Synergistic interaction between gefitinib (Iressa, ZD1839) and paclitaxel against human gastric carcinoma cells

Jong-Kook Park[a], Sang-Hak Lee[a], Jin-Hyoung Kang[b], Kazuto Nishio[c], Nagahiro Saijo[c] and Hyo-Jeong Kuh[a]

We have evaluated the antitumor effects of gefitinib (Iressa, ZD1839) in SNU-1 human gastric cancer cells (hMLH1-deficient and epidermal growth factor receptor-overexpressed) when given alone or as a doublet with oxaliplatin (LOHP), 5-fluorouracil (5-FU) or paclitaxel (PTX). The four drugs showed IC_{50}s ranging from 1.81 nM to 13.2 μM. LOHP and PTX induced G_2/M arrest, 5-FU increased S phase, and gefitinib increased G_1 in a concentration-dependent manner. The analysis using the previously developed cytostatic TP_i model showed that 64 and 80% of the overall growth inhibition was attributed to cell cycle arrest in cells exposed to 7.55 μM of LOHP or 10 nM of PTX for 72 h, respectively. PTX + gefitinib showed greatest synergism as determined by combination index analysis and apoptosis induced by PTX was potentiated by the co-administration of gefitinib. LOHP + gefitinib showed a similar, although to a lesser degree, synergistic effect. This study demonstrates the antitumor activity and the significant cell cycle arrest induced by gefitinib in SNU-1 human gastric carcinoma cells, and its synergistic interaction with LOHP and PTX. *Anti-Cancer Drugs* 15:809–818 © 2004 Lippincott Williams & Wilkins.

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Introduction

The contemporary combination regimens for treatment of gastric cancer usually contain cisplatin and 5-fluorouracil (5-FU). Recently, the triplet combination of cisplatin, 5-FU and paclitaxel (PTX) has become one of the most highly active regimens against advanced gastric carcinoma [1].

Tumor tissues from gastric cancer patients show a high incidence of epidermal growth factor (EGF) and its receptor (EGFR) overexpression, both of which play a promotional role in the development of gastric cancer cooperatively with other members of the EGFR gene family, c-erbB-2 and c-erbB-3 [2]. The EGF and EGFR gene families have been associated with the growth regulation and gastric wall invasion in gastric cancers [3,4], and seem to be involved in determining the chemosensitivity of human cancer cells to chemotherapy [5]. Recently, it was also reported that inhibition of the EGFR cascade abrogated *Helicobacter pyroli*-induced up-regulation of vascular endothelial growth factor in gastric cancer cells [6]. A novel approach for the therapeutic blockade of EGFR signaling in human cancer has been recently developed based on the discovery of low-molecular-weight compounds that selectively inhibit the ligand-induced activation of EGFR tyrosine kinase (TK) and its receptor-mediated intracellular signaling [7]. Among various quinazoline-derived compounds tested as new anticancer drugs, gefitinib ([4-(3-chloro-4-fluoro-anilino)-7-methoxy-6-(3-morpholinopropoxy) quinazoline], also 'Iressa', ZD1839) has shown impressive preclinical activity in various tumor models *in vitro* and *in vivo* [7]. It is an orally active, selective EGFR-TK inhibitor that blocks the signal transduction pathways implicated in the proliferation and survival of cancer cells, and is currently under phase III clinical trial [7,8].

In addition, the methylation of DNA mismatch repair (MMR) genes has been observed in many human cancers, including gastric cancers. The methylation of the hMLH-1 promoter region has been shown to be involved in the mechanism of low or undetectable hMLH-1 protein expression in gastric tumors [9,10]. In addition to predisposing oncogenesis, the loss of MMR activity is related to drug resistance, since the MMR proteins play important roles in mediating the activation of cell cycle checkpoints and apoptosis in response to DNA damage induced by anticancer agents. This drug resistance extends to a variety of alkylating anticancer agents including platinum compounds, such as cisplatin and carboplatin [11]. Moreover, it has been shown that *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG)-resistant

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human gastric cancer cells have very low or undetectable levels of hMLH-1 protein, which plays a key role with hMSH2 in the MMR system [12].

Oxaliplatin (LOHP) has a spectrum of activity that differs from that of cisplatin or carboplatin, suggesting that it has different molecular targets and/or different mechanism of resistance. It has been reported that MMR deficiencies do not induce similar resistance to LOHP [13], and because of this decreased possibility of resistance development, LOHP may serve as a good candidate for first-line treatment as a monotherapy or in combination with other agents in gastric cancer. Hence, LOHP may effectively substitute for cisplatin in the platinum-based triplet combination with 5-FU and PTX, as mentioned above.

In many studies, gefitinib in combination with radiation as well as a variety of cytotoxic agents, including taxanes and platinum compounds, has shown synergistic and supra-additive interactions in many types of cancers, such as colon, lung, breast, prostate and ovarian cancer [14]. However, no studies have been conducted on the antitumor effects of gefitinib given alone or in combination with cytotoxic agents against human gastric cancer cells.

In the present study, we evaluated the growth-inhibitory and cell cycle arrest effects of LOHP, 5-FU and PTX, which are promising cytotoxic drugs for the treatment of gastric carcinoma, and of a target-based cytostatic drug, gefitinib, in SNU-1 human gastric carcinoma cells that show MMR deficiency and EGFR overexpression. We also determined whether simultaneous EGFR blockade by gefitinib could improve the anticancer activities of these cytotoxic drugs. Our results show that the gefitinib + PTX combination had the greatest synergistic interaction. Gefitinib was found to potentiate the apoptosis induced by PTX.

Materials and methods

Chemicals

Clinical grade gefitinib and LOHP were kindly provided by AstraZeneca Pharmaceuticals (Macclesfield, UK) and Sanofi-Synthelabo (Malvern, PA), respectively. PTX and 5-FU were provided by the Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, Division of Cancer Treatment and Diagnosis, NCI (Bethesda, MD). Other drugs and reagents, unless otherwise stated, were purchased from Sigma (St Louis, MO).

