Available online at www.sciencedirect.com
SCIENCE DIRECT
Biochemical Pharmacology 68 (2004) 135–144
Biochemical Pharmacology
www.elsevier.com/locate/biochempharm

The schedule-dependent enhanced cytotoxic activity of 7-ethyl-10-hydroxy-camptothecin (SN-38) in combination with Gefitinib (Iressa™, ZD1839)

Amalia Azzariti[a], Jian-Ming Xu[a,b], Letizia Porcelli[a], Angelo Paradiso[a,*]

[a]*Clinical Experimental Oncology Laboratory, National Cancer Institute, Bari, Italy*
[b]*Beijing 307 Hospital Cancer Center, Beijing, China*

Received 3 November 2003; accepted 17 March 2004

Abstract

The combination of the topoisomerase I (Topo I) inhibitor CPT-11 with the anti-epidermal growth factor receptor (EGFR) agent Gefitinib (Iressa™, ZD1839) represents a promising medical approach for colorectal cancer patients. In this report, we provide pre-clinical evidences for their optimal combination schedule in HT-29 and LoVo human colon cancer cell lines. We analyzed the different effects that three different combination schedules of SN-38 (the active CPT-11 metabolite) and Gefitinib (Gefitinib before; Gefitinib simultaneously; Gefitinib after SN-38) have on cell growth, cell cycle, apoptosis, and expression/phosphorylation of EGFR, Topo I and some steps of the signal transduction pathway. We first determined the IC_{50} of each drug choosing the 5 days exposure for Gefitinib (0.6 and 3.8 μM for LoVo and HT-29 cells, respectively) and 1 day exposure for SN-38 (0.31 and 0.5 μM for LoVo and HT-29 cells, respectively). The different drug combination schedules were tested in various concentrations by using equiactive concentrations of the two drugs. The cytotoxicity of Gefitinib and SN-38 combination was schedule- and concentration-dependent but not cell line-specific. The most synergistic schedule was Gefitinib given after SN-38, with combination indexes (CI) of 0.007 and 0.454 in HT-29 and LoVo, respectively. Analysis of bio-molecular targets showed that Gefitinib was able to modulate SN-38 ability to inhibit Topo I, to accumulate cells in S-phase, and to induce apoptosis. Interestingly, SN-38 was able to activate EGFR and its signal transduction pathway. Confirming preliminary clinical experience of Gefitinib with other cytotoxic drugs, it seems that Gefitinib after SN-38 represents the best cytotoxic combination schedule but the biomolecular basis for this synergism remain to be completely elucidated.

Keywords: Colon cancer; Combination effect; Gefitinib; SN-38; Cell cycle; EGFR

Some new drugs have become recently available for the treatment of colorectal cancer patients. Among these, CPT-11 has shown encouraging clinical efficacy but the survival of these patients remains still poor [1,2]. CPT-11 acts as a prodrug being converted by carboxylesterase into its active metabolite 7-ethyl-10-hydroxy-camptothecin (SN-38). It binds the transient cleavable complex between DNA and topoisomerase I (Topo I) enzyme preventing dissociation of the DNA–Topo I complex and thereby inhibiting enzyme activity [3–5].

In recent years, the increasing understanding of the role that the tyrosine kinase (TK) epidermal growth factor receptor (EGFR) family plays in cancerogenesis and progression of several epithelial cancers led to the hypothesis that these membrane receptors could represent an effective target for new anticancer treatments [6]. In particular, epidermal growth factor receptor 1 is widely present in human colorectal cancer being high tumor cell expression associated with a poorer prognosis [7,8].

Gefitinib (Iressa™, ZD1839) an orally active EGFR-TK inhibitor, blocks signal trasduction pathways implicated in

Abbreviations: CI, combination index; DMSO, dimethyl sulfoxide; EDTA, ethylenediaminetetraacetic acid; EGFR, epidermal growth factor receptor; 5-FU, 5-fluorouracil; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazoliumbromide; NB, neutralizing buffer; PBS, phosphate buffered saline; PMSF, phenylmethylsulfonyl fluoride; RIPA, radio-immunoprecipitation assay; SDS, sodium dodecyl sulfate; SN-38, 7-ethyl-10-hydroxy-camptothecin; TKI, tyrosine kinase inhibitor; Topo I, topoisomerase I

* Corresponding author. Tel.: +39-080-5555561; fax: +39-080-5555561.
E-mail address: paradiso.io.bari@virgilio.it (A. Paradiso).

0006-2952/$ – see front matter © 2004 Elsevier Inc. All rights reserved.
doi:10.1016/j.bcp.2004.03.014

proliferation and survival of cancer cells and other host-dependent processes promoting cancer growth [9–11]. In vitro antitumor activity of Gefitinib has been demonstrated in a range of human tumor xenografts [9–11] but its clinical activity has not been demonstrated to be directly related to the amount of EGFR protein [12]. Gefitinib monotherapy has demonstrated clinically meaningful antitumor activity in Phase II trials of patients with recurrent or refractory non-small-cell lung cancer (NSCLC) [13,14] and has now been approved in several countries, including Australia, Japan, and the USA.

In vitro and in animal experiments have shown that this drug has a synergistic effect with paclitaxel and other chemotherapeutic drugs [15]. Consequently, some clinical trials have investigated the combination of cytotoxic drugs with Gefitinib in various tumors and specifically in lung cancer [16,17] and colorectal cancers patients [11,18]. All these trials utilized empirical schedules, essentially based on the different cell effect the two drugs have (cytotoxic for common drugs and cytostatic for Gefitinib) without considering their timely-dependent effect and possible interaction at specific cell target level.

The objective of our study was to provide pre-clinical evidence for optimizing the combination schedule of CPT-11 plus Gefitinib considering specific biomolecular effects the different combinations could have. To interpret these results, we analyzed the capability of the drugs, either alone or in combination, to modulate EGFR, Topo I, some steps of the signal transduction pathway, and to perturb the cell cycle and apoptosis human cell characteristics. The study was performed by using the same in vitro model we employed in a previous study analyzing Gefitinib- and oxaliplatin combinations [19,20].

1. Materials and methods

1.1. Drugs and chemicals

Gefitinib was provided by Astra-Zeneca Pharmaceuticals (London, UK). Stock solutions were prepared at 20 mM in dimethyl sulfoxide (DMSO) and stored in aliquots at −20 °C. SN-38 (the active metabolite of CPT-11) was kindly provided by Aventis Pharma (New York, USA) and dissolved at 20 mM in DMSO, with aliquots kept at −20 °C. Further dilutions were made in medium supplemented with 10% fetal bovine serum, 2 mM glutamine, 50,000 UL^{-1} penicillin, and 80 μM streptomycin.

1.2. Cell lines

Two colon cancer cell lines of human origin were used, LoVo and HT-29. Cells were routinely cultured in Ham F-12 (LoVo) and McCoy's (HT-29) supplemented with 10% fetal bovine serum, 2 mM glutamine, 50,000 UL^{-1} penicillin, and 80 μM streptomycin in a humidified incubator at 37 °C with an atmosphere containing 5% CO_2. Cells were trypsinized once a week with trypsin/ethylenediaminetetraacetic acid (EDTA) (0.25%/0.02%) and medium was changed twice a week. Doubling times were 20 ± 1 h for HT-29 and 24 ± 1 h for LoVo.

