Cooperative inhibitory effect of ZD1839 (Iressa) in combination with trastuzumab (Herceptin) on human breast cancer cell growth

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Background: Co-expression of the epidermal growth factor receptor (EGFR) and of ErbB-2 is found in a subset of primary human breast cancer.

Materials and methods: The antiproliferative effects of anti-EGFR and anti-ErbB-2 agents were evaluated using a monolayer assay. The effects of these agents on the activation of EGFR, ErbB-2, AKT and p42/p44 MAP kinases (MAPK) were investigated by western blot analysis.

Results: We found that both ZD1839 (Iressa), a specific EGFR tyrosine kinase inhibitor, and trastuzumab (Herceptin) (TRA), a humanized anti-ErbB-2 monoclonal antibody, were able to inhibit the growth of SK-Br-3 and BT-474 breast carcinoma cells, which express both EGFR and ErbB-2. Treatment of breast carcinoma cells with a combination of ZD1839 and TRA resulted in a synergistic inhibitory effect. Treatment of SK-Br-3 cells with ZD1839 produced a significant, dose-dependent reduction of the tyrosine phosphorylation of both EGFR and ErbB-2. Phosphorylation of MAPK and AKT were significantly reduced in SK-Br-3 cells following treatment with ZD1839, whereas treatment with TRA produced a reduction of AKT but not MAPK phosphorylation. Finally, treatment with ZD1839, but not with TRA, produced a significant increase in fragmented DNA in breast carcinoma cells. However, a more pronounced increase in the levels of fragmented DNA was observed following combined treatment with ZD1839 and TRA.

Conclusions: These data suggest that combined treatment with drugs that target EGFR and ErbB-2 might result in an efficient inhibition of tumor growth in those breast carcinoma patients whose tumors co-express both receptors.

Key words: antibodies, breast cancer, EGF receptor, ErbB-2, therapy, tyrosine kinase inhibitors

Introduction

Epidermal growth factor receptor (EGFR) and c-ErbB-2 are involved in the pathogenesis of human breast carcinoma [1]. In fact, overexpression of EGFR has been found in 14% to 91% of human primary breast carcinomas, with a median value of 48% [1]. Expression of EGFR ligands such as transforming growth factor-α (TGF-α) or amphiregulin (AR) has been detected in 50% to 90% of human primary breast carcinomas [2–4]. Furthermore, co-expression of EGFR and its ligands has also been found in primary breast carcinomas, suggesting that an autocrine loop may be operating in these tumors [1]. Expression of the EGFR also occurs in a majority of human breast carcinoma cell lines. It has been shown that blockade of EGFR activation using either anti-EGFR monoclonal antibodies or specific tyrosine kinase inhibitors significantly inhibits the in vitro and/or in vivo growth of human breast carcinoma cells [5, 6]. In this regard, it has been demonstrated recently that the anilinoquinazoline ZD1839 (Iressa), an orally active, specific EGFR tyrosine kinase inhibitor (TKI), is able to inhibit the growth of human breast cancer cells [7]. Preliminary data show that ZD1839 has promising clinical activity in patients with a wide range of tumor types [6].

The expression of ErbB-2 in primary breast carcinomas is more restricted, as compared with EGFR. In fact, ErbB-2
overexpression is reported in ~20% of human primary breast cancers with a wide interstudy range of 9% to 39% [1]. No ligand for ErbB-2 has been identified yet. However, it has been shown that ErbB-2 functions as a ‘preferred partner’ and forms heterodimers with other members of the ErbB family of receptors, thereby functioning as a co-receptor [8]. Therefore, overexpression of ErbB-2 on the cell membrane of tumor cells might represent a mechanism for amplifying the response to exogenous stimuli carried by ligands of the EGFR or other ErbB-receptors. In fact, it has been reported that overexpression of ErbB-2 can enhance the binding affinities of both EGF and heregulin β1, through deceleration of ligand dissociation rates [9]. Specific antisense oligonucleotides or anti-receptor monoclonal antibodies directed against ErbB-2 have been shown to significantly inhibit the growth of several human breast carcinoma cell lines that express this protein [10–13]. In this regard, phase II studies using a recombinant humanized anti-ErbB-2 monoclonal antibody (trastuzumab; Herceptin™) (TRA) in patients with metastatic breast cancer have shown clinical efficacy of this compound [14, 15]. Furthermore, objective responses to treatment with TRA were observed in women with ErbB-2 overexpressing, metastatic breast cancer that had progressed after chemotherapy for metastatic disease [16]. More recently, a phase III study has demonstrated that Herceptin increases the clinical benefit of first-line chemotherapy in patients with metastatic breast cancer that overexpresses ErbB-2 [17].

