Sequence-dependent, synergistic antiproliferative and proapoptotic effects of the combination of cytotoxic drugs and enzastaurin, a protein kinase Cβ inhibitor, in non-small cell lung cancer cells

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Abstract

Enzastaurin, an acyclic bisindolymaleimide, is a potent and selective competitive inhibitor of protein kinase Cβ, which has been shown to inhibit cancer cell proliferation and angiogenesis in human cancer cell lines. Gemcitabine and pemetrexed are two cytotoxic drugs that are currently used in non-small cell lung cancer (NSCLC) therapy. In this study, we have investigated whether the addition of enzastaurin to gemcitabine or to pemetrexed is able to increase their antitumor activity to establish an effective schedule of combined treatment. The effects on cancer cell proliferation, cell cycle distribution, intracellular mitogenic and antiapoptotic signaling pathways, and induction of apoptosis were evaluated in three different combination sequences (concomitant treatment, sequential treatment with the cytotoxic drug followed by enzastaurin, or sequential treatment with enzastaurin followed by the cytotoxic drug) in a panel of human NSCLC cell lines. The combination of enzastaurin with either gemcitabine or pemetrexed caused different antiproliferative and proapoptotic effects depending on the treatment schedule. A synergistic antiproliferative and proapoptotic activity was only obtained when chemotherapy was followed by treatment with enzastaurin. These effects were accompanied by the arrest of the surviving cancer cells in the S phase, thus limiting their ability to proceed through the cell cycle, and by a maximum inhibition in the activated, phosphorylated forms of Akt and mitogen-activated protein kinase. In contrast, the concomitant treatments or the sequential treatments, in which enzastaurin was given before chemotherapy, resulted in significant antagonistic effects. [Mol Cancer Ther 2008;7(6):1698–707]

Introduction

Despite recent advances in early diagnosis and treatment, non-small cell lung cancer (NSCLC) is a disease with a poor prognosis. Extensive clinical studies showed that chemotherapy increases survival in the adjuvant setting and in patients with advanced, metastatic disease (1). Nonetheless, in stage IV metastatic disease, objective clinical responses are observed in ~25% to 30% of patients, with a median survival of 8 to 10 months, thus emphasizing the need for new effective drugs and combination regimens (2). However, the rationale for chemotherapy combinations has remained mostly empirical, determined by the antitumor activity of each agent and the lack of overlapping toxicities, despite many attempts to discover models for rational selection of mechanistic, pharmacokinetic, or pharmacodynamic drug interactions.

Gemcitabine (2,2-difluorodeoxycytidine) is an antimetabolite, a deoxycytidine analogue, with a broad spectrum of anticancer activity against several solid tumors in preclinical models, and it is an established, effective agent in the treatment of several malignancies, particularly of NSCLC and pancreatic cancer (3). Gemcitabine is a prodrug that is transported into the cell, where it requires intracellular phosphorylation to its active metabolite, 2,2-difluoro-dCTP, for being incorporated into DNA and thus leading to DNA chain termination and cell death (4). The rate-limiting step in the activation of the drug is catalyzed by deoxycytidine kinase, whereas 5-nucleotidase and cytidine deaminase are the main inactivating enzymes (5).

Antimetabolites are widely used in chemotherapy combination regimens because of their ability to biochemically modulate the cytotoxicity of other drugs (6). In this respect, pemetrexed is an anti-folate inhibitor of thymidylate synthase, dihydrofolate reductase, and glycinamide ribonucleotide formyltransferase, with activity against a wide spectrum of human cancer cell lines, including NSCLC (7). The results of a large prospective randomized study comparing pemetrexed with docetaxel in the second-line treatment of 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

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with toxicity profiles favoring pemetrexed (8). The ability of pemetrexed to deplete cellular nucleotide pools, to modulate cell cycle, and to induce apoptosis render this drug an interesting cytotoxic agent for polychemotherapy regimens (9, 10).

The development of drug resistance is a major problem with chemotherapy agents. The activation of specific intracellular signaling pathways in cancer cells could be responsible for the gain of escape mechanisms by which the cancer cell could overcome cytotoxic damage and survive. In this respect, the phosphatidylinositol 3-kinase (PI3K)/Akt pathway stimulates cell cycle progression, suppresses apoptosis, and is frequently activated by different mechanisms in a variety of human cancers (11, 12). An area of clinical research of increasing interest is the development of rationale combinations of cytotoxic drugs with molecularly targeted therapies to increase the therapeutic potential by blocking cancer cell survival mechanisms (13).

However, this approach has failed to improve survival in unselected patients with advanced NSCLC in several large, randomized, controlled studies of combination of chemotherapy with anti–epidermal growth factor receptor (EGFR) drugs (14–17). A reason may be represented by the induction of G_1 arrest in tumor cells by the continuous administration of the anti-EGFR drug, which could block the antiproliferative effects of subsequent administration of cytotoxic agents. In support to this hypothesis, a negative interaction between erlotinib and gefitinib, the most clinically developed EGFR tyrosine kinase inhibitors, or vandetanib (ZD6474), a dual EGFR-vascular endothelial growth factor receptor tyrosine kinase inhibitor, and chemotherapeutics, such as paclitaxel and docetaxel (18) in cancer cells, has been reported when exposure to the biological agent precedes chemotherapy treatment. On the contrary, treatment with the biological agent after cytotoxic drug exposure showed a synergistic interaction by potentiating a G_2-M-phase cell cycle arrest and by further limiting the ability of the cells to repair damaged DNA and therefore to proceed through the cell cycle. Therefore, the definition of the optimal schedule of administration of chemotherapy with molecularly targeted therapeutic agents largely remains an open clinical issue.