1.3. Evaluation of cytotoxicity

Determination of IC_{50} was performed using the 3-[4, 5-dimethylthiazol-2-yl]-2,5-diphenyltetrazoliumbromide (MTT) assay. On day 1, 10,000 cells per well in a volume of 200 μl were plated in 96-well plates. In each plate, one column contained cells not exposed to drug (control), and seven columns contained cells exposed to increasing concentrations of drug. Each drug or drug combination was repeated in six identical wells. On day 2, Gefitinib (0.05, 0.1, 0.5, 1, 5, 10, and 20 μM) or SN-38 (0.005, 0.01, 0.05, 0.1, 0.5, 1, and 5 μM) was added with different times of exposure (1 h to 5 days). For each drug, results were expressed as dose–effect curves with a plot of the fraction of unaffected (surviving) cells versus drug concentration. The IC_{50} was defined as the drug concentration yielding a fraction of affected (no surviving) cells = 0.5, compared with untreated controls. Each experiment was done in triplicate.

1.4. Drug combination studies

To define the best schedule for the combination of the two drugs, either simultaneous or sequential exposures were tested. According to the expected biologic effects, and the clinical efficacy, toxicity, and pharmacodynamic data already available [3], we decided to use SN-38 with a short time-exposure (1 day) and Gefitinib with a long time-exposure (5 days), in three different combination schedules:

1. Simultaneous drug exposure followed by Gefitinib alone: Gefitinib and SN-38 were given simultaneously for 1 day, then, after two wash-steps performed with the medium, Gefitinib was given alone for 4 days
2. Gefitinib after SN-38: SN-38 was given for 1 day, then, after two wash-steps performed with the medium, Gefitinib was given for 5 days
3. Gefitinib before SN-38: Gefitinib was given for 5 days, then, after two wash-steps performed with the medium, SN-38 was given for 1 day.

Each drug combination was tested in seven different concentrations, using a constant ratio of 1:1 with respect to their equiactive concentrations (IC_{50}).

Growth inhibition by MTT assay was analyzed with the Median Drug Effect Analysis software [21] to determine the interaction between Gefitinib and SN-38. The cytotoxicity of the combination was compared with the cytotoxicity of each drug alone in each experiment, and each experiment was performed at least three times.

Dose–response interactions (antagonism, additivity, and synergism) were expressed as a non-exclusive case combination index (CI) for every fraction affected [22], using CalcuSyn software (Biosoft) [23,24], based on the methods of Chou and Talalay [21].

1.5. Western blot

LoVo cells were exposed to 0.6 μM Gefitinib and 0.31 μM SN-38 alone or combined in one of the three schedules—simultaneous drug exposure, Gefitinib after, and Gefitinib before—with 1 day of exposure time for each drug. Proteins were extracted from 3×10^6 cells by homogenization in radioimmunoprecipitation assay (RIPA) buffer (0.5 M NaCl, 1% Triton X-100, 0.5% NP40, 1% deoxycolic acid, 3.5 mM sodium dodecyl sulfate [SDS], 8.3 mM Tris HCl, pH 7.4, 1.6 mM Tris base) and treated with 20% protease inhibitor cocktail (Sigma, Missouri, USA). Total proteins were measured by Bradford method and 25–50 μg were electrophoretically separated on 10–12.5% acrylamide gel (SDS–PAGE by Laemli). Signal was detected by chemoluminescence assay (ECL-Plus, Amersham Life Science, UK). Expression level was evaluated by densitometric analysis using Multi-Analyst software (Bio-Rad, Hercules, CA, USA) and β-actin expression level was used to normalize the sample values.

1.6. Antibodies

The monoclonal antibody 6B5 [25], kindly provided by Dr. A Scovassi (Ist. di Genetica Biochimica ed Evoluzionistica-CNR, Pavia, Italy) was used to detect DNA–Topo I; clone 13G8 (Alexis Corporation, USA), which recognizes the COOH domain epitope, was used to detect EGFR; antiphosphotyrosine antibody PY99 (Santa Cruz Biotechnology, USA) was used for the immunoprecipitation assay; the AKT antibody, the phosphoAKT antibody, the ERK1/2 antibody, and the phospho-ERK1/2 antibody were provided by Cell Signaling, USA. Mouse and rabbit horseradish peroxidase (Amersham Pharmacia Biotech, Upsala, Sweden) were used as secondary antibodies.

1.7. Immunoprecipitation assay

3×10^6 LoVo cells were exposed to 0.6 μM Gefitinib and 0.31 μM SN-38 alone or combined in one of the three schedules (simultaneous drug exposure, Gefitinib after, and Gefitinib before) with 1 day of exposure time for each drug. Cells were lysed in RIPA-IP buffer (140 mM NaCl, 20 mM Tris–HCl, 10 mM EDTA, pH8, 10% glycerol, 1% NP40, 1 mM Na-deoxycolic acid, 1 mM phenylmethylsulfonyl fluoride [PMSF]) passed through a 22-gauge syringe and cleared by centrifugation at $10{,}000 \times g$ at 4 °C for 10 min. Proteins were immunoprecipitated by incubating 0.1–0.3 mg of total cell lysate with 0.2 μg of antiphosphotyrosine antibody or EGFR antibody for 1 h at 4 °C. 2–5 μl of protein A/G agarose (Santa Cruz Biotechnology, USA) were incubated overnight at 4 °C. The cell suspension was centrifuged at 2600 rpm and the pellet was washed three times with phosphate buffered saline and then resuspended in 10 μl of Laemli buffer. Each sample was separated on 10% acrylamide gel and Western blot was performed as described above.

1.8. Cell-cycle perturbation

LoVo cells were exposed to 0.6 μM Gefitinib and 0.31 μM SN-38 alone or combined in one of the three schedules with 1 day of exposure time for each drug. Cells were harvested, washed twice in ice-cold PBS (pH 7.4), fixed in 4.5 ml of 70% ethanol at –20 °C and then washed once again in ice-cold PBS. The pellet was resuspended in PBS containing 1 mg/ml RNase and 0.01% NP40, and the cellular DNA was stained with 50 μg/ml propidium iodide (Sigma). Cells were stored in ice for 30 min prior to analysis. Cell-cycle determinations were performed using a FACScan flow cytometer (Becton Dickinson), and data were interpreted using the CellFit software, provided by the manufacturer.

