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

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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; dFdC, 2',2'-difluorodeoxycytidine; dFdCTP, 2',2'-difluorodeoxycytidine triphosphate; 5'-NT, 5'-nucleotidase; CDA, cytidine deaminase; RR, ribonucleotide reductase; RRM1, regulatory and RRM2 catalytic subunits of RR; PBS, phosphate-buffered saline; P-Ser473 Akt, activated, phosphorylated form of Akt at Ser473; dNTPs, deoxynucleotide triphosphates mix; MMLV-RT, Moloney murine leukemia virus reverse transcriptase; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; PI3K-Akt, phosphatidylinositide 3-kinase/Akt; PKB/Akt, protein kinase B/Akt.

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 on gemcitabine cytotoxicity was examined with inhibitors of deoxycytidine kinase (dCK), 5'-nucleotidase and cytidine deaminase, while the role of pemetrexed targets, thymidylate synthase (TS), dihydrofolate reductase (DHFR) and glycinamide ribonucleotide formyltransferase (GARFT), on drug chemosensitivity was analysed in cytotoxicity rescue studies. The effect of gemcitabine and pemetrexed on Akt phosphorylation was investigated with ELISA, while quantitative 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 of the human nucleoside equilibrative transporter 1 (hENT1) in all cell lines. PCR demonstrated a correlation between dCK expression and gemcitabine sensitivity, while 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 suppression of Akt phosphorylation and induction of apoptosis; (2) 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.

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Introduction

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 below 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, based on 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, dFdC) is a deoxycytidine analogue with broad spectrum of anticancer activity against several solid tumors in preclinical models, and it is 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 equilibrative nucleoside transporter 1 (hENT1) and then requires intracellular phosphorylation to its active metabolite, 2',2'-difluorodeoxycytidine triphosphate (dFdCTP) to be incorporated into DNA, leading to chain termination (Bergman et al., 2002).

The rate-limiting step in the activation of the drug is catalysed by deoxycytidine kinase (dCK), while 5'-nucleotidase (5'-NT) and cytidine deaminase (CDA) are the main inactivating enzymes (Galmarini et al., 2001). In addition to be 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., 2001; 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., 2000, Teicher et al., 1999; Tonkinson et al., 1999), while clinical trials showed response rates of 20% with single agent pemetrexed and 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 advanced NSCLC patients, indicated similar response rates (9.1 vs 8.8%) and median survival outcome (7.9 *vs* 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 (Tonkinson et al., 1997; Shih 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 estabilish a correlation between cytotoxicity and gene expression of selected genes.

Materials and Methods

Drugs and Chemicals. Gemcitabine, pemetrexed and 6*R*-2',5'-thienyl-5,10-dideazatetrahydrofolic acid (LY309887) were generous gifts from Eli Lilly (Indianapolis, IN). Drugs were dissolved in sterile distilled water and diluted in culture medium immediately before use. RPMI, McCoy's and MEM media, fetal bovine serum (FBS), L-glutamine (2 mM), penicillin

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(50 IU/mL) and streptomycin (50 µg/mL) were from Gibco (Gaithersburg, MD). All other chemicals were from Sigma Chemical Co. (St. Louis, MO).

Cell Culture. The NSCLC cell line A549 (adenocarcinoma) was obtained from American Type Culture Collection (Manassas, VA), while Calu-1 (epidermoid carcinoma) and Calu-6 (anaplastic carcinoma) cell lines were generously provided by Prof. F. Basolo, University of Pisa, Italy. Cells were maintained as monolayer cultures respectively in RPMI, McCoy's and MEM medium, with 10% FBS, 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 harvested with trypsin-EDTA when they were in logarithmic growth.

Assay of Cytotoxicity. Cells were plated in 24-well sterile plastic plates (Costar, Cambridge, MA) at 5×10^4 cells/well and allowed to attach for 24 hours. Cells were treated with: 1) gemcitabine 0.1 ng/ml (0.33 nM) - 100 µg/ml (333 µM) for 1 hour; 2) pemetrexed 0.1 ng/ml (0.21 nM) - 100 µg/ml (212 µM) for 24 hours; 3) gemcitabine for 1 hour followed by a 24-hour washout in drug-free medium and then pemetrexed for 24 hours; 4) pemetrexed for 24 hours followed by a 24-hour washout in drug-free medium and then gemcitabine for 1 hour. 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 dNTP pool depletion occur during the first 30 minutes and reach the maximum effect within 2 hours (Symon et al., 2002). Cytotoxicity was determined after 1 hour treatment with gemcitabine and 24 hours incubation with pemetrexed, using concentrations that are comparable to 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 additional 24 hours in drug-free medium and the growth inhibition by drugs was assessed by counting cells. The 50% inhibitory concentration of cell growth (IC_{50}) relative to untreated cultures was calculated by non-linear least squares curve fitting.

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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 by the Calcusyn software (Biosoft, Oxford, UK).

Modulation of Gemcitabine Metabolism and Cytotoxicity. Cells were plated in 24-well plates, as described in "Assay of Cytotoxicity", and treated with gemcitabine 0.1 ng/ml (0.33 nM) - 10 μg/ml (33 μM) for 24 hours alone or in combination with 10 μM 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} was 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 in "Assay of Cytotoxicity", treated with pemetrexed 0.1 ng/ml (0.21 nM) - 100 μg/ml (212 μM) for 24 hours and rescued by simultaneous supplementation of thymidine (5 μM) and hypoxanthine (100 μM), 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 - 100 μg/ml) and the GARFT selective inhibitor LY309887 (0.1 ng/ml - 100 μg/ml).

Cell Cycle Analysis. Cells were plated at 1×10^6 in 100-mm plastic dishes (Costar) and allowed to attach for 24 hours. After treatment with gemcitabine (1 hour), pemetrexed (24 hours) and their combinations at their IC_{50} levels, followed by a 24-hour 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 μg/ml), RNase (1 mg/ml) and Nonidet-P40 (0.1%) and samples were kept on ice for 30 min. Cytofluorimetry was performed using a FACScan (Becton Dickinson, San Jose, CA) and data analysis was carried out with CELLQuest software, while cell cycle distribution was determined using Modfit software (Verity Software, Topsham, ME).