Cell culture conditions

The human gastric cancer cell lines, SNU-1 and MKN-45, human lung adenocarcinoma cell line, A549, and human epidermoid carcinoma cell line, A431 were obtained from the Korean Cell Line Bank (Seoul, South Korea). Cells were maintained in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum, 100 mg/ml of streptomycin and 100 U/ml penicillin in humidified air containing 5% (v/v) CO_2 at 37°C.

Western blotting

Total cell protein extracts were obtained as previously described [15]. Briefly, cells were lysed with lysis buffer [20 mM Tris–HCl (pH 7.5), 150 mM NaCl, 0.1% SDS, 1% Triton X-100, 1% sodium deoxycholate]. The lysate, containing 30 μg of total protein, was then mixed with 2 × SDS–PAGE sample buffer, boiled for 5 min and electrophoresed in 8% SDS gels under reducing conditions. The separated proteins were then electrophoretically transferred to PVDF membranes (Millipore, Bedford, MA) and the membranes were probed with a primary antibody against EGFR or hMLH1 (anti-human EGFR rabbit polyclonal antibody and anti-human hMLH1 rabbit polyclonal antibody; Santa Cruz Biotechnology, Santa Cruz, CA) at 1:1000 dilution. Immunoreactive proteins were detected by using an enhanced chemiluminescence detection system (Amersham Pharmacia Biotech, Little Chalfont, UK).

Measurement of growth inhibition

Growth inhibitory effects were measured by MTT assay and by direct cell counting [16]. For MTT assay, cells were plated in 96-well microtiter 24 h prior to treatment (4000 cells/well). Cells were exposed to various concentrations of the tested agents for 72 h. The absorbance of the reaction mixture was measured at 540 nm and the IC_{50} defined as the drug concentration required to reduce the absorbance to 50% of the control in each test was determined using an E_{max} model:

$$\%\ \text{Cell viability} = (100 - R) \times \left(1 - \frac{[D]^m}{K_d^m + [D]^m}\right) + R \quad (1)$$

where D is the drug concentration, K_d is the concentration of the drug that produces a 50% reduction in absorbance (i.e. IC_{50}), m is the Hill-type coefficient and R is the residual unaffected fraction (the resistant fraction). The Sigma Plot regression function was used for model fitting.

For direct cell counting, cells were seeded at a density of 1×10^6 in 100- or 150-mm Petri dishes at least 24 h prior to drug exposure and were exposed to two different concentrations of the drug for up to 72 h. The concentrations of each drug were 0.75 and 7.55 μM for LOHP, 9 and 65 μM for 5-FU, 2.5 and 10 nM for PTX, and 13 and 38 μM for gefitinib. At predetermined times, cells were harvested by resuspending then in PBS and then the total cell number was determined using a Counter (Coulter Electronics, Luton, UK). Trypan blue

exclusion under the microscope was used to determine the viable cell fraction. The remainder of the cell suspension samples was used for the cell cycle study (see below). For the combination study, gefitinib was given simultaneously with either LOHP, 5-FU or PTX for 72 h. Drugs were combined at equitoxic ratios (i.e. doses were applied in combinations that would have produced the same cytotoxic effect if the drugs were administered separately to produce a 50% growth inhibition, as determined by MTT assay). The cytotoxicities of the two-drug combinations were determined by MTT assay using the same procedure as used for single treatments. Each experiment was performed in triplicate.

Determination of combination effects

The cytotoxic effects obtained with two-drug combinations were analyzed using the Chou and Talalay method [17]. The interaction between two drugs was assessed by using combination index (CI) (2). CI was calculated for a cell death range of 20–80 %, i.e. CI_{20}–CI_{80}.

$$CI_x = \frac{(D)_A}{(D_x)_A} + \frac{(D)_B}{(D_x)_B} + \alpha \cdot \frac{(D)_A (D)_B}{(D_x)_A (D_x)_B} \qquad (2)$$

where CI_x is the CI for a fixed effect, x [fraction affected $(f_a)\cdot 100$], for a combination of drug A and drug B, $(D_x)_A$ is the concentration of drug A alone giving an effect x, $(D_x)_B$ is the concentration of drug B alone giving an effect x, $(D)_A$ is the concentration of drug A in combination A + B giving an effect x, $(D)_B$ is the concentration of drug B in combination A + B giving an effect x, and α is a parameter with value 0 when A and B are mutually exclusive and 1 when A and B are mutually non-exclusive. A CI_x between 0.8 and 1.2 was categorized as additive, less than 0.8 as synergistic, and greater than 1.2 as antagonistic.

Measurement of cell cycle effect

Cells were plated and treated as described above (see measurement of growth inhibition by direct cell counting). For the combination of PTX and gefitinib, cells were exposed to 1.25 nM of PTX and 8.5 μM of gefitinib or 0.6 nM of PTX and 4 μM of gefitinib. After harvesting, the cells were fixed in 10 ml of 70% cold ethanol while vortexing, and cells were kept at 4°C for 1 h and stored at –20°C until analysis. Upon analysis, fixed cells were washed and resuspended in 1 ml of PBS containing 50 μg/ml RNase A and 50 μg/ml propidium iodide. After 20 min incubation at 37°C, cells were analyzed for DNA content by flow cytometry (FACSVantage; Becton Dickinson Immunocytometry Systems, San Jose, CA). For each sample, 10 000 events were acquired. Cell cycle distribution was determined using cell cycle analysis software (Modfit; Verity, Topsham, ME).