1.9. Analysis of apoptosis

Drug-dependent apoptosis was evaluated with the Cell Death Detection ELISA^{PLUS} kit (Roche) and a radioactive assay, which measured the percentage of DNA ladder using [^{3}H] thymidine. In a preliminary phase, the correlation between the two tests was analyzed, showing a correlation factor of 0.95. Experiments performed with the kit were carried out following the standard protocol. For the radioactive assay, on day 0, 15,000 cells per well were plated in 96-well plates in the presence of [^{3}H] thymidine. In each plate, one column contained cells not exposed to drug (control) while in the other columns the LoVo cells were exposed to 0.6 μM Gefitinib and 0.31 μM SN-38 alone or combined in one of the three schedules. The drug exposure time was 1 day and all the experiments were carried out in the presence of [^{3}H] thymidine. To measure apoptosis, the cellular membrane was broken with Lysis Buffer (0.5% Triton X-100; 20 mM EDTA; 5 mM Tris–HCl pH 7.5) for 10 min and we quantified the DNA ladder, in the cytoplasm, with respect to the total DNA (nuclear and cytosolic one) with the Top Count. For each schedule, results were expressed as percentage of DNA ladder/total DNA. Each experiment was done in triplicate.

1.10. Biostatistical analysis

All of the in vitro experiments were performed in triplicate and shown as mean ± standard deviation (S.D.), unless otherwise indicated. The statistical significance of the apoptotic results was determined by the Student's *t*-test.

2. Results

2.1. Cell-growth inhibition by Gefitinib and/or SN-38 in colon cancer cell lines

To provide pre-clinical evidence for optimizing the combination schedule of CPT-11 and Gefitinib, we first determined the IC_{50} of each drug. A marked time-dependent effect was observed with Gefitinib, with a 5-days IC_{50} exposure nearly 50 times lower than that of 1-day exposure. Time dependency was also evident for SN-38, with 3-days IC_{50} exposure about 5–10 times lower than that of 1-day exposure (Table 1). The LoVo cell line was five times more sensitive to the biologic drug than the HT-29 cell line.

In both cell lines, exposure to SN-38 together with or followed by Gefitinib was more efficient than SN-38 alone; conversely, exposure to SN-38 after Gefitinib was antagonistic (Fig. 1). In LoVo cells, CIs at 50% cell-growth inhibition demonstrated that when SN-38 was given before or simultaneously with Gefitinib, the activity of the two drugs was synergistic (CI = 0.6 and 0.5, respectively) while, in contrast, SN-38 given after Gefitinib produced a strong antagonist effect (CI = 4.2) (Table 2). In HT-29 cells, the combination of the two drugs was more active (Table 2).

Table 1
IC_{50} of Gefitinib and SN-38 in LoVo and HT-29 cells at various times of incubation

Time of incubation	LoVo		HT-29	
	SN-38 (μM)	Gefitinib (μM)	SN-38 (μM)	Gefitinib (μM)
18 h	0.35 ± 0.12	48.5 ± 2.5	0.77 ± 0.20	>100
1 day	0.31 ± 0.09	29 ± 3.1	0.50 ± 0.12	>100
2 days	0.14 ± 0.05	16.5 ± 1.5	0.26 ± 0.04	>100
3 days	0.033 ± 0.005	7.3 ± 0.9	0.13 ± 0.01	23.6 ± 4.1
4 days		2.6 ± 0.62		7.7 ± 2.1
5 days		0.6 ± 0.01		3.8 ± 0.95

Each experiment was carried out in triplicate.

To determine if the effect of the multi-drug therapy was Gefitinib concentration-dependent, we analyzed cell-growth inhibition using three different ratios of drug concentration ($IC_{50SN\text{-}38}$:$IC_{50Gefitinib}$ = 1:0.1; 1:1; 1:10) in which SN-38 concentration was constant while that of Gefitinib changed. The best ratio in each of the three schedules was 1:1 (Table 2).

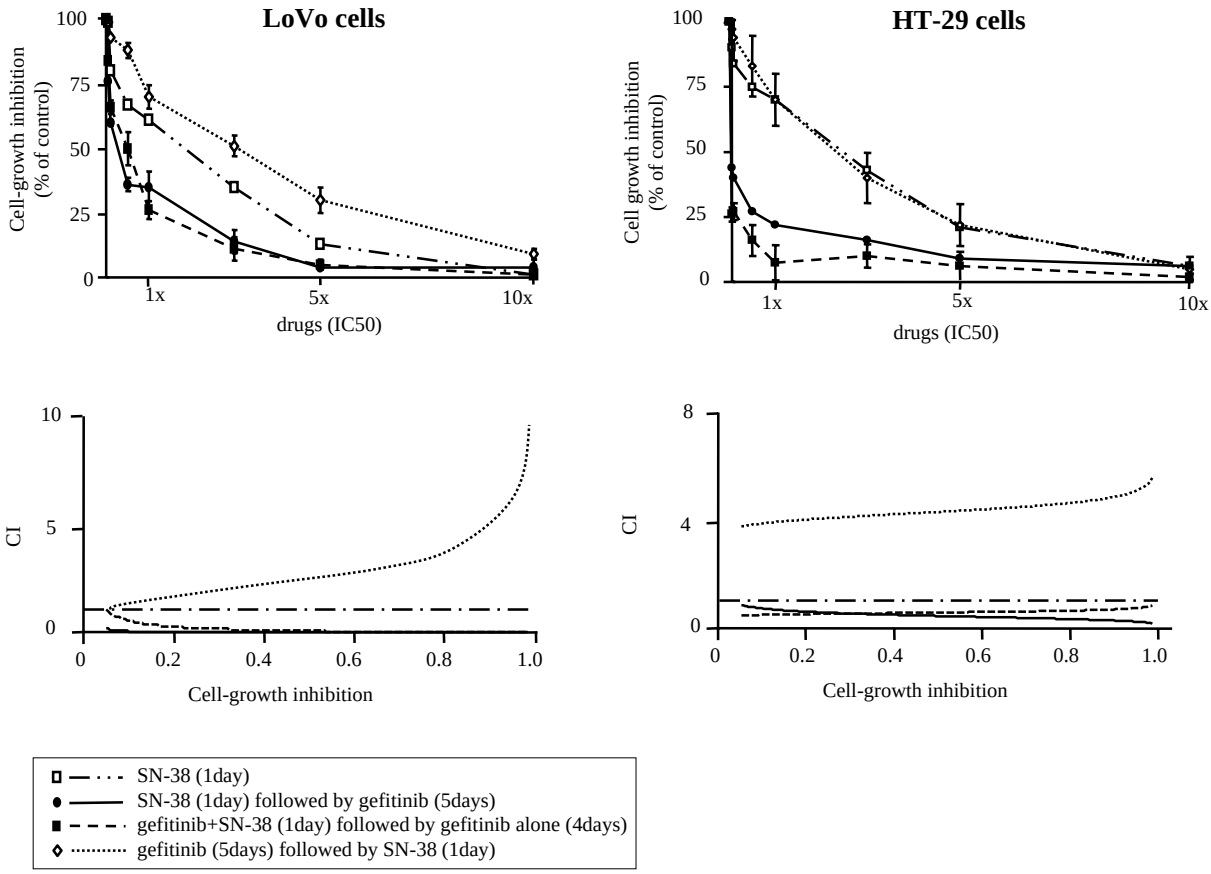

Fig. 1. Cytotoxicity of SN-38 and Gefitinib in combination. LoVo and HT-29 cells were incubated with Gefitinib and SN-38 in combination utilizing three different schedules: Gefitinib after SN-38, simultaneous drug exposure followed by Gefitinib alone, and Gefitinib before SN-38. Each drug combination was tested in seven different concentrations by using equiactive concentrations of the two drugs.