The protein kinase C (PKC) family of isoenzymes is involved in key cellular processes including cell proliferation, apoptosis, and differentiation (19) by the promotion of survival through the PI3K/Akt pathway. Different isoforms of PKC may exert both positive and negative effects on cell proliferation and survival, and altered levels of individual isoforms are implicated in cancer cell progression (20). The actual mechanism by which PKC contributes to tumorigenesis is unclear. Moreover, PKC has also been implicated in chemoresistance (21). The role of PKC in tumorigenesis suggests that combining PKC inhibitors with conventional cytotoxics may be an effective anticancer treatment. Evidence from preclinical studies with human cancer cell lines and from early clinical trials suggests that this could be a suitable and promising clinical approach (22–25). In addition, it has been shown that selective inhibition of PKCβ isoform sensitizes transformed murine fibroblasts to radiation-induced apoptosis (26) and suppresses tumor growth and tumor-induced angiogenesis (27). In this respect, activated PKCβ is expressed in a broad range of human cancers, including NSCLC, mesothelioma, brain, prostate, pancreas, colorectal, hepatocellular, and ovarian cancer (28).

Enzastaurin (LY317615), an acyclic bisindolymaleimide, is a potent and selective competitive inhibitor of PKCβ (29) at low concentrations (IC_{50}, 6 nmol/L), but it also inhibits other PKC isoenzymes at higher concentrations that are, however, within the plasma concentrations achievable in humans (1-4 μmol/L). Treatment with enzastaurin inhibits the growth of several NSCLC cancer cell lines (30, 31). In this respect, enzastaurin treatment induces apoptosis and suppresses cancer cell proliferation through inhibition of critical target proteins involved in malignant transformation, such as the PI3K/Akt pathway, the glycogen synthase kinase-3β, and the ribosomal protein S6 (32).

Enzastaurin has also been evaluated in combination with anticancer agents in cancer cell lines. The combination of pemetrexed with enzastaurin was found to be synergistic in thyroid cancer cells (33). An additive and even synergistic effect was observed in NSCLC and small-cell lung cancer cell lines (34). Finally, enzastaurin was found to exert antitumor activity in taxane-resistant ovarian cancer cells, suggesting a potential activity in drug resistance (35).

Based on these reasons, the present study was undertaken to evaluate the antiproliferative and proapoptotic activity of the combination of enzastaurin with gemcitabine or with pemetrexed in a panel of NSCLC cell lines. To establish a correct schedule of treatment, a series of experiments were done in which the cytotoxic drug and the molecularly targeted agent were combined in different sequences. In fact, a better understanding of the interactions of chemotherapy and agents that target selective cancer cell survival pathways could lead to the development of rational combination cancer therapies.

Materials and Methods

Drugs and Chemicals

Enzastaurin, gemcitabine, and pemetrexed were generous gifts of Eli Lilly. Gemcitabine and pemetrexed were dissolved in sterile distilled water and diluted in culture medium immediately before use. Enzastaurin was dissolved in DMSO. RPMI, fetal bovine serum, horse serum, L-glutamine (2 mmol/L), penicillin (50 IU/mL), and streptomycin (50 g/mL) were from Life Technologies. All other chemicals were from Sigma.

Cell Lines and Cell Cultures

Calu-3, H460, A549, and H1299 NSCLC cell lines (American Type Culture Collection) were grown in RPMI with 10% fetal bovine serum, glutamine, and penicillin-streptomycin. None of these cell lines harbor mutation in the EGFR gene; K-Ras gene is mutated in H1299, A549, and H460 cell lines. Only H1299 cell line harbors a p53 gene mutation. Cells were cultivated in 75-cm^2 flasks (Costar) at

37°C in 5% CO_2 and 95% air and were harvested with EDTA when they were in logarithmic growth.

Evaluation of Cytotoxicity

Cells (3×10^3 per well) of the indicated NSCLC cell lines were plated in 96-multiwell cluster dishes (Becton Dickinson). Cancer cells were exposed to different concentrations of single-agent enzastaurin (0.00005-5 μmol/L enzastaurin in a final DMSO concentration of 0.1% for 48 h) or gemcitabine or pemetrexed (0.0001-10 μg/mL of each cytotoxic drug for 24 h). In the combination treatment experiments, cancer cells were treated according to each of the following three sequences: (*a*) gemcitabine or pemetrexed followed by enzastaurin: cells were exposed to either cytotoxic drug for 24 h and then the medium containing drug was removed and cells were rinsed with PBS and treated with enzastaurin for additional 48 h; (*b*) enzastaurin followed by gemcitabine or pemetrexed: cells were treated with enzastaurin for 48 h and then the medium containing drug was removed and cells were rinsed with PBS and treated with either cytotoxic agent for additional 24 h; and (*c*) concurrent treatment: cells were exposed to both enzastaurin (for 48 h) and either cytotoxic drug (for the first 24 h). At the end of the treatment period, cell proliferation was measured by using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. The drug concentrations required to inhibit cell growth by 50% (IC_{50}) were determined by interpolation from the dose-response curves. Eight replicate wells were used for each analysis, and at least three independent experiments were done. The results of the combined treatment were analyzed according to the method of Chou and Talalay by using the Calcusyn software program (Biosoft; ref. 36). The resulting combination index (CI) is a quantitative measure of the degree of interaction between different drugs. If CI = 1, it denotes additivity; if CI > 1, it denotes antagonism; and if CI < 1, it denotes synergism.

Evaluation of Cell Cycle Distribution and Apoptosis

To evaluate the effects on cell cycle and the induction of apoptosis, NSCLC cell lines were plated in 100-mm tissue culture dishes (Becton Dickinson) and treated with the indicated concentrations of either enzastaurin, gemcitabine, pemetrexed alone, or the three sequence combinations as above described. After additional 24 h, both adherent and detached cells were harvested. Flow cytometric analysis of apoptotic cell death was done on cell pellets that were fixed in 70% ethanol, washed in PBS, and mixed with RNase (Sigma) and propidium iodide (Sigma) solution as reported previously (37). DNA content was analyzed by a FACScan flow cytometer (Becton Dickinson) coupled with a Hewlett-Packard computer, and the percent of apoptotic cells was calculated by gating the hypodiploid region on the DNA content histogram using the LYSYS software (Becton Dickinson) as reported previously (37). Cell cycle data analysis was done using the CellFit software (Becton Dickinson) as reported previously (37).