Cytostatic model analysis

In order to predict the contribution of cell cycle arrest to the overall growth inhibition induced by a cytotoxic agent, we used the cytostatic TP_i model as described previously [18]. In brief, the model assumptions were: (1) exponential growth of a cell population with a growth rate constant (k); (2) all cells were in cycle, i.e. no cell deaths and no G_0 phase arrest; (3) that the distribution of cell numbers in a cell cycle follows the age structure of a simple exponential population. $TP_i(t)$, the transition probability for i phase at time t, was defined as $(\alpha_i(t)-\alpha_i(t+\Delta t))/\alpha_i(t)/\Delta t$, where $\alpha_i(t) = [N_i(t)-(\#$ cells already exiting from i phase at time $t)]/N_i(t)$ and $N_i(t)$ is the number of cells in i phase at time t. The transition probability for each cell cycle check point, i.e. $TP_{G1}(t)$, $TP_S(t)$ and $TP_{G2/M}(t)$, was calculated using $F_i(t)$, the fraction of cells in the G_1, S and G_2/M phases at time t, and $k(t)$, the growth rate constant. The simulation of cell population growth over time was performed using a numerical method based on the cell population growth algorithm using $TP_i(t)$ and $F_i(t)$. This model assumes no cell death during cell cycle progression; hence, the simulation result represents a reduction in the number of cells resulting from cell cycle arrest (or disturbed cell cycle progression) only. The model should underestimate growth inhibition in the presence of cell death and the difference between the model-predicted and the observed growth curve of a treated cell population represents the growth inhibition resulting from cell death in the population.

Simultaneous measurement of drug-induced apoptosis and cell cycle distribution

For simultaneous determination of cell cycle contents and apoptosis, the user's manual of Apo-Direct kit (PharMingen, San Diego, CA) was followed. Briefly, after harvest, cells were fixed in 1% paraformaldehyde/PBS on ice for 15 min and resuspended in 70% ice-cold ethanol. Cells were then incubated in 50 μg of solution containing terminal deoxynucleotidyltransferase and FITC-conjugated dUTP deoxynucleotides 1:1 in reaction buffer for 2 h at 37°C in the dark. After washing in PBS containing 0.1% Triton X-100, the cells were stained with 5 μg of propidium iodide and 10 kU of RNase in 1 ml of PBS for 20 min at 37°C. Flow cytometric analysis was performed with FL1 (FITC) and FL2 (propidium iodide) and data acquisition and analysis were done using CellQuest software (Becton Dickinson Immunocytometry Systems).

Statistical analysis

Statistical comparisons were completed using Student's paired t-test; $p < 0.05$ was considered statistically significant.

Results

hMLH-1 and EGFR expression in SNU-1 and MKN45

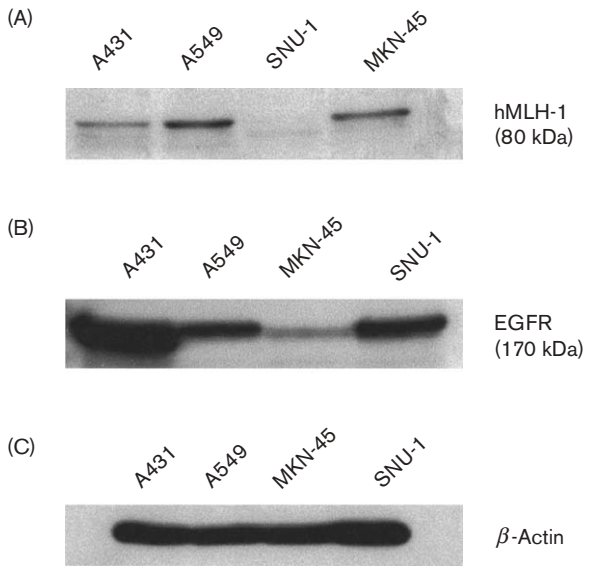
We evaluated the antitumor activities of the three cytotoxic drugs, LOHP, 5-FU or PTX, and that of a cytostatic drug, gefitinib, alone and in doublet combinations. We selected SNU-1 human gastric carcinoma cells because they are known to be MMR deficient due to a

missense mutation in hMLH-1 [12], which was confirmed in this study (Fig. 1A). EGFR expression was also examined and significant expression was observed in these cells. The level of expression was higher than those in MKN-45, another human gastric cancer cell line, and in A549, a human lung cancer cell line (Fig. 1B and 1C). Hence, SNU-1 cells were considered to represent an *in vitro* gastric cancer model that may have intrinsic chemoresistance related to both MMR deficiency and EGFR overexpression.

Cytotoxicity of LOHP, 5-FU, PTX and gefitinib in SNU-1 cells

Dose–response curves were analyzed using an E_{max} model with a resistant fraction (R), which represents the fraction of cells insensitive to the drug (Table 1). Significant R values were obtained for PTX (mean of 32%), whereas the other three drugs showed a full dose–response curve with percentage cell viabilities decreasing almost to the base line level (< 10%). The IC_{50} showed a wide range from 1.81 nM to 13.2 μM, i.e. 0.788 μM for LOHP, 9.35 μM for 5-FU, 1.81 nM for PTX and 13.2 μM for gefitinib. The antiproliferative activity of these agents was confirmed by direct cell counting. Drugs were given at two different concentrations, i.e. at the IC_{50} and IC_{80} levels. For all agents, 72 h exposure exhibited 50–60 and 80–90% growth inhibition at the IC_{50} and IC_{80} drug concentrations, respectively (Fig. 2). For PTX, 10 nM induced 68% growth inhibition when measured by MTT assay, with no further inhibition at higher concentrations, nonetheless, 89% inhibition was observed by direct cell counting (Fig. 2C).

Fig. 1



Western blot analysis of hMLH-1 (A) and EGFR (B) expression in A549, A431, SNU-1 and MKN-45 cells. The relative expression levels of EGFR relative to β-actin (C) were compared for these four cell lines.

