Cancer Chemother Pharmacol (2009) 63:973–981
DOI 10.1007/s00280-008-0820-9

ORIGINAL ARTICLE

Dual targeting of EGFR and HER-2 in colon cancer cell lines

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Received: 21 May 2008 / Accepted: 1 August 2008 / Published online: 27 August 2008
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Abstract
Purpose A number of studies have revealed that coexpression of EGFR and HER-2 has been found in a subset of colon cancers and may cooperatively promote tumor cell growth and survival. In the present work, two tyrosine kinase inhibitors, gefitinib and lapatinib, together with trastuzumab, raised a monoclonal antibody against HER-2 were evaluated in two colon cancer cell lines, DLD-1 and Caco-2. The aim of the study was to investigate their effect on tumor cell proliferation and apoptosis.
Methods Cell proliferation was assessed using the MTT assay and apoptosis was evaluated by DNA fragmentation and the Annexin V binding assay. EGFR and HER-2 protein and mRNA levels were evaluated by immunoblotting and quantitative RT-PCR, respectively.
Results Treatment of cells with each agent alone resulted in inhibition of cell proliferation after 48 h in a dose-dependent manner except for trastuzumab, which did not alter cell proliferation of DLD-1. Apoptosis increased in DLD-1 cells, after 24 h treatment with gefitinib. None of the tested agents altered apoptosis in Caco-2 cells. HER-2 and EGFR protein levels did not follow the changes of mRNA levels after treatment with the tested agents.
Conclusions The inhibitory effect of these agents on cell proliferation and the induction of apoptosis differ for the two colon cancer cell lines under consideration. Further studies are necessary to investigate the way they exert their antitumor effect.

Keywords Gefitinib · Lapatinib · Trastuzumab · Caco-2 · DLD-1

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Introduction

The ErbB family of tyrosine kinase receptors comprises four known members, namely epidermal growth factor receptor (EGFR), HER-2, HER-3, and HER-4. These are often deregulated in neoplasms and can promote proliferation, migration, angiogenesis, stromal invasion, and resistance to apoptosis [1]. Colorectal cancer is the second most frequent cause of cancer mortality in Western countries. It is frequently associated with the overexpression of EGFR suggesting more aggressive disease and poor prognosis [2]. Similarly, the expression of HER-2 and HER-3 is often elevated as HER-4 has not yet been extensively studied [3]. Inhibition of EGFR and HER-2 can be achieved either by antibodies targeting the extracellular domain of the receptor or by soluble tyrosine kinase inhibitors [4–7]. At present, a number of studies have focused on the application of monoclonal antibodies or tyrosine kinase inhibitors of ErbB family receptors [8–12].

Trastuzumab (Herceptin), a chimeric HER-2-specific monoclonal antibody, has already been approved for the management of patients with breast cancer. With respect to colorectal cancer, Mann et al. [13] have shown that trastuzumab inhibited colony formation of the HCA-7 colon cancer cell line and HCA-7 tumor xenografts. Gefitinib (Iressa) is a tyrosine kinase inhibitor of EGFR, which has been approved for the treatment of non-small cell lung

cancer in USA and Japan [14]. The inhibitory effect of gefitinib has been previously shown in human lung [15], breast [16], prostate [17], and in several colon cancer cell lines [18]. Moreover, data are available regarding the synergistic effect of gefitinib with oxaliplatin in colon cancer cell lines [10]. Lapatinib is a dual tyrosine kinase inhibitor of both EGFR and HER-2 approved for the management of patients with trastuzumab refractory breast cancer [19]. An important inhibitory effect of lapatinib has been demonstrated in certain cell lines including human breast [20] and prostate cancer [21]. To our knowledge, there is only one study on the inhibitory effect of lapatinib in the human colon cancer cell line GEO [22]. Although the efficacy of each one of the tyrosine kinase inhibitors combined with other agents such as gefitinib and oxaliplatin [10] or lapatinib and tamoxifen [20] has been studied, little is currently known about the efficacy of the combination of two tyrosine kinase inhibitors. To this end, only one study has compared the activity of gefitinib and lapatinib on a human prostate cancer cell line, suggesting that lapatinib is a more potent inhibitor than gefitinib [21].

The purpose of the present study was to investigate the effect of trastuzumab, gefitinib, and lapatinib, alone and in combination, on two human colon cancer cell lines, Caco-2 and DLD-1. More specifically, we investigated the effect of these agents on proliferation and apoptosis as well as on HER-2 and EGFR expression. Caco-2 and DLD-1 cells were chosen as they originate from well and poorly differentiated colon carcinomas, and have different expression patterns of proteins involved in cell proliferation. A proteome study on Caco-2 and DLD-1 cells revealed differences in the expression of 18 proteins. Among them are proteins that regulate cell proliferation (nm23H1G and cell cycle protein), differentiation (CAH1), and drug resistance (cytokeratin 18) [22]. The above agents were chosen as they are commercially available and they target differently EGFR and HER-2. Therefore, we hypothesized that their combination may prove to be more efficient than each agent alone.