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Analysis of Apoptosis. Cells were treated with gemcitabine, pemetrexed and their combinations at their IC_{50} levels, as described in "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 further 15 min in a solution containing 8 μg/ml bisbenzimide HCl. Cells were spotted on glass slides and examined by fluorescence microscopy (Leica, 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 in "Cell Cycle Analysis" were collected, washed twice with PBS and fixed with ice-cold 70% ethanol for 1 hour. 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 quantitated from the sub-G1 signal, measured with the Modfit software.

Assay of Akt Phosphorylation. Akt protein activation by phosphorylation after gemcitabine and pemetrexed treatment was assayed with a 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 μl of extraction buffer for 30 min on ice, while vortexing. A volume of 5 μL of cell extract was diluted to 100 μL with sodium azide (NaN_3 15 mM), centrifuged at 15000 *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 of phosphorylated full lenght human recombinant Akt (P-Ser473 Akt) and 20, 10, 5, 2.5, 1.25, 0.6 and 0.3 ng/mL of human recombinant total Akt. After overnight incubation at 4° C, the solution was aspirated from wells and 100 μL of rabbit anti-P-Ser473 Akt and biotin-conjugated anti-total Akt was added into each well of P-Ser473

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Akt and Akt total ELISA, respectively. Plates were incubated at room temperature for 1 hour, washed 4 times and 100 µL of a working solutions of horseradish HRP-labeled anti-rabbit IgG and HRP-labeled streptavidin were 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 µL 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. In order to establish a correlation between drug effect and modulation of gene expression, drug concentrations corresponding to the IC_{25}, IC_{50} and IC_{75} of gemcitabine, pemetrexed and their combinations were studied. Moreover, to evaluate the time course modulation of gene expression, QRT-PCR analysis was performed at 6, 12, 24 and 48 hours after completion of drug treatment. Total RNA was extracted from cells using the TRI REAGENT LS (Sigma). RNA was dissolved in 10 mM dithiothreitol and 200 U/ml of RNase inhibitor in RNase free-water, and measured at 260 nm. One µg of RNA was reverse transcribed at 37° C for 1 hour in 100-µl reaction volume containing 0.8 mM dNTPs, 200 U of MMLV-RT, 40 U of RNase inhibitor, and 0.05 µg/ml of 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 µl of cDNA, 12.5 µl of TaqMan Universal PCR Master Mix, 2.5 µl of probe and 2.5 µl of forward and reverse primers in a final volume of 25 µl. 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 sec followed by annealing and extension at 60° for 1 min.

Forward (F) and reverse (R) primers and probes (P) 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, while primers and probes for the regulatory (RRM1) and catalytic

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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 $2^{-\Delta\Delta C_T}$.

Preliminary experiments were carried out with dilutions of cDNA obtained from Quantitative PCR Human Reference Total RNA (Stratagene, La Jolla, CA, USA) 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 *vs.* 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 repeated at least three times. Data were expressed as mean values ± S.E. and were analysed by Student's *t*-test or ANOVA 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}s of 0.13±0.02 and 0.25±0.03 μg/ml (A549), 5.28±1.25 and 34.13±5.78 μg/ml (Calu-1) and 1.66±0.36 and 4.84±0.60 μg/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}s of gemcitabine to 3.5±1.0, 130.5±27.8 and 5.4±1.7 ng/ml in A549, Calu-1 and

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Calu-6 cells, while the IC_{50}s 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 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 μM for 24 hours, a 6-12 fold increase in IC_{50}s in all cell lines was observed. In contrast, there was a 2-6 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, while partially prevented the inhibition of cell growth by pemetrexed, as demonstrated by a 1.2 to 3.4 fold increase in the IC_{50} (Table 2). Similar results were obtained with hypoxanthine that, alone, reduced the cytotoxicity of LY309887, albeit 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 hours, while a modest increase was detected in Calu-1 cells (from 25.6 to 38.2%, P=NS). The same effect on cell cycle was observed after a 1-hour treatment with gemcitabine in A549 (from 6.0 to 18.0%, P<0.05), and Calu-1 cells (from 25.7 to 30.7%, P=NS). In contrast, in Calu-6 cells, flow cytometry studies demonstrated that pemetrexed and gemcitabine blocked cells in the G1-S boundary. In particular, pemetrexed caused a 1.5-fold increase in the population of cells in

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the G1-phase, from 50.5% to 74.2%. The G1 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 G2 phase 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 fragmentation, and rupture of cells into debris, after 24-hour washout. In all cell lines, 5-7% 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 as compared to gemcitabine in A549 and Calu-6 cells (12.2±0.6% and 8.61 ±1.6% *vs* 22.2±5.4% and 16.5±4.9%, respectively), while Calu-1 cells were the least sensitive and both sequences were equivalent (9.6±1.2% *vs* 10.8±1.1% and 9.8±1.6%) (Fig. 3).

These results were confirmed by the enhancement of the sub-G1 region on the DNA content histograms demonstrating that, after drug treatments, cell cycle modulation was accompanied by induction of apoptosis. The A549 cells treated with the sequential administration of pemetrexed→gemcitabine exhibited the largest sub-G1 signal (25.2%), while a minor increase in the proportion of hypodyploid cells with respect to controls was observed in Calu-1 cells after pemetrexed exposure (Table 3).

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

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The sensitivity of A549 cells to gemcitabine was correlated with low expression of RRM1/M2 and high expression of dCK and hENT1, while the lower chemosensitivity of Calu-1 cells appeared 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, while at IC_{25} there was only a minimal enhancement of hENT1 expression in A549 cells (Fig. 5). Similar results were observed at pemetrexed concentration corresponding to IC_{50} and IC_{75} 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), while dCK gene expression was not modulated in Calu-1 cells at IC_{50}, although there was a +23.8% enhancement at IC_{75}. Furthermore, each drug combination at IC_{50}, using the fixed combination ratios where gemcitabine and pemetrexed are equipotent, induced both hENT1 and dCK expression in all cell lines, while 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 hours after the end of pemetrexed treatment, demonstrating that their upregulation occurred mostly between the 12 and 48 hour time points.