Co-expression of EGFR and ErbB-2 has been observed in 10% to 36% of primary human breast carcinomas [18–20]. However, expression of both receptors probably occurs in a higher percentage of breast carcinoma patients. In fact, most of the above mentioned results have been obtained by using immunohistochemical techniques, that are semi-quantitative techniques with low sensitivity. In this regard, quantitative measurement of ErbB-2 expression in human breast carcinomas by radioimmunochemical revealed overexpression of ErbB-2 in 91% of the carcinoma samples, as compared with non-transformed breast epithelial cells [21]. The presence of both EGFR and ErbB-2 might result in cooperation between these two oncogenes in sustaining the autonomous proliferation of breast cancer cells. In fact, it has been demonstrated that overexpression of both c-ErbB-2 and EGFR is associated with a poorer prognosis than overexpression of either oncogene alone in breast carcinoma patients [18–20].

In this report we show the effects of simultaneous blockade of the EGFR and ErbB-2 on cell proliferation, cell survival and signal transduction in breast cancer cells that co-express both receptors. For this purpose, two specific drugs currently under clinical evaluation, the EGFR tyrosine kinase inhibitor ZD1839 and the anti-ErbB-2 humanized antibody TRA, were employed.

Materials and methods

Materials

ZD1839 was kindly provided by AstraZeneca (Macclesfield, UK). Herceptin (trastuzumab) was kindly provided by Genentech Inc. (San Francisco, CA, USA).

Cell culture

SK-BR-3 and BT-474 human breast cancer cell lines were procured from the American Type Culture Collection (Rockville, MD, USA). Cells were routinely maintained as described previously [22].

Anchorage dependent growth assays

Cells (10⁴ cells/well) were seeded into 48-multiwell cluster dishes (Becton Dickinson, Milan, Italy), and treated every 24 h with the indicated concentrations of ZD1839 and/or TRA. After 5 days of growth, the cells were trypsinized and counted with an hemocytometer.

Analysis of combination effect

Combination analysis was performed by using the method as described by Chou and Talalay [23], and the CalcuSyn software program (Biosoft, Cambridge, UK) for automated analysis.

Flow cytometry

Indirect immunofluorescence assay was performed on live cells using purified, specific anti-EGFR or anti-ErbB-2 MAbs [24, 25]. Cells were incubated with antibodies (10 µg/ml) for 30 min at 4°C, washed twice, and then incubated with FITC-labeled goat anti-mouse (1:100) (Kirkegaard Perry Laboratories, Inc., Gaithersburg, MD, USA) for 30 min at 4°C. Cells were washed again and resuspended in PBS. Fluorescence was evaluated by a FACScan using LYSIS II software (Becton Dickinson, Mountain View, CA, USA).

