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# Cellular and Pharmacogenetics Foundation of Synergistic Interaction of Pemetrexed and Gemcitabine in Human Non–Small-Cell Lung Cancer Cells

Elisa Giovannetti, Valentina Mey, Sara Nannizzi, Giuseppe Pasqualetti, Luca Marini, Mario Del Tacca, and Romano Danesi

*Division of Pharmacology and Chemotherapy, Department of Oncology, Transplants and Advanced Technologies in Medicine, University of Pisa, Pisa, Italy (E.G., V.M., S.N., G.P., M.D.T., R.D.); and Medical Department, Lilly Oncology, Florence, Italy (L.M.)*

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## ABSTRACT

Gemcitabine and pemetrexed are effective agents in the treatment of non–small-cell lung cancer (NSCLC), and the present study investigates cellular and genetic aspects of their interaction against A549, Calu-1, and Calu-6 cells. Cells were treated with pemetrexed and gemcitabine, and their interaction was assessed using the combination index. The role of drug metabolism in gemcitabine cytotoxicity was examined with inhibitors of deoxycytidine kinase (dCK), 5′-nucleotidase, and cytidine deaminase, whereas the role of pemetrexed targets, thymidylate synthase (TS), dihydrofolate reductase (DHFR), and glycinamide ribonucleotide formyltransferase (GARFT) in drug chemosensitivity was analyzed in cytotoxicity rescue studies. The effect of gemcitabine and pemetrexed on Akt phosphorylation was investigated with enzyme-linked immunosorbent assay, whereas quantitative polymerase chain reaction (PCR) was used to study target gene-expression profiles and its modulation by each drug. Synergistic cytotoxicity was demonstrated, and pemetrexed significantly decreased the amount of phosphorylated Akt, enhanced apoptosis, and increased the expression of dCK in A549 and Calu-6 cells, as well as the expression of the human nucleoside equilibrative transporter 1 (hENT1) in all cell lines. PCR demonstrated a correlation between dCK expression and gemcitabine sensitivity, whereas expression of TS, DHFR, and GARFT was predictive of pemetrexed chemosensitivity. These data demonstrated that 1) gemcitabine and pemetrexed synergistically interact against NSCLC cells through the suppression of Akt phosphorylation and induction of apoptosis; 2) the gene expression profile of critical genes may predict for drug chemosensitivity; and 3) pemetrexed enhances dCK and hENT1 expression, thus suggesting the role of gene-expression modulation for rational development of chemotherapy combinations.





Despite recent advances in early diagnosis and treatment, non–small-cell lung cancer (NSCLC) is a disease with a grim prognosis. Extensive clinical studies demonstrated that chemotherapy increases survival in the adjuvant setting (Arriagada et al., 2004) and in patients with advanced disease (Reck and Gatzemeier, 2004). Nonetheless, response rates remain lower than 15%, and median survival is less than 6 months, thus emphasizing the need for new effective drugs and combination regimens (Rosell and Crinò, 2002). However, the rationale for chemotherapy combinations has remained mostly empirical, determined from the antitumor activity of each agent and the lack of overlapping toxicities, despite many attempts to discover preclinical models for rational selection of drug interactions.

Gemcitabine (2′,2′-difluorodeoxycytidine) is a deoxycytidine analog with a broad spectrum of anticancer activity against several solid tumors in preclinical models, and it is

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**ABBREVIATIONS:** NSCLC, non–small-cell lung cancer; dCK, deoxycytidine kinase; TS, thymidylate synthase; DHFR, dihydrofolate reductase; GARFT, glycinamide ribonucleotide formyltransferase; hENT1, human nucleoside equilibrative transporter 1; 5′-NT, 5′-nucleotidase; CDA, cytidine deaminase; RR, ribonucleotide reductase; RRM1, regulatory subunit of ribonucleotide reductase; RRM2, catalytic subunit of ribonucleotide reductase; PBS, phosphate-buffered saline; P-Ser473 Akt, activated, phosphorylated form of Akt at Ser473; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; ELISA, enzyme-linked immunosorbent assay; LY309887, 6*R*-2′,5′-thienyl-5,10-dideazatetrahydrofolic acid; PCR, polymerase chain reaction; CI, combination index; QRT-PCR, quantitative real-time polymerase chain reaction.

now an established, effective agent in the treatment of malignancies, particularly NSCLC and pancreatic cancer (Noble and Goa, 1997; Li et al., 2004). Gemcitabine is a prodrug that is transported into the cell mostly by human equilibrative nucleoside transporter 1 (hENT1) and then requires intracellular phosphorylation to its active metabolite, 2′,2′-difluorodeoxycytidine triphosphate, to be incorporated into DNA, leading to chain termination (Bergman et al., 2002).

The rate-limiting step in the activation of the drug is catalyzed by deoxycytidine kinase (dCK), whereas 5′-nucleotidase (5′-NT) and cytidine deaminase (CDA) are the main inactivating enzymes (Galmarini et al., 2001). In addition to being incorporated into DNA, gemcitabine exerts its cytotoxicity by inhibiting ribonucleotide reductase (RR); therefore, a mechanism for gemcitabine resistance, other than decreased activity of dCK and enhanced activity of 5′-NT and CDA, could be a mutation or overexpression of RR (Bergman et al., 2002).

Antimetabolites are widely used in combination regimens because of their ability to biochemically modulate the cytotoxicity of other drugs (Peters et al., 2000). In particular, preclinical studies on gemcitabine in combination with cisplatin (van Moorsel et al., 1999), carboplatin, or paclitaxel (Theodossiou et al., 1998; Edelman et al., 2001) and topotecan (Tolis et al., 1999) have shown schedule-dependent drug interaction in several human lung cancer cell lines.

Pemetrexed is an antifolate inhibitor of thymidilate synthase (TS), dihydrofolate reductase (DHFR), and glycinamide ribonucleotide formyltransferase (GARFT), with activity against a wide spectrum of tumor cell lines, including NSCLC (Britten et al., 1999; Teicher et al., 2000). Preclinical studies suggested that the combinations of pemetrexed with cisplatin, as well as taxanes and gemcitabine, produce additive or synergistic cytotoxicity (Teicher et al., 1999, 2000; Tonkinson et al., 1999), whereas clinical trials showed response rates of 20% with single-agent pemetrexed and of approximately 40% in combination with cisplatin (Rusthoven et al., 1999; Manegold et al., 2000; Shepherd et al., 2001). Moreover, the results of a large prospective randomized study comparing pemetrexed with docetaxel in the second-line treatment of 571 patients with advanced NSCLC indicated similar response rates (9.1 versus 8.8%) and median survival outcome (7.9 versus 8.5 months) for the two agents with toxicity profiles favoring pemetrexed (Hanna et al., 2004). The ability of pemetrexed to deplete cellular nucleotide pools, modulate cell cycle, and induce apoptosis makes this drug a new, attractive cytotoxic agent for polychemotherapy regimens (Shih et al., 1997; Tonkinson et al., 1997). In particular, dCTP depletion and GARFT inhibition by pemetrexed may enhance the expression of the key genes hENT1 and dCK as a compensatory mechanism, thus potentially favoring gemcitabine activity. For these reasons, the present study was performed in NSCLC cell lines to investigate the ability of the drugs to synergistically interact and to establish a correlation between cytotoxicity and gene expression of selected genes.