Table 2
Effects and CIs of the three sequences of Gefitinib/SN-38 in HT-29 and LoVo cells

Scheme of drug administration	Ratio	HT-29		LoVo	
	IC_{50SN}:IC_{50Gef}	Effect	CI	Effect	CI
Gefitinib before SN-38	1:0.1	Ant	3.223 ± 1.02	Strong ant	4.798 ± 2.7
	1:1	Strong ant	4.624 ± 0.23	Strong ant	4.195 ± 0.89
	1:10	Ant	3.190 ± 0.89	Strong ant	7.585 ± 2.19
Simultaneous drug exposure followed by Gefitinib alone	1:0.1	Ant	2.014 ± 1.01	Slight syn	0.871 ± 0.09
	1:1	Very strong syn	0.085 ± 0.006	Syn	0.588 ± 0.14
	1:10	Nearly add	1.096 ± 0.96	Moderate syn	0.783 ± 0.25
Gefitinib after SN-38	1:0.1	Very strong syn	0.085 ± 0.08	Nearly add	1.051 ± 0.36
	1:1	Very strong syn	0.007 ± 0.001	Syn	0.464 ± 0.08
	1:10	Syn	0.385 ± 0.034	Strong ant	3.821 ± 1.09

CI value at 50% of cell growth inhibition; SN: SN-38; Gef: Gefitinib; ant: antagonism; syn: synergism; add: additive.

2.2. Intracellular activity of Gefitinib and/or SN-38

In order to interpret our growth inhibition results, we analyzed the more sensitive LoVo cell line for the capability of each drug to modulate its specific cell target (EGFR for Gefitinib and Topo I for SN-38), the main steps of the EGFR signal transduction pathway (EGFR, ERK1/2, and Akt) and cell cycle and apoptosis processes.

2.2.1. EGFR and the signal transduction pathway

EGFR expression was partially inhibited after 1 day of Gefitinib exposure, and after 5 days of continuous drug exposure the receptor recovered to the baseline level (data not shown). As expected, the phosphorylated form was completely inhibited by Gefitinib and complete restoration of the receptor occurred 1 day after drug washout. Consequently, Gefitinib was able to partially inhibit ERK1/2 and Akt, with a complete recovery 1 day after drug washout (Fig. 2). Surprisingly, SN-38 was also able to inhibit EGFR and after 1 and 2 days of drug washout, the receptor recovered and reached approximately two-fold baseline levels. This increased p-EGFR level induced an increase in p-ERK1/2, more evident after 1 day of drug washout, while, conversely, p-Akt was partially inhibited, in agreement with the apoptotic activity of SN-38.

The analysis of the effect of the three different combination schedules showed that if Gefitinib was given after SN-38, it was able to strongly reduce the capability of SN-38 to increase p-EGFR; furthermore, p-Akt was inhibited with all different combination schedules but the entity of this inhibition was higher (60 and 30%) with synergistic combinations with respect to the antagonistic one (20%). Interestingly, this did not occur with p-ERK1/2; instead the addition of Gefitinib after SN-38 induced a stronger increase in p-ERK1/2. Simultaneous exposure to the two drugs completely inhibited p-EGFR and additive effects on p-ERK1/2 and p-Akt were observed. The schedule of Gefitinib followed by SN-38 strongly reduced the capability of cells to recover to baseline p-EGFR; the effect on p-Akt was additive, while p-ERK1/2 was increased in a faster and stronger way compared with SN-38 alone.

2.3. Topo I

SN-38 acts as a potent Topo I inhibitor by stabilizing the formation of the DNA–Topo I cleavable complex. Our analysis of SN-38 activity on Topo I confirms our previous results [26], which showed that significant depletion of Topo I was reached 12–24 h after exposure to SN-38, with recovery starting after 1 day, and baseline levels reached 2 days after the removal of SN-38 (Fig. 2). Conversely, Gefitinib alone did not show any effect on Topo I. To explore whether the modulation of SN-38-dependent cytotoxicity by Gefitinib could be related to SN-38-dependent Topo I inhibition, we studied the effect on this enzyme of exposure to the three different drug combinations. Gefitinib, given simultaneously with or after SN-38, was not able to reduce SN-38-dependent Topo I inhibition (Fig. 2) conversely, Gefitinib given before SN-38 induced a reduction of SN-38-dependent Topo I inhibition.

2.4. Cell-cycle analysis

Successively we determined if and how Gefitinib, which induces a light block in G0/G1 phase, could modulate SN-38-dependent cell arrest into the S-phase. The cell-cycle analysis of LoVo cells exposed to the drugs alone or in combination confirmed that SN-38 induced a prominent S-phase arrest with a decrease in the percentage of cells in G0/G1 phase; Gefitinib induced only a small increase in G0/G1 phase and a late apoptosis after 3 days of drug exposure (Fig. 3a). When the two drugs were used in combination, Gefitinib was not able to deeply modify SN-38 activity if given before, whereas the simultaneous schedule slowed down cell cumulating in S-phase (Fig. 3a) The same experiments were carried out using different