Immunoprecipitation

Cell lines were treated with different concentrations of ZD1839 for 3 days, prior lysis with lysis buffer (50 mM Tris–HCl pH 7.5, 150 mM NaCl, 5 mM EDTA, 1% Triton, 10% glycerol, 1 mM phenylmethyl-sulphonyl-fluoride, 2 mM Na-orthovanadate, 10 mM leupeptin, 100 mM Na-fluoride, 10 mM Na-pyrophosphate). Immunoprecipitations were carried out as described earlier [26]. Briefly, protein extracts were immunoprecipitated for 3 h using specific antibodies and 20 µl of protein-A/G sepharose beads (Amersham Pharmacia Biotech, Milan, Italy). Precipitates were then separated on 8% SDS–PAGE, transferred onto nitrocellulose membrane (Amersham Pharmacia Biotech) and probed with the following antibodies: (i) a monoclonal anti-phosphotyrosine antibody (UBI, Lake Placid, NY, USA); (ii) a monoclonal anti-ErbB-2 antibody (Oncogene Research, Boston, MA, USA); and (iii) a monoclonal anti-EGFR antibody (Transduction Laboratories, Lexington, KY, USA). Proteins were detected using chemiluminescence ECL western blotting kit (Amersham Pharmacia Biotech) following the manufacturer’s instructions.

Western blot analysis

Whole protein extracts were prepared and analyzed by western blotting using the chemiluminescence ECL western blotting kit (Amersham Pharmacia Biotech), as described previously [27]. The following antibodies from New England Biolabs were used: (i) a rabbit polyclonal anti-phospho p44/p42 MAPK antibody; (ii) a rabbit polyclonal anti-p44/p42 MAPK.
antibody; (iii) a rabbit polyclonal anti-phospho AKT antibody; and (iv) a rabbit polyclonal anti-AKT antibody.

Apoptotic death assays

DNA fragmentation was used as a criteria for assessing apoptotic cell death. For demonstration of internucleosomal DNA fragmentation, 4 × 10⁵ cells were plated in 60 mm cell culture dishes. After appropriate treatment, the cells were harvested, washed with PBS solution at 4°C, and suspended in lysis buffer (10 nM Tris-HCl, pH 7.5, 10 mM EDTA, and 0.2% Triton X100). After incubation for 15 min at 4°C, samples were centrifuged at 13,000 g for 10 min at 4°C. The supernatant containing the fragmented DNA was precipitated with NaCl 0.5 M and 1 volume of isopropanol for at least 1 h at −70°C. The precipitates were dissolved in 10 µl TE-RNase (0.1 mg/ml) and incubated at 37°C for 30 min. Finally, the samples were electrophoresed through a 1.5% agarose gel. Quantitation of DNA fragmentation was determined by the Cell Death Detection ELISA Plus Kit (Boheringer Mannheim, Indianapolis, IN, USA), as suggested by the manufacturer and published previously [28].

Results

Expression of EGFR and ErbB-2 in human breast cancer cells

It has been demonstrated previously that SK-Br-3 and BT-474 human breast carcinoma cells express high levels of the ErbB-2 tyrosine kinase receptor, and moderate levels of EGFR [29]. We assessed the levels of expression of both ErbB-2 and EGFR in SK-Br-3 and BT-474 cells by flow cytometry (Figure 1). Our results basically confirmed the above mentioned earlier findings.

Effect of ZD1839 and TRA on SK-Br-3 and BT-474 cell growth

We evaluated the effects of the EGFR tyrosine kinase inhibitor ZD1839 and of the anti-ErbB-2 humanized antibody TRA on the anchorage dependent growth (ADG) of SK-Br-3 and BT-474 cells. A dose-dependent growth inhibition was observed following treatment of breast carcinoma cells with both drugs (Figure 2). In particular, 30% and 40% growth inhibition was observed, respectively, following treatment of SK-Br-3 or BT-474 cells with 20 µg/mL TRA (Figure 2). Treatment of SK-Br-3 and BT-474 cells with 2 µM ZD1839 resulted in nearly 50% and 60% growth inhibition, respectively (Figure 2).