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, while the sequence pemetrexed→gemcitabine caused an additive-synergistic

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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, owing to 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), while 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 pre-clinical sequence-dependent synergism prompted a three-arm randomized phase II study of pemetrexed plus gemcitabine as front-line 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; Tonkinson et al., 1999). Since gemcitabine is a 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 dFdCTP 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

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cells are not fully characterized, apoptosis does indeed play a role in cell death by both agents (Tolis et al., 1999; Bandala et al., 2001; Tonkinson et al., 1997). 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 when compared to controls and cells treated with single agents. A similar observation has been reported in WiDr colon cancer cells and in pancreatic cancer cells, where a few apoptotic cells were observed after gemcitabine treatment, whereas a significantly higher percentage was found after treatment with gemcitabine-pemetrexed combination (Tesei et al., 2002; Giovannetti et al., 2004). Moreover, a recent investigation showed that the reduction of phosphorylated PKB/Akt correlated with the enhancement of gemcitabine-induced apoptosis and antitumor activity, suggesting that the PI3K-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.

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 as a possible determinant of drug efficacy (Mackey et al., 1998). Because TS inhibitors upregulate 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 upregulation 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

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(Bergman et al., 2002, Blackstock et al., 2001). Moreover, pre-treatment 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, while transcriptome analysis suggested the predictive value of expression of dCK in particular and also of RR, 5'-NT and CDA. As previously reported 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, while in Calu-1 cells dCK gene expression was not apparently modulated, possibly because of higher levels of target enzymes of pemetrexed and the need of higher concentrations of the drug to upregulate dCK, as demonstrated with the exposure to IC_{75}. This result is in agreement with a previous study which indicated that inhibitors of DNA biosynthesis widely differ in their stimulatory effect on dCK, and there are also "responsive" and "non-responsive" cells with respect to dCK activation (Spasokoukotskaja et al., 1999). Moreover, PCR analysis of cells exposed to different concentration 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

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pemetrexed in the sequence pemetrexed followed by 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 cells response to gemcitabine and pemetrexed. In particular, A549 sensitivity could be explained by the favorable dCK/RR ratio, as described 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, while 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, of 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 modulation of cell cycle, 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 considered 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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References

Adjei AA, Erlichman C, Sloan JA, Reid JM, Pitot HC, Goldberg RM, Peethambaram P, Atherton P, Hanson LJ, Alberts SR, and Jett J (2000) Phase I and pharmacologic study of sequences of gemcitabine and the multitarget antifolate agent in patients with advanced solid tumors. *J Clin Oncol* **18:**1748-1757.

Adjei AA, Nair S, Reuter N, Mandrekar S, Kuross S, Rowland LM, Steen P, Hillman S, Schild S and Jett J (2004) Pemetrexed (Pem)/gemcitabine (Gem) as front-line therapy for advanced NSCLC: A randomized, phase II trial of three schedules. *J Clin Oncol* (*2004 ASCO Annual Meeting Proceedings)* **22**, 14S:abstract 7070

Arriagada R, Bergman B, Dunant A, Le Chevalier T, Pignon JP, and Vansteenkiste J (2004) The International Adjuvant Lung Cancer Trial Collaborative Group. Cisplatin-based adjuvant chemotherapy in patients with completely resected non-small cell lung cancer. *N Engl J Med* **350:**351-360.

Bandala E, Espinosa M, Maldonado V, and Melendez-Zajgla J (2001) Inhibitor of apoptosis-1 (IAP-1) expression and apoptosis in non-small cell lung cancer cells exposed to gemcitabine. *Biochem Pharmacol* **62:**13-19.

Bergman AM, Pinedo HM, and Peters GJ (2002) Determinants of resistance to 2',2'-difluorodeoxycytidine (gemcitabine). *Drug Resist Updat* **5:**19-33.

Blackstock AW, Lightfoot H, Case LD, Tepper JE, Mukherji SK, Mitchell BS, Swarts SG, and Hess SM (2001) Tumor uptake and elimination of 2',2'-difluorodeoxycytidine (gemcitabine) after deoxycytidine kinase gene transfer: correlation with *in vivo* tumor response. *Clin Cancer Res* **7:**3263-3268.

Britten CD, Izbicka E, Hilsenbeck S, Lawrence R, Davidson K, Cerna C, Gomez L, Rowinsky EK, Weitman S, and Von Hoff DD (1999) Activity of the multitargeted antifolate LY231514 in the human tumor cloning assay. *Cancer Chemother Pharmacol* **44:**105-110.

Chou TC, Motzer R, Tong Y, and Bosl G (1994) Computerized quantitation of synergism and antagonism of taxol, topotecan, and cisplatin against human teratocarcinoma cell growth: a rational approach to clinical protocol design. *J Natl Cancer Inst* **86:**1517-1524.

Eda H, Ura M, Ouchi KF, Tanaka Y, Miwa M, and Ishitsuka H (1998) The antiproliferative activity of DMDC is modulated by inhibition of cytidine deaminase. *Cancer Res* **58:**1165-1169.

Edelman MJ, Quam H, and Mullins B (2001) Interactions of gemcitabine, carboplatin and paclitaxel in molecularly defined non-small cell lung cancer cell lines. *Cancer Chemother Pharmacol* **48:**141-144.

Galmarini CM, Mackey JR, and Dumontet C (2001) Nucleoside analogues: mechanisms of drug resistance and reversal strategies. *Leukemia* **6**:875-890.

Giovannetti E, Mey V, Danesi R, Mosca I, and Del Tacca M (2004) Synergistic cytotoxicity and pharmacogenetics of gemcitabine and pemetrexed combination in pancreatic cancer cell lines *Clin Cancer Res* **10:**2936-2943.

Hanna N, Shepherd FA, Fossella FV, Pereira JR, De Marinis F, von Pawel J, Gatzemeier U, Tsao TC, Pless M, Muller T, Lim HL, Desch C, Szondy K, Gervais R, Shaharyar, Manegold C, Paul S, Paoletti P, Einhorn L, and Bunn PA Jr (2004) Randomized phase III trial of pemetrexed versus docetaxel in patients with non-small cell lung cancer previously treated with chemotherapy. *J Clin Oncol* **22:**1589-1597.

Hicks-Berger CA, Yang F, Smith TM, and Kirley TL (2001) The importance of histidine residues in human ecto-nucleoside triphosphate diphosphohydrolase-3 as determined by site-directed mutagenesis. *Biochim Biophys Acta* **1547:**72-81.