## Materials and Methods

**Drugs and Chemicals.** Gemcitabine, pemetrexed, and LY309887 were generous gifts from Eli Lilly & Co. (Indianapolis, IN). Drugs were dissolved in sterile distilled water and diluted in culture medium immediately before use. RPMI 1640, McCoy's, and minimal essential medium, fetal bovine serum, L-glutamine (2 mM), penicillin (50 IU/ml), and streptomycin (50 $\mu$g/ml) were from Invitrogen (Carlsbad, CA). All other chemicals were from Sigma-Aldrich (St. Louis, MO).

**Cell Culture.** The NSCLC cell line A549 (adenocarcinoma) was obtained from American Type Culture Collection (Manassas, VA), and Calu-1 (epidermoid carcinoma) and Calu-6 (anaplastic carcinoma) cell lines were generously provided by Professor F. Basolo (University of Pisa, Pisa, Italy). Cells were maintained as monolayer cultures, respectively, in RPMI, McCoy's, and minimal essential medium with 10% fetal bovine serum, glutamine, and penicillin-streptomycin. Cells were cultivated in 75-cm$^2$ tissue culture flasks (Costar, Cambridge, MA) at 37°C in 5% $CO_2$ and 95% air and were harvested with trypsin/EDTA when they were in logarithmic growth.

**Assay of Cytotoxicity.** Cells were plated in 24-well sterile plastic plates (Costar) at $5 \times 10^4$ cells/well and were allowed to attach for 24 h. Cells were treated with 1) gemcitabine 0.1 ng/ml (0.33 nM) to 100 $\mu$g/ml (333 $\mu$M) for 1 h; 2) pemetrexed 0.1 ng/ml (0.21 nM) to 100 $\mu$g/ml (212 $\mu$M) for 24 h; 3) gemcitabine for 1 h, followed by a 24-h washout in drug-free medium and then pemetrexed for 24 h; and 4) pemetrexed for 24 h followed by a 24-h washout in drug-free medium and then gemcitabine for 1 h. The experimental conditions adopted in this study, including gemcitabine concentrations and time of exposure, are similar to those selected in previous studies (Tonkinson et al., 1999; Giovannetti et al., 2004). Moreover, the effects of gemcitabine on deoxynucleotide triphosphate pool depletion occur during the first 30 min and reach the maximum effect within 2 h (Symon et al., 2002). Cytotoxicity was determined after 1 h of treatment with gemcitabine and 24 h of incubation with pemetrexed using concentrations that are comparable with the drug exposure observed in the clinical setting (Noble and Goa, 1997; Thödtmann et al., 1999). After drug treatments were completed, cells were cultured for an additional 24 h in drug-free medium, and the growth inhibition by drugs was assessed by counting cells. The $IC_{50}$ values relative to untreated cultures were calculated by nonlinear least-squares curve-fitting.

Drug interaction between gemcitabine and pemetrexed was assessed at a fixed concentration ratio using the combination index, where CI $<$ 1, CI $=$ 1, and CI $>$ 1 indicate synergistic, additive, and antagonistic effects, respectively (Chou et al., 1994). Data analysis was performed with the use of Calcusyn software (Biosoft, Oxford, UK).

**Modulation of Gemcitabine Metabolism and Cytotoxicity.** Cells were plated in 24-well plates as described under "Assay of Cytotoxicity" and were treated with gemcitabine 0.1 ng/ml (0.33 nM) to 10 $\mu$g/ml (33 $\mu$M) for 24 h alone or in combination with 10 $\mu$M 2′-deoxycytidine, diethylpyrocarbonate, and tetrahydrouridine to inhibit drug activation by phosphorylation (Eda et al., 1998) or drug inactivation by dephosphorylation (Hicks-Berger et al., 2001) and deamination (Eda et al., 1998), respectively. $IC_{50}$ values were calculated as described above.

**Rescue Studies of Pemetrexed Cytotoxicity.** To gain further insight into the mechanism of action of pemetrexed, cells were plated in 24-well plates as described under "Assay of Cytotoxicity", treated with pemetrexed 0.1 ng/ml (0.21 nM) to 100 $\mu$g/ml (212 $\mu$M) for 24 h, and rescued by simultaneous supplementation of thymidine (5 $\mu$M) and hypoxanthine (100 $\mu$M), with the end products of the enzymatic steps inhibited by pemetrexed. Moreover, rescue experiments with thymidine and hypoxanthine were performed with the selective TS inhibitor 5-fluorouracil (0.1 ng/ml to 100 $\mu$g/ml) and the GARFT-selective inhibitor LY309887 (0.1 ng/ml to 100 $\mu$g/ml).

**Cell-Cycle Analysis.** Cells were plated at $1 \times 10^6$ in 100-mm plastic dishes (Costar) and were allowed to attach for 24 h. After treatment with gemcitabine (1 h), pemetrexed (24 h), and their combinations at their $IC_{50}$ levels followed by a 24-h washout, cells were harvested with trypsin/EDTA and washed twice with phosphate-buffered saline (PBS). DNA was stained with a solution containing propidium iodide (25 $\mu$g/ml), RNase (1 mg/ml), and Nonidet

P-40 (0.1%), and samples were kept on ice for 30 min. Cytofluorimetry was performed using a FACScan (BD Biosciences, San Jose, CA), and data analysis was carried out with CELLQuest software, whereas cell-cycle distribution was determined using ModFit software (Verity Software House, Topsham, ME).

**Analysis of Apoptosis.** Cells were treated with gemcitabine, pemetrexed, and their combinations at their $IC_{50}$ levels as described under "Assay of Cytotoxicity". At the end of incubation, cells were washed twice with PBS and fixed in 4% buffered paraformaldehyde for 15 min. Cells were resuspended and incubated for a further 15 min in a solution containing 8 $\mu$g/ml bisbenzimide HCl. Cells were spotted on glass slides and examined by fluorescence microscopy (Leica, Wetzlar, Germany). A total of 200 cells from randomly chosen microscopic fields were counted, and the percentage of cells displaying chromatin condensation and nuclear fragmentation relative to the total number of counted cells (apoptotic index) was calculated.

Apoptosis induced by gemcitabine, pemetrexed, and their combinations was also studied by flow cytometry. Cells treated as described under "Cell-Cycle Analysis" were collected, washed twice with PBS, and fixed with ice-cold 70% ethanol for 1 h. Fixed cells were washed with PBS, resuspended in 0.2 M phosphate-citrate buffer at pH 7.8, and then stained with propidium iodide as described previously. The percentage of apoptotic cells was quantified from the sub-$G_1$ signal, measured with the ModFit software.