Cytostatic model analysis

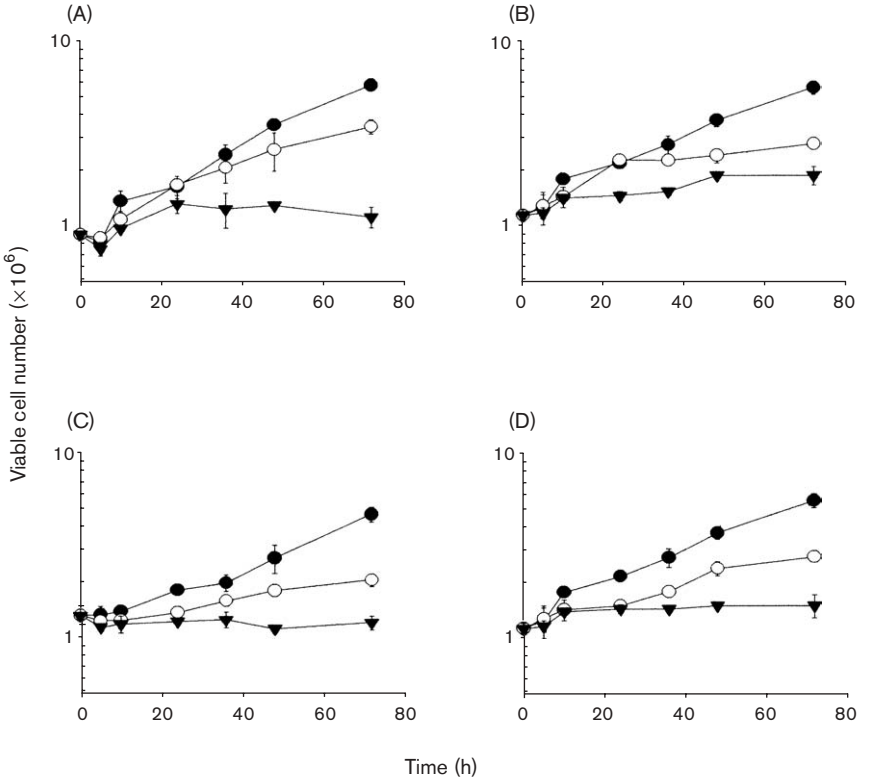
Growth inhibition, i.e. the reduction in the growth rate of a cell population following drug treatment, is the result of cell cycle arrest (cytostatic effect) and cell death (cytotoxic effect). As previously reported, we have developed a computational model (a cytostatic model, because the model assumes no cell death to predict the growth inhibition resulting from cell cycle arrest only) to assess the respective contributions of cell cycle arrest and cell death to the overall growth inhibition induced by cytotoxic anticancer agents [18]. We used this cytostatic model to analyze the contribution of cell cycle arrest to overall growth inhibition when SNU-1 cells were treated with each cytotoxic agent. The time course of cell cycle distribution was determined in SNU-1 cells exposed to LOHP, 5-FU and PTX at IC_{80} levels, respectively, and used in model simulation (part of data shown in Fig. 3). Since the model uses the percentage of cells in each phase to simulate the growth of a cell population, predictions cannot be made with 0% in any phase at anytime. For this reason, this cytostatic model was used only for LOHP and PTX, but not for 5-FU, because in this case the percentage of cells in the G_2/M phase was zero after 12 h exposure (data not shown). The cytostatic computational model predicted 64% of the overall inhibition from the cell cycle arrest induced by LOHP after 72 h exposure at 7.55 μM. For PTX given for 72 h at 10 nM and 80% of the overall inhibition was attributed to cell cycle arrest by the model. These results indicate that the reduction in population growth rate caused by cell

Table 1 **Parameters of the antiproliferative activities of LOHP, 5-FU, PTX and gefitinib against SNU-1 human gastric carcinoma cells**

	LOHP	5-FU	PTX	Gefitinib
IC_{50}[a]	0.788 ± 0.142	9.35 ± 1.57	1.81 ± 0.67	13.2 ± 0.33
R[b]	5.00 ± 4.48	8.53 ± 7.64	32.0 ± 11.1	0
m[c]	0.811 ± 0.135	0.793 ± 0.125	5.02 ± 0.80	1.14 ± 0.10

Each value represents mean ± SD of three independent experiments.
[a]IC_{50} is the concentration of the drug that kills 50% of cancer cells compared to the control after 72 h of continuous exposure. Expressed in μM, except for paclitaxel, which is in nM.
[b]R is the residual unaffected fraction (resistance fraction) (1) and is equal to $(100 - E_{max})$.
[c]m is the Hill-type coefficient (1).

Fig. 2



Growth curves of SNU-1 cells when incubated with (open circles, solid triangles) and without (solid circles) drug treatment: LOHP (A), 5-FU (B), PTX (C) and gefitinib (D). Cells were treated at two different concentrations near the IC_{50} (open circles) and the IC_{80} (solid triangles). The concentrations were: 0.75 and 7.55 μM for LOHP, 9 and 65 μM for 5-FU, 2.5 and 10 nM for PTX, and 13 and 38 μM for gefitinib. For cell number counting, cells were harvested by resuspending in medium and counted using a Coulter counter. Trypan blue exclusion was used for the determination of viable cell fraction.

cycle arrest effects contributes significantly to the overall growth inhibition induced by LOHP and PTX.