Evaluation of Protein Expression by Western Blotting

NSCLC cells were treated with the indicated concentrations of enzastaurin, gemcitabine, pemetrexed alone, or the three different sequence combinations. The cells were harvested and then incubated with radioimmunoprecipitation assay buffer [50 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, 1 mmol/L phenylmethylsulfonyl fluoride, 1 mmol/L EDTA, 5 μg/mL aprotinin, 5 μg/mL leupeptin, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS] and homogenized by sonication (4×15 s) in an ice bath, incubated on ice for 30 min, and centrifuged ($15{,}000 \times g$, 30 min). The supernatants corresponding to the protein extracts were then collected for protein analysis. The following antibodies and working dilutions were used for Western blotting: rabbit polyclonal antibodies against human pAkt (Ser^{473}; 1:1,000) and Akt (1:1,000); mouse monoclonal antibodies against human p44/42 mitogen-activated protein kinase (pp44/42 mitogen-activated protein kinase; Thr^{202}/Tyr^{204}; 1:500; Cell Signaling Technology) and Bcl-2 (1:1,000; Santa Cruz Biotechnology); goat polyclonal antibodies against p44/42 mitogen-activated protein kinase (1:1,000; Cell Signaling Technology); goat polyclonal antibodies against p21 (1:1,000; Santa Cruz Biotechnology), p27 (1:1,000; Santa Cruz Biotechnology), and Bcl-xL (1:1,000; Santa Cruz Biotechnology); goat polyclonal antibody against β-actin (1:4,000; Santa Cruz Biotechnology); rabbit anti-mouse IgG-horseradish peroxidase conjugate (1:2,000; DakoCytomation); donkey anti-rabbit IgG-horseradish peroxidase conjugate (1:2,000; Santa Cruz Biotechnology); and rabbit anti-goat IgG-horseradish peroxidase conjugate (1:2,000; Santa Cruz Biotechnology). The protein-antibody complexes were detected by using an enhanced chemiluminescence kit (Amersham) according to the manufacturer's recommended protocol.

Results

Sequence Dependence of Chemotherapy Plus Enzastaurin Effects on Proliferation of NSCLC Cells

To evaluate the antiproliferative effects of chemotherapy and enzastaurin treatment alone or in combination using the three different schedule sequences, we did a series of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide cell growth assays. Treatment with enzastaurin alone for 48 h resulted in a dose-dependent inhibition of cancer cell growth with an IC_{50} value between 1.5 and 2.5 μmol/L for H1299, A549, and H460 cells and a less pronounced antiproliferative effect in Calu-3 cells (IC_{50}, 7 μmol/L; Table 1A).

In a previous phase I clinical trial of enzastaurin in patients with advanced solid tumors, at a daily dose of 525 mg, the mean plasma exposure was ~2 μmol/L (38). Therefore, 2 μmol/L was considered as clinically achievable and a relevant enzastaurin plasma level in cancer patients and this concentration was chosen as the dose for the subsequent experiments.

A dose-dependent inhibition of growth by each cytotoxic drug was observed with an IC_{50} of 0.5 to 5 μg/mL for pemetrexed and 1 to 2 μg/mL for gemcitabine, respectively (Table 1A). According to these results, 1 μg/mL was chosen as the dose of gemcitabine and pemetrexed for the subsequent experiments. These concentrations are much

Table 1. Effects of gemcitabine, pemetrexed, or enzastaurin and their combinations on human NSCLC cancer cell line growth

(A)

Cancer cell line	Gemcitabine IC_{50} (μg/mL)	Pemetrexed IC_{50} (μg/mL)	Enzastaurin IC_{50} (μmol/L)
A549	1	2	2
Calu-3	1.5	5	7
H1299	2	0.5	2.5
H460	1	2	1.5

(B)

	CI at fa_{50}	CI at fa_{75}	CI at fa_{90}
Gemcitabine followed by enzastaurin			
A549	0.003	0.007	0.03
Calu-3	0.13	0.25	0.48
H1299	0.006	0.04	0.32
H460	0.011	0.005	0.006
Gemcitabine in combination with enzastaurin			
A549	1.4	1.5	1.3
Calu-3	1.7	2	1.4
H1299	1.5	1.9	3
H460	2.1	1.4	1.7
Enzastaurin followed by gemcitabine			
A549	3.7	2.8	3.9
Calu-3	3	2.6	2.8
H1299	2.7	2.4	2.6
H460	3.1	3.6	2.7
Pemetrexed followed by enzastaurin			
A549	0.001	0.005	0.11
Calu-3	0.039	0.038	0.037
H1299	0.001	0.005	0.065
H460	0.001	0.007	0.184
Pemetrexed in combination with enzastaurin			
A549	1.6	1.8	2
Calu-3	1.4	2.1	16
H1299	1.9	1.7	2.2
H460	2	2	2.4
Enzastaurin followed by pemetrexed			
A549	3.3	3.6	3.2
Calu-3	2.7	2.9	2.2
H1299	2.9	3	3.3
H460	3.7	3.2	2.6

NOTE: The evaluation of the cell growth-inhibitory effects of gemcitabine, pemetrexed, or enzastaurin, given as single agents, was done by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. IC_{50} values for each drug were calculated by performing dose-response experiments. Cells were treated for 24 h with gemcitabine (0.0001-10 μg/mL) or with pemetrexed (0.0001-10 μg/mL) or for 48 h with enzastaurin (0.00005-10 μmol/L). Evaluation of the growth-inhibitory effects by the combined treatment with gemcitabine or pemetrexed and enzastaurin using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Treatment combinations and sequences are described in Materials and Methods. CI values were calculated according to the Chou and Talalay mathematical model for drug interactions using the Calcusyn software for different fractions affected (fa). CI is a quantitative measure of the degree of interaction between different drugs. If CI = 1, it denotes additivity; if CI > 1, it denotes antagonism; if 1 < CI > 0.7, it denotes slight synergism; if CI = 0.7-0.3, it denotes synergism; if CI < 0.3, it denotes strong synergism. Results are the median of three independent experiments, each done in eight replicate wells for experimental point.

lower than the mean peak plasma concentration of gemcitabine and pemetrexed achievable in patients, indicating a high *in vitro* sensitivity of these four NSCLC cells to these drugs (38).