Kroep JR, Loves WJ, van der Wilt CL, Alvarez E, Talianidis L, Boven E, Braakhuis BJ, van Groeningen CJ, Pinedo HM, and Peters GJ (2002) Pretreatment deoxycytidine kinase levels predict *in vivo* gemcitabine sensitivity. *Mol Cancer Ther* **1:**371-376.

Li D, Xie K, Wolff R, and Abbruzzese JL (2004) Pancreatic cancer. *Lancet* **363**:1049-1057.

Mackey JR, Mani RS, Selner M, Mowles D, Young JD, Belt JA, Crawford CR, and Cass CE (1998) Functional nucleoside transporters are required for gemcitabine influx and manifestation of toxicity in cancer cell lines. *Cancer Res* **58:**4349-4357.

Manegold C, Gatzemeier U, von Pawel J, Pirker R, Malayeri R, Blatter J, and Krejcy K (2000) Front-line treatment of advanced non-small cell lung cancer with MTA (LY231514, pemetrexed disodium, ALIMTA) and cisplatin: a multicenter phase II trial. *Ann Oncol* **11:**435-440.

Ng SSW, Tsao MS, Nicklee T, and Hedley DW (2001) Wortmannin inhibits PKB/Akt phosphorylation and promotes Gemcitabine antitumor activity in orthotopic human pancreatic cancer xenografts in immunodeficient mice. *Clin Cancer Res* **7:**3269-3275.

Noble S and Goa KL (1997) Gemcitabine. A review of its pharmacology and clinical potential in non-small cell lung cancer and pancreatic cancer. *Drugs* **54**:447-472.

Peters GJ, van der Wilt CL, van Moorsel CJ, Kroep JR, Bergman AM, and Ackland SP (2000) Basis for effective combination cancer chemotherapy with antimetabolites. *Pharmacol Ther* **87:**227-253.

Pressacco J, Wiley JS, Jamieson GP, Erlichman C, and Hedley DW (1995) Modulation of the equilibrative nucleoside transporter by inhibitors of DNA synthesis. *Br J Cancer* **72:**939-942.

Rauchwerger DR, Firby PS, Hedley DW, and Moore MJ (2000) Equilibrative-sensitive nucleoside transporter and its role in gemcitabine sensitivity. *Cancer Res* **60:**6075-6079.

Reck M and Gatzemeier U (2004) Chemotherapy in stage-IV NSCLC. *Lung Cancer* **45** (Suppl 2):S217-222.

Rosell R and Crinò L (2002) Pemetrexed combination therapy in the treatment of non-small cell lung cancer. *Semin Oncol* **29** (2 Suppl 5):23-29.

Rusthoven JJ, Eisenhauer E, Butts C, Gregg R, Dancey J, Fisher B, and Iglesias J (1999) Multitargeted antifolate LY231514 as first-line chemotherapy for patients with advanced non-small cell lung cancer: A phase II study. *J Clin Oncol* **17:**1194-1199.

Shepherd FA, Dancey J, Arnold A, Neville A, Rusthoven J, Johnson RD, Fisher B, and Eisenhauer E (2001) Phase II study of pemetrexed disodium, a multitargeted antifolate, and cisplatin as first-line therapy in patients with advanced non-small cell lung carcinoma: a study of the National Cancer Institute of Canada Clinical Trials Group. *Cancer* **92:**595-600.

Shih C, Chen VJ, Gossett LS, Gates SB, MacKellar WC, Habeck LL, Shackelford KA, Mendelsohn LG, Soose DJ, Patel VF, Andis SL, Bewley JR, Rayl EA, Moroson BA, Beardsley GP, Kohler W, Ratnam M, and Schultz RM (1997) LY231514, a pyrrolo [2,3-d]pyrimidine-based antifolate that inhibits multiple folate-requiring enzymes. *Cancer Res* **57:**1116-1123.

Sigmond J, Backus HH, Wouters D, Temmink OH, Jansen G, and Peters GJ (2003) Induction of resistance to the multitargeted antifolate Pemetrexed (ALIMTA) in WiDr human colon cancer cells is associated with thymidylate synthase overexpression. *Biochem Pharmacol* **66:**431-438.

Spasokoukotskaja T, Sasvari-Szekely M, Keszler G, Albertioni F, Eriksson S, and Staub M (1999) Treatment of normal and malignant cells with nucleoside analogues and etoposide enhances deoxycytidine kinase activity. *Eur J Cancer* **35:**1862-1867.

Symon Z, Davis M, McGinn CJ, Zalupski MM, and Lawrence TS (2002) Concurrent chemoradiotherapy with gemcitabine and cisplatin for pancreatic cancer: from the laboratory to the clinic. *Int J Radiat Oncol Biol Phys* **53**:140-145.

Teicher BA, Alvarez E, Liu P, Lu K, Menon K, Dempsey J, and Schultz RM (1999) MTA (LY231514) in combination treatment regimens using human tumor xenografts and the EMT-6 murine mammary carcinoma. *Semin Oncol* **26** (2 Suppl 6):55-62.

Teicher BA, Chen V, Shih C, Menon K, Forler PA, Phares VG, and Amsrud T (2000) Treatment regimens including the multitargeted antifolate LY231514 in human tumor xenografts. *Clin Cancer Res* **6:**1016-1023.

Tesei A, Ricotti L, De Paola F, Amadori D, Frassinetti GL, and Zoli W (2002) *In vitro* schedule-dependent interactions between the multitarget antifolate LY231514 and gemcitabine in human colon adenocarcinoma cell lines. *Clin Cancer Res* **8:**233-239.

Theodossiou C, Cook JA, Fisher J, Teague D, Liebmann JE, Russo A, and Mitchell JB (1998) Interaction of gemcitabine with paclitaxel and cisplatin in human tumor cell lines. *Int J Oncol* **12:**825-8327.

Thödtmann R, Depenbrock H, Dumez H, Blatter J, Johnson RD, van Oosterom A, Hanauske AR (1999) Clinical and pharmacokinetics phase I study of multitargeted antifolate (LY231514) in combination with cisplatin. *J Clin Oncol* **17**:3009-3016

Tolis C, Peters GJ, Ferreira CG, Pinedo HM, and Giaccone G (1999) Cell cycle disturbances and apoptosis induced by topotecan and gemcitabine on human lung cancer cell lines. *Eur J Cancer* **35:**796-807.