Material and methods

Cell culture and reagents

DLD-1 and Caco-2 colon cancer cell lines were purchased from the American Type Culture Collection. DLD-1 cells were cultured in RPMI 1640 medium with 2 mM L-glutamine and supplemented with 10 mM HEPES, 1 mM sodium pyruvate, 4.5 g/l glucose, 1.5 g/l sodium bicarbonate, and 10% fetal bovine serum as recommended by ATCC. Caco-2 cells were cultured in Eagle's Minimum Essential medium with Earle's BSS and 2 mM L-glutamine (EMEM) and supplemented with 1.0 mM sodium pyruvate, 0.1 mM nonessential amino acids, 1.5 g/l sodium bicarbonate, and 20% fetal bovine serum as recommended by ATCC. Cells were cultured at 37°C, 5% CO2 and 100% humidity.

The monoclonal antibody trastuzumab (Herceptin) was purchased from Roche. The inhibitors gefitinib (Iressa) and lapatinib (Tykerb) were kindly provided by AstraZeneca and GlaxoSmithKline, respectively. As HER-2 and EGFR are predominantly localized to the basolateral membranes of colonic epithelial cells and Caco-2 cells, it can form polarized monolayers; trastuzumab was added to Caco-2 cells while they were transiently suspended before plating [23], to ensure access to HER-2. Gefitinib and lapatinib had no such restrictions and were applied after cell attachment to both cell lines. All agents were tested for their antiproliferative effect in a range of concentrations according to the current bibliography [24, 25]. Trastuzumab was applied at concentrations: 0, 10, 20, 40, 80 and 100 μg/ml. Gefitinib and lapatinib were applied at doses of 0, 0.1, 0.5, 1, 5, 10 μM and 0, 1, 2, 10, 20, 25 μM, respectively. All tested agents were diluted in phosphate buffered saline (PBS) pH 7.6 with dimethyl sulfoxide (DMSO). The final concentration of DMSO in the culture medium was 0.5% which is not toxic to cells. The culture medium of untreated cells contained the same final concentration of DMSO, as control [26].

Cell proliferation assay

To determine whether these agents affect the proliferation of DLD-1 and Caco-2 colon cancer cell lines, the 3-[4,5-dimethylthiazol-2-yl]-2,5-dimethyltetrazolium bromide (MTT) assay was used, as previously described [27].

DNA fragmentation

DLD-1 and Caco-2 cell lines were plated at 5×10^5 cells per 25-cm^2 flask. Twenty-four hours after treatment with the agents, cells were trypsinized and subjected to a standard DNA isolation procedure. Briefly, cells were resuspended in 20 μl of lysis buffer (50 mM Tris–HCl pH 8.0, 10 mM EDTA, 0.5% SDS and 0.5 mg/ml proteinase K) and incubated at 55°C for 60 min. Each sample was mixed with 5 μl of 1 mg/ml RNase A stock solution and incubated at 55°C for another 60 min. The samples were heated at 70°C for a few minutes and were analyzed in a 1.2% agarose gel containing 0.5 μg/ml ethidium bromide for 4 h at 40 V. The gel was visualized by ultraviolet transillumination and a Kodak camera was used to capture the images.

Apoptosis assay

Both colon cancer cell lines were plated at 1×10^5 cells per well in 6-well plates. Tested agents were added as previously

described. At the end of a 24 h incubation, cells were washed twice with PBS, trypsinized for 6 min and centrifuged for 4 min at 166×*g*. Cells were resuspended in 195 µl 1X binding buffer (10 mM HEPES pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$) to a final concentration of 5×10^5 cells/ml. The cell suspension was incubated with 5 µl Annexin V-FITC in the dark at 25°C, for 10 min. Then, 10 µl of the 20 µg/ml propidium iodide stock solution was added followed by 200 µl of binding buffer and the cells were analyzed by flow cytometry [23] immediately (EPICS-XL of Coulter) according to the manufacturer's instructions (rh Annexin V/FITC kit, Bender MedSystems).

Immunoprecipitation

DLD-1 and Caco-2 cell lines were plated at 1×10^6 cells per 75 cm^2. Tested agents were added as described earlier and the incubation of cells was terminated 48 h later by adding 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 and 10 mM leupeptin). Protein concentration was determined by Bradford assay and 1 mg of total protein was immunoprecipitated with a monoclonal anti-EGFR antibody (Upstate, Lake Placid, NY) or a polyclonal anti-HER-2 antibody (Upstate, Lake Placid, NY) overnight at 4°C, under continuous agitation. In each sample, 50 µl of protein-A sepharose beads (Sigma, Amersham biosciences) were added and samples were incubated for 4 h, at 4°C, under continuous agitation. Precipitates were washed twice with ice-cold lysis buffer and sepharose beads were resuspended in 50 µl 2X sample buffer (0.5 M Tris–HCl pH 6.8, 20% glycerol, 2% SDS and 2% bromophenol blue, 10% *β*-mercaptoethanol). Samples were heated for 5 min at 95°C and analyzed by immunoblotting [28].