We next assessed the combination of enzastaurin and each cytotoxic drug in the three different treatment sequences. As shown in Fig. 1A to D, a 24-h exposure to gemcitabine or pemetrexed followed by a 48-h exposure to enzastaurin resulted in a significant synergistic antiproliferative effect in all four NSCLC cell lines, with a CI ranging between 0.001 and 0.1 (Table 1B and C). This was significantly different for the reverse sequence (enzastaurin followed by chemotherapy), which was clearly antagonistic in all cancer cell lines, with CI ranging between 2.7 and 3.7

(Table 1B and C). Likewise, the concurrent treatment schedule was also less than additive or frankly antagonistic with CI ranging between 1.4 and 1.7 (Table 1B and C). These data show a clear synergistic inhibitory effect on cell growth only for the sequence in which chemotherapy is followed by enzastaurin treatment. Similar results in all four NSCLC cell lines were obtained when these experiments were done in monolayer cultures at clonogenic cell density (data not shown).

Sequence-Dependent Changes in Cell Cycle Distribution by the Combination of Cytotoxic Treatments and Enzastaurin

To explore the mechanism(s) of the sequence-dependent synergistic antiproliferative activity, we investigated the effects of gemcitabine, pemetrexed, and enzastaurin in the three different exposure sequences on cell cycle distribution. As shown in Fig. 2 in Calu-3 cells, treatment with gemcitabine for 24 h resulted in S-phase arrest (63% of cells) compared with control untreated cells (8% of cells). Treatment with enzastaurin induced accumulation in the G_1 phase of the cell cycle (80% of cells) when compared with controls (68% of cells). Interestingly, the gemcitabine-induced block in the S phase was maintained only by the gemcitabine followed by enzastaurin sequence (60% of cells), whereas it was completely antagonized in the enzastaurin followed by gemcitabine sequence (18% of cells) or in the concurrent exposure (14% of cells; Fig. 2). Similarly, pemetrexed single-agent treatment induced a block in G_2 and S phases of the cell cycle. In contrast, the sequence pemetrexed followed by enzastaurin completely blocked cells in S phase (96% of cells), and this effect was antagonized in the reverse enzastaurin followed by pemetrexed sequence (14% of cells), whereas the concurrent treatment mostly accumulated cells in the G_2-M phases (Fig. 2). Similar results with the three different schedules of treatment were observed with H1299, A549, and H460 cells (data not shown). Taken together, these results indicate that the sequential treatment with enzastaurin after gemcitabine