Treatment of SK-Br-3 and BT-474 cells with combinations of ZD1839 and TRA resulted in a more significant growth inhibition, as compared with treatment with a single drug (Figure 2A and C). In fact, a 65% to 70% growth inhibition was observed when SK-Br-3 or BT-474 cells were treated with 20 µg/ml (0.13 µM) TRA and 2 µM ZD1839. In order to evaluate the nature of the interaction between TRA and ZD1839 (additive or synergistic), combination analysis was performed by using the Combination Index (CI) isobologram analysis according to the method of Chou and Talalay (Figure 2B and D). Breast carcinoma cells were treated with serial dilutions of TRA and ZD1839 or with a combination of the two drugs at a fixed 1:15 (TRA:ZD1839) molar ratio. CI was calculated by using the CalcuSyn software. According to the
Chou and Talalay method, CI values <1 indicate synergism [23]. CI values for the combination ZD1839 + TRA were below 0.7 in both cell lines (Figure 2B and D). These values clearly suggest that this drug combination produces a synergistic growth inhibitory effect in breast carcinoma cells.

**Effect of treatment with ZD1839 on tyrosine phosphorylation of EGFR and ErbB-2**

We assessed the effect of ZD1839 on tyrosine phosphorylation of both EGFR and ErbB-2 in SK-Br-3 cells. Treatment of SK-Br-3 cells with concentrations of ZD1839 as low as 1 µM produced a significant reduction in the levels of tyrosine phosphorylation of both receptors (Figure 3A). No tyrosine phosphorylation of either EGFR or ErbB-2 was detectable in SK-Br-3 cells following treatment with 5 µM ZD1839 (Figure 3A). Treatment of SK-Br-3 cells with concentrations up to 10 µM of ZD1839 did not produce significant changes in the endogenous levels of EGFR and ErbB-2, as assessed by flow cytometry (data not shown). In order to evaluate the specificity of ZD1839 for EGFR, we treated NIH/3T3 fibroblasts that were transfected with a human ErbB-2 expression vector (NIH 3T3/ErbB-2) with similar concentrations of ZD1839. No changes in ErbB-2 tyrosine phosphorylation were observed in NIH 3T3/ErbB-2 cells that were treated with up to 5 µM ZD1839 (Figure 3B).

**Effect of treatment with ZD1839 and/or TRA on the phosphorylation of AKT and p42/p44 MAP kinases (MAPK)**

We next evaluated the effects of treatment of SK-Br-3 cells with TRA and ZD1839 on the activation of intracellular transducers of growth factor signaling, such as p42/p44 MAP kinases (MAPK) and AKT. We found that treatment of SK-Br-3 cells for 72 h with 2 µM ZD1839 produced a significant reduction in the levels of phosphorylation of both p42/p44 MAPK and AKT (Figure 4A). However, treatment of breast carcinoma cells for a similar period of time with 20 µg/mL of TRA produced a significant reduction of AKT

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Figure 2. Effect of treatment with TRA, or ZD1839, or combinations of TRA and ZD1839 on the ADG of SK-Br-3 cells (A) and BT-474 cells (C). Cells (1 x 10⁴ cells/well) were seeded into 48-multiwell cluster dishes, and treated every 24 h with the indicated concentrations of either TRA or ZD1839 for 4 days. After 5 days of growth, the cells were trypsinized and counted with an hemocytometer. Combination analysis was performed using the method described by Chou and Talalay. CI, Combination index. (B) SK-Br-3 cells; (D) BT-474 cells.
but not MAPK phosphorylation (Figure 4C). The levels of AKT phosphorylation in SK-Br-3 cells following treatment with a combination of TRA and ZD1839 were comparable to those observed in cells treated with ZD1839 alone. Treatment with ZD1839 or TRA did not produce any significant changes in the endogenous levels of p42/p44 MAPK and AKT (Figure 4B and D).

