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ORIGINAL ARTICLE

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The effect of gefitinib (Iressa, ZD1839) in combination with oxaliplatin is schedule-dependent in colon cancer cell lines

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Abstract *Background*: Clinical trials of gefitinib (Iressa, ZD1839) in combination with cytotoxic agents have been carried out or are ongoing in several varieties of tumor. To provide a rationale for future clinical trials, the effects of combining gefitinib with oxaliplatin in different sequences of administration and different dose ratios in two colon cancer cell lines were evaluated. *Materials and methods*: The colon cancer cell lines HT-29 and LoVo were used. The methods consisted of median effect and combination index analysis, Western blot, mass spectrometry, and a cell death ELISA. *Results*: In vitro analysis demonstrated that the combination effects of the two agents were sequence-dependent. Changing the sequence of administration from gefitinib first to gefitinib last changed the combination effect from antagonism to synergy. The dose ratio between the two agents affected the combination effects. When equiactive doses of the two agents were used with the sequence gefitinib following oxaliplatin, the greatest level of synergism was obtained ($CI = 0.6 \pm 0.2$, $P = 0.032$). Further evaluation revealed that gefitinib significantly inhibited removal of Pt-DNA adducts ($P < 0.05$), providing a potential explanation for the sequence-dependent synergy observed with gefitinib following oxaliplatin. However, this effect was not dose-dependent. Additional studies demonstrated that gefitinib enhanced the effects of oxaliplatin by maintaining oxaliplatin-induced apoptosis, and equiactive dose of gefitinib following oxaliplatin induced prominent enhancement of apoptosis. *Conclusions*: Oxaliplatin followed by an equiactive relative dose of gefitinib is an appropriate combination for evaluation in colon cancer.

Keywords Epidermal growth factor receptor · Gefitinib (Iressa, ZD1839) · Oxaliplatin · Pt-DNA adducts · Apoptosis · Colon cancer

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Introduction

Colorectal cancer is frequently associated with the expression of high levels of the epidermal growth factor receptor (EGFR), and this high expression is associated with more aggressive disease and a poor prognosis [1, 2, 3, 4]. For these reasons, the blockade of EGFR activation and/or function has been proposed as a potential therapeutic strategy for colorectal cancer [5, 6]. More recently, various anti-EGFR blocking monoclonal antibodies (mAbs) and small molecule inhibitors of EGFR tyrosine kinase enzymatic activity have been developed [7, 8, 9]. One of these agents, gefitinib, an anilinoquinazoline, is an orally active EGFR tyrosine kinase inhibitor (EGFR-TKI). Gefitinib has shown antitumor activity in a range of human cancer cell lines and xenografts [10, 11, 12]. It has shown promising clinical efficacy, and phase II and phase III clinical evaluation in cancer patients has been started [13].

In experimental preclinical models, gefitinib has shown a synergistic effect with chemotherapeutic drugs [14, 15]. This provides the rationale for combining gefitinib and some chemotherapeutic drugs for clinical trial in colorectal cancer. Clinical investigation of gefitinib in combination with various chemotherapeutic drugs has been reported [13]. Oxaliplatin, a new platin analogue, has shown significant single-agent activity in advanced colorectal cancer, with relative absence of cross-resistance with cisplatin and carboplatin [16, 17]. Its response rates range from 10% to 20%, depending on the dose

administered and whether the patient has previously received chemotherapy [18, 19]. A direct comparison of the preclinical activity of the combination of gefitinib and oxaliplatin has not yet been reported. The present study aimed to determine whether gefitinib in combination with oxaliplatin caused effects in colon cancer cell lines that were dependent on the sequence and dose ratio of administration.

Materials and methods

Materials

Gefitinib was kindly provided by AstraZeneca. Stock solutions were prepared at 10 m*M* in dimethylsulfoxide (DMSO) and stored in aliquots at −20°C. Oxaliplatin was purchased from Sanofi (Milan, Italy) and dissolved in phosphate-buffered saline (PBS) to prepare a 10 m*M* stock solution. These drugs were further diluted with culture medium immediately before use. HindIII and RNase A were purchased from TaKaRa (Kyoto, Japan).