Tonkinson JL, Marder P, Andis SL, Schultz RM, Gossett LS, Shih C, and Mendelsohn LG (1997) Cell cycle effects of antifolate antimetabolites implications for cytotoxicity and cytostasis. *Cancer Chemother Pharmacol* **39:**521-531.

Tonkinson JL, Worzalla JF, Teng CH, and Mendelsohn LG (1999) Cell cycle modulation by a multitargeted antifolate, LY231514, increases the cytotoxicity and antitumor activity of gemcitabine in HT29 colon carcinoma. *Cancer Res* **59:**3671-3676.

van Moorsel CJ, Pinedo HM, Veerman G, Bergman AM, Kuiper CM, Vermorken JB, van der Vijgh WJ, and Peters GJ (1999) Mechanism of synergism between cisplatin and gemcitabine in ovarian and non-small cell lung cancer cell lines. *Br J Cancer* **80:**981-990.

Footnotes

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Legends to Figures

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

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

Fig. 3. Induction of apoptosis by gemcitabine, pemetrexed and their combinations, after 24-hour washout. Cancer cells were exposed to 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*, SE. * $P<0.05$ with respect to gemcitabine treatment.

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.

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

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

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Tables

Table 1. Effects of deoxycytidine (dCyd), diethylpyrocarbonate (DEPC) and tetrahydrouridine (THU) on gemcitabine cytotoxicity

	IC_{50} (ng/ml)[a]			
	Gemcitabine	+dCyd	+DEPC	+THU
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

[a]Mean values ± SE of at least three independent experiments

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Table 2. Effects of thymidine and hypoxanthine on 5-fluorouracil, LY309887 and pemetrexed IC_{50}s (μg/ml)[a]

	A549	Calu-1	Calu-6
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

[a]Mean values ± SE of at least three independent experiments

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Table 3. Effects of gemcitabine and pemetrexed on cell cycle and induction of apoptosis of NSCLC cell lines

Cells	Treatment[a]	G1 (%)[b]	S (%)	G2 (%)	Sub-G1 (%)
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

[a]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

[b]Mean percent values of total number of cells examined in three independent experiments

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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

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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 $2^{-\Delta\Delta C_T}$

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

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Table 6. Effects of pemetrexed, at IC_{50} level, on dCK and hENT1 gene expression of NSCLC cell lines 6,12, 24 and 48 hours after the end of treatment. The amount of target gene, normalized to GAPDH and relative to the calibrator (untreated control cells), is given as $2^{-\Delta\Delta C_T}$

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

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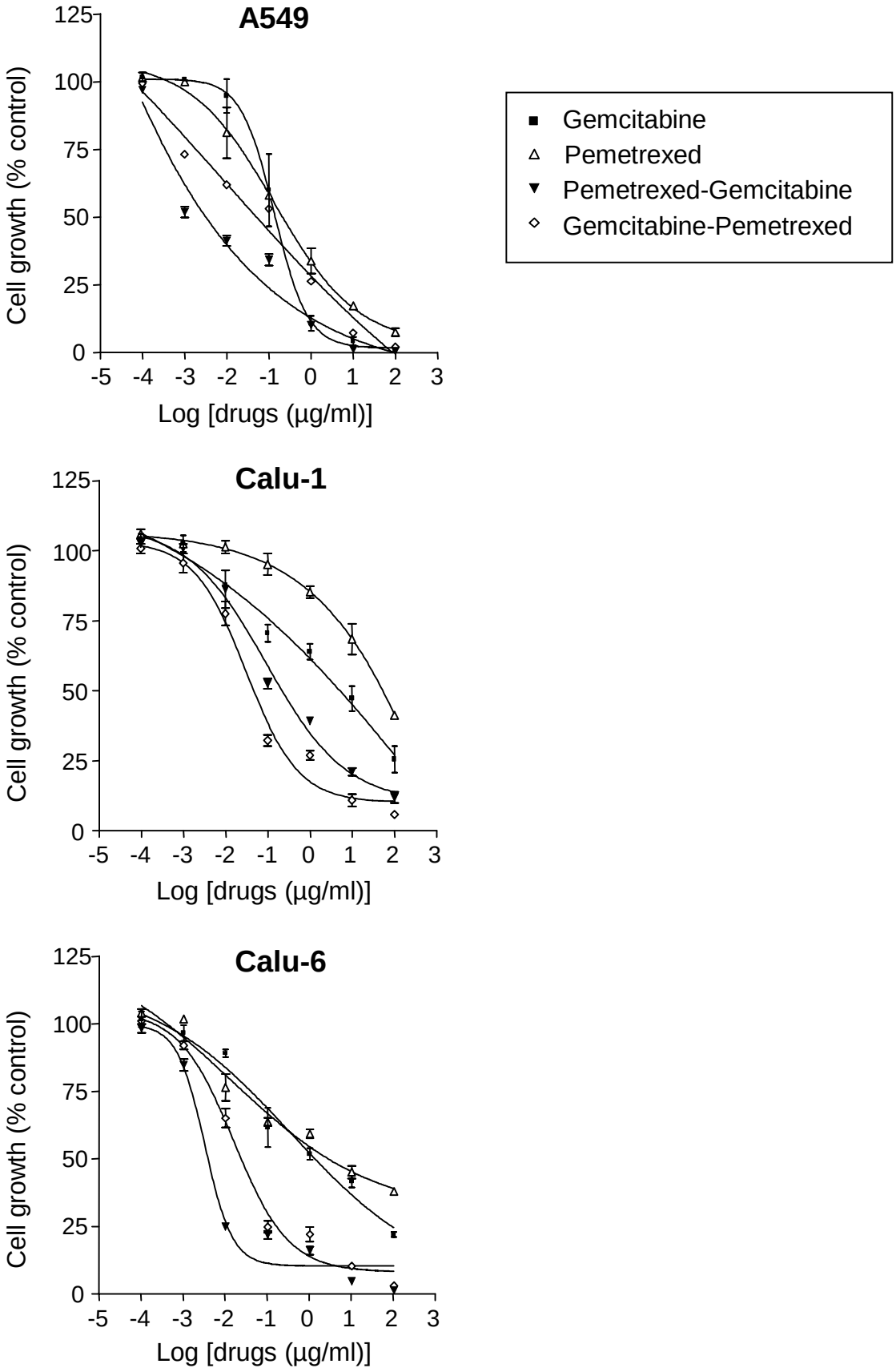