Immunoblotting

Immunoprecipitates and control samples for actin (40 µg of total protein lysed in 2X sample buffer) were analyzed by SDS-PAGE electrophoresis in 7 and 10% gels, respectively and transferred to nitrocellulose membranes. Membranes were blocked in 5% (w/v) non-fat dry milk in Tris-buffered saline pH 7.4 containing 0.05% Tween 20 (TBS-T) for 1 h at room temperature and were then incubated with a monoclonal anti-EGFR antibody (Upstate, Lake Placid, NY, 1 µg/ml), a polyclonal anti-HER-2 antibody (Upstate, Lake Placid, NY, 1 µg/ml) or a monoclonal anti-actin antibody (Chemicon, dilution 1:1,000) for 2 h, at room temperature. After three washes in TBS-T, membranes were further incubated with horseradish peroxidase conjugated goat anti-mouse IgG (Upstate, Lake Placid, NY, dilution 1:2,000) or goat anti-rabbit IgG (Upstate, Lake Placid, NY, dilution 1:5,000) for 1.5 h at room temperature. Detection of the immunoreactive proteins was performed by chemiluminescence horseradish peroxidase substrate SuperSignal (Pierce), according to the manufacturer's instructions.

RNA isolation and cDNA synthesis

DLD-1 and Caco-2 cells were treated with the tested agents at the indicated concentrations. Cells were harvested as before and total RNA was isolated using Absolutely RNA (Stratagene, La Jolla, USA) according to the manufacturer's instructions. The integrity of RNA was confirmed by visualization of ribosomal bands in EtBr stained agarose gels. Total RNA was quantified using Ribogreen (Molecular Probes, Leiden, The Netherlands) and the MX3000p (Stratagene, La Jolla, USA) according to the manufacturer's instructions. cDNA was synthesized from 1.6 µg of total RNA from each sample using random nonamers (ITE, Crete, Greece) and 50 U Stratascript reverse transcriptase (Stratagene, La Jolla, USA). In addition, Human Reference RNA (Stratagene, La Jolla, USA) was used as a calibrator sample to allow adjustment from run to run variation. A no enzyme control was also included.

Real time PCR

EGFR and HER-2 mRNA was quantified using Taqman chemistry and Brilliant quantitative PCR core reagents (Stratagene). TBP (TATA box binding protein) was used as a reference gene and TBP mRNA was quantified using SYBR Green I intercalation dye in Brilliant Sybr Green QPCR Master Mix (Stratagene). Primers and Taqman probes were designed using Primer 3 and were synthesized at ITE (Crete, Greece) and DNA Technology A/S (Aarhus C. Denmark), respectively [18].

All reactions were performed in MX3000p (Stratagene), in triplicates, and contained 5-carboxy-x-rhodamine (ROX) as a passive reference dye and cDNA equivalent to 100 ng of RNA. A standard curve was always included for assay validation. In addition, a melting curve analysis was performed for the SYBR Green I assay. The relative abundance of mRNA of the gene of interest was deducted from the cycle number at which fluorescence increased above background level (Ct) in the exponential phase of the PCR reaction, after normalization to the Ct of the calibrator sample.

Statistical analysis

Differences between groups and controls were tested by unpaired *t* test (one way ANOVA). Each experiment included at least triplicate measurements for each condition tested. All results are expressed as mean ± SEM from at

least three independent experiments. Data were analyzed using the SPSS (version 11) software.

Results

The effect of lapatinib, gefitinib and trastuzumab on cell proliferation

Initially, we studied the effect of lapatinib, gefitinib, and trastuzumab on the proliferation of DLD-1 and Caco-2 cells. Lapatinib inhibited cell proliferation in both cell lines in a dose-dependent manner. At 1 μM, inhibition was greatest, reaching 35 ± 3% in DLD-1 cells and 27 ± 3% in Caco-2 cells (Figs. 1a, 2a). Gefitinib inhibited cell proliferation in a dose-dependent manner in both cell lines. The maximum inhibition was 40 ± 4% at 20 μM for DLD-1 cells and 35 ± 5% at 10 μM for Caco-2 cells as shown in Figs. 1b and 2b, respectively. Furthermore, we found that trastuzumab did not affect cell proliferation of DLD-1 (data not shown) as it has previously been described [23], but inhibited cell proliferation of Caco-2 in a dose-dependent manner (Fig. 2c). The maximum inhibition was observed at 40 μg/ml and was 24 ± 5%.