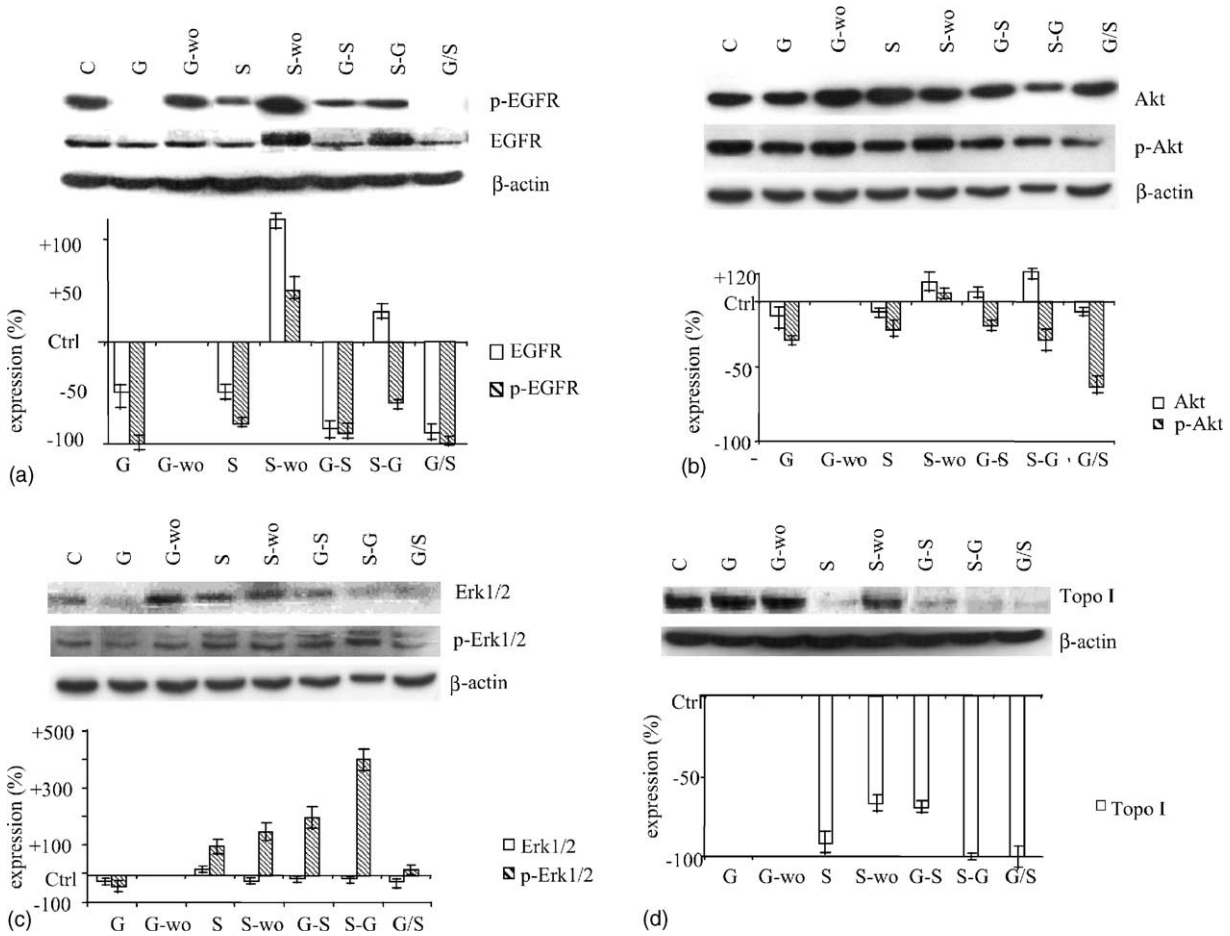

Fig. 2. Target modulations with different combination schedules of Gefitinib and SN-38. Cells were incubated with Gefitinib 0.6 μM and/or SN-38 0.31 μM alone or in the three different schedules, as reported in Section 1. All the data are shown as respect to the cell basal level (control = 0). (a) EGFR modulation; (b) Akt modulation; (c) Erk1/2 modulation; (d) Topo I modulation. G: Gefitinib (1 day); S: SN-38 (1 day); G/S: the two drugs together for 1 day; wo: drug washout (1 day); Ctrl: control.

Gefitinib concentrations showing that Gefitinib activity was concentration-dependent (Fig. 3b).

2.5. Apoptosis analysis

Both SN-38, which stabilizes the DNA–Topo I complex thus inhibiting DNA repair and replication, and Gefitinib, which prevents cell survival by activating the pro-apoptotic protein BAD, are pro-apoptotic drugs [27,28]. LoVo cells began to die by apoptosis after only 1 day of SN-38 exposure, while no apoptotic phenomenon was observed after 1 day of exposure to Gefitinib, in agreement with the late apoptosis seen after 3 days of Gefitinib exposure (Fig. 3a) and reported elsewhere [29]. We carried out an experiment to determine if Gefitinib was able to modulate the SN-38-dependent induction of apoptosis. As expected, when Gefitinib was given in sequence (before or after) with SN-38, apoptosis was not modulated while if the two drugs were given together the apoptosis was significantly reduced, in agreement with our hypothesis of the capability of Gefitinib to modulate SN-38 activity (Fig. 4).

3. Discussion

In vitro and in vivo studies have shown that Gefitinib sensitizes a variety of human cancer cell lines to the cytotoxicity of cisplatin, doxorubicin, paclitaxel, topotecan, and to radiotherapy [30–34]. Promising preliminary results were also observed in clinical trials when Gefitinib was combined with carboplatin/paclitaxel in NSCLC patients [35], and with 5-FU [11] or oxaliplatin [18] in patients with advanced colorectal cancer. However, two large, multicenter, Phase III studies of Gefitinib in combination with cytotoxic drugs (carboplatin/paclitaxel or cisplatin/gemcitabine) as first-line treatment in inoperable stage III and IV NSCLC patients have shown no added survival benefit [16,17]. All these trials utilized empirical schedules based on the different effect of the two drugs on the tumor cell without considering the possibility of an interaction at cell-cycle or target-drug level. We hypothesized that a pre-clinical kinetic and biologic study of these drugs alone or in combination could explain the failure of the clinical therapies and give some information to optimize the schedule of drug administration.

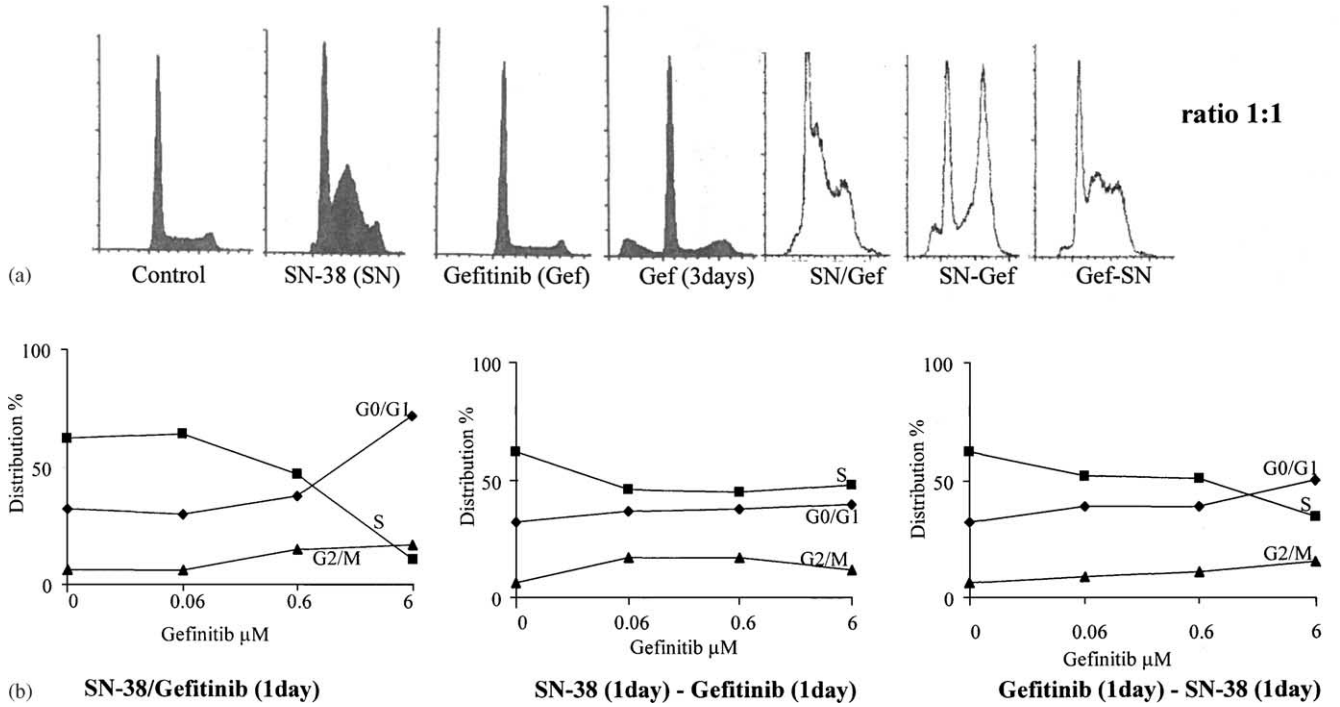

Fig. 3. Gefitinib and/or SN-38-dependent modulation of cell cycle. LoVo cells were incubated with Gefitinib 0.6 μM and/or SN-38 0.31 μM for different times and the cell cycle was analyzed by flow cytometry analysis as described in Section 1. (a) Histograms show cell cycle of LoVo cells (control) and of cells exposed to SN-38 (1 day), Gefitinib (1 day), Gefitinib (3 day), SN/Gef: the two drugs together for 1 day, SN-Gef: Gefitinib after SN-38 and Gef-SN: Gefitinib before SN-38. (b) Plots of G0/G1-, S- and G2/M-phase distribution (%) vs. Gefitinib concentration (μM) in the three schedule of drugs combination.