Fig. 1, ↑

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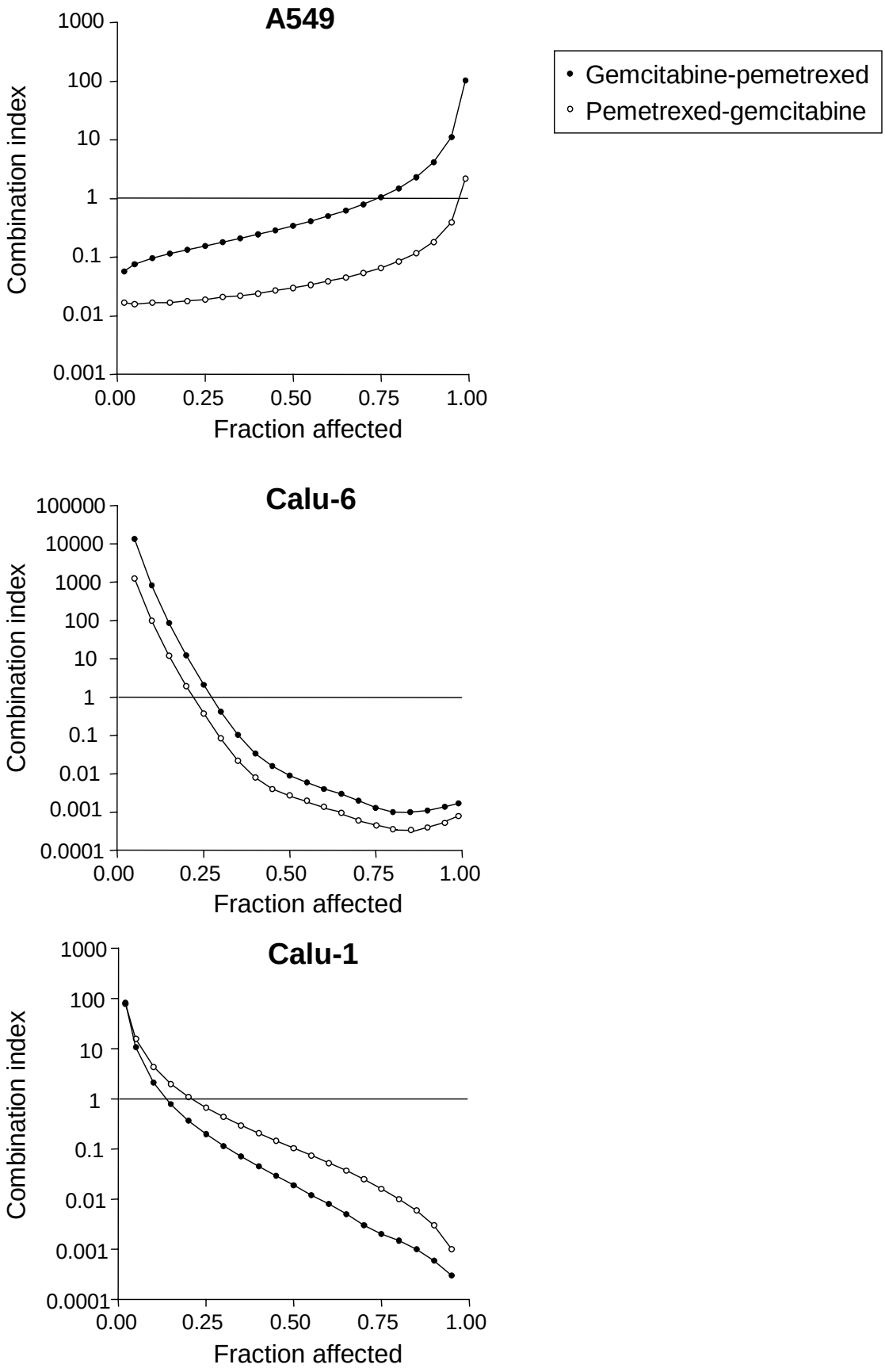

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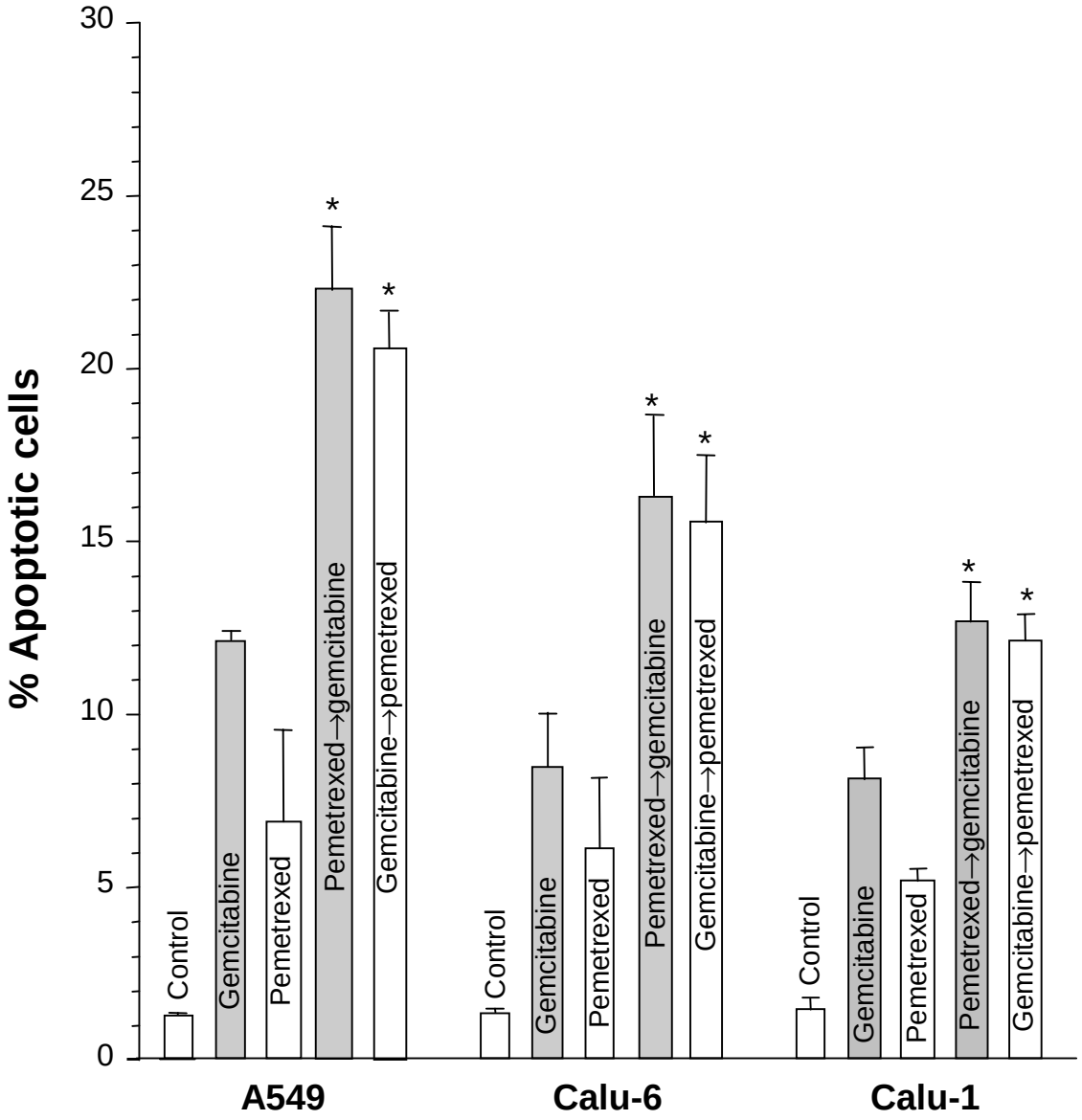