Effect of treatment with ZD1839 and/or TRA on the survival of human breast cancer cells

A number of floating cells was observed when SK-Br-3 and BT-474 cells were treated with high concentrations (2 µM) of ZD1839. In fact, a characteristic DNA ladder was observed in SK-Br-3 cells following treatment with either ZD1839 (2 µM) or with a combination of TRA (20 µg/ml) and ZD1839 (2 µM), but not with TRA alone (Figure 5A). A quantitative assessment of fragmented DNA was also performed by using a commercially available ELISA kit. Treatment with ZD1839 (2 µM), but not with TRA, produced an ∼3-fold increase in fragmented DNA in SK-Br-3 cells (Figure 5B). However, a more pronounced increase (3.5-fold) in the levels of fragmented DNA in SK-Br-3 cells was observed following combined treatment with ZD1839 and TRA, as compared with treatment with ZD1839 alone (Figure 5B). This difference was statistically significant as assessed by Student’s t-test (P <0.01). Similar findings were observed in BT-474 cells following treatment with ZD1839 (2 µM) or with the combination of ZD1839 (2 µM) and TRA (20 µg/ml) (data not shown).

Discussion

Breast cancer cells are relatively independent from exogenous growth factors when compared with their normal counterpart [1]. This phenomenon is due, at least in part, to the ability of breast cancer cells to produce high levels of growth factors and/or receptors for growth factors [1]. In this context, it has been demonstrated previously that co-expression of several, different EGF-like peptides and/or ErbB receptors occurs in human breast carcinoma cells [2–4, 18–20]. Therefore, it is conceivable that a network consisting of multiple ligands and receptor molecules is sustaining the autonomous proliferation and/or the survival of human breast carcinoma cells.

This study is the first to demonstrate that a synergistic growth inhibition occurs when human breast cancer cells that co-express EGFR and ErbB-2 are treated with a combination of the humanized anti-ErbB-2 antibody TRA (Herceptin) and the anilinoquinazoline EGFR-specific TKI ZD1839 (Iressa). We have demonstrated previously that treatment of breast carcinoma cells with combinations of antisense oligonucleotides directed against the EGF-related peptides AR, CRIPTO-1 (CR-1) or TGF-α results in a larger degree of growth inhibition as compared with treatment with a single antisense oligonucleotide [28]. Taken together, these findings suggest strongly that simultaneous blockade of different growth factor-driven signal-transduction pathways might result in a more significant antitumor effect.

These results may be important for future clinical applications. In fact, several studies have shown the clinical efficacy of TRA in breast cancer patients [14–17]. Furthermore, preliminary data have also shown that ZD1839 has promising clinical activity in patients with a wide range of tumor types [6]. Therefore, the findings that are shown in this paper could

Figure 3. (A) Effects of treatment with ZD1839 on tyrosine phosphorylation of EGFR and ErbB-2 in SK-Br-3 cells. SK-Br-3 cells were treated for 3 days with different concentrations of ZD1839. EGFR (lanes 1–3) or ErbB-2 (lanes 4–6) were immunoprecipitated with specific antibodies. Western blot analysis was then performed with a specific anti-phosphotyrosine antibody. Lanes 1 and 4, untreated SK-Br-3 cells; lanes 2 and 5, cells treated with 1 µM ZD1839; lanes 3 and 6, cells treated with 5 µM ZD1839. (B) Effects of treatment with ZD1839 on tyrosine phosphorylation of ErbB-2 in NIH/3T3/ErbB-2 cells. Immunoprecipitation of ErbB-2 and evaluation of its phosphorylation were performed as described above. Lane 1, untreated NIH/3T3/ErbB-2 cells; lane 2, cells treated with 1 µM ZD1839; lane 3, cells treated with 5 µM ZD1839.

Figure 4. Western blot analysis for phosphorylated (A) or total (B) p42/p44 MAPK, and for phosphorylated (C) or total (D) AKT expression in SK-Br-3 cells. Lane 1, control untreated SK-Br-3 cells; lane 2, cells treated for 72 h with 20 µg/mL TRA; lane 3, cells treated for 72 h with 2 µM ZD1839; lane 4, cells treated with 20 µg/mL TRA plus 2 µM ZD1839.
be immediately transferred in the clinical setting. This concept could also be applied to other carcinoma types that co-express EGFR and c-ErbB-2 [1]. In this context, it has been shown previously that combined treatment with both TRA and the humanized anti-EGFR antibody C225 results in additive anti-proliferative effects in ovarian carcinoma cells [30].