The effect of the combination of the tested agents on DLD-1 and Caco-2 proliferation was also studied. Combining lapatinib and gefitinib had no additional effect on DLD-1 proliferation (data not shown), while a synergistic effect was observed in Caco-2 proliferation with a reduction of 55 ± 5% (Fig. 2d). This synergistic effect was validated by

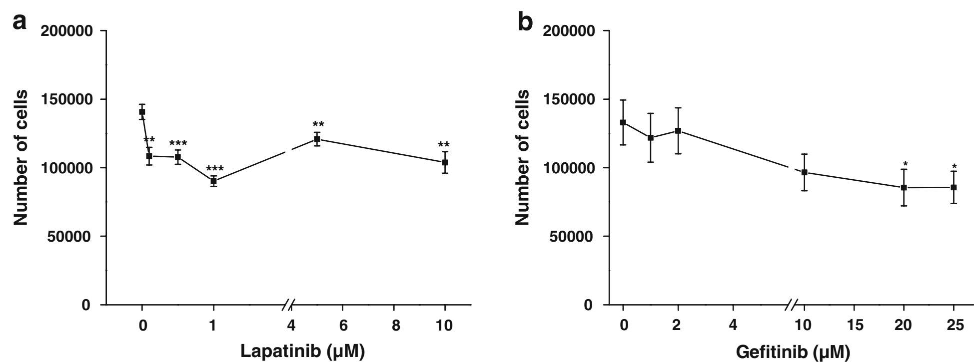

Fig. 1 The effect of lapatinib (**a**) and gefitinib (**b**) on DLD-1 proliferation. Different doses of lapatinib or gefitinib were applied on DLD-1 cells. Results are expressed as mean ± SEM of the number of cells. *Asterisks* denote a statistically significant difference (unpaired *t* test) compared to untreated cells. *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$

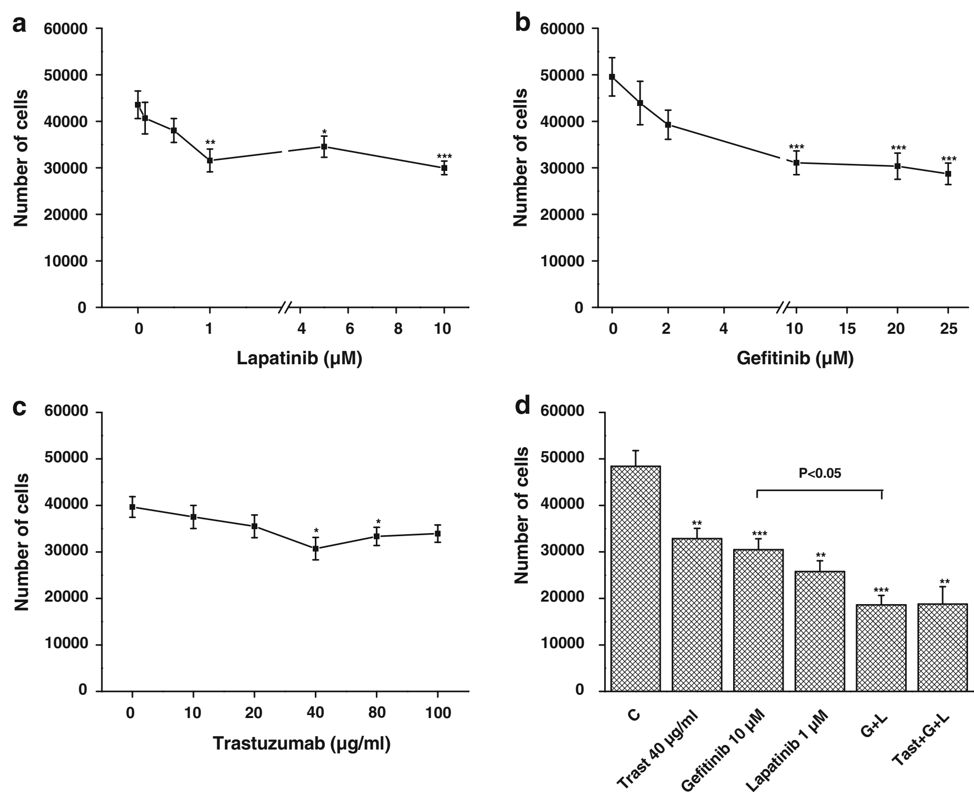

Fig. 2 The effect of lapatinib (**a**), gefitinib (**b**), trastuzumab (**c**) and their combination (**d**) on Caco-2 proliferation. Different doses of lapatinib, gefitinib or trastuzumab were applied on Caco-2 cells. Results are expressed as mean ± SEM of the number of cells. *Asterisks* denote a statistically significant difference (unpaired *t* test) compared to untreated cells. * $P < 0.05$, **$P < 0.01$ and ***$P < 0.001$