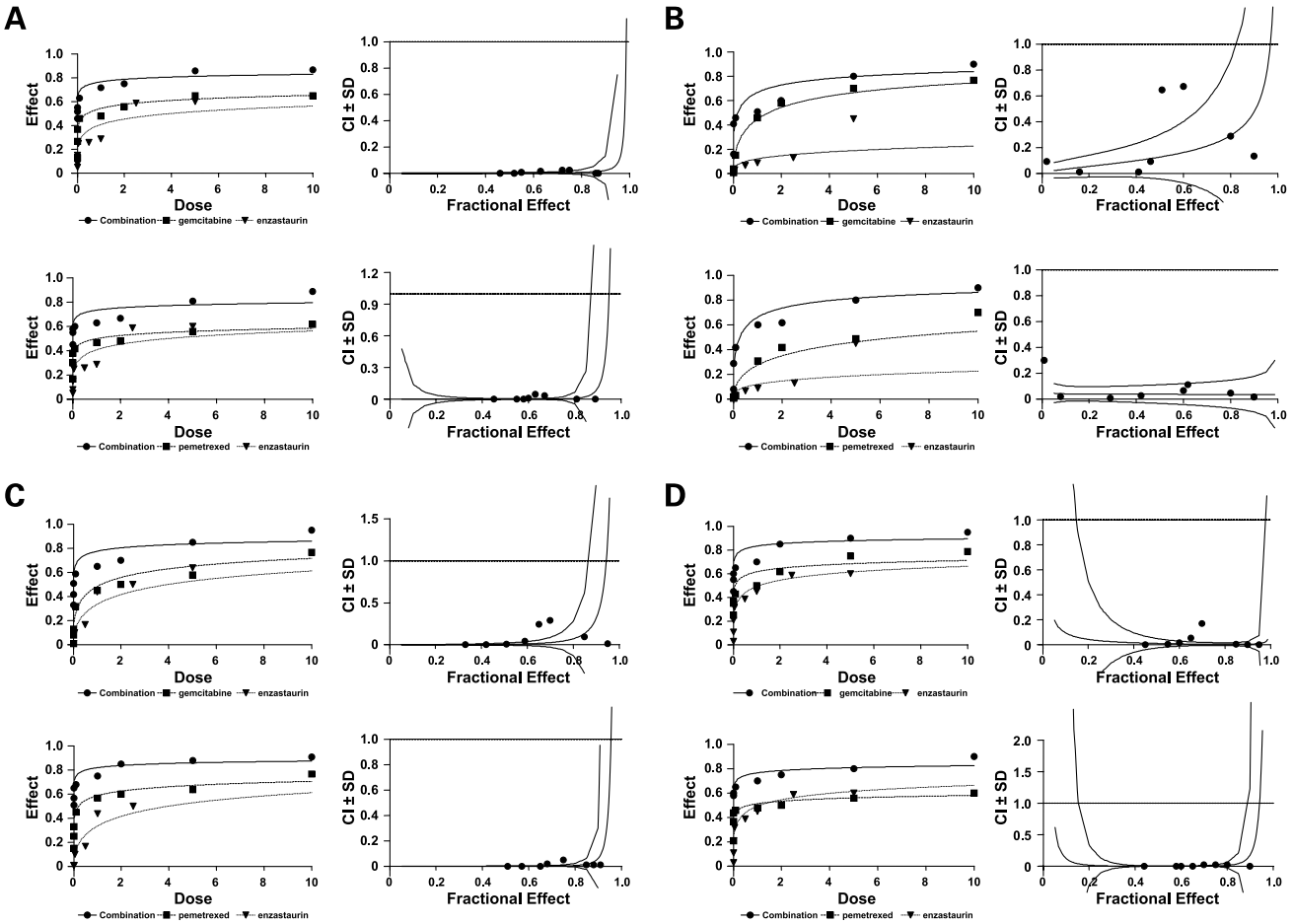

Figure 1. Effects on A549 (**A**), Calu-3 (**B**), H1299 (**C**), and H460 (**D**) cell growth of the combination of enzastaurin and cytotoxic dugs. Cell viability was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Treatment combinations and sequences are described in Materials and Methods. Results are the median of three independent experiments, each done in eight replicate wells for experimental point. CI values were calculated according to the Chou and Talalay mathematical model for drug interactions using the Calcusyn software. CI is a quantitative measure of the degree of interaction between different drugs. If CI = 1, it denotes additivity; if CI > 1, it denotes antagonism; if CI < 1, it denotes synergism.

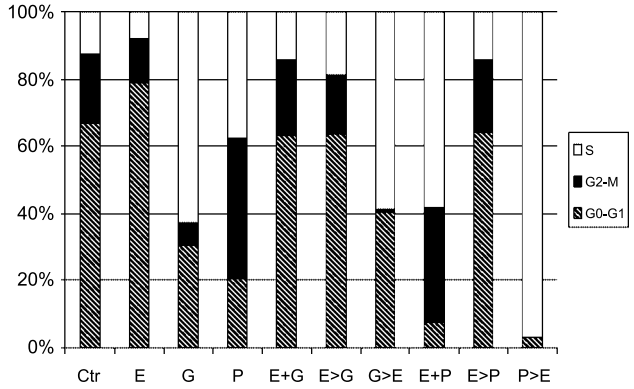

Figure 2. Cell cycle distribution of Calu-3 cells after treatment with cytotoxic drugs and/or enzastaurin. Treatment combinations and sequences are described in Materials and Methods. Cell cycle analysis of surviving cells was done as described in Materials and Methods. Each experiment was done in duplicate.

or pemetrexed induces a maximum accumulation of the cancer cell population in the S phase and limits the ability of cells to proceed through the cell cycle.

Sequence-Dependent Effects on Intracellular Signaling Pathways and Programmed Cell Death by the Combination of Cytotoxic Treatments and Enzastaurin

To further elucidate the mechanism responsible for these effects on induction of cell death by these different sequences of treatment, we next evaluated the effects of each drug alone and/or the three sequence combinations on the expression of proteins and their activated forms, known to be important steps in key intracellular signaling survival pathways (Figs. 3 and 4). Moreover, to better study the effects of these treatments on the induction of apoptosis, we evaluated the expression of Bcl-xL, Bcl-2, and the cyclin-dependent kinase inhibitors, p27 and p21 (Figs. 3 and 4). Because enzastaurin selectively inhibits PKCβ, we sought to examine whether intracellular signaling pathways, which are known to be influenced by PKC activity, could be affected in human NSCLC cells by enzastaurin treatment. PKC activity has been connected to several intracellular cascades, including the Ras-extracellular signal-regulated kinase (ERK) signaling axis and the PI3K/Akt pathway (11, 12). Treatment of Calu-3 cells with increasing doses of enzastaurin (0.005-5 μmol/L) for 48 h slightly decreased ERK1/2 activity as detected by Western blot analysis of activated, pERK1/2 protein (Fig. 3). In contrast, enzastaurin treatment caused a significant, dose-dependent reduction of Akt activity as detected by Western blot analysis of activated, pAkt protein, suggesting a predominant role of the PKCβ on the PI3K/Akt pathway in these cancer cells in basal conditions. Furthermore, enzastaurin treatment increased p27 expression while having little or no effect on Bcl-2 and Bcl-xL expression (Fig. 3). These effects, however, did not induce any significant increase in programmed cell death as assessed by flow cytometry (Fig. 5). Treatment with increasing doses (0.001-10 μg/mL) of gemcitabine or pemetrexed, as single agents, had no major effect on total Akt protein levels but determined a slight increase of its phosphorylation, whereas it caused a decrease in the expression levels of the antiapoptotic protein Bcl-2 (Fig. 3). Interestingly, the antiapoptotic protein Bcl-xL and the phosphorylation of ERK1/2 protein were induced by gemcitabine or pemetrexed single-agent treatment (Fig. 3), suggesting a prosurvival stress response to cytotoxic agent treatment in Calu-3 cancer cells. These effects were associated with 27% and 55% of the cancer cell population being apoptotic following gemcitabine or pemetrexed treatment, respectively (Fig. 5).

Concurrent treatment with enzastaurin in combination with gemcitabine or with pemetrexed inhibited the phosphorylation of Akt and prevented the increased activation of ERK1/2 by single-agent cytotoxic treatment (Fig. 4A). However, the levels of Bcl-xL protein expression were not altered compared with treatment with chemotherapeutic drug alone (Fig. 5).