Aim of the present paper has been to acquire pre-clinical data on the optimum way to combine CPT-11 and Gefitinib. The study was carried out utilizing SN-38 (the CPT-11 active metabolite) and two colon cancer cell lines, HT-29 and LoVo, which show only small differences in their EGFR expression (LoVo cells having a slightly higher EGFR expression than HT-29 cells). Starting from the scheme and the times of drug exposure commonly used in clinical trials [36], we exposed cells to SN-38 for 1 day and to Gefitinib for 5 days finding IC_{50} values similar to those previously reported [29].

The kinetic analysis of the cell growth inhibition by Gefitinib and SN-38 in three different schedules was performed using three different ratios of drug concentration ($IC_{50SN-38}$:$IC_{50Gefitinib}$ = 1:0.1; 1:1; 1:10 with SN-38 constant and Gefitinib changing). Our results provided

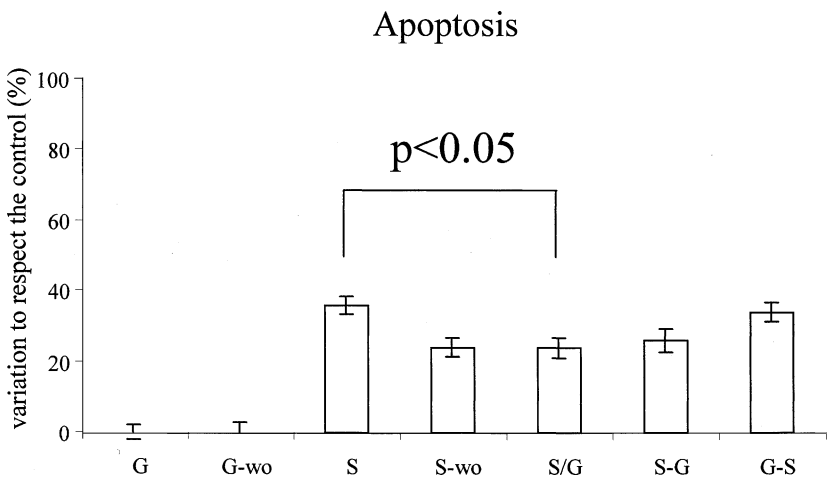

Fig. 4. Gefitinib and/or SN-38-dependent induction of apoptosis. LoVo cells were incubated with Gefitinib 0.6 μM and/or SN-38 0.31 μM and the apoptosis was determined through the DNA ladder analysis, as described in Section 1. The statistical analysis utilised the Student's *t*-test. C: control; G: Gefitinib (1 day); S: SN-38 (1 day); G/S: the two drugs together for 1 day; wo: drug washout (1 day).

evidences of strong synergy between the two drugs when Gefitinib was given simultaneously with or following SN-38; conversely, Gefitinib given before SN-38 produced strong antagonistic effects [37]. Moreover, this phenomenon was shown to be concentration-dependent (the best ratio was 1:1) and not cell line-specific. Thus, our study demonstrates that the synergism, evident when SN-38 was given sequentially to or simultaneously with Gefitinib followed by the biologic drug alone, is in agreement with the schedules employed by clinics [36].

To acquire information on how to further optimize the schedule of SN-38 plus Gefitinib, we carried out a biological analysis of their specific targets of inhibition, signal transduction pathways [38], cell-cycle modulation, and apoptosis induction by these two drugs. We confirm the capability of the single drug to modulate its own target is in agreement with Xu et al. [26], Ciardiello et al. [29], and Shimoyama et al. [39]; in particular, Gefitinib was able to completely inhibit EGFR phosphorylation. However, after 1 day of Gefitinib exposure, p-ERK1/2 and p-Akt were only partially inhibited. These data are not in agreement with Sugiyama et al. [40] and Miyazaki et al. [41], who reported that these steps of the signal transduction pathway were completely inhibited after Gefitinib administration in some NSCLC cell lines that overexpressed EGFR. We suggest that, as already reported [12], EGFR level and Gefitinib efficacy could not be directly related in other than lung cancer model. Surprisingly, SN-38 was able to reduce EGFR and, after 1–2 days of drug wash out, the receptor levels increased about two-fold than the control. Previous studies have indicated that the inhibition of Topo I by camptothecin induced an increase of phosphorylated ERK1/2 [42], which could be a consequence of increased EGFR. Moreover, EGFR increase was found in some cancer cell lines with multidrug-resistant phenotype [43,44], further suggesting that persistent depletion of EGFR could induce a subsequent increase of the receptor through a feedback mechanism. These preliminary results demonstrate that the signaling pathways modulated by these two drugs are not completely independent and further support the idea that Gefitinib and some cytotoxic drugs may target the same cells where they can largely interact [45].

Analysis of the modulation of p-EGFR, p-Akt, p-ERK1/2, and Topo I provide some informations useful to interpret why the schedules of SN-38 given before or simultaneously with Gefitinib result in a synergistic cytotoxic effect. The pre-exposure of cell to SN-38 seemed to induce a less evident inhibitory effect of Gefitinib on p-EGFR expression. The analysis of apoptosis did not provide particularly relevant information perhaps depending on the Gefitinib exposure-time utilized that was too short to generate a strong apoptosis modulation [29]. However, we showed a significant reduction of apoptosis (Fig. 4) when the two drugs were given simultaneously, data that could justify the higher synergism for SN-38 given before Gefitinib rather than simultaneously.

The antagonism observed when Gefitinib was given before SN-38 could be explained by the reduction of SN-38-dependent inhibition of Topo I and the increase of EGFR expression. This Gefitinib-dependent inhibition of SN-38 activity could be related to the inhibition of SN-38 transport into cells by Gefitinib as reported in lung cancer cells by Nakamura et al. [46]. The reduced capability of Gefitinib to completely inhibit EGFR when given after SN-38, suggests that it may be beneficial to wait some time between administration of the two drugs. This hypothesis is also supported by flow cytometry analysis which showed that, even after SN-38 removal, subsequent Gefitinib exposure increased the cell movement to G2/M phase, so facilitating the repair of SN-38-induced DNA damage. One further possibility for the antagonistic effect of the Gefitinib before SN-38 could be represented by the quantitative different inhibitory effect this schedule has on p-Akt with respect to the synergistic ones (Fig. 2b).