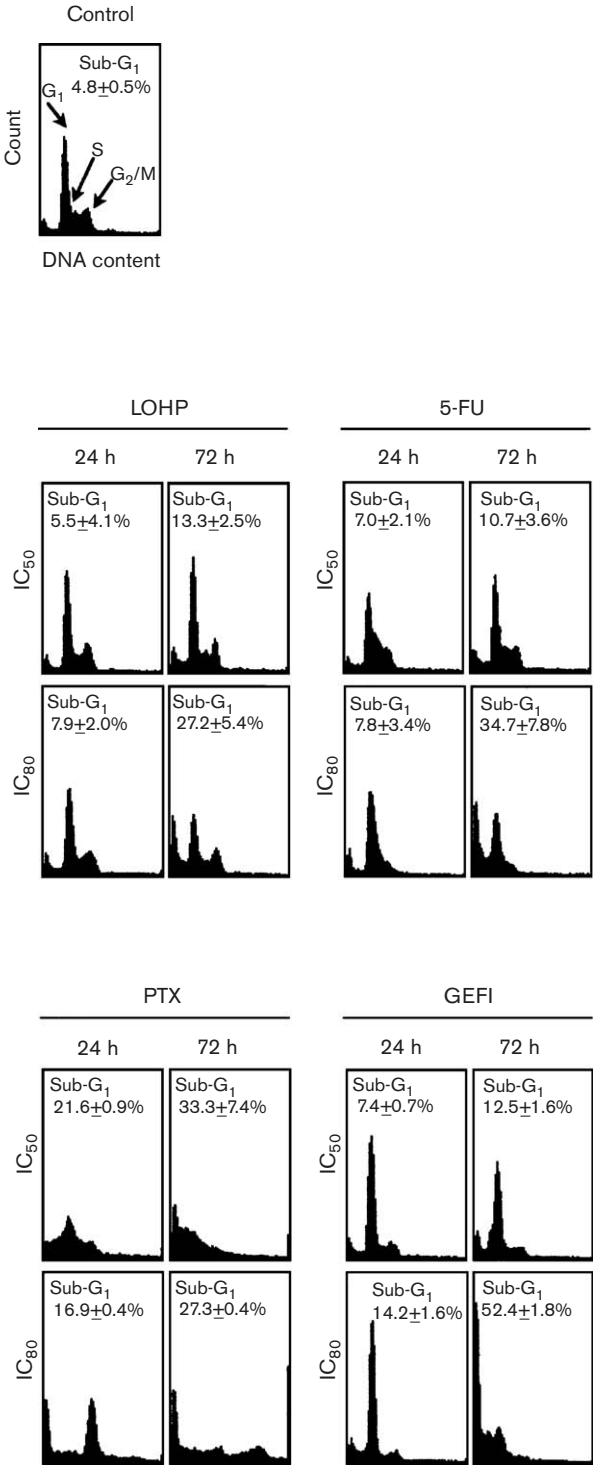
Cell cycle arrest effect with single drug treatment

The cell cycle arrest effect of the four agents was studied following a single drug exposure at two different concentrations for 72 h, i.e. around IC_{50} and IC_{80} as determined from the MTT dose–response curves. For LOHP, exposure to IC_{50} level concentrations, 0.75 μM did not induce significant changes in the cell cycle distribution (Fig. 3). When exposed to IC_{80} level concentrations, 7.55 μM of LOHP showed a moderate S phase decrease and G_2/M phase increase. Exposure to 5-FU (9 and 65 μM) resulted in an S phase increase along with G_2/M phase decreases in a concentration dependent manner: the cell cycle change following 5-FU treatment at the higher concentration (IC_{80}) was more pronounced. For LOHP and 5-FU, the sub-G_1 population, representative of cells that had undergone apoptosis, increased with time- and concentration-dependent manner. For PTX, cell cycle effect was dependent on drug concentration. At IC_{50} (2.5 nM), the number of cells in G_1 phase decreased with rapid accumulation of cells in sub-G_1 phase. At 10 nM, however, most cells were blocked in G_2/M phase ($69.5 \pm 0.8\%$) and a parallel decrease of the G_1 population was observed at 24 h, and a significant increase of the sub-G_1 population ($27.4 \pm 0.4\%$) and polyploid cells with $\geq 4n$ at 72 h. Gefitinib (13 and 38 μM) also showed a concentration dependent pattern of G_1 phase cell cycle arrest. The sub-G_1 population was induced after 72 h exposure at IC_{50} concentration ($12.5 \pm 1.6\%$) and increased to 52% after 72 h exposure at the concentration of 38 μM.

Evaluation of synergism

We evaluated the synergistic interaction between the cytotoxic agents, LOHP, 5-FU or PTX, and the cytostatic agent, gefitinib. All combinations were given at equitoxic ratios at the 50% inhibition levels of each drug, i.e. IC_{50} of drug A:IC_{50} of drug B. The dose–response curves are shown in Fig. 4 and the CI_x values calculated for $0.2 \leq f_a \leq 0.8$ (i.e. $20 \leq x \leq 80$) are shown in Fig. 5. CI_x values for the combination LOHP + gefitinib varied with f_a: CI_x decreased from 1.74 at $f_a = 0.2$ to 0.67 at $f_a = 0.8$. In the clinically relevant range of $f_a \geq 0.5$, hence, LOHP + gefitinib was considered to be synergistic to

Fig. 3



DNA histogram analysis in cells exposed to LOHP, 5-FU, PTX and gefitinib (GEFI) single treatment at IC_{50} and IC_{80}. Representative histograms are shown for 24 and 72 h post-treatment with the percentage of cells in sub-G_1 phase. Cells were harvested and fixed with ethanol before treated with RNase. Cells were then stained with propidium iodide and analyzed by flow cytometry. The concentrations were: 0.75 and 7.55 μM for LOHP, 9 and 65 μM for 5-FU, 2.5 and 10 nM for PTX, and 13 and 38 μM for GEFI.