In contrast, induction of apoptosis was maximum in the synergistic antiproliferative combination sequence (when enzastaurin was added 24 h after gemcitabine or

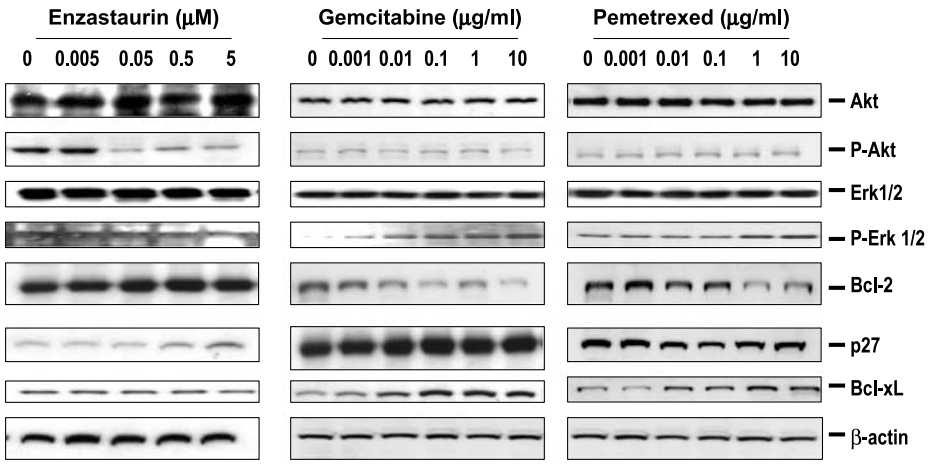

Figure 3. Western blot analysis of protein expression in Calu-3 cells following treatment with enzastaurin for 48 h (range, 0.005-5 μmol/L), gemcitabine (range, 0.001-10 μg/mL), or pemetrexed for 24 h (range, 0.001-10 μg/mL). For the evaluation of ERK1/2, pERK1/2, Akt, pAkt, Bcl-2, Bcl-xL, p27, p21, and β-actin protein expression, total cell protein extracts were fractionated through 4% to 20% SDS-PAGE, transferred to nitrocellulose filters, and incubated with the appropriate antibodies as described in Materials and Methods. Immunoreactive proteins were visualized by enhanced chemiluminescence.