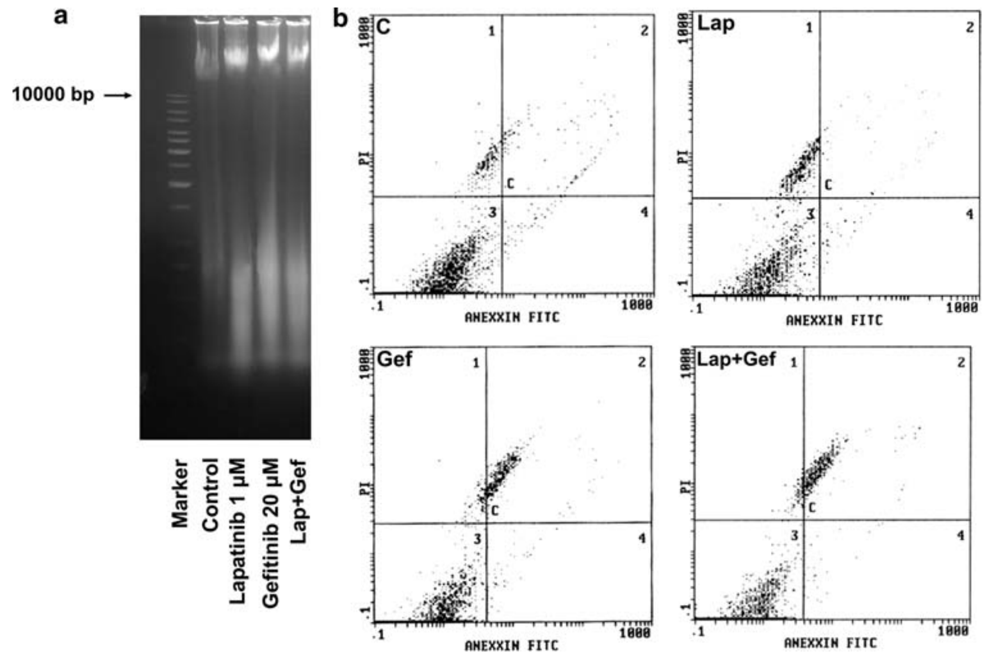

Fig. 3 The effect of gefitinib and lapatinib on DLD-1 apoptosis. DLD-1 cells were treated with 20 μM gefitinib and 1 μM lapatinib and 24 h later (**a**) DNA was extracted and analyzed in a 1.2% agaroze gel (Marker: 1-kb DNA ladder) or (**b**) cells were stained with Annexin/Propidium iodide and analyzed with flow cytometry. **c** Untreated DLD-1 cells, *Lap* DLD-1 cells treated with 1 μM lapatinib, *Gef* DLD-1 cells treated with 20 μM gefitinib, *Lap + Gef* DLD-1 cells treated with 1 μM lapatinib and 20 μM gefitinib

a formula that Fischel et al. have previously described [29]. The addition of trastuzumab to lapatinib and gefitinib did not alter the inhibitory effect of the combination (Fig. 2d).

The effect of lapatinib, gefitinib and trastuzumab on cell apoptosis

DLD-1 and Caco-2 cells were treated with the tested agents at the concentrations with the maximum inhibitory effect on cell proliferation. Twenty-four hours later, apoptosis was evaluated by DNA fragmentation and the Annexin V binding assay. Gefitinib alone and in combination with lapatinib increased DNA fragmentation in DLD-1 cells, whereas lapatinib alone exhibited no effect (Fig. 3a). These findings are in line with the results from the annexin/propidium iodide assay (Figs. 3b, 4). In contrast, gefitinib, lapatinib, and trastuzumab had no effect on Caco-2 cell apoptosis alone or in combination either by evaluating DNA fragmentation, or using Annexin V binding assay (data not shown).