An additional important finding of this paper is that a significant reduction in the levels of tyrosine phosphorylation of both EGFR and ErbB-2 was observed in breast cancer cells following treatment with ZD1839. It is conceivable that ZD1839 can block the trans-phosphorylation of ErbB-2 that follows autocrine activation of the EGFR in breast cancer cells. Therefore, overexpression of the ErbB-2 receptor might represent a mechanism which is able to amplify the response to autocrine or paracrine stimuli that activate the EGFR. In this regard, we have shown previously that antisense oligonucleotides directed against TGF-α are able to inhibit significantly the growth of SK-Br-3 cells [28]. Furthermore, a comparable inhibition of SK-Br-3 cell growth has been described previously following treatment of these cells with a different, specific EGFR-TKI, PD153035 [31]. These findings also suggest that the ability of ZD1839 to block the proliferation of tumor cells might not only depend on the levels of expression of EGFR, but also on the expression in the target cells of ErbB-2 and/or other ErbB receptors that can be trans-activated by the EGFR. Alternatively, a direct inhibition of ErbB-2 tyrosine kinase activity by ZD1839 might occur. However, no reduction of ErbB-2 phosphorylation following treatment with ZD1839 was observed in NIH/3T3 cells that overexpress ErbB-2 but do not express the EGFR.

Activation of the EGFR provides a protective role against apoptosis in non-transformed cells and in tumor cells [28, 32–35]. In this context, we found that treatment of SK-Br-3 or BT-474 cells with ZD1839 was able to induce programmed cell death, as assessed by an increase in DNA fragmentation and nucleosome formation. A recent report has shown that treatment with ZD1839 induces DNA fragmentation in several carcinoma cell lines [7]. AKT has been identified as a key effector of PI3K-mediated cell survival [36, 37]. In this regard, the ability of ZD1839 to induce apoptosis in human carcinoma cells might be due, in part, to the reduction of AKT phosphorylation that we have observed to occur following treatment with ZD1839. However, blockade of p42/p44 MAPK activation or other additional intracellular signals might also be required to induce apoptosis in breast carcinoma cells. In fact, the blockade of AKT phosphorylation produced by treatment with TRA was not sufficient to induce apoptosis. Furthermore, the levels of AKT phosphorylation in SK-Br-3 cells treated with a combination of TRA and ZD1839 were comparable with those observed in cells treated with ZD1839 alone. Therefore, cooperation between these two oncogenes in controlling cell survival might operate through signals other than AKT.

Taken together, these findings provide a strong rationale for the combined treatment of breast carcinoma patients whose tumors co-express EGFR and ErbB-2 with both TRA and ZD1839. Furthermore, this work provides an example of the potential for novel therapeutic approaches to the treatment of human carcinomas, based on the use of a combination of molecules able to block different growth factor-driven signal transduction pathways. The relative amount and the type of

Figure 5. (A) Electrophoretic pattern of DNA isolated from SK-Br-3 cells. Lane 1, control untreated SK-Br-3 cells; lane 2, cells treated for 72 h with 20 µg/mL TRA; lane 3, cells treated for 72 h with 2 µM ZD1839; and lane 4, cells treated with 20 µg/mL TRA plus 2 µM ZD1839. M, amplisize DNA size standard (Biorad). (B) Effect of treatment with TRA, or with ZD1839, or with combinations of TRA and ZD1839 on DNA fragmentation in SK-Br-3 cells. Cells were treated for 72 h with: 20 µg/mL TRA; or with 2 µM ZD1839; or with a combination of 20 µg/mL TRA plus 2 µM ZD1839. DNA fragmentation was measured as described in the Materials and methods, and data were expressed as Relative Death as compared with untreated control cells.
molecules to be used should depend on the levels of expression of the specific target in the primary tumor. Interestingly, pre-clinical and clinical data have shown that this novel therapeutic approach can be combined with conventional anti-cancer drugs.

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