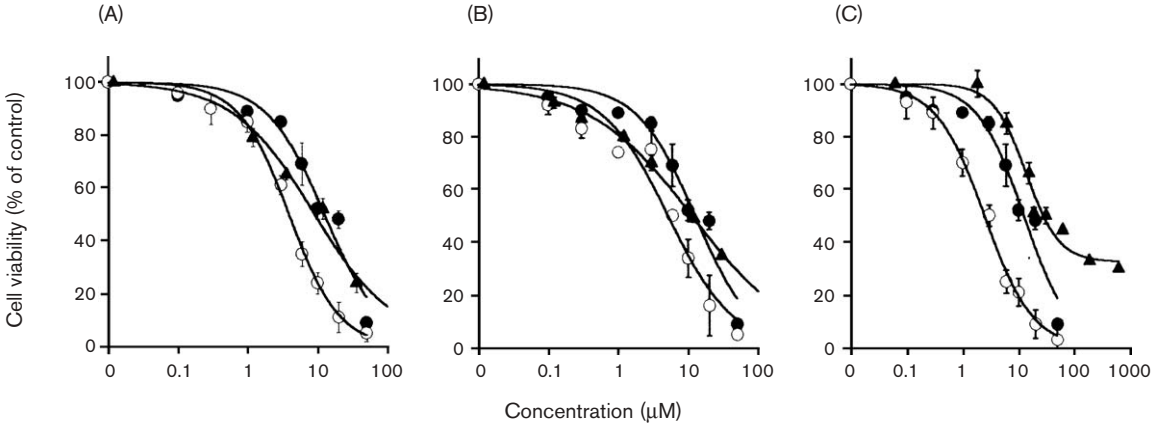
additive. 5-FU + gefitinib was found additive with CI values ranging from 1.06 to 1.24. The combination of PTX + gefitinib showed greatest synergism: with CI values of less than 1.0 (0.28–0.99) for the whole f_a range; in particular, the resistant fraction associated with PTX single treatment was abrogated when combined with gefitinib, indicating a greater advantage compared to the other combinations (Fig. 4). For PTX + gefitinib, the most synergistic combination, the cell cycle arrest and apoptosis induction were studied in cells exposed to the simultaneous treatment of PTX and gefitinib at two different concentrations, i.e. 0.62 nM PTX + 4 μM gefitinib (combination IC_{50}) and 1.25 nM PTX + 8.5 μM gefitinib (combination IC_{65}) (Fig. 6). No significant changes in the cell cycle distribution were observed at the combination IC_{50} level until 72 h. At the higher concentration, i.e. 1.25 nM PTX + 8.5 μM gefitinib (around IC_{65}), a significant decrease in G_1 phase cells occurred with rapid increase in sub-G_1 cells. The simultaneous staining of DNA content and DNA strand breaks were used to discern the apoptotic cells as well as necrotic cells from viable cells (Fig. 6). The combination of PTX and gefitinib at the IC_{50} and IC_{65} level induced 100 and 35% increase ($p < 0.05$) in apoptosis (TUNEL-positive cells), respectively, compared to the single treatment, supporting the synergism between these two drugs.

Discussion

Systemic chemotherapy for the treatment of gastric carcinomas includes mitomycin C, anthracyclines, alkylating agents and 5-FU. Among these drugs, cisplatin and 5-FU are most commonly used in combination regimens. Recently, PTX has been added and a triplet combination of PTX, 5-FU and cisplatin has also been evaluated for the treatment of advanced gastric cancer [20]. In addition, a new platinum compound, LOHP, may replace cisplatin due to its reduced toxicity and decreased possibility of resistance development related to MMR deficiency. Hence, we undertook to evaluate in human gastric cancer cells the antitumor activities of LOHP, 5-FU and PTX, and the potential synergistic interactions between these cytotoxic agents individually and a newly developed target-based (cytostatic) drug, gefitinib, to provide preclinical data for the future clinical development of these agents in a combination setting for the treatment of advanced gastric carcinomas.

The *in vitro* antitumor activity of the four agents was evaluated by MTT assay. SNU-1 cells showed differential sensitivity toward these agents, and the rank order of sensitivities was PTX (1.81 nM) > LOHP (0.788 μM) > 5-FU (9.35 μM) > gefitinib (13.2 μM). Among these four agents, PTX showed the greatest cytotoxicity with an IC_{50} in the nanomolar range; however a significant

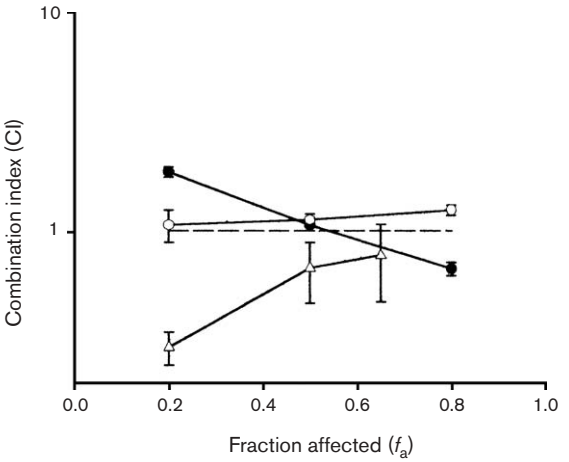
Fig. 4



Representative dose–response curves of LOHP, 5-FU, PTX and gefitinib administered alone and in combination. (A) Gefitinib alone (solid circles), LOHP alone (solid triangles), LOHP + gefitinib (open circles); (B) gefitinib alone (solid circles), 5-FU alone (solid triangles), 5-FU + gefitinib (open circles); and (C) gefitinib alone (solid circles), PTX alone (solid triangles), PTX + gefitinib (open circles). Cells were simultaneously exposed to each treatment regimen for 72 h and cell viability was determined by MTT assay. The *x*-axis is [gefitinib] × 1, [LOHP] × 12, [5–FU] × 1.2 and [PTX] × 6250.

fraction of resistant cells was found (Table 1). Such PTX-resistant fractions have been observed in other cell lines, such as A549, a human lung adenocarcinoma cell line, and in FaDu, a pharynx squamous carcinoma cell line, when cell viability was measured by the MTT or the SRB assay (unpublished data). In the case of PTX, the growth-inhibitory effect as measured by two different methods produced different results, i.e. MTT versus direct cell counting (Table 1 and Fig. 2). The exposure of cells to 10 nM of PTX for 72 h induced around 90% growth inhibition, when determined by direct cell counting, whereas 68% growth inhibition was expected based on MTT data. In the cases of the other three agents, the MTT data agreed with direct cell counting. Therefore, the resistant fraction obtained in the MTT assay seemed to be associated with the assay method, especially for PTX, suggesting that the experimental data obtained by widely used viability assays, such as MTT and SRB, should be interpreted with caution when determining the cytotoxicity of PTX in monolayer cultures. However, in the present study, this did not affect the degree of synergy calculated for gefitinib + PTX since the maximum concentration of PTX used for the CI calculation was 2.5 nM, where no difference was observed between the cell counting and MTT results (Fig. 2).