**Assay of Akt Phosphorylation.** Akt protein activation by phosphorylation after gemcitabine and pemetrexed treatment was assayed with an ELISA specific for P-Ser473 Akt and normalized to the total Akt content (BioSource International, Camarillo, CA). Cells were treated as described above for apoptosis analysis. At the end of incubation, cells were washed twice with PBS, harvested by centrifugation (1000*g* for 5 min at 4°C), and resuspended in 25 $\mu$l of extraction buffer for 30 min on ice while vortexing. A volume of 5 $\mu$l of cell extract was diluted to 100 $\mu$l with 15 mM $NaN_3$, centrifuged at 15,000*g* for 10 min at 4°C, and transferred to microtiter plates coated with a monoclonal antibody specific for total Akt. A standard curve was run with each assay using 100, 50, 25, 12.5, 6.25, 3.12, and 1.6 U/ml phosphorylated full-length human recombinant Akt (P-Ser473 Akt) and 20, 10, 5, 2.5, 1.25, 0.6, and 0.3 ng/ml human recombinant total Akt. After overnight incubation at 4°C, the solution was aspirated from wells, and 100 $\mu$l of rabbit anti-P-Ser473 Akt and biotin-conjugated anti-total Akt was added into each well of P-Ser473 Akt and Akt total ELISA, respectively. Plates were incubated at room temperature for 1 h, washed four times, and 100 $\mu$l of a working solution of horseradish peroxidase-labeled anti-rabbit IgG and horseradish peroxidase-labeled streptavidin was added into each well of P-Ser473 Akt and total Akt ELISA assay, respectively. After 30 min, a chromogen solution was added; 20 min later, the reactions were stopped with 100 $\mu$l of a stop solution, and the absorbance was read at 450 nm at 20-min intervals for 120 min to construct a plot of absorbance increase as a function of time. To calculate P-Ser473 Akt and Akt total concentrations, a standard curve method was used. Values of P-Ser473 Akt, calculated from the standard curve, were then normalized for total Akt and protein content.

**QRT-PCR Analysis.** To establish a correlation between drug effect and modulation of gene expression, drug concentrations corresponding to the $IC_{25}$, $IC_{50}$, and $IC_{75}$ values of gemcitabine, pemetrexed, and their combinations were studied. Moreover, to evaluate the time-course modulation of gene expression, QRT-PCR analysis was performed 6, 12, 24, and 48 h after completion of drug treatment. Total RNA was extracted from cells using the TRI REAGENT LS (Sigma-Aldrich). RNA was dissolved in 10 mM dithiothreitol and 200 U/ml RNase inhibitor in RNase-free water and measured at 260 nm. One microgram of RNA was reverse-transcribed at 37°C for 1 h in a 100-$\mu$l reaction volume containing 0.8 mM deoxynucleotide triphosphates, 200 U of Moloney murine leukemia virus reverse transcriptase, 40 U of RNase inhibitor, and 0.05 $\mu$g/ml random primers. The cDNA was amplified by quantitative, real-time PCR with the Applied Biosystems 7900HT sequence detection system (Applied Biosystems, Foster City, CA). QRT-PCR reactions were performed in triplicate using 5 $\mu$l of cDNA, 12.5 $\mu$l of TaqMan Universal PCR Master Mix, 2.5 $\mu$l of probe, and 2.5 $\mu$l of forward and reverse primers in a final volume of 25 $\mu$l. Samples were amplified using the following thermal profile: an initial incubation at 50°C for 5 min followed by incubation at 95°C for 10 min, 40 cycles of denaturation at 95°C for 15 s, followed by annealing and extension at 60°C for 1 min.

Forward and reverse primers and probes were designed with Primer Express 2.0 (Applied Biosystems) on the basis of dCK, 5′-NT, CDA, TS, DHFR, and GARFT gene sequence obtained from the GeneBank, whereas primers and probes for the regulatory (RRM1) and catalytic subunits (RRM2) of RR and for hENT1 were obtained from Applied Biosystems Assay-on-Demand Gene expression products (Hs00168784, Hs0035724, and Hs00191940). Amplifications were normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH), and quantification of gene expression in treated cells was performed using the $\Delta\Delta C_T$ calculation, where $C_T$ is the threshold cycle; the amount of target gene, normalized to GAPDH and relative to the calibrator (untreated control cells), is given as $2^{-\Delta\Delta CT}$.

Preliminary experiments were carried out with dilutions of cDNA obtained from Quantitative PCR Human Reference Total RNA (Stratagene, La Jolla, CA) to determine the primer concentrations that give the minimum standard deviation between $C_T$ values and to demonstrate that the efficiencies of amplification of the target (dCK, 5′-NT, CDA, RRM1, RRM2, TS, DHFR, and GARFT) and reference (GAPDH) genes are approximately equal. The absolute value of the slope of standard cDNA concentration versus $C_T$ were −3.06 (dCK), −3.04 (5′-NT), −3.52 (CDA), −3.12 (RRM1), −3.24 (RRM2), −2.98 (hENT1), −4.00 (TS), −3.23 (DHFR), −2.94 (GARFT), and −3.32 (GAPDH); thus, the PCR efficiency was better than 88%.

**Statistical Analysis.** All experiments were performed in triplicate and were repeated at least three times. Data were expressed as mean values ± S.E. and were analyzed by Student's *t* test or analysis of variance followed by the Tukey's multiple comparisons; the level of significance was set at $P < 0.05$.

## Results

**Cytotoxicity of Gemcitabine and Pemetrexed.** A dose-dependent inhibition of cell growth was observed with gemcitabine and pemetrexed (Fig. 1), with $IC_{50}$ values of 0.13 ± 0.02 and 0.25 ± 0.03 $\mu$g/ml (A549), 5.28 ± 1.25 and 34.13 ± 5.78 $\mu$g/ml (Calu-1), and 1.66 ± 0.36 and 4.84 ± 0.60 $\mu$g/ml (Calu-6), respectively. On the basis of these results, combination studies were performed at fixed concentration ratios (1:2, 1:3, and 1:6 for gemcitabine/pemetrexed) in A549, Calu-6, and Calu-1 cells, respectively. The sequential exposure of cell lines to pemetrexed followed by gemcitabine reduced the $IC_{50}$ values of gemcitabine to 3.5 ± 1.0, 130.5 ± 27.8, and 5.4 ± 1.7 ng/ml in A549, Calu-1, and Calu-6 cells, whereas the $IC_{50}$ values resulting from the reverse sequence were 30.4 ± 11.2, 47.1 ± 10.3, and 23.4 ± 7.3 ng/ml, respectively. The calculation of the CI value showed that, at effect levels between 0.25 and 0.75, both schedules of gemcitabine and pemetrexed demonstrated synergism in all cell lines; however, although the differences were not marked, the sequence of pemetrexed→gemcitabine proved to be the most effective against A549 and Calu-6 cells (Fig. 2).

**Modulation of dCK, 5′-NT, CDA, and Gemcitabine Cytotoxicity.** A key role for dCK on sensitivity to gemcitabine of NSCLC cell lines was demonstrated. After simultaneous treatment with gemcitabine and 2′-deoxycytidine 10 $\mu$M for 24 h, a 6- to 12-fold increase in $IC_{50}$ values in all cell lines was observed. In contrast, there was a 2- to 6-fold

decrease in $IC_{50}$ values by inhibition of 5′-NT and CDA (Table 1), suggesting that inactivating enzymes may play an additional role in modulating gemcitabine cytotoxicity.

**Rescue Studies of Pemetrexed Cytotoxicity.** Thymidine completely reversed the cytotoxicity of the TS inhibitor 5-fluorouracil in all cell lines, whereas it partially prevented the inhibition of cell growth by pemetrexed, as demonstrated by a 1.2- to 3.4-fold increase in the $IC_{50}$ value (Table 2). Similar results were obtained with hypoxanthine that, alone, reduced the cytotoxicity of LY309887, although it was less effective with pemetrexed (Table 2). However, only the combination of thymidine and hypoxanthine totally protected cells from the antiproliferative effect of pemetrexed (Table 2).