Fig. 3, ↑

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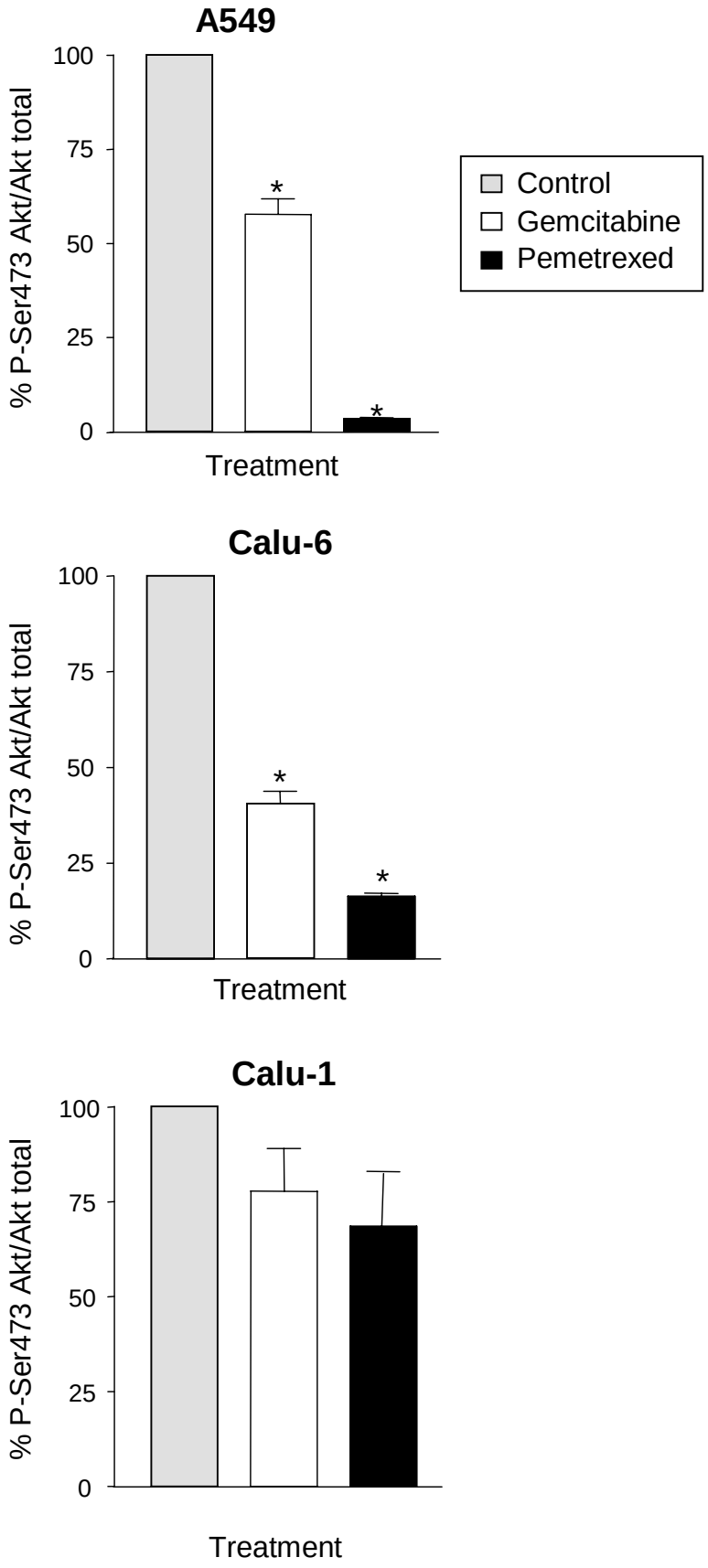