Fig. 5

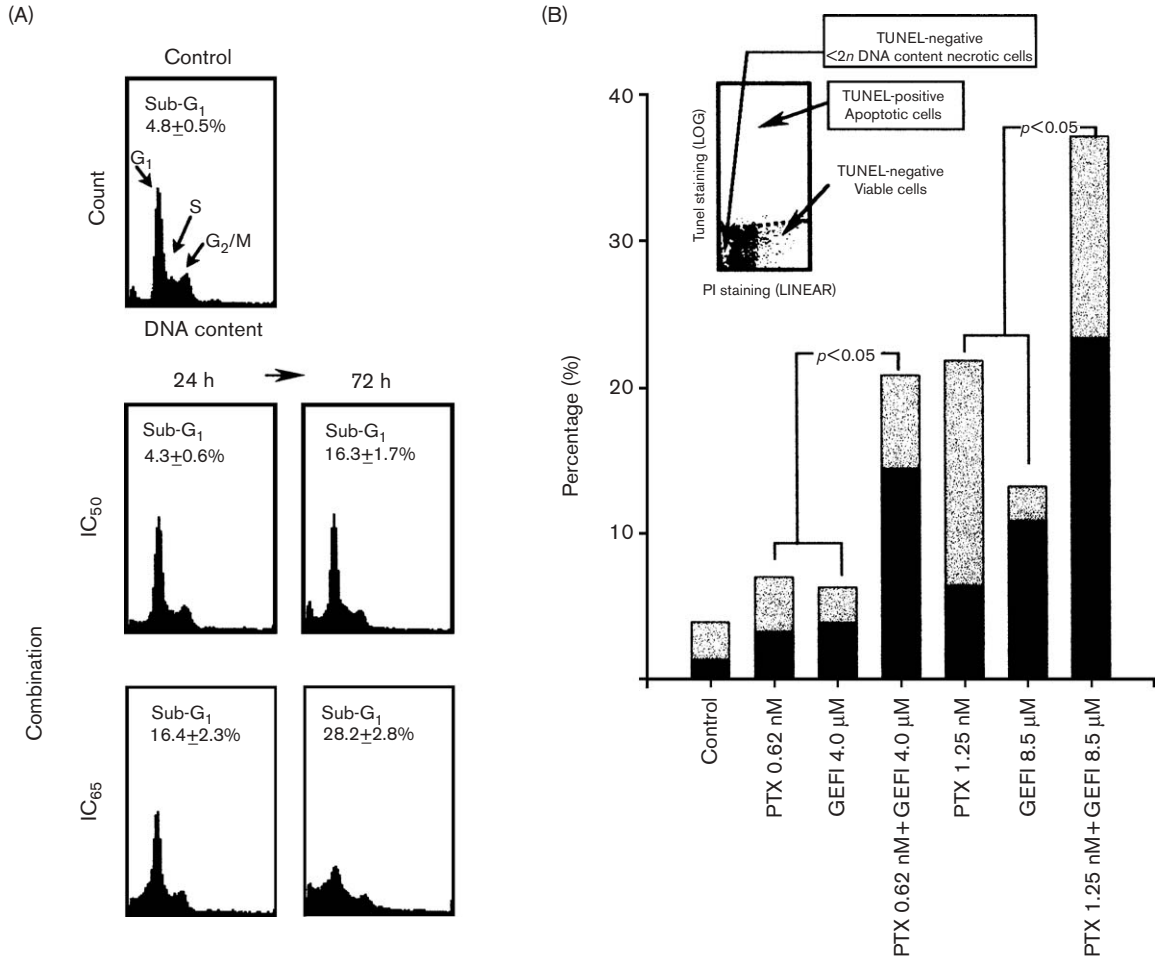


Combination index (CI_x) versus affected fraction (f_a) plots for LOHP + gefitinib (solid circles), 5-FU + gefitinib (open circles) and PTX + gefitinib (open squares) in SNU-1 cells. Cells were treated with LOHP or 5-FU or PTX + gefitinib at fixed equitoxic ratios. CI < 0.8, CI = 1 and CI > 1.2 indicate synergism, additivity and antagonism, respectively. LOHP and gefitinib were treated at a 0.083:1 molar ratio, 5-FU and gefitinib at 0.83:1, and PTX and gefitinib at 0.00016:1. CI_x was calculated using E_{max} model parameters obtained from the MTT data shown in Fig. 4.

SNU-1 cells showed significant resistance to 5-FU, i.e. the IC_{50} of 5-FU in SNU-1 cells was rather high (9.35 μM), which is close to that of gefitinib, a known cytostatic drug. MMR-proficient MKN-45 cells showed about 3 times higher sensitivity to 5-FU than SNU-1 cells (unpublished data). Hence, this intrinsic resistance of SNU-1 to 5-FU may be related to its MMR deficiency [15].