**Cell-Cycle Effects of Gemcitabine and Pemetrexed.** Both pemetrexed and gemcitabine were able to affect the cell cycle of NSCLC cells (Table 3). In particular, the percentage of A549 cells in the S phase significantly increased from 6.0 to 32.5% ($P < 0.05$), after treatment with pemetrexed for 24 h, whereas a modest increase was detected in Calu-1 cells (from 25.6 to 38.2%, $P$ = not significant). The same effect on cell cycle was observed after a 1-h treatment with gemcitabine in A549 cells (from 6.0 to 18.0%, $P < 0.05$) and Calu-1 cells (from 25.7 to 30.7%, $P$ = not significant). In contrast, in Calu-6 cells, flow-cytometry studies demonstrated that pemetrexed and gemcitabine blocked cells in the $G_1$-S boundary. In particular, pemetrexed caused a 1.5-fold increase in the population of cells in the $G_1$ phase, from 50.5 to 74.2%. The $G_1$ arrest was overcome by gemcitabine, and cell-cycle distribution analysis of drug combinations demonstrated that both schedules enhanced the percentage of cells in S and $G_2$ phases in all cell lines (Table 3).

**Induction of Apoptosis by Gemcitabine and Pemetrexed.** Cells exposed to pemetrexed, gemcitabine, and their combinations presented typical apoptotic morphology with cell shrinkage, nuclear condensation and fragmenta-

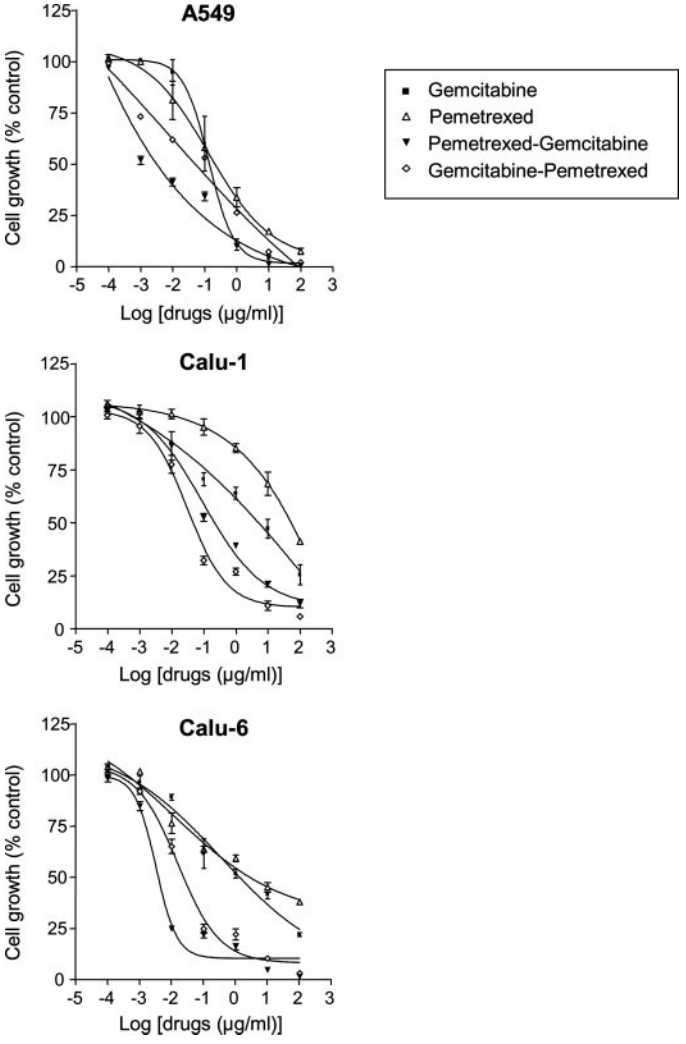


**Fig. 1.** Inhibitory effect of gemcitabine, pemetrexed and their combinations on proliferation of NSCLC cells. Points, mean values obtained from three independent experiments; bars, S.E.

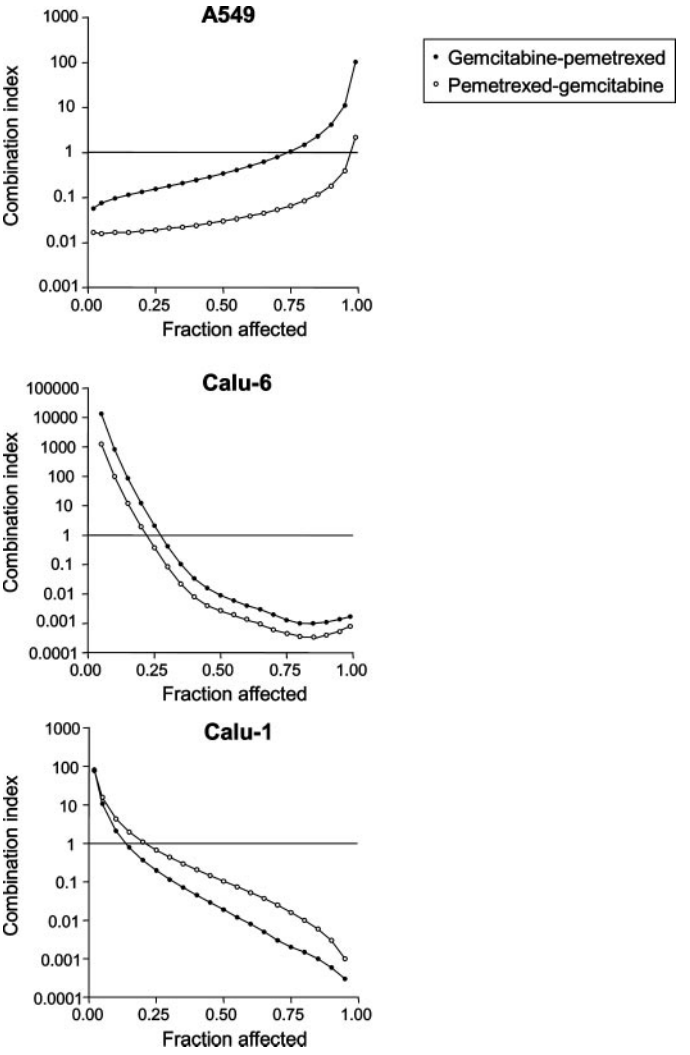


**Fig. 2.** CI plots of pemetrexed-gemcitabine combinations in A549, Calu-1, and Calu-6 cells.

TABLE 1

Effects of deoxycytidine (dCyd), diethylpyrocarbonate (DEPC), and tetrahydrouridine (THU) on gemcitabine cytotoxicity

Values are presented as mean ± S.E. of at least three independent experiments.

| Cells | $IC_{50}$ Values | | | |
|---|---|---|---|---|
| | Gemcitabine | +dCyd | +DEPC | +THU |
| | *ng/ml* | | | |
| A549 | 30.31 ± 1.73 | 378.6 ± 16.24 | 16.65 ± 1.21 | 4.80 ± 0.71 |
| Calu-1 | 117.50 ± 27.71 | 699.72 ± 14.80 | 36.60 ± 3.07 | 42.81 ± 2.12 |
| Calu-6 | 194.85 ± 25.23 | 1505.1 ± 54.21 | 77.33 ± 6.50 | 93.50 ± 32.71 |

tion, and rupture of cells into debris after a 24-h washout. In all cell lines, 5 to 7% of apoptotic cells were observed after pemetrexed treatment, whereas gemcitabine exposure was associated with a higher percentage (8–12%) of apoptotic cells; in each case, the drug combinations significantly increased the apoptotic index with respect to control cells (Fig. 3). In particular, the sequential administration of pemetrexed→gemcitabine produced the highest apoptotic index compared with gemcitabine in A549 and Calu-6 cells (12.2 ± 0.6 and 8.61 ± 1.6% versus 22.2 ± 5.4 and 16.5 ± 4.9%, respectively), whereas Calu-1 cells were the least sensitive, and both sequences were equivalent (9.6 ± 1.2 versus 10.8 ± 1.1 and 9.8 ± 1.6%) (Fig. 3).