Cell lines

The colon cancer cell lines HT-29 and LoVo were obtained from the American Type Culture Collection and were maintained at 37°C in an incubator under an atmosphere containing 5% carbon dioxide. HT-29 cells and LoVo cells were routinely cultured in McCoy's 5A medium and Ham's F12 medium, respectively, supplemented with 10% calf serum (Life Technologies).

Evaluation of cytotoxicity

Cells were seeded in 96-well microtiter plates (100 µl/well) to obtain exponential growth for the whole duration of the experiment (the initial cell number was 1×10^4 and 2×10^4 viable cells per well for HT-29 and LoVo, respectively). The cells had attached to the bottom of the plate 24 h later. Cells were then exposed to different concentrations of drugs. At the end of the indicated incubation period, growth inhibition was determined by MTT assay as described previously [20]. For each drug, the results are expressed as the relative percent absorbance compared with controls without drug. Cell sensitivity to drug is expressed as the drug concentrations that yielded 50% cell inhibition (IC_{50}). Experimental conditions were tested in sextuplicate (six wells of the 96-well plate per experimental condition). Dose-response curves were generated. All of the experiments were performed in triplicate.

Median effect and combination index (CI) analysis

In order to determine the effects of sequencing, cells were treated with serial dilutions of the two drugs sequentially or simultaneously at a fixed drug exposure time. After incubation with one drug, or gefitinib plus oxaliplatin, cells were washed three times and then incubated with the other drug for the indicated time.

Cells were exposed to oxaliplatin and gefitinib in the following sequences:

- Sequence I: gefitinib for 5 days followed by oxaliplatin for 1 day
- Sequence II: gefitinib plus oxaliplatin for 1 day followed by gefitinib for 4 days
- Sequence III: oxaliplatin for 1 day followed by gefitinib for 5 days

To determine the relative contribution of gefitinib to the combination effect, three sequences were evaluated at equiactive doses of the two agents (oxaliplatin/gefitinib IC_{50} 1:1), at higher relative doses of gefitinib (oxaliplatin/gefitinib IC_{50} 1:10), and at lower relative doses of gefitinib (oxaliplatin/gefitinib IC_{50} 1:0.1).

Fractional survival (f) was calculated by dividing the mean optical density (OD) value in the drug-treated group by the OD value in the control group. The data from the combined drug effects were analyzed by the method of Chou and Talalay [21]. In brief, log[(1/f)−1] was plotted against log(drug dose). From the resulting median effect lines, the *x* intercept [log(IC_{50})] and slope *m* were calculated for each drug and for the combination by the method of least squares. These parameters were then used to calculate the doses of individual drugs and the combination required to produce various levels of cytotoxicity according to the following equation:

$$\mathrm{Dose_f} = \mathrm{Dose_{IC50}}[(1-\mathrm{f})/\mathrm{f}]^{1/\mathrm{m}} \quad (1)$$

Because the two drugs were administered at a fixed ratio, the dose of the combination required to produce fractional survival f could be divided into the component doses $(D)_1$ and $(D)_2$ of drugs 1 and 2, respectively. For each level of cytotoxicity, the CI was then calculated according to following equation:

$$\mathrm{CI} = (D)_1/(D_f)_1 + (D)_2/(D_f)_2 + \alpha(D)_1(D)_2/(D_f)_1(D_f)_2 \quad (2)$$

Where $(D)_1$ and $(D)_2$ are the concentrations of the combination required to produce survival f, and $(D_f)_1$ and $(D_f)_2$ are the concentrations of the individual drugs required to produce survival f. The CIs were calculated based on the most conservative assumption of mutually nonexclusive drug interactions ($\alpha = 1$) since oxaliplatin has a different mechanism of action from gefitinib. In this method, the CI indicates antagonism ($CI > 1$), additivity ($CI = 1$), or synergism (CI). The linear correlation coefficient *r* was generated for each curve to determine the applicability of the data to this method of analysis. In all experiments, *r* was > 0.9.