It has been suggested that the inhibition of EGFR-TK is an effective antiproliferative principle in EGFR-positive human gastric cancer cells [4]. Recently, gefitinib showed antiangiogenic and antiproliferative activity in a variety of human cancer cells *in vitro*, including human gastric cancer cells (KATO III and N87) [21]. The cytostatic growth inhibitory activity of gefitinib has been

Fig. 6



Cell cycle distribution and apoptosis induction during simultaneous treatment with PTX and gefitinib in SNU-1 cells. The drug concentrations used were 0.6 nM PTX + 4 μM (IC_{50}) or 1.25 nM PTX + 8.5 μM gefitinib (IC_{65}). (A) Representative histograms are shown for 24 and 72 h post-treatment with the percentage of cells in the sub-G_1 phase. At predetermined times following drug exposure, cells were harvested, fixed, stained with propidium iodide (PI) and analyzed by flow cytometry. (B) The bivariate analysis of DNA content and apoptosis in cells exposed to the indicated drug treatment for 72 h. Cells were treated for 72 h and processed for the double staining of TUNEL/PI and analyzed by flow cytometry. Solid box: TUNEL-negative necrotic cells; gray box: TUNEL-positive apoptotic cells. Statistical analysis: sum of PTX and gefitinib alone versus combination: $p<0.05$.

demonstrated in a wide range of human cancer cell lines, and the reported IC_{50}s of gefitinib vary by cell line and by the assay method used. A soft agar colony assay showed an IC_{50} range of 0.05–2.5 μM for gefitinib in breast, colon and gastric cancer cells [8,21]. On the other hand, an IC_{50} range of 6–30 μM was reported in human head/neck and colon cancer cells by MTT [22,23]. Our results on the antitumor activity of gefitinib in SNU-1 cells are comparable with those obtained using the same viability assay method, i.e. MTT (Table 1).

A few studies have demonstrated an inverse correlation between growth IC_{50} and EGFR expression level [22], whereas contradictory data have been reported by others [7]. SNU-1 cells express moderate levels of EGFR, which may explain its moderate IC_{50} compared to other studies, which used highly overexpressing cell lines, such as A431. The EGFR signaling pathway involves the activation of several nuclear proteins, including cyclin D_1, via the activation of *ras* and mitogen-activated protein kinase [21]. Since EGFR activates cyclin D_1, and cyclin D_1 is required for cell cycle progression from G_1 to the S phase, EGFR signaling is critical for cell proliferation and its inhibition causes G_1 arrest in human cancer cells [21]. Our results also demonstrate that the inhibition of EGFR signaling by gefitinib induces G_1 arrest in human gastric cancer cells in a concentration dependent manner (Fig. 3).

In common with other cytostatic agents, gefitinib is expected to be a good candidate for combination regimens with cytotoxic agents. The combination of gefitinib + PTX has been shown to induce dose-depen-

dent cooperative growth inhibition and the potentiation of apoptosis *in vitro* [8,21], and to induce complete regression in some human tumor xenograft models [7]. Gefitinib + LOHP has been found to be supra-additive in human ovarian, breast and colon cells [14,24]. The sequence-dependent synergy appeared to be cell-line specific, i.e. gefitinib followed by cisplatin/5-FU was synergistic in head/neck cell cancer cell line [22], whereas gefitinib followed by oxaliplatin was antagonistic in human colon cancer cell line [25]. The simultaneous exposure was additive to synergistic in both studies, hence, the simultaneous schedule was to chosen to evaluate the synergy in the present study. We did not evaluate the sequence-dependent interactions because SNU-1 cells grow as a suspension and, hence, are not suitable for such experiments. The sequence dependency should be investigated using another human gastric cancer cell line.

In the present study, CI_x was calculated for the range of $0.2 \leq f_a \leq 0.8$ (Fig. 5), but the combination effect on cell cycle distribution and apoptosis induction was evaluated for the range of $f_a \geq 0.5$, i.e. at IC_{50} and IC_{80} levels (Fig. 6). Considering the fact that the maximum effect is needed in the clinical situation, it should be more relevant to focus on the effect above IC_{50} level [22]. Preclinical studies (especially, animal models) commonly use lower doses of chemotherapy to observe greater synergy; however, this often do not translate to the clinic, where maximum therapeutic doses are used [26].

In our study, the potentiation of antitumor activity was greatest for PTX + gefitinib, which had the lowest CI_{50} value among the three combinations and moreover the resistant fraction in the PTX single treatment was completely abrogated (Figs 4 and 5). LOHP + gefitinib is also a promising combination regimen because it produced a very similar level of synergism to PTX + gefitinib at $f_a = 0.8$ and additive effects at the $f_a = 0.5$ level. Gefitinib combined with PTX resulted in enhanced drug-induced apoptosis (Fig. 6). It is conceivable that the cytotoxicity of PTX is potentiated by the effective inhibition of survival signals upregulated by the EGFR signal network. The elucidation of the mechanism of this interaction requires further investigation.

Results of phase III lung trials for gefitinib + cytotoxics were disappointing and can be attributed to many factors including the following: (i) due to the lack of correlation between the apparent expression of EGFR and sensitivity, the responding phenotype was not known and patient selection could not be made, (ii) it is believed that triplet regimen of conventional chemotherapy are not superior to doublets in non-small cell lung cancer, and (iii) patients (in IDEAL phase II trials) who were already heavily treated with, and refractory to, chemotherapy may have been more sensitive to the inhibition of the EGFR pathway by gefitinib [27]. Despite the negative results of phase III lung trials, the rationales for studying the combination of gefitinib with cytotoxics in gastric cancers are 3-fold. (i) EGFR levels were associated with poor prognosis in gastric cancer patients [28,29], (ii) gefitinib is given orally, hence, higher drug concentration can be obtained in the gastric tissues and (iii) gastric cancers are relatively easy for biopsy study through which the responding phenotype can be identified.

In summary, the present study demonstrates that the antitumor activity of gefitinib, against human gastric carcinoma cells, is accompanied by significant cell cycle arrest and apoptosis. We also found that the antiproliferative effects of the cytotoxic drugs, LOHP and PTX, could be greatly enhanced when combined with gefitinib. The suppression of growth by gefitinib may be of clinical importance as the prolonged administration of orally active gefitinib could offer long-term control of gastric tumor growth and metastasis. This study provides preclinical data supporting the clinical development of gefitinib and its use in combination with PTX or LOHP against MMR-deficient human gastric cancers that express EGFR. Moreover, this study shows that gefitinib warrants further evaluation *vis-à-vis* its use in other gastric cancer cells/tumors.

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