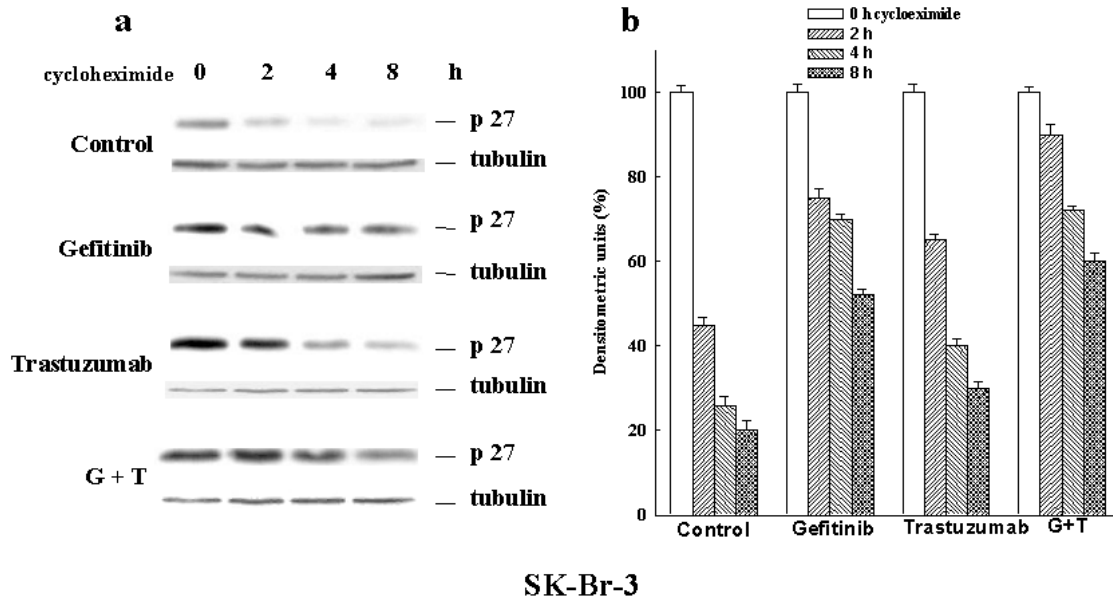


Fig. 5

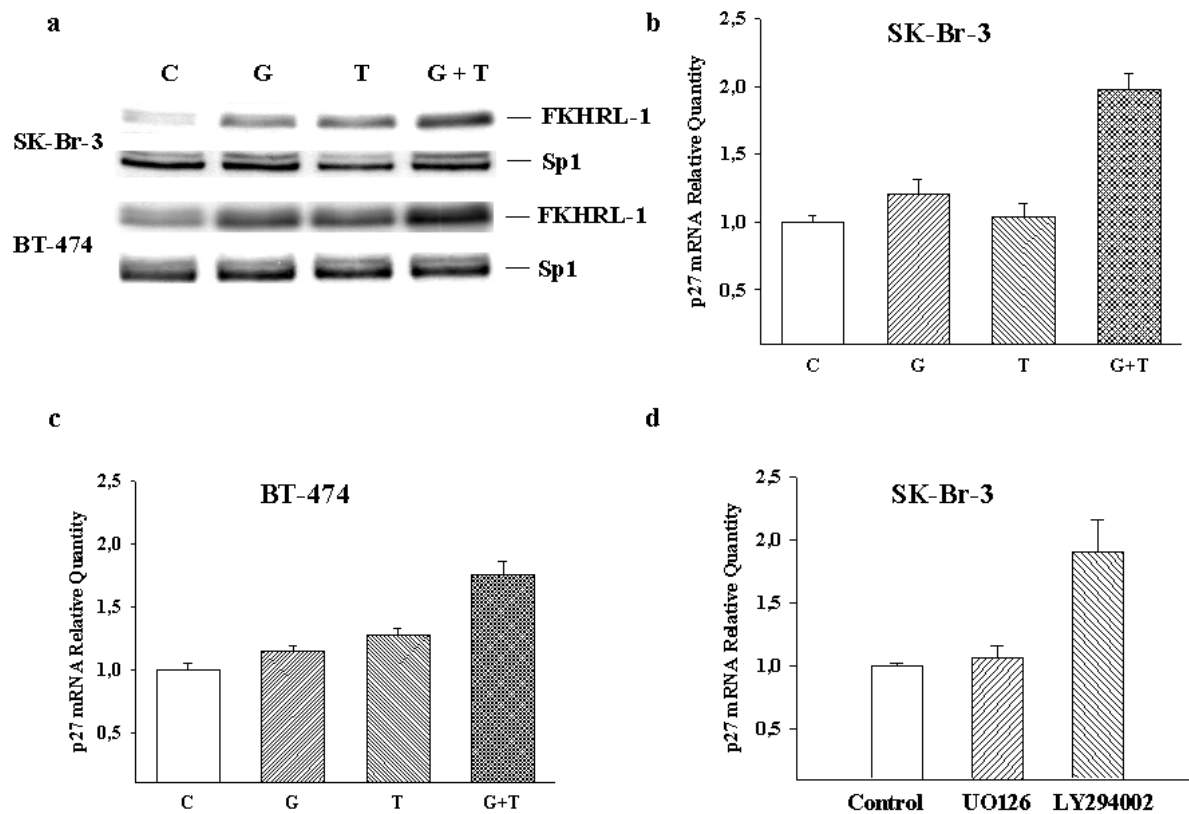


Fig. 6

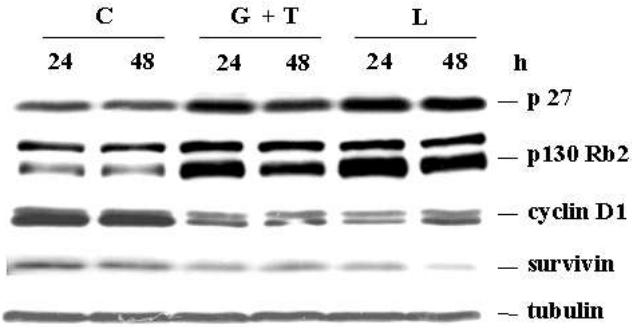


Fig. 7