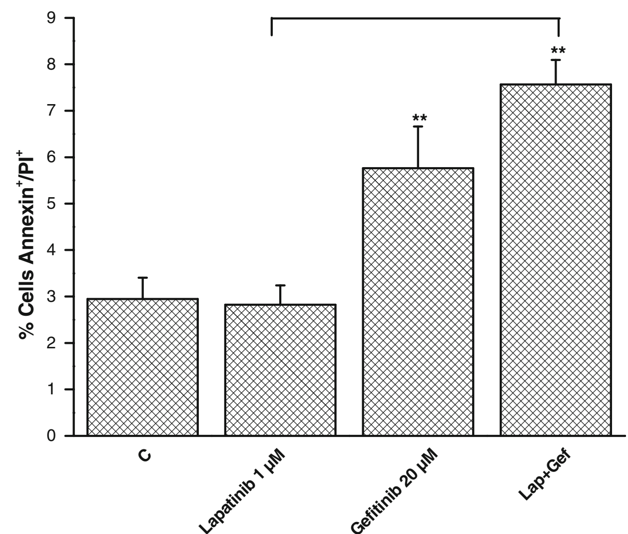

Fig. 4 Effect of lapatinib and gefitinib on apoptosis of DLD-1 cell line. DLD-1 cells were treated with 20 μM gefitinib and 1 μM lapatinib and 24 h later the samples were analyzed with flow cytometry. Results are expressed as the percentage of $Annexin^+$/Propidium $iodide^+$ cells ± SEM of the number of cells. *Asterisks* denote a statistically significant difference (unpaired *t* test) compared to untreated cells (C). $^{**}P < 0.01$

The effect of lapatinib, gefitinib and trastuzumab on EGFR and HER-2 protein levels

DLD-1 and Caco-2 cells were treated with the tested agents at the concentrations with the maximum inhibitory effect on cell proliferation. Forty-eight hours later, samples were immunoprecipitated with anti-HER-2 or anti-EGFR antibodies before assessing protein expression by immunoblotting. Immunoprecipitation is recommended for the detection of HER-2 or/and EGFR since direct western blot is not efficient for their detection [28]. We found that HER-2 protein levels decreased in DLD-1 cells after treatment with the combination of gefitinib and lapatinib, while lapatinib alone did not alter the protein levels (Fig. 5). As shown in Fig. 5, treatment of Caco-2 cells with gefitinib decreased HER-2 protein levels, while treatment of cells with trastuzumab increased HER-2 protein levels. In contrast, gefitinib alone and in combination with lapatinib as well as lapatinib with trastuzumab resulted in a decrease of HER-2

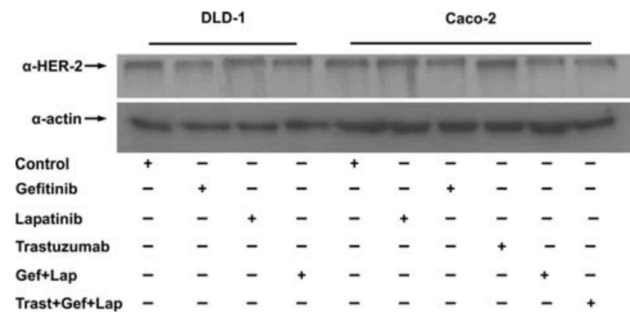

Fig. 5 Effect of lapatinib, gefitinib and trastuzumab on HER-2 protein levels in DLD-1 and Caco-2 cells. DLD-1 cells were treated with 20 μM gefitinib and 1 μM lapatinib and Caco-2 cells were treated with 10 μM gefitinib, 1 μM lapatinib and 40 μg/ml trastuzumab. The figure is a representative from at least three independent experiments (± indicate the presence/absence of the tested agent, respectively)

protein levels. Furthermore, EGFR protein levels decreased after treatment of both cell lines with combinations of gefitinib and lapatinib as well as gefitinib plus lapatinib and trastuzumab (Fig. 6), while each agent alone did not alter EGFR protein levels.

The effect of lapatinib, gefitinib and trastuzumab on EGFR and HER-2 mRNA levels

EGFR and HER-2 mRNA expression was assessed in DLD-1 and Caco-2 cells and both genes were found to be overexpressed in both cell lines compared to normal colonic tissue (data not shown). mRNA levels of EGFR and HER-2 were assessed by quantitative RT-PCR in DLD-1 and Caco-2 cells, 48 h after treatment of cells with or without the tested agents. Notable changes were recorded only in the following cases. In DLD-1 cells EGFR mRNA levels increased after treatment of cells with lapatinib (51 ± 5%) but decreased when it was combined with gefitinib (52 ± 4%) (Fig. 8). In Caco-2 cells, HER-2 mRNA levels were decreased with gefitinib (45 ± 7%) (Fig. 7) as also

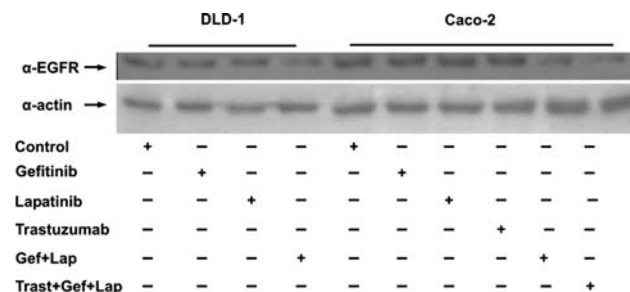

Fig. 6 Effect of lapatinib, gefitinib, and trastuzumab on EGFR protein levels in DLD-1 and Caco-2 cells. DLD-1 cells were treated with 20 μM gefitinib and 1 μM lapatinib and Caco-2 cells were treated with 10 μM gefitinib, 1 μM lapatinib and 40 μg/ml trastuzumab. The figure is a representative from at least three independent experiments (± indicate the presence/absence of the tested agent, respectively)