These results were confirmed by the enhancement of the sub-$G_1$ region on the DNA content histograms demonstrating that, after drug treatments, cell-cycle modulation was accompanied by the induction of apoptosis. The A549 cells treated with the sequential administration of pemetrexed→gemcitabine exhibited the largest sub-$G_1$ signal (25.2%), whereas a minor increase in the proportion of hypodyploid cells with respect to controls was observed in Calu-1 cells after pemetrexed exposure (Table 3).

TABLE 2
Effects of thymidine and hypoxanthine on 5-fluorouracil, LY309887, and pemetrexed $IC_{50}$ values
Shown are mean values ± S.E. of at least three independent experiments.

| | A549 | Calu-1 | Calu-6 |
|---|---|---|---|
| | *μg/ml* | *μg/ml* | *μg/ml* |
| 5-Fluorouracil | 1.04 ± 0.11 | 0.57 ± 0.04 | 0.47 ± 0.03 |
| + Thymidine | >100 | >100 | >100 |
| + Hypoxanthine | 1.26 ± 0.17 | 0.56 ± 0.02 | 0.77 ± 0.04 |
| LY309887 | 0.02 ± 0.01 | 1.64 ± 0.13 | 0.16 ± 0.05 |
| + Thymidine | 0.02 ± 0.01 | 1.86 ± 0.25 | 0.16 ± 0.03 |
| + Hypoxanthine | >100 | >100 | >100 |
| Pemetrexed | 0.25 ± 0.03 | 34.13 ± 5.78 | 4.83 ± 0.60 |
| + Thymidine | 0.85 ± 0.14 | 55.32 ± 4.41 | 5.96 ± 0.75 |
| + Hypoxanthine | 0.28 ± 0.04 | 36.23 ± 3.94 | 4.57 ± 0.56 |
| + Thymidine/ hypoxanthine | >100 | >100 | >100 |

TABLE 3
Effects of gemcitabine and pemetrexed on cell cycle and induction of apoptosis of NSCLC cell lines
Cancer cells were exposed to $IC_{50}$ values of drugs alone and in combination at fixed 1:2, 1:6, and 1:3 (gemcitabine/pemetrexed) concentration ratios in A549, Calu-1, and Calu-6 cells, respectively. Values are presented as the mean percentage of the total number of cells examined in three independent experiments.

| Cell Treatment | $G_1$ | S | $G_2$ | Sub-$G_1$ |
|---|---|---|---|---|
| | | % | | |
| A549 | | | | |
| Control | 90.71 | 5.95 | 3.34 | 1.8 |
| Gemcitabine | 68.53 | 18.02 | 13.46 | 12.9 |
| Pemetrexed | 58.83 | 32.48 | 8.70 | 6.9 |
| Gemcitabine→pemetrexed | 49.59 | 40.89 | 9.51 | 21.0 |
| Pemetrexed→gemcitabine | 45.25 | 39.68 | 15.07 | 25.1 |
| Calu-1 | | | | |
| Control | 59.58 | 25.68 | 14.74 | 2.1 |
| Gemcitabine | 57.65 | 30.70 | 11.66 | 9.3 |
| Pemetrexed | 54.22 | 38.42 | 2.66 | 5.4 |
| Gemcitabine→pemetrexed | 39.00 | 35.57 | 25.43 | 10.6 |
| Pemetrexed→gemcitabine | 45.26 | 36.42 | 18.32 | 12.0 |
| Calu-6 | | | | |
| Control | 50.46 | 35.01 | 14.54 | 5.3 |
| Gemcitabine | 64.10 | 31.62 | 4.27 | 9.4 |
| Pemetrexed | 74.20 | 25.75 | 5.95 | 6.2 |
| Gemcitabine→pemetrexed | 20.49 | 54.03 | 25.48 | 17.1 |
| Pemetrexed→gemcitabine | 27.97 | 44.85 | 27.18 | 18.8 |

**Inhibition of Akt Phosphorylation.** Gemcitabine and pemetrexed were able to significantly reduce the amount of phosphorylated Akt in A549 and Calu-6 cells ($P < 0.05$), with pemetrexed being more potent than gemcitabine. In Calu-1 cells, the amount of the phosphorylated form of Akt was decreased up to −31.5% by pemetrexed and up to −22.2% by gemcitabine compared with controls (Fig. 4).

**Correlation between Gene Expression and Chemosensitivity.** The relative expression of dCK was remarkably higher than 5′-NT, CDA, RRM1, RRM2, and hENT1 in all cell lines (Table 4). The sensitivity of A549 cells to gemcitabine was correlated with low expression of RRM1/M2 and high expression of dCK and hENT1, whereas the lower chemosensitivity of Calu-1 cells seemed mostly dependent on high expression of the gene encoding the inactivating enzymes CDA and 5′-NT (Table 4). A similar correlation was found between the $IC_{50}$ values of pemetrexed and the expression of target enzymes TS, DHFR, and GARFT; the least sensitive cell line (Calu-1) was found to overexpress TS, DHFR, and GARFT with respect to the other cell lines (Table 4).

**Modulation of dCK and hENT1 Gene Expression.** Pemetrexed, at its $IC_{50}$ and $IC_{75}$ levels, significantly increased hENT1 expression in all cell lines, whereas at the $IC_{25}$ level, there was only a minimal enhancement of hENT1 expression in A549 cells (Fig. 5). Similar results were observed at a pemetrexed concentration corresponding to $IC_{50}$ and $IC_{75}$ levels for dCK, whose expression was increased by pemetrexed up to +92.40 and +83.61% (A549 cells) and +40.69 and +47.10% (Calu-6 cells), respectively (Fig. 6), whereas dCK gene expression was not modulated in Calu-1 cells at the $IC_{50}$ level, although there was a

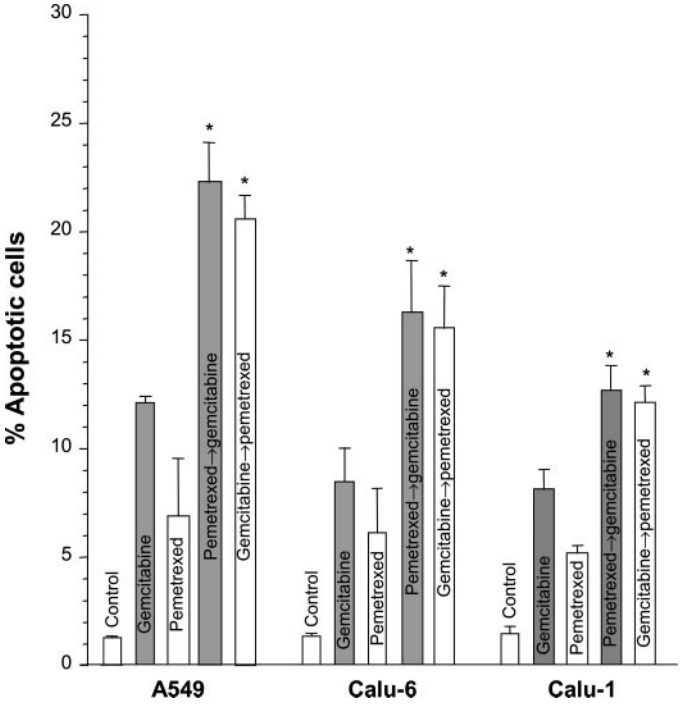


**Fig. 3.** Induction of apoptosis by gemcitabine, pemetrexed, and their combinations after a 24-h washout. Cancer cells were exposed to the $IC_{50}$ value of drugs alone and in combination at fixed 1:2, 1:6, and 1:3 (gemcitabine/pemetrexed) concentration ratios. Columns, mean values obtained from three independent experiments; bars, S.E. ⋆, $P < 0.05$ with respect to gemcitabine treatment.