Fig. 4, ↑

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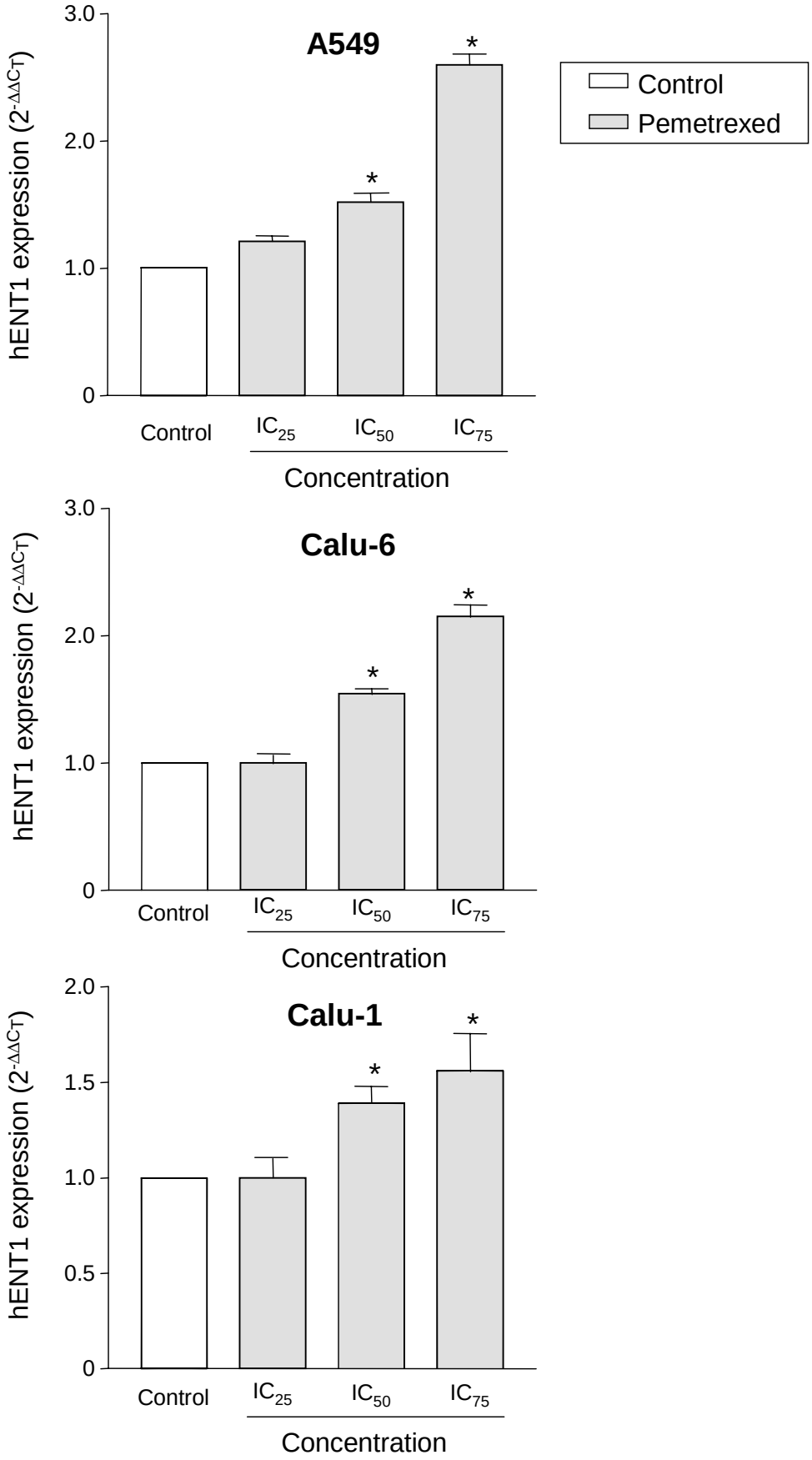

Fig. 5, ↑

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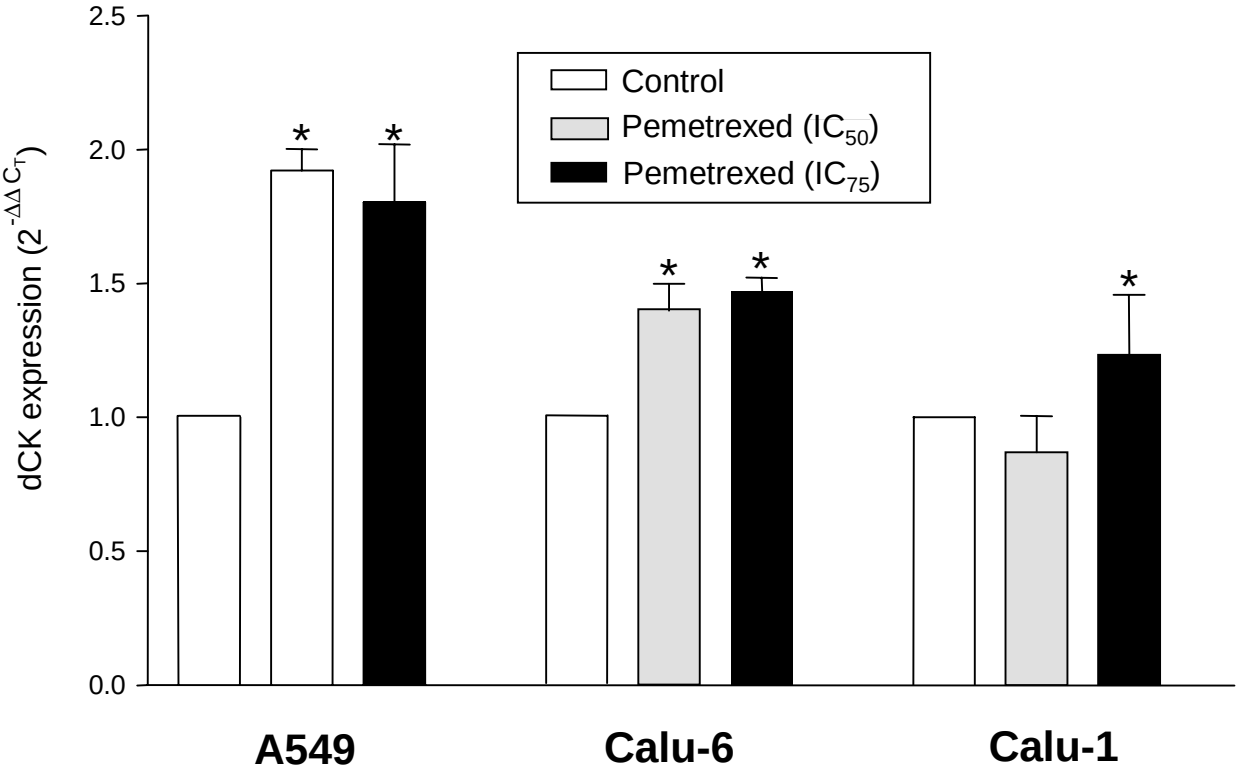

Fig. 6, ↑