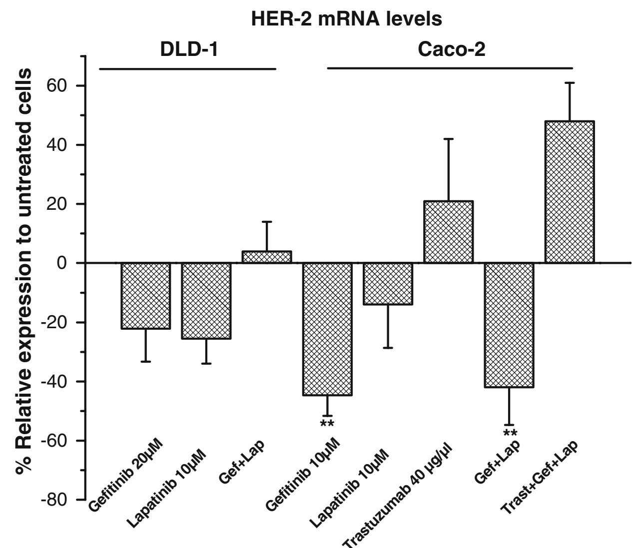

Fig. 7 HER-2 gene expression. DLD-1 cells were treated with 20 μM gefitinib and 1 μM lapatinib and Caco-2 cells were treated with 10 μM gefitinib, 1 μM lapatinib and 40 μg/ml trastuzumab. Results are expressed as relative expression and normalized to untreated cells. Asterisks denote a statistically significant difference (unpaired *t*-test) compared to untreated DLD-1 or Caco-2 respectively. *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$

when it was combined with lapatinib (42 ± 13%). EGFR levels decreased with trastuzumab alone and with the combination of gefitinib and lapatinib (40 ± 16% and 64 ± 8%, respectively) (Fig. 8).

Discussion

The present study sought to investigate the effect of gefitinib, lapatinib, and trastuzumab on two colon cancer cell lines, DLD-1 and Caco-2, by evaluating changes in cell proliferation, apoptosis, and expression of EGFR and HER-2.

Gefitinib decreased cell proliferation in a dose-dependent manner and increased apoptosis in DLD-1 cells. Previous studies have shown that gefitinib induces apoptosis in PCa, a prostate cancer cell line by downregulating the PI3K pathway through PTEN expression. It is known that EGFR can activate two critical signaling cascades: the Ras-Raf-MEK-ERK pathway and the PI3K-PDK1-Akt pathway [29]. Inhibition of these pathways results in a decrease in cell proliferation and an increase in apoptosis, respectively. Gefitinib may act in a similar manner in DLD-1 cells. Moreover, HER-2 mRNA levels were not altered by gefitinib while protein levels decreased. In contrast, EGFR mRNA levels increased while protein levels were not affected. The interaction between EGFR and HER-2 [30, 31] may explain why a drug that targets EGFR affects protein levels of HER-2. The increase in the mRNA levels of

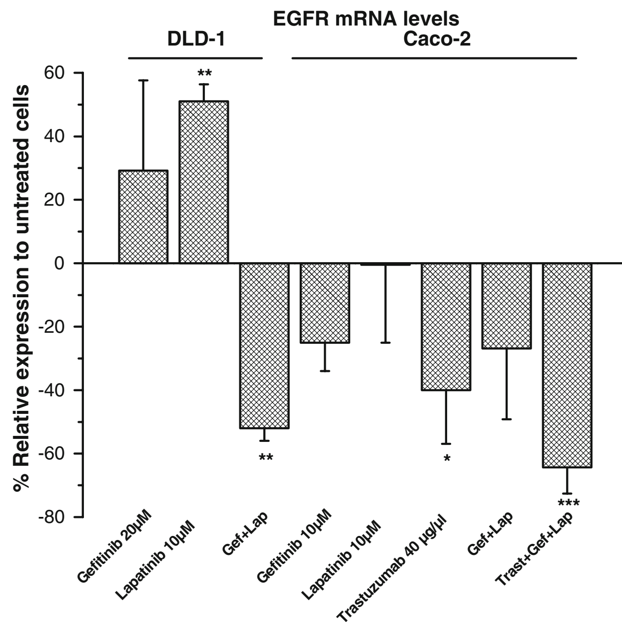

Fig. 8 EGFR gene expression. DLD-1 cells were treated with 20 μM gefitinib and 1 μM lapatinib and Caco-2 cells were treated with 10 μM gefitinib, 1 μM lapatinib and 40 μg/ml trastuzumab. Results are expressed as relative expression and normalized to untreated cells. Asterisks denote a statistically significant difference (unpaired t test) compared to untreated DLD-1 or Caco-2, respectively. $*P < 0.05$, $**P < 0.01$ and $***P < 0.001$

EGFR may represent the effort of the cell to overcome the inhibition of EGFR by activating its transcription [32].