+23.8% enhancement at $IC_{75}$. Furthermore, each drug combination at the $IC_{50}$ level, using the fixed combination ratios in which gemcitabine and pemetrexed are equipotent, induced both hENT1 and dCK expression in all cell lines, whereas at 0.25 effect levels, dCK gene expression was not significantly modulated in Calu-6 and Calu-1 cells (Table 5). Finally, Table 6 shows the expression levels of dCK and hENT1 6, 12, 24, and 48 h after the end of pemetrexed treatment, demonstrating that their up-regulation occurred mostly between the 12- and 48-h time points.

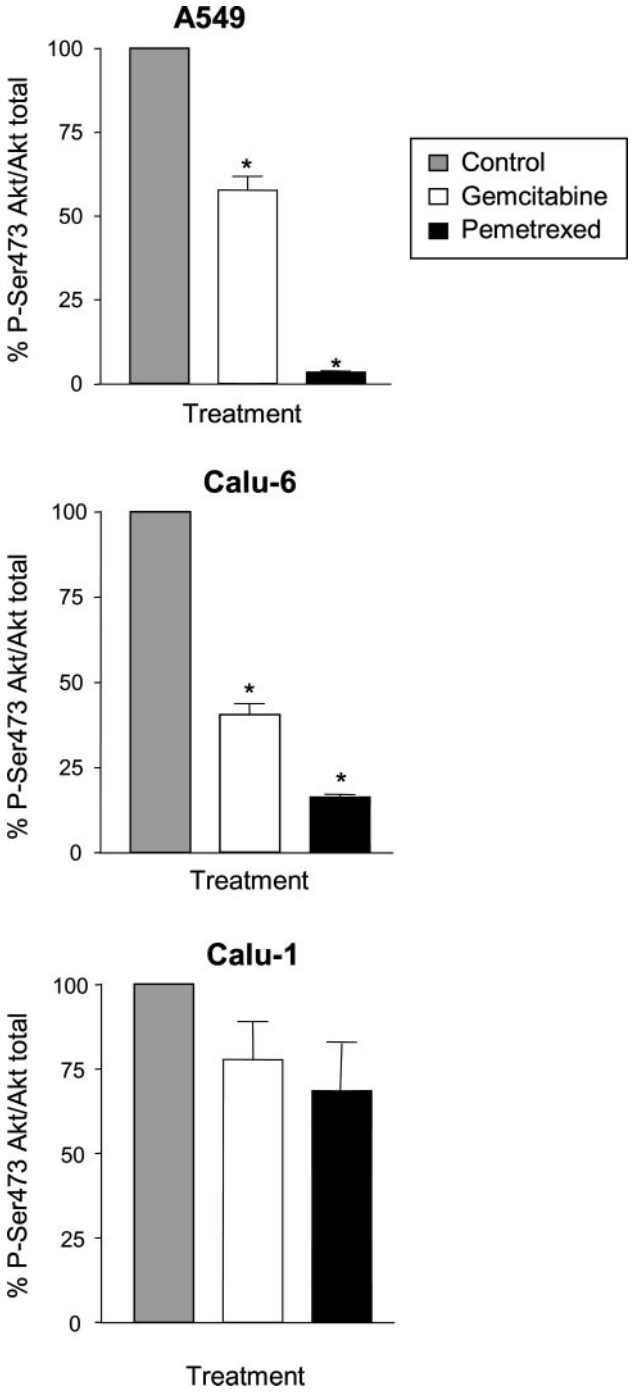


**Fig. 4.** Reduction of P-Ser473 Akt by gemcitabine and pemetrexed in A549, Calu-6, and Calu-1 cells. ⋆, $P < 0.05$ with respect to control.

## Discussion

The present study demonstrates that gemcitabine and pemetrexed are synergistic against NSCLC cell lines A549, Calu-6, and Calu-1. These findings are novel because in preclinical studies, the combination of pemetrexed and gemcitabine yielded conflicting results. A recent study on colorectal cancer cell lines showed a synergistic cytotoxicity of gemcitabine followed by pemetrexed in HCT-8 cells (Adjei et al., 2000), and similar results were obtained in LoVo, WiDr, and LRWZ cells, whereas the sequence pemetrexed→gemcitabine caused an additive-synergistic effect (Tesei et al., 2002). On the contrary, other studies demonstrated that the schedule-dependent synergism was maximal when pemetrexed preceded gemcitabine in HT29 colon cancer cells (Tonkinson et al., 1999) and in MIA PaCa-2, PANC-1, and Capan-1 pancreatic cancer cells (Giovannetti et al., 2004). Because of the inherent limitations of translating in vitro data to in vivo models, because of the pharmacokinetic and pharmacodynamic complexity of living systems, in vivo studies were performed to test the reproducibility of drug effects. Indeed, the combination of these two drugs also showed a schedule-dependent interaction in vivo. An additive cytotoxic activity with both the sequence gemcitabine→pemetrexed and the simultaneous administration was demonstrated in H460 NSCLC xenograft (Teicher et al., 2000), whereas a synergistic interaction of sequential administration of pemetrexed followed by gemcitabine was shown in HT29 xenograft. Thus, the synergistic activity of these agents in cell culture translated into enhanced antitumor activity in vivo (Tonkinson et al., 1999). The evidence of preclinical sequence-dependent synergism prompted a three-arm randomized phase II study of pemetrexed plus gemcitabine as frontline therapy for advanced NSCLC, and preliminary efficacy and toxicity data indicated that pemetrexed followed by gemcitabine on day 1 was the optimal schedule (Adjei et al., 2004).

Recent studies have shown the importance of modulating cell cycle to exploit the effect of drug combinations (Peters et al., 2000). In the present study, flow cytometry demonstrated that in A549 and Calu-1 cells, pemetrexed and gemcitabine caused an accumulation of cells in the S phase as a result of the inhibition of DNA synthesis. This finding is in agreement with previous data obtained in A549 cells treated with gemcitabine (Bandala et al., 2001) and in CCRF-CEM and HT29 cells, which were synchronized after a 24-h pemetrexed exposure (Tonkinson et al., 1997, 1999). Because gemcitabine is

TABLE 4
Relative expression of dCK, 5′-NT, CDA, RRM1, RRM2, hENT1, TS, DHFR, and GARFT with respect to the quantitative PCR human reference total RNA calibrator and GAPDH

| | Gene Expression | | |
|---|---|---|---|
| | A549 | Calu-1 | Calu-6 |
| dCK | 1526.76 | 1401.22 | 1461.44 |
| 5′-NT | 2.78 | 6.99 | 1.98 |
| CDA | 5.62 | 20.84 | 0.07 |
| RRM1 | 0.11 | 2.62 | 4.40 |
| RRM2 | 0.03 | 1.15 | 1.45 |
| hENT1 | 0.58 | 0.10 | 0.12 |
| TS | 0.06 | 65.98 | 1.31 |
| DHFR | 0.17 | 132.71 | 1.62 |
| GARFT | 0.08 | 86.28 | 0.38 |

an S-phase–specific drug, the increase of activity of the schedule pemetrexed→gemcitabine may be the result of a modulation of cell cycle, potentially facilitating 2′,2′-difluorodeoxycytidine triphosphate incorporation into DNA.