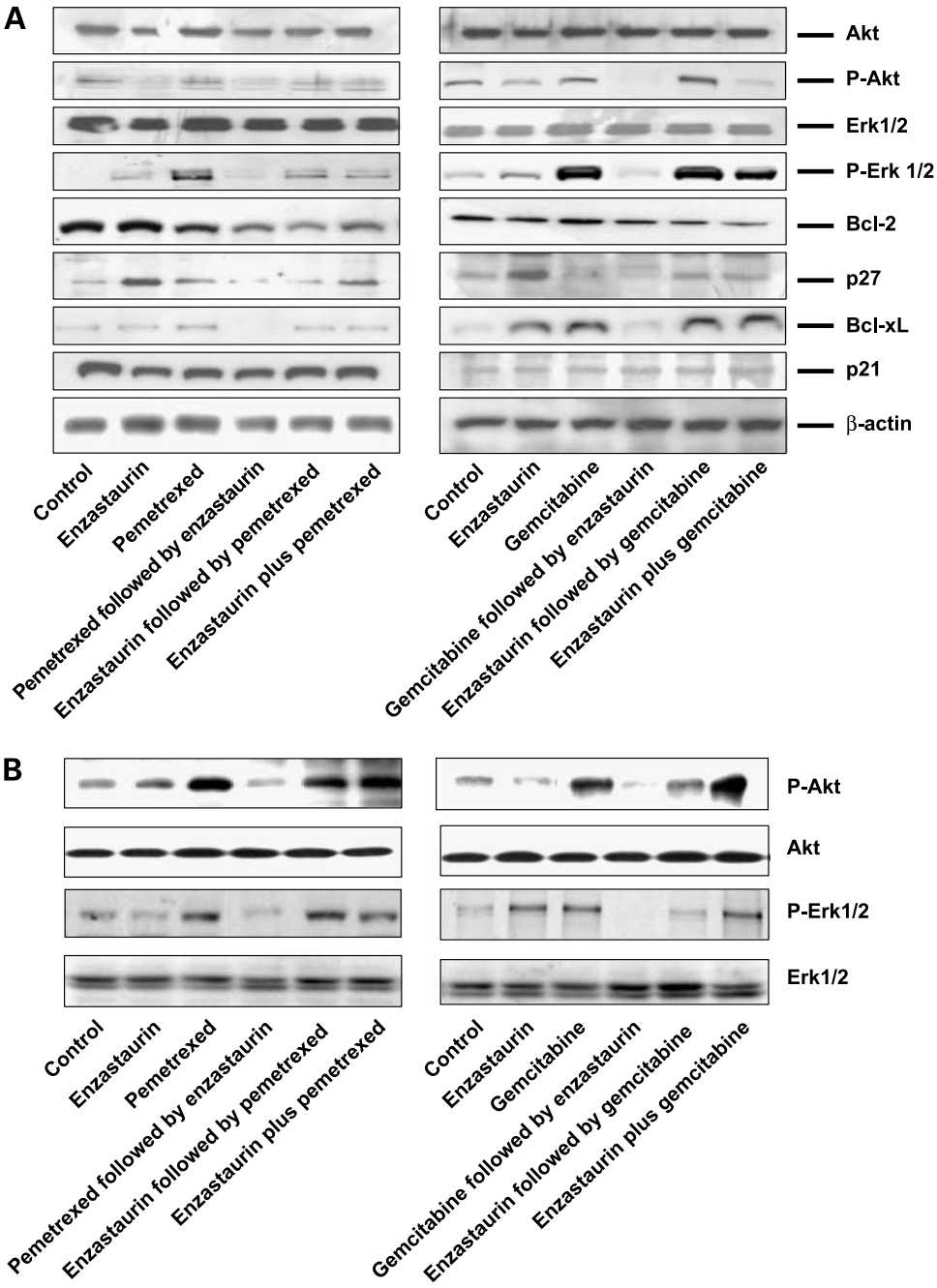

Figure 4. **A,** Western blot analysis of protein expression in Calu-3 cells following treatment with enzastaurin for 48 h, gemcitabine, or pemetrexed for 24 h alone or the three sequence schedules. *Lane 1,* control untreated cells; *lane 2,* enzastaurin (2 μmol/L); *lane 3,* pemetrexed (1 μg/mL; *right*) or gemcitabine (1 μg/mL; *left*); *lane 4,* pemetrexed (1 μg/mL; *right*) or gemcitabine (1 μg/mL; *left*) for 24 h followed by enzastaurin (2 μmol/L) for 48 h; *lane 5,* enzastaurin (2 μmol/L) for 48 h followed by pemetrexed (1 μg/mL; *right*) or gemcitabine (1 μg/mL; *left*) for 24 h; *lane 6,* enzastaurin (2 μmol/L) for 48 h + pemetrexed (1 μg/mL; *right*) or gemcitabine (1 μg/mL; *left*). For the evaluation of ERK1/2, pERK1/2, Akt, pAkt, Bcl-2, Bcl-xL, p27, p21, and β-actin protein expression, total cell protein extracts were fractionated through 4% to 20% SDS-PAGE, transferred to nitrocellulose filters, and incubated with the appropriate antibodies as described in Materials and Methods. Immunoreactive proteins were visualized by enhanced chemiluminescence. **B,** Western blot analysis of protein expression in A549 cells following treatment with enzastaurin for 48 h, gemcitabine, or pemetrexed for 24 h alone or the three sequence schedules. *Lane 1,* control untreated cells; *lane 2,* enzastaurin (2 μmol/L); *lane 3,* pemetrexed (1 μg/mL; *right*) or gemcitabine (1 μg/mL; *left*); *lane 4,* pemetrexed (1 μg/mL; *right*) or gemcitabine (1 μg/mL; *left*) for 24 h followed by enzastaurin (2 μmol/L) for 48 h; *lane 5,* enzastaurin (2 μmol/L) for 48 h followed by pemetrexed (1 μg/mL; *right*) or gemcitabine (1 μg/mL; *left*) for 24 h; *lane 6,* enzastaurin (2 μmol/L) for 48 h + pemetrexed (1 μg/mL; *right*) or gemcitabine (1 μg/mL; *left*).

pemetrexed treatment) compared with control or single-agent treatments for both gemcitabine and pemetrexed combinations (Fig. 5) and it was associated with the maximum inhibition of Akt and ERK1/2 phosphorylation and by the strongest reduction in Bcl-xL and p27 expression (Fig. 4A).

When the sequence of treatment was reversed and enzastaurin treatment preceded chemotherapy treatment, the fraction of cancer cells that underwent apoptosis was only 7.3% for the sequence enzastaurin followed by gemcitabine and only 11.5% for the sequence enzastaurin followed by pemetrexed (Fig. 5), with an incomplete inhibition of Akt and ERK1/2 phosphorylation (Fig. 4A), indicating a clear antagonism for this sequence combination. In addition, p27 expression was not significantly changed when chemotherapeutics were given simultaneously or 24 h before enzastaurin treatment (Fig. 4A). Moreover, p21 protein levels did not change under any of the treatment conditions. The reduction in Bcl-2 protein levels, which was induced by single-agent cytotoxic

treatment, was similarly obtained in the concurrent combination schedules and the chemotherapy followed by enzastaurin schedules (Fig. 4A).

To investigate whether Ras gene mutations and/or p53 status could alter cancer cell sensitivity to the induction of apoptosis, we evaluated the effects of each drug alone and/or the three sequence combinations on the expression of the same proteins and their activated forms in NSCLC cell lines, which harbor K-Ras mutations (H1299 and A549) or p53 mutations (H1299). In both A549 (Fig. 4B) and H1299 (data not shown) cancer cells, the effects of each drug alone and/or the three sequence combinations on the expression of Akt and ERK1/2 and on the phosphorylation of their activated forms were similar to those observed in Calu-3 cancer cells, which harbor wild-type K-Ras and p53 genes.

Discussion

The balance between cell survival and cell death is a complex issue, and there is considerable effort to understand how cancer cells regulate the decision point between these critical pathways. In this context, the possibility of combining conventional cytotoxic drugs with novel agents that specifically interfere with key pathways controlling cancer cell survival, proliferation, invasion, and/or metastatic spreading has generated a wide interest (13).