In Caco-2 cells, gefitinib caused a decrease in cell proliferation in a dose-dependent manner without affecting apoptosis. The failure of gefitinib to augment apoptosis in Caco-2 cells may be associated with the lack of PTEN from lipid rafts involved in PI3K pathway [33]. HER-2 mRNA and protein levels were decreased after treatment of cells with gefitinib, whilst EGFR mRNA and protein levels were not affected. This may be attributable to the effect of gefitinib at both the translational and post-translational levels [34].

Lapatinib affected DLD-1 cells and Caco-2 cells in the same manner. It decreases cell proliferation in a dose-dependent manner but with no effect on apoptosis. Cells may be less sensitive to this molecule, because lapatinib is unable to downregulate the PI3K pathway. There are data regarding the failure of lapatinib to induce apoptosis in head and neck cancer cell line HN5, although the same molecule induces apoptosis in breast cancer cell line BT474, and it has an important role in the management of patients with trastuzumab refractory breast carcinoma [35, 36]. Moreover, lapatinib did not affect HER-2 and EGFR mRNA and protein expression.

Trastuzumab did not affect DLD-1 cell proliferation. The resistance of cells to this agent could be explained by a constitutive activation of HER-2 inactivating the receptor. Alternatively, cells may utilize other kinases to control cell proliferation and survival [23].

In Caco-2 cells, trastuzumab decreased cell proliferation in a dose-dependent manner without affecting apoptosis. Recent studies have shown that this antibody promoted cell-cycle arrest in breast cancer cells [36–38]. Furthermore, although HER-2 mRNA levels were not affected, protein levels increased suggesting a stabilization of the protein induced by the binding of the antibody to the extracellular domain of the receptor. In this way, the antibody inactivates the receptor, but also inhibits its degradation by metalloproteinases [37]. Although trastuzumab is a monoclonal antibody against HER-2, it decreased EGFR mRNA levels without affecting protein levels. The cross talk between EGFR and HER-2 may explain these changes.

The dual targeting of EGFR and HER-2 with the combination of gefitinib and lapatinib showed no further inhibition in cell proliferation compared to each agent alone. Instead, dual targeting of EGFR and HER-2 promoted apoptosis probably due to the presence of gefitinib and decreased the protein levels of both receptors that corresponded to a decrease in mRNA levels only in the case of EGFR. This observation strengthens the hypothesis that the antitumor activity of both agents is independent of the protein levels of HER-2 and EGFR. In all likelihood, the dual targeting of EGFR by two different agents could be more effective than each agent alone.

In Caco-2 cells, the combination of gefitinib and lapatinib exhibited a synergistic effect on cell proliferation, which may be related to the downregulation of EGFR and HER-2 protein levels. The downregulation of protein levels may be at the transcription level for HER-2 since a decrease was noted in HER-2 mRNA levels, although for EGFR the regulation may occur at the protein level as EGFR mRNA levels did not change. Apoptosis was not affected probably due to the absence of PTEN [33] as discussed earlier, whereas the effect on cell proliferation may be attributed to a cell-cycle arrest.

The combination of trastuzumab, gefitinib, and lapatinib was studied only in Caco-2 cells since trastuzumab had no effect on DLD-1 cell proliferation. This combination did not decrease cell proliferation or increase apoptosis more than the combination of gefitinib and lapatinib. Similar results were observed for EGFR and HER-2 mRNA and protein levels. Although the combination of trastuzumab, gefitinib, and lapatinib targets the extracellular and the intracellular domain of HER-2 receptor and the intracellular domain of EGFR receptor, the efficacy of this combination was not improved compared to the combination of gefitinib and lapatinib.

In conclusion, single blockade of EGFR and/or HER-2 had an antitumor effect on both colon cancer cell lines, while the dual targeting of EGFR and HER-2 improved the

antiproliferative effect only in Caco-2 cells. Furthermore, the changes in HER-2 or EGFR protein and mRNA levels seem to be—at least in certain cases—independent of the antitumor effect of the tested agents. However, the expression of these molecules may be important for long term effects. Moreover, the dual targeting of both the extracellular and the intracellular domain of HER-2 receptor did not improve the antitumor effect.

The divergent response of the two cell lines to treatment may reflect the differences in their genetic background and, therefore, the signaling pathways activated. Further studies are required to explore the way the current agents exert their antitumor effect through HER- dependent and independent signaling pathways.

Acknowledgments Efstathia Giannopoulou and Anna Antonacopoulou were supported by Hellenic Cooperative Oncology Group (HECOG).

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