The triggering of apoptotic machinery may be crucial to improve the therapeutic activity of gemcitabine. Although the cell-killing mechanisms of gemcitabine and pemetrexed against NSCLC cells are not fully characterized, apoptosis does indeed play a role in cell death by both agents (Tonkinson et al., 1997; Tolis et al., 1999; Bandala et al., 2001). In the present in vitro study, A549, Calu-1, and Calu-6 cells were exposed to gemcitabine and pemetrexed in combination, and an enhancement of apoptosis was observed in treated cells compared with controls and cells treated with single agents. A similar observation has been reported in WiDr colon cancer cells and in pancreatic cancer cells, in which a few apoptotic cells were observed after gemcitabine treatment, whereas a significantly higher percentage was found after treatment with the gemcitabine-pemetrexed combination (Tesei et al., 2002; Giovannetti et al., 2004). Moreover, a recent investigation showed that the reduction of phosphorylated protein kinase B/Akt correlated with the enhancement of gemcitabine-induced apoptosis and antitumor activity, suggesting that the phosphatidylinositide 3-kinase/Akt pathway plays a significant role in mediating drug resistance in human cancer cells (Ng et al., 2001). Our study demonstrates for the first time that gemcitabine and, more effectively, pemetrexed decreased the amount of the activated form of Akt; thus, the reciprocal improvement of their therapeutic potential may depend on drug-induced apoptosis.

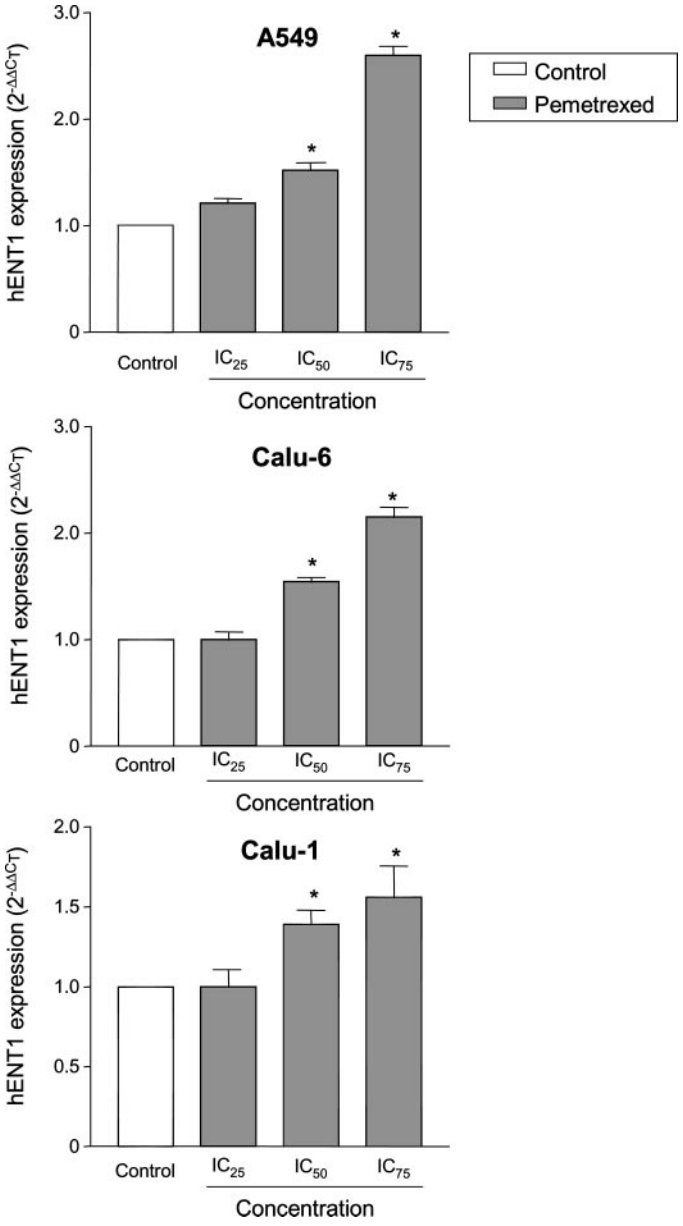


**Fig. 5.** Modulation of hENT1 gene expression by pemetrexed at $IC_{25}$, $IC_{50}$, and $IC_{75}$ concentration levels in A549, Calu-1, and Calu-6 cells. ⋆, $P < 0.05$ with respect to control.

Like other nucleoside analogs, gemcitabine is hydrophilic, and it is transported into the cell by concentrative and equilibrative nucleoside carriers. In particular, gemcitabine is most efficiently transported by hENT1, and activity of this protein is considered to be a possible determinant of drug efficacy (Mackey et al., 1998). Because TS inhibitors up-regulate hENT1 and increase gemcitabine sensitivity by depleting intracellular nucleotide pools (Pressacco et al., 1995; Rauchwerger et al., 2000), the present study analyzed the modulation of hENT1 expression by pemetrexed and demonstrated a significant up-regulation of this carrier, potentially facilitating gemcitabine cytotoxicity.

Several studies have suggested that dCK, a key enzyme of the nucleoside salvage pathway, is a limiting factor for the antitumor effect of gemcitabine because its deficiency is critically involved in acquired resistance to nucleoside analogs in vitro; indeed, the sensitivity to these drugs in general and to gemcitabine in particular was restored by transfection with wild-type dCK (Blackstock et al., 2001; Bergman et al., 2002). Moreover, the pretreatment dCK expression level could be used as a predictive parameter of tumor sensitivity. Recent data showed a clear correlation between dCK activity and gemcitabine sensitivity in human tumor xenografts (Kroep et al., 2002). The crucial role of dCK was confirmed in the present work by the marked reduction of gemcitabine activity with 2′-deoxycytidine, whereas transcriptome analysis suggested the predictive value of expression of dCK in particular and also of RR, 5′-NT, and CDA. As reported previously in colon cancer cells, a similar correlation was found between TS and chemoresistance to pemetrexed (Sigmond et al., 2003). Moreover, cytotoxicity rescue experiments suggested that purine de novo biosynthetic pathway is an important target for pemetrexed in addition to TS, and the analysis of gene expression of DHFR and GARFT demonstrated that their expression levels were related to $IC_{50}$ values of pemetrexed in the NSCLC cell lines examined in the present study.