In summary, our study demonstrates that SN-38 followed by Gefitinib, which has already been indicated by clinical experience [36], could be the optimal sequence to combine these two drugs. Our results also suggest that it may be important to delay the second drug administration because the two agents could largely interact at kinetic and molecular tumor cell level.

'Iressa' is a trademark of the Astra-Zeneca group of companies.

Acknowledgments

We are grateful to Dr. Scovassi for providing the monoclonal Topo I antibody. This work was supported by grants from the Italian Association for Cancer Research (AIRC, P.F. 1363, 2002).

References

[1] Rothenberg ML, Eckardt JR, Kuhn JG, Burris 3rd HA, Nelson J, Hilsenbeck SG, et al. Phase II trial of irinotecan in patients with progressive or rapidly recurrent colorectal cancer. J Clin Oncol 1996; 14:1128–35.
[2] Rougier P, Bugat R, Douillard JY, Culine S, Suc E, Brunet P, et al. Phase II study of irinotecan in the treatment of advanced colorectal cancer in chemotherapy-naive patients and patients pretreated with fluorouracil-based chemotherapy. J Clin Oncol 1997;15:251–60.
[3] Paradiso A, Simone G, Petroni S, Leone B, Vallejo C, Lacava J, et al. Thymidilate synthase and p53 primary tumour expression as predictive factors for advanced colorectal cancer patients. Br J Cancer 2000;82:560–7.
[4] Kaneda N, Nagata H, Furuta T, Yokokura T. Metabolism and pharmacokinetics of the camptothecin analogue CPT-11 in the mouse. Cancer Res 1990;50:1715–20.
[5] Rivory LP, Robert J. Molecular, cellular, and clinical aspects of the pharmacology of 20(S)camptothecin and its derivatives. Pharmacol Ther 1995;68:269–96.
[6] Carpenter G. Receptors for epidermal growth factor and other polypeptide mitogens. Annu Rev Biochem 1987;56:881–914.

[7] Salomon DS, Brandt R, Ciardiello F, Normanno N. Epidermal growth factor-related peptides and their receptors in human malignancies. Crit Rev Oncol Hematol 1995;19:183–232.
[8] Prewett MC, Hooper AT, Bassi R, Ellis LM, Waksal HW, Hicklin DJ. Enhanced antitumor activity of anti-epidermal growth factor receptor monoclonal antibody IMC-C225 in combination with irinotecan (CPT-11) against human colorectal tumor xenografts. Clin Cancer Res 2002;8:994–1003.
[9] Wakeling AE, Barker AJ, Davies DH, Brown DS, Green LR, Cartlidge SA, et al. Specific inhibition of epidermal growth factor receptor tyrosine kinase by 4-anilinoquinazolines. Breast Cancer Res Treat 1996;38:67–73.
[10] Woodburn JR, Barker AJ, Gibson KH, Ashton SE, Wakeling AE, Curry BJ, et al. ZD1839, an epidermal growth factor tyrosine kinase inibitor selected for clinical development. Proc Am Assoc Cancer Res 1997;38:633 [Abstract 4251].
[11] Hammond LA, Figueroa J, Schwartzberg L, Ochoa L, Hidalgo M, Olivo N, et al. Feasibility and pharmacokinetic (PK) trial of ZD1839 (Iressa™), an epidermal growth factor receptor tyrosine kinase inhibitor (EGFR-TKI), in combination with 5-fluorouracil (5-FU) and leucovorin (LV) in patients with advanced colorectal cancer. Proc Am Soc Clin Oncol 2001;20:137a [Abstract 544].
[12] Albanell J, Rojo F, Baselga J. Pharmacodynamic studies with the epidermal growth factor receptor tyrosine kinase inhibitor ZD1839. Semin Oncol 2001;28(Suppl 16):56–66.
[13] Fukuoka M, Yano S, Giaccone G, Tamura T, Nakagawa K, Douillard J-Y, et al. Multi-institutional randomized phase II trial of Gefitinib for previously treated patients with advanced non-small-cell lung cancer. J Clin Oncol 2003;21:2237–46.
[14] Kris MG, Natale RB, Herbst RS, Lynch TJ, Prager D, Belani CP, et al. Efficacy and safety of Gefitinib (Iressa, ZD1839), an inhibitor of the epidermal growth factor receptor tyrosine kinase, in symptomatic patients with advanced non-small cell lung cancer. J Am Med Assoc 2003;290(16):2149–58.
[15] Sirotnak FM, Zakowski MF, Miller WA, Scher HI, Kris MG. Efficacy of cytotoxic agents against human tumor xenografts is markedly enhanced by coadministration of ZD1839 (Iressa), an inhibitor of EGFR tyrosine kinase. Clin Cancer Res 2000;6:4885–92.
[16] Giaccone G, Johnson DH, Manegold C, Scagliotti GV, Rosell R, Wolf M, et al. A phase III clinical trial of ZD1839 ('Iressa') in combination with gemcitabine and cisplatin in chemotherapy-naive patients with advanced non-small-cell lung cancer (INTACT 1). Ann Oncol 2002;13(Suppl 5):2 [Abstract 40].
[17] Johnson DH, Herbst R, Giaccone G, Schiller J, Natale RB, Miller V, et al. ZD1839 (''Iressa'') in combination with paclitaxel, ZD1839 (''Iressa'') in combination with paclitaxel & carboplatin in chemotherapy-naive patients with advanced non-small-cell lung cancer (NSCLC): results from a phase III clinical trial (INTACT 2). Ann Oncol 2002;13(Suppl 5):127 [Absract 4680].
[18] Cho CD, Fisher GA, Halsey J, Jambalos CN, Advani RH, Wakelee H, et al. A phase II study of Gefitinib in combination with FOLFOX-4 (IFOX) in patients with unresectable or metastatic colorectal cancer. Proc Am Soc Clin Oncol 2003;22:265 [Abstract 1062].
[19] Xu JM, Azzariti A, Severino M, Lu B, Colucci G, Paradiso A. Characterization of sequence-dependent synergy between ZD1839 (''Iressa'') and oxaliplatin. Biochem Pharmacol 2003;66:551–63.
[20] Xu JM, Azzariti A, Colucci G, Paradiso A. Effect of Gefitinib ('Iressa', ZD1839) in combination with oxaliplatin is schedule-dependent in colon cancer cell lines. Cancer Chemother Pharmacol 2003;52(6):442–8.
[21] Chou TC, Talalay P. Quantitative analysis of dose–effect relationships: the combined effects of multiple drugs or enzyme inhibitors. Adv Enzyme Regul 1984;22:27–55.
[22] Chou TC, Rideout D, Chou J. Chemotherapeutic synergism, potentiation and antagonism. In: Dulbecco R, editor. Encyclopedia of human biology, vol. 2. New York: Academic Press; 1991. p. 371–9.
[23] Chou J, Chou TC. Dose effect analysis with microcomputers. Cambridge: Biosoft; 1987.
[24] Chou TC. The median-effect principle and the combination index for quantitation of synergism and antagonism. In: Chou TC, Rideout D, editors. Synergism and antagonism in chemotherapy. New York: Academic Press; 1991. p. 61–102.
[25] Codegoni AM, Castagna S, Mangioni C, Scovassi AI, Broggini M, D'Incalci M. DNA–Topoisomerase I activity and content in epithelial ovarian cancer. Ann Oncol 1998;9:313–9.
[26] Xu JM, Azzariti A, Tommasi S, Lacalamita R, Colucci G, Johnston PG, et al. Combination of 5-fluorouracil and irinotecan on modulation of thymidylate synthase and topoisomerase I expression and cell cycle regulation in human colon cancer LoVo cells: clinical relevance. Clin Colorectal Cancer 2002;2:182–8.
[27] Oizumi S, Isobe H, Ogura S, Ishida T, Yamazaki K, Nishimura M, et al. Topoisomerase inhibitor-induced apoptosis accompanied by down-regulation of Bcl-2 in human lung cancer cells. Anticancer Res 2002;22:4029–37.
[28] Gilmore AP, Valentijn AJ, Wang P, Ranger AM, Bundred N, O'Hare MJ, et al. Activation of BAD by therapeutic inhibition of epidermal growth factor receptor and transactivation by insulin-like growth factor receptor. J Biol Chem 2002;277:27643–50.
[29] Ciardiello F, Caputo R, Bianco R, Damiano V, Pomatico G, De Placido S, et al. Antitumor effect and potentiation of cytotoxic drugs activity in human cancer cells by ZD-1839 (Iressa), an epidermal growth factor receptor-selective tyrosine kinase inhibitor. Clin Cancer Res 2000; 6:2053–63.
[30] Williams KJ, Telfer BA, Stratford IJ, Wedge SR. ZD1839 ('Iressa'), a specific oral epidermal growth factor receptor-tyrosine kinase inhibitor, potentiates radiotherapy in a human colorectal cancer xenograft model. Br J Cancer 2002;86:1157–61.
[31] Ciardiello F, Caputo R, Borriello G, Del Bufalo D, Biroccio A, Zupi G, et al. ZD1839 (IRESSA), an EGFR-selective tyrosine kinase inhibitor, enhances taxane activity in bcl-2 overexpressing, multidrug-resistant MCF-7 ADR human breast cancer cells. Int J Cancer 2002; 98:463–9.
[32] Raben D, Helfrich BA, Chan D, Johnson G, Bunn Jr PA. ZD1839, a selective epidermal growth factor receptor tyrosine kinase inhibitor, alone and in combination with radiation and chemotherapy as a new therapeutic strategy in non-small cell lung cancer. Semin Oncol 2002;29(Suppl 4):37–46.
[33] Magne N, Fischel JL, Dubreuil A, Formento P, Marcie S, Lagrange JL, et al. Influence of epidermal growth factor receptor (EGFR), p53 and intrinsic MAP kinase pathway status of tumour cells on the antiproliferative effect of Gefitinib (''Iressa''). Br J Cancer 2002; 86:819–27.
[34] Tortora G, Caputo R, Damiano V, Fontanini G, Melisi D, Veneziani BM, et al. Oral administration of a novel taxane, an antisense oligonucleotide targeting protein kinase A, and the epidermal growth factor receptor inhibitor Iressa causes cooperative antitumor and antiangiogenic activity. Clin Cancer Res 2001;7:4156–63.
[35] Miller VA, Johnson D, Heelan RT, Pizzo BA, Perez WJ, Bass A, et al. A pilot trial demonstrates the safety of ZD1839 ('Iressa'), an oral epidermal growth factor receptor tyrosine kinase inhibitor (EGFR-TKI), in combination with carboplatin (C) and paclitaxel (P) in previously untreated advanced non-small cell lung cancer (NSCLC). Proc Am Soc Clin Oncol 2001;20:326a [Abstract 1301].
[36] Kindler HL, Shulman KL. Metastatic colorectal cancer. Curr Treat Options Oncol 2001;2:459–71.
[37] Paradiso A, Xu JM, Azzariti A, Colucci G. Synergy between Gefitinib ('Iressa', ZD1839) and conventional cytotoxic agents (oxaliplatin or CPT-11) in colon cancer cell lines is dependent on the sequence of drug application. Proc Am Assoc Cancer Res 2003;44:867 [Abstract 3791].
[38] Kometani T, Hirata A, Miyagawa M, Kuwano M, Ono M. Drug sensitivity of Gefitinib ('Iressa') correlates with Ras-Map kinase