Immunoprecipitation and Western blotting

Total cell lysates were prepared by homogenizing the cell pellets in lysis buffer (1% Triton, 50 m*M* Tris-HCl, pH 7.4, 150 m*M* NaCl, 5 m*M* EDTA, 10% glycerol, 1 m*M* phenylmethylsulfonyl fluoride, 2 m*M* Na-orthovanadate, 10 m*M* leupeptin, 100 m*M* Na-fluoride, 10 m*M* Na-pyrophosphate) as described previously [22] of cells that had been treated with the gefitinib and/or oxaliplatin schedules. Protein concentrations were determined using the Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, Calif.). For EGFR autophosphorylation analysis, protein extracts were immunoprecipitated for 6 h with anti-EGFR mAb and 20 µl of protein-A/G Sepharose beads (Amersham Pharmacia Biotech, Little Chalfont, UK). Precipitates were then separated by 8% SDS-PAGE and transferred onto a nitrocellulose membrane. The filters were blocked in 5% skimmed milk for 1 h before probing with P-Tyr mAb at a dilution of 1:200. For Western blot analysis of EGFR expression, aliquots containing 50 µg protein were loaded onto a 10% SDS-PAGE and probed with anti-EGFR mAb (BD Bioscience) at a dilution of 1:200 for 1 h. After the incubations with the primary mAbs, the filter was washed and incubated with secondary anti-mouse IgG horseradish peroxidase-linked antibody (Sigma Chemical Company, St Louis, Mo.) at a dilution of 1:20,000 for 1 h. After additional washes, the blots were developed by an enhanced chemiluminescence detection system (ECL-plus, Amersham Pharmacia Biotech).

Measurement of Pt-DNA adducts

Pt-DNA adducts were measured as described by Walker et al. [23]. HT-29 cells grown to 70–80% confluence on four 100-mm tissue culture dishes were treated with drugs as indicated. At the completion of the incubation, cells were scraped from the plates without removal of drug-containing medium, sedimented at 200 *g* for

10 min, washed three times with ice-cold PBS, and lysed in 5 ml of TEN buffer [10 m*M* Tris-HCl (pH 7.4 at 21°C), 10 m*M* EDTA, 150 m*M* NaCl] supplemented with 0.4% SDS and 1 mg/ml of proteinase K. After incubation at 50°C for 16 h, highly purified DNA samples were prepared by extraction with phenol/$CHCl_3$ and $CHCl_3$, ethanol precipitation, RNAse A treatment, phenol/$CHCl_3$ and $CHCl_3$ extraction, and HindIII digestion. After aliquots (2 μg DNA) had been subjected to electrophoresis on agarose minigels to confirm complete removal of RNA and digestion of DNA, DNA was re-extracted with phenol-$CHCl_3$ and $CHCl_3$, ethanol-precipitated, resuspended in 750 μl 0.6 *M* HCl, and heated to 95°C for 30 min. The DNA concentration was estimated by measuring absorbance at 260 nm.

Elemental Pt was assayed by inductively coupled plasma mass spectrometry as described previously [23]. In brief, a Gilson AS90 autosampler was used to infuse samples at a rate of 0.5 ml/min into a Perkin-Elmer Sciex 6000 mass spectrometer operating at the following settings: nebulizer flow rate 0.9 l/min, inductively coupled plasma RF power 1200 W, lens voltage 8.0 V, analog stage voltage −2100 V, pulse stage voltage 1700 V, and dwell time 100 ns/amu. Platinum is expressed as the sum of platinum species detected at 194 and 195 amu using a program that swept from 1 to 263 amu 50 times per reading. Platinum standards (0.2 to 20 ng/ml in 0.6 *M* HCl) were utilized to confirm the linearity of the assay ($R \geq 0.999$), and rhodium-103 served as an internal standard. Each experiment was performed three times.

Apoptosis analysis

Cells were plated at 5×10^4 cells/well in a 96-well microtiter plate and grown for 24 h. After 24 h, the cells were treated with gefitinib and/or oxaliplatin as indicated, samples harvested and analyzed for histone-associated DNA fragments using a Cell Death ELISA^{PLUS} kit according to the manufacturer's instructions (Roche Molecular Biochemicals, Shanghai, China). The test is based on the detection of mono- and oligonucleosomes in the cytoplasmic fraction of cell lysates by biotinylated antihistone-coupled antibodies, and their enrichment in the cytoplasm is calculated as absorbance of sample cells/absorbance of control cells. The enrichment factor was used as a measure of apoptosis and is shown on the *y*-axis as mean ± SD.

Statistical analysis

Differences between the mean values of the two subgroups were evaluated using Student's *t*-test. Differences between the mean values of three subgroups were compared by one-way analysis of variance (ANOVA). Two-sided *P* values ≤ 0.05 were considered statistically significant.