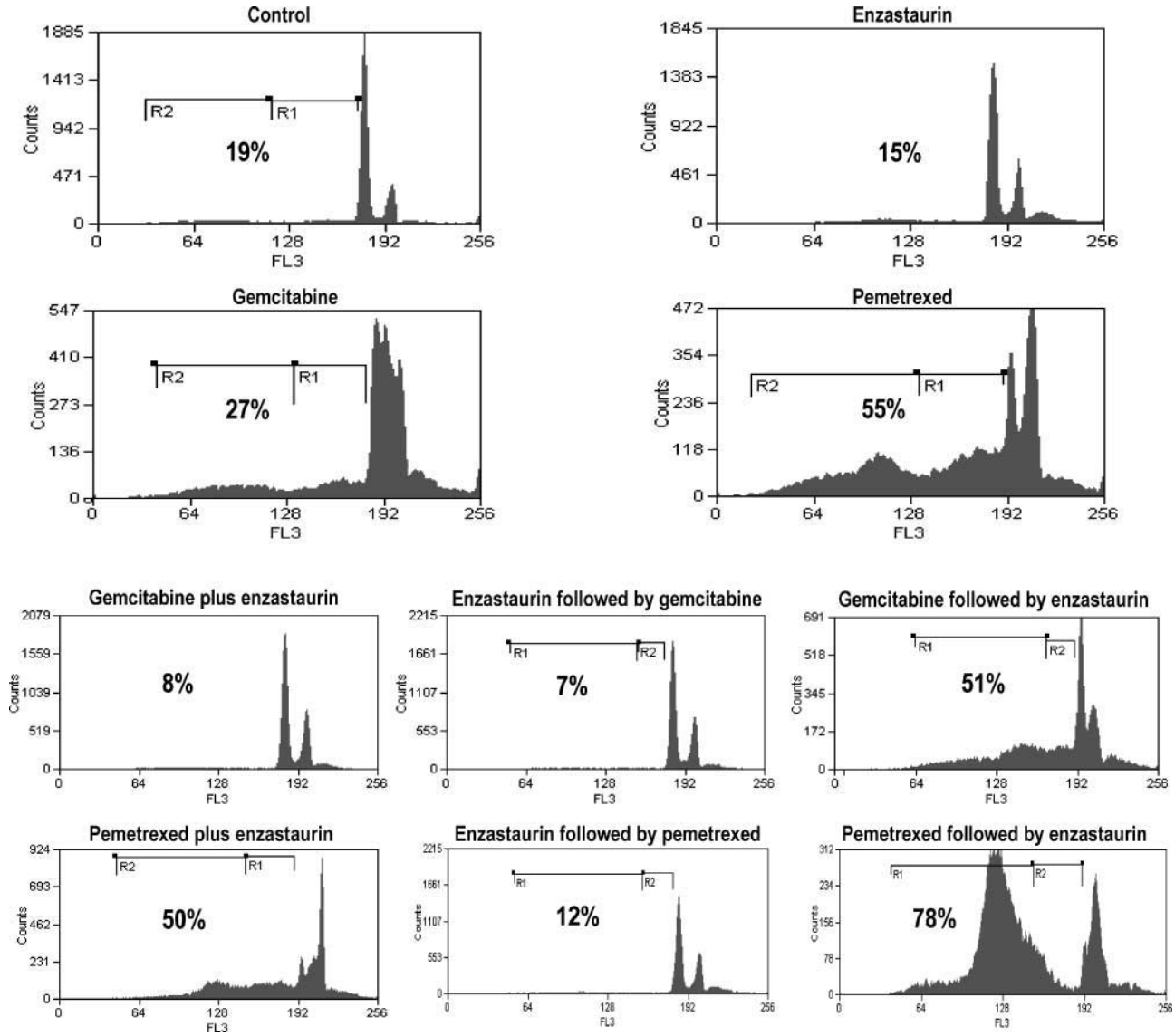

Figure 5. Calu-3 cells were treated with enzastaurin (2 μmol/L), gemcitabine (1 μg/mL), and pemetrexed alone (1 μg/mL) or the three schedule sequences. Both adherent and detached cells were harvested and flow cytometric analysis of cell death was done as described in Materials and Methods. In each panel, the number represents the percent of apoptotic cells. Each experiment was done in duplicate.

Unfortunately, four large, randomized phase III randomized trials of continuous daily administration of EGFR tyrosine kinase inhibitors in combination with conventional chemotherapy doublets as first-line therapy have failed to show an improvement in survival in patients with advanced NSCLC (14–17), leading to the conclusion that an EGFR tyrosine kinase inhibitor cannot be usefully combined with cytotoxic therapies in NSCLC. However, there is a growing experimental evidence of a possible sequence-dependent interaction between molecularly targeted and cytotoxic agents. In this context, a G_1-phase-induced cell arrest by treatment of cancer cells with molecularly targeted agents against growth factor receptors could protect cancer cells from the cytotoxic effects of chemotherapy (39).

The present study provides evidence that modulation of both survival and cell cycle regulation plays a role in the interaction between two cytotoxic drugs, such as gemcitabine and pemetrexed, and enzastaurin, in terms of enhanced apoptosis and cell growth inhibition in NSCLC cell lines. Enzastaurin is a selective, orally active, PKCβ inhibitor. Tumor-induced angiogenesis requires the activation of PKCβ and enzastaurin was originally evaluated in human tumor xenograft models in mice for its antiangiogenic activity on PKCβ inhibition in endothelial cells (40). However, in addition to its antiangiogenic effects, enzastaurin, at concentrations that could be achieved in cancer patients, directly inhibits proliferation and induces apoptosis in human cancer cells through the suppression of phosphorylation of Akt and its downstream effectors glycogen synthase kinase-3β and ribosomal protein S6 (41, 42). Furthermore, although enzastaurin was originally developed as a relatively specific PKCβ inhibitor, subsequent studies have shown that it has additional activity on PKCα at clinically relevant plasma concentration (43). Because PKCβ and PKCα are both overexpressed in lung cancer cells (44), it may represent the reason of the direct antitumor activity of enzastaurin in lung cancer.

The mitogen-activated protein kinase/extracellular signal regulated kinase and the PI3K/Akt pathways regulate fundamental cellular functions such as cell proliferation, survival, differentiation, and motility (45). In addition to their contribution to cell proliferation, persistently active ERK and Akt pathways may protect cells from apoptosis induced by cytotoxic agents. Recent investigations showed that the reduction of pAkt correlated with the enhancement of gemcitabine- and pemetrexed-induced apoptosis and antitumor activity, suggesting that the PI3K/Akt pathway plays a significant role in mediating drug resistance in human cancer cells (46).

In the present study, we provide experimental evidence that gemcitabine and pemetrexed, two S-phase-specific cytotoxic drugs, when used