and PI3-Akt kinase pathways in non-small cell lung cancer cells. Proc Am Assoc Cancer Res 2003;44:1074 [Abstract 4681].

[39] Shimoyama T, Koizum F, Saijo N, Nishio K. Synergistic interaction between the EGFR tyrosine kinase inhibitor Gefitinib ('Iressa') and the topoisomerase I inhibitor CPT-11 (irinotecan) in human colorectal cancer cells. Proc Am Assoc Cancer Res 2003;44:866 [Abstract 3786].

[40] Sugiyama K, Yamashita K, Nishio K, Akinga S, Kanazawa J. Synergistic combined effect of vinorelbine and Gefitinib (Iressa) in NSCLC cell lines which overexpress the phosphorylated EGFR and EebB2. Proc Am Assoc Cancer Res 2003;44:625 [Abstract 2737].

[41] Miyazaki M, Tamura K, Kurata T, Uejima H, Nogami T, Yonesaka K, et al. Synergistic antitumor activity of Gefitinib ('Iressa'), an epidermal growth factor receptor tyrosine kinase inhibitor (EGFR-TKI), in combination with vinorelbine in human lung cancer xenografts. Proc Am Assoc Cancer Res 2003;44:851 [Abstract 3720].

[42] Lee S, Lee HS, Baek M, Lee DY, Bang YJ, Cho HN, et al. ERK1/2 signaling is involved in camptothecin-induced cell death. Mol Cells 2002;14:348–54.

[43] Takeda Y, Nishio K, Niitani H, Sanijo N. Reversal of multidrug resistance by tyrosine-kinase inhibitors in a non-*P*-glycoprotein-mediated multidrug-resistant cell line. Int J Cancer 1994;57:229–39.

[44] Meyers MB, Merluzzi VJ, Spengler BA, Biedler JL. Epidermal growth factor receptor is increased in multidrug-resistant Chinese hamster and mouse tumor cells. Proc Natl Acad Sci USA 1986;83:5521–5.

[45] Kurata T, Tamura K, Kaneda H, Nogami T, Uejima H, Asai G, et al. Effect of re-treatment with gefinitib (iressa, ZD1839) after acquisition of resistance. Ann Oncol 2004;15:173–7.

[46] Nakamura Y, Oka M, Shiozawa K, Nakatomi K, Yoshikawa M, Kawabata S, et al. Gefitinib ('Iressa', ZD1839) inhibits topotecan-efflux in small-cell lung cancer cells overexpressing breast cancer resistance protein (BCRP/ABCG2). Proc Am Assoc Cancer Res 2003;44:1073 [Abstract 4679].