Results

Response of colon cancer cell lines to single agents

Cells were treated with gefitinib (0.01–20 μ*M*) or oxaliplatin (0.01–10 μ*M*) alone at various concentrations for different times. As shown in Table 1, markedly time-dependent activity of gefitinib was observed, with the IC_{50} values of HT-29 and LoVo cells after a 3-day exposure more than 6-fold and 12-fold higher than after a 5-day exposure, respectively. However, the time-dependent cytotoxicity of oxaliplatin was less pronounced, with the IC_{50} values of HT-29 and LoVo cells after a 1-day exposure only 3.5-fold and 3-fold higher than after a 5-day exposure.

Table 1 IC_{50} values of single drug oxaliplatin and gefitinib to HT-29 and LoVo cells with different exposure times. Each value is the mean ± SD of at least three experiments (*ND* not determined; above the range of concentrations tested)

Drug	Exposure (days)	IC_{50} (μ*M*)	
		HT-29	LoVo
Oxaliplatin	1	15.1 ± 2.3	10.4 ± 2.1
	3	8.2 ± 1.1	6.0 ± 0.8
	5	4.0 ± 0.2	3.1 ± 0.3
Gefitinib	1	ND	ND
	2	ND	16.5 ± 1.6
	3	23.6 ± 1.9	7.3 ± 0.6
	4	7.7 ± 0.8	2.6 ± 0.5
	5	3.8 ± 0.4	0.6 ± 0.1

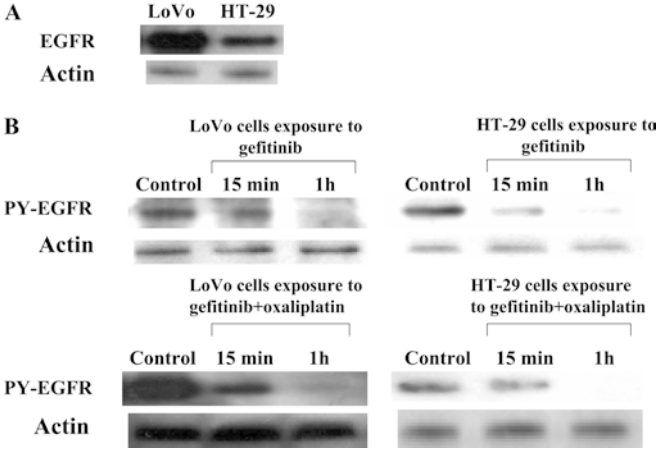


Fig. 1 **A** Western blot analysis of EGFR expression in LoVo and HT-29 cell lines. **B** Effects of gefitinib alone or gefitinib in combination with oxaliplatin on EGFR phosphorylation. LoVo and HT-29 cells were treated with 0.6 μ*M* and 3.8 μ*M* gefitinib alone, respectively, or the relevant concentrations of gefitinib in combination with 10.4 μ*M* oxaliplatin, respectively, for the indicated times. At the completion of the incubation, samples were prepared for determination of EGFR phosphorylation

EGFR expression and effects of gefitinib on EGFR phosphorylation

As shown in Fig. 1A, different expression levels of EGFR were detected in LoVo and HT-29 cells, with LoVo cells expressing higher levels of EGFR than HT-29 cells. When LoVo and HT-29 cells were treated with gefitinib for 5 days alone or in combination with oxaliplatin for 1 day at their respective IC_{50} values, similar time-dependent inhibitions of EGFR tyrosine phosphorylation were obtained, with both treatments resulting in nearly complete inhibition of EGFR phosphorylation in 1 h (Fig. 1B).

Evaluation of the combination effect of gefitinib and oxaliplatin

HT-29 and LoVo cells were treated with serial dilutions of oxaliplatin that corresponded to 1/20, 1/10, 1/5, 1/2,

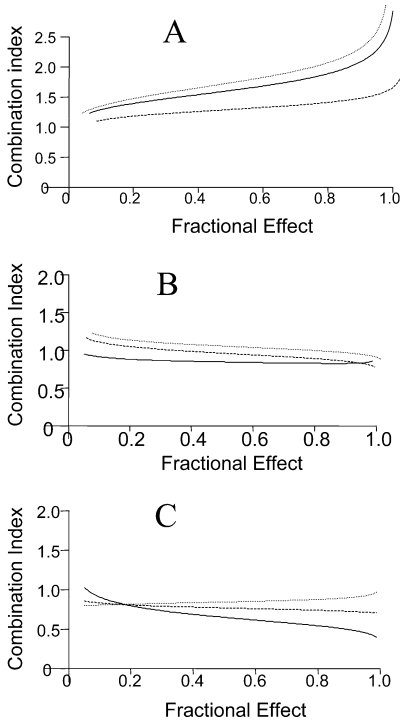


Fig. 2A–C Effect of sequential treatments with gefitinib and oxaliplatin at different molar ratios: oxaliplatin/gefitinib 1:0.025, 1:0.25 and 1:2.5 (*dashed line*, *solid line*, and *dotted line*, respectively). **A–C** CI plots with the assumption that the effects of the drugs were mutually nonexclusive. HT-29 cells were treated with (**A**) gefitinib for 5 days followed by oxaliplatin for 1 day (sequence I), (**B**) gefitinib plus oxaliplatin for 1 day followed by gefitinib for 4 days (sequence II), and (**C**) oxaliplatin for 1 day followed by gefitinib for 5 days (sequence III). The results presented are representative of three independent experiments

1, 1.5 and 2 times the individual IC_{50} values. Because the IC_{50} values of oxaliplatin 1-day exposure in HT-29 and in LoVo cells were fourfold and tenfold higher than those of gefitinib 5-day exposure, respectively, the molar ratios used to analyze the combination effect were:

- For equiactive doses: oxaliplatin/gefitinib 1:0.25 in HT-29 cells, 1:0.1 in LoVo cells
- For higher relative doses of gefitinib: oxaliplatin/gefitinib 1:2.5 in HT-29 cells, 1:1 in LoVo cells
- For lower relative doses of gefitinib: oxaliplatin/gefitinib 1:0.025 in HT-29 cells, 1:0.01 in LoVo cells

Therefore, the serial dilutions of gefitinib were modified according to the molar ratios.

In HT-29 cells, sequence I produced an antagonistic effect at broad effect ranges and all three dose ratios tested (Fig. 2A and Table 2). This antagonism was particularly pronounced at high effect levels, with a CI of 1.9 ± 0.3 ($P < 0.001$) at the IC_{50}. The data in Fig. 2A show a trend for CI values to decrease as the relative dose of gefitinib changed from higher relative doses to lower relative doses. Sequence II resulted in additive effects or nearly additive effects, with the CI values at the IC_{50} values tending to decrease from higher relative doses to equiactive relative doses of gefitinib ($P > 0.05$; Fig. 2B and Table 2). For sequence III, synergy was obtained at all dose ratios tested, with the CI values at all effect levels being < 1 (Fig. 2C and Table 2). However, the synergistic effect was greatest at the IC_{50} when an equiactive dose of gefitinib was used ($CI = 0.6 \pm 0.2$, $P = 0.032$), and the higher relative dose of gefitinib in the combination resulted in a lower degree of synergism. These observations argue against the possibility that subsequent gefitinib augmenting the effects of oxaliplatin is dose-dependent.

Table 2 CI results in HT-29 and LoVo cell lines exposed to different schedules of oxaliplatin and gefitinib. Cells were exposed to the two agents at the different dose ratios.*Sequence I*gefitinib for 5 days followed by oxaliplatin for 1 day; *sequence II* simultaneous gefitinib and oxaliplatin for 1 day followed by gefitinib for 4 days; *sequence III*oxaliplatin for 1 day followed by gefitinib for 5 days. Each value is the mean of at least three experiments carried out in triplicate. CI values of the three groups were compared by one-way analysis of variance (*CI at IC_{50}* combination index indicating the combined amount of the two agents necessary to inhibit 50% of the cells)

Cell line	Dose ratio (oxaliplatin/gefitinib)	CI at IC_{50}, mean (SD)		
		Sequence I	Sequence II	Sequence III
HT-29	1:2.5	1.9 (0.3)	1.1 (0.2)	0.8 (0.1)
	1:0.25	1.7 (0.2)	1.0 (0.1)	0.6 (0.2)
	1:0.025	1.4 (0.1)	1.0 (0.1)	0.7 (0.2)
P value		0.000	0.157	0.032
LoVo	1:1	1.6 (0.2)	1.1 (0.2)	0.7 (0.1)
	1:0.1	1.4 (0.2)	1.0 (0.1)	0.5 (0.2)
	1:0.01	1.2 (0.1)	1.1 (0.2)	0.6 (0.1)
P value		0.000	0.168	0.008

Similar to the results with HT-29 cells, schedule-dependent synergy was observed in LoVo cells (Table 2). Less than additive and additive effects were observed with sequence I and sequence II, respectively. The CI value with sequence I was > 1 at the IC_{50} and with sequence II approached 1 at the IC_{50} at different dose ratios. Synergistic effects were obtained with sequence III at all dose ratios, with the equiactive dose of gefitinib resulting in a greater potentiation at broad effect ranges ($CI = 0.5 \pm 0.2$ at the IC_{50}, $P = 0.008$)

Effects of dose of gefitinib on inhibition of removal of Pt-DNA adducts

DNA is recognized as the primary cellular target of platinum. Therefore, to explain the observed synergy with sequence III and the greatest synergism at equiactive relative doses of gefitinib, the removal of Pt-DNA adducts was measured in the absence and presence of gefitinib. For this experiment, after 1 day of exposure to 15.1 μM oxaliplatin, HT-29 cells were incubated with oxaliplatin-free medium in the absence or presence of moderate doses (3.8 μM), or lower (0.38 μM) or higher (38 μM) relative doses of gefitinib for 1 day. As shown in Fig. 3, in the absence of gefitinib, 44.0% of Pt-DNA adducts were removed. However, in the presence of

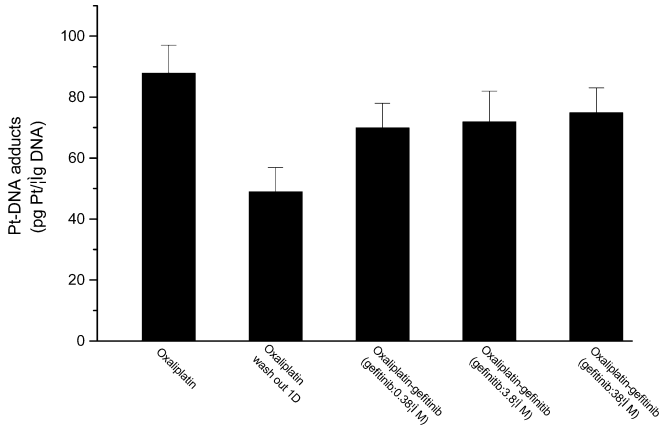


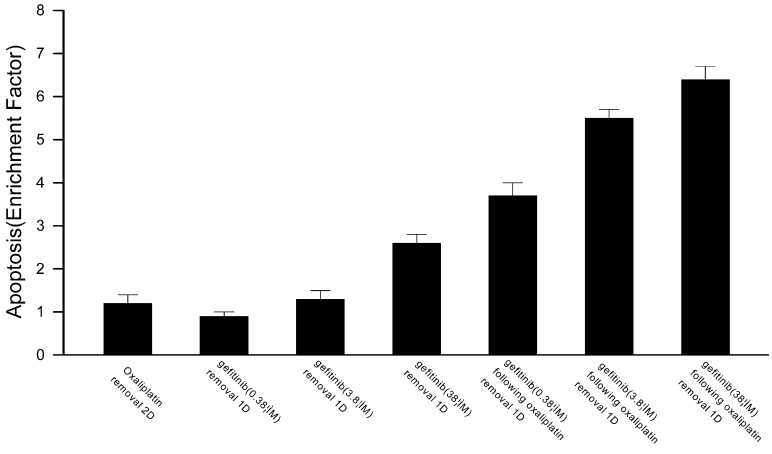
Fig. 3 Effect of gefitinib and oxaliplatin on Pt-DNA adducts. HT-29 cells were treated with oxaliplatin alone or oxaliplatin followed by gefitinib. After exposure to oxaliplatin for 1 day, cells were incubated in the absence or presence of different concentrations of gefitinib (0.38 μ*M*, 3.8 μ*M*, or 38 μ*M*) for 1 day and then harvested for measurement of Pt-DNA adducts. The results presented are means ± SD (*bars*) of three independent experiments performed in triplicate

lower, moderate or higher relative doses of gefitinib, 20.5%, 18.2% and 14.8% of Pt-DNA adducts were removed, respectively. These results indicate that gefitinib significantly inhibited removal of Pt-DNA adducts ($P = 0.032$, $P = 0.024$, $P = 0.016$, respectively), and increasing gefitinib from lower to higher relative doses did not result in a marked enhancement in inhibition of removal of Pt-DNA adducts ($P = 0.753$).

Effects of gefitinib on oxaliplatin-induced apoptosis

Since the higher relative dose of gefitinib following oxaliplatin did not result in pronounced inhibition of removal of Pt-DNA adducts, studies were performed to determine whether the enhancement of oxaliplatin-induced apoptotic events by gefitinib was dose-dependent. Cells were treated with 15.1 μ*M* oxaliplatin alone for 1 day, washed three times, then incubated with 0.38 μ*M*, 3.8 μ*M* or 38 μ*M* of gefitinib for 1 day. As shown in Fig. 4, subsequent gefitinib increased apoptotic maintenance. Notably, moderate and higher relative doses of gefitinib resulted in marked increases in apoptosis compared with the lower relative dose of gefitinib. However, the higher relative dose of gefitinib did not potentiate apoptosis significantly compared with the moderate dose of gefitinib. These results provide a potential explanation for the greatest synergism with the moderate dose of gefitinib following oxaliplatin.

Fig. 4 Effects of gefitinib and oxaliplatin on apoptosis. HT-29 cells were incubated with oxaliplatin alone (15.1 μ*M*), gefitinib alone (0.38 μ*M*, 3.8 μ*M*, or 38 μ*M*) for 1 day, with oxaliplatin for 1 day followed by different concentrations of gefitinib (0.38 μ*M*, 3.8 μ*M*, and 38 μ*M*) for 1 day. After completion of the treatments, cells were washed and incubated for 1 or 2 days in drug-free medium and the degree of apoptosis evaluated using an apoptosis kit. The results presented are means ± SD (*bars*) of triplicate determinations



Discussion

In the present study, we demonstrated that oxaliplatin produces sequence-dependent cytotoxicity when combined with gefitinib in HT-29 and LoVo cells. In particular, sequential exposure to oxaliplatin for 1 day followed by gefitinib for 5 days produced additive and synergistic effects, while sequential exposure to gefitinib for 5 days followed by oxaliplatin for 1 day showed mainly antagonistic effects. Overall, the results show that changing sequences from gefitinib first to gefitinib last resulted in a change from antagonism to synergy.

This sequence dependency was also consistent among the three dose ratios tested, with the equiactive dose of gefitinib producing the greatest absolute level of synergism following sequential exposure to oxaliplatin then gefitinib. These results suggest that the enhancement of synergy between the two agents was not gefitinib dose-dependent. In this regard, not only the appropriate sequence but also the appropriate dose ratio of gefitinib in combination with oxaliplatin should be considered in the investigation of cellular interaction between these two agents.

Although it has been shown that a high level of EGFR expression is associated with a drug-resistant cell line [24], in this study, a 1.5-fold increase in sensitivity to

a 1-day exposure to oxaliplatin was obtained in LoVo cells as compared with HT-29 cells. This indicates that factors other than simply the amount of EGFR may influence the schedule-dependent synergy of oxaliplatin in combination with gefitinib. Furthermore, gefitinib alone or gefitinib combined with oxaliplatin at their relevant IC_{50} values markedly inhibited EGFR tyrosine phosphorylation in 1 h in both LoVo and HT-29 cells. This observation rules out the possibility that oxaliplatin is potentiating the inhibition of EGFR phosphorylation by gefitinib.

Previous studies have indicated that the level of Pt-DNA adducts is related to oxaliplatin cytotoxicity, and the decrease in Pt-DNA adducts results from an increase in DNA repair [25, 26]. These observations raise the possibility that an agent that diminishes the repair of Pt-DNA adducts may play a role in potentiating oxaliplatin-induced cytotoxicity. Our further experiments revealed that gefitinib inhibited removal of Pt-DNA adducts, providing a potential explanation for the synergistic effects observed when gefitinib was added to cells after oxaliplatin. Subsequent studies demonstrated that the removal of Pt-DNA adducts was even markedly inhibited by the lower relative dose of gefitinib, and that higher relative doses of gefitinib did not markedly potentiate the inhibition of removal of Pt-DNA adducts. These results do not explain the observations that the greatest synergy was obtained with the moderate dose of gefitinib following oxaliplatin. This prompted us to further examine apoptotic events induced by gefitinib following oxaliplatin.