Being an inhibitor of de novo purine biosynthesis, because of the blockade of the key enzyme GARFT (Shih et al., 1997), pemetrexed may increase the expression of dCK as a compensatory mechanism. The present study confirms this hypothesis in A549 and Calu-6 cells, in which the highest synergism was observed with the sequence of pemetrexed→gemcitabine, whereas in Calu-1 cells, dCK gene expression was not apparently modulated, possibly because of higher levels of target enzymes of pemetrexed and the need for higher concentrations of the drug to up-regulate dCK, as demonstrated with the exposure to the $IC_{75}$ level. This result

is in agreement with those of a previous study which indicated that inhibitors of DNA biosynthesis widely differ in their stimulatory effect on dCK, and there are also "responsive" and "nonresponsive" cells with respect to dCK activation (Spasokoukotskaja et al., 1999). Moreover, PCR analysis of cells exposed to different concentrations of drug combinations revealed a good relationship between modulation of dCK gene expression and the type of drug interaction. Gemcitabine-pemetrexed combinations at concentrations resulting in synergistic drug interaction ($IC_{50}$ levels) increased dCK expression in all cell lines. On the contrary, dCK gene expression was not significantly modulated in Calu-6 and Calu-1 cells in conditions of drug antagonism (0.25 effect level). Therefore, enhancement of hENT1 and dCK expression by pemetrexed in the sequence pemetrexed→gemcitabine strongly supports this combination, with upregulation of key genes dCK-hENT1 being a marker of their synergistic interaction.

The present in vitro experimental findings also suggest that the pharmacogenetic profiling may contribute to the assessment of tumor-cell response to gemcitabine and pemetrexed. In particular, A549 sensitivity could be explained by the favorable dCK/RR ratio, as shown in pancreatic cancer cell lines (Giovannetti et al., 2004), particularly for the low levels of the target RR, as well as of TS, DHFR, and GARFT, whereas the lower sensitivity of Calu-1 cells could be dependent on increased activity of the gemcitabine catabolic pathway and high levels of pemetrexed targets. Moreover, enhancement of hENT1 and dCK expression by pemetrexed could be responsible, at least in part, for the synergistic interaction obtained with the sequential exposure to gemcitabine in NSCLC cell lines, thus underlying the importance of modulation of gene expression for rational development of cytotoxic drug combinations.

In conclusion, several factors involving the modulation of cell cycle, the induction of apoptosis, and expression of critical genes involved in drug activity may contribute to the synergistic effect between gemcitabine and pemetrexed against in vitro models of lung cancer. Although the extrapolation of in vitro data to the in vivo setting should be con-

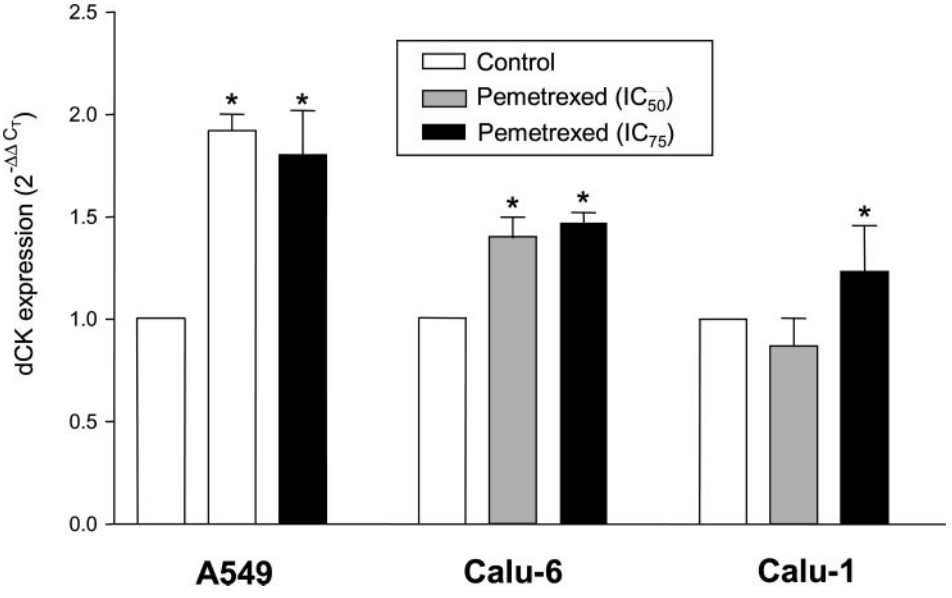


**Fig. 6.** Modulation of dCK expression by pemetrexed ($IC_{50}$ and $IC_{75}$ levels) in comparison with controls in A549, Calu-1, and Calu-6 cells. Columns, mean values obtained from two independent experiments; bars, S.E. ⋆, $P < 0.05$ with respect to control.

TABLE 5

Effects of gemcitabine-pemetrexed combinations on dCK and hENT1 gene expression of NSCLC cell lines

The amount of target gene, normalized to GAPDH and relative to the calibrator (untreated control cells), is given as $2^{-\Delta\Delta CT}$.

| Effect Levels | Treatments | dCK | hENT1 |
|---|---|---|---|
| A549 Cells | | | |
| | Control | 1.00 | 1.00 |
| 0.25 | Gemcitabine-pemetrexed | 1.37 | 1.38 |
| 0.25 | Pemetrexed-gemcitabine | 1.42 | 1.51 |
| 0.50 | Gemcitabine-pemetrexed | 1.45 | 2.80 |
| 0.50 | Pemetrexed-gemcitabine | 3.01 | 3.19 |
| Calu-1 Cells | | | |
| | Control | 1.00 | 1.00 |
| 0.25 | Gemcitabine-pemetrexed | 1.05 | 1.42 |
| 0.25 | Pemetrexed-gemcitabine | 1.12 | 1.38 |
| 0.50 | Gemcitabine-pemetrexed | 1.37 | 1.36 |
| 0.50 | Pemetrexed-gemcitabine | 1.59 | 1.73 |
| Calu-6 Cells | | | |
| | Control | 1.00 | 1.00 |
| 0.25 | Gemcitabine-pemetrexed | 0.93 | 1.54 |
| 0.25 | Pemetrexed-gemcitabine | 1.07 | 1.66 |
| 0.50 | Gemcitabine-pemetrexed | 1.87 | 1.80 |
| 0.50 | Pemetrexed-gemcitabine | 1.95 | 2.02 |

TABLE 6

Effects of pemetrexed, at $IC_{50}$ level, on dCK and hENT1 gene expression of NSCLC cell lines 6,12, 24, and 48 h after the end of treatment

The amount of target gene, normalized to GAPDH and relative to the calibrator (untreated control cells), is given as $2^{-\Delta\Delta CT}$.

| Times | dCK | hENT1 |
|---|---|---|
| A549 Cells | | |
| 6 h | 1.24 | 1.11 |
| 12 h | 1.52 | 1.78 |
| 24 h | 1.92 | 1.52 |
| 48 h | 1.97 | 1.29 |
| Calu-1 Cells | | |
| 6 h | 0.76 | 1.31 |
| 12 h | 0.95 | 1.34 |
| 24 h | 0.87 | 1.39 |
| 48 h | 1.01 | 1.71 |
| Calu-6 Cells | | |
| 6 h | 0.51 | 1.18 |
| 12 h | 1.77 | 1.51 |
| 24 h | 1.41 | 1.51 |
| 48 h | 2.12 | 0.76 |

sidered with caution, these findings may have implications for rational design of future drug regimens incorporating gemcitabine and pemetrexed for the treatment of NSCLC.

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**Address correspondence to:** Dr. Elisa Giovannetti, Division of Pharmacology and Chemotherapy, Department of Oncology, Transplants and Advanced Technologies in Medicine, 55, Via Roma, 56126 Pisa, Italy. E-mail: elisagio@tiscalinet.it