Some studies have demonstrated that the blockade of EGFR signaling in combination with cytotoxic agents can cause irreparable damage to cancer cells, leading to increased apoptosis [27, 28]. Given the mechanism of the synergistic effects of cytotoxic agents in combination with EGFR inhibitor, the synergistic effects observed with sequence III in the present study suggest that gefitinib might be enhancing the action of oxaliplatin by maintaining oxaliplatin-induced apoptosis. Further experiments revealed that the moderate dose of gefitinib following oxaliplatin induced marked enhancement of apoptosis as compared with that induced by the lower relative dose of gefitinib. These results are in agreement with those of previous studies indicating that the antiproliferative effect of the higher dose of gefitinib is accompanied by induction of apoptosis [29]. However, this dose-dependent potentiation is limited: with the higher relative dose of gefitinib, the apoptosis was no longer enhanced significantly. Taken together, the results of these studies provide a potential explanation for the greatest synergy observed with the moderate dose of gefitinib following oxaliplatin.

Since an important mechanism of synergism for an EGFR inhibitor following a cytotoxic agent could be the potentiation and maintenance of apoptosis, several factors regarding the most effective use of gefitinib should be considered. First, the maximum tolerated dose of cytotoxic agent will be administered since the essential prerequisite for synergy of these agents is that the cytotoxic drug induces maximal cell damage, so that the simultaneous or subsequent receptor blockade could interfere with repair of the damage. Second, the timing of gefitinib administration is important. Since the optimal time to give gefitinib is when maximal cell damage has been obtained, adjusting the timing of gefitinib administration for an individual tumor based on its response to chemotherapy may actually achieve its potential. If the tumor does not respond to the cytotoxic agent, subsequent gefitinib will not be expected to achieve its potential synergy with chemotherapy. With these considerations, these studies provide some basic information for the rational design of a clinical protocol of cytotoxic agents in combination with gefitinib. Recently, the final efficacy analysis of the initial two large trials [30, 31] has demonstrated that there is no clinical benefit when gefitinib is added to chemotherapy simultaneously in non-small-cell lung cancer. Whether the negative results simply indicate a lack of efficacy of gefitinib in combination with the two chemotherapeutic regimens in advanced non-small-cell lung cancer or are a result of poor patient selection requires further study.

The clinically relevant conditions used to explore the synergistic mechanism of agents have important practical implications. In the clinic, oxaliplatin is usually administered at a dose level of 130 mg/m^2 as a 4-h infusion on day 1, and during the terminal elimination phase, plasma levels of free platin decline with a half-life of up to 24 h [32]. In two phase III trials of gefitinib in combination with carboplatin/paclitaxel or gemcitabine/cisplatin in patients with non-small-cell lung cancer, gefitinib was administered as once-daily oral doses of 250 mg and 500 mg. The pharmacokinetic results showed that, following administration of 225–525 mg/day of gefitinib, biologically relevant plasma concentrations of gefitinib ranging from 150 ng/ml to 469 ng/ml can be maintained for more than 24 h [33, 34]. The in vitro IC_{50} values of gefitinib following a 5-day exposure obtained in HT-29 and LoVo cells are right in the plasma concentration range of gefitinib. Therefore, the findings in this study that used clinically relevant condition have important practical implications.

Our results demonstrated that the combination of oxaliplatin with gefitinib not only displays synergism when oxaliplatin is followed by gefitinib in these two cell lines, but also that greater synergistic effects occur at equiactive doses of gefitinib. Further analyses demonstrated that gefitinib inhibits removal of Pt-DNA adducts, which was not dose-dependent. However, gefitinib maintains oxaliplatin-induced apoptosis, which was dose-dependent in a limited dose range. These findings suggest that oxaliplatin followed by an equiactive or moderate relative dose of gefitinib is an appropriate combination for evaluation in colon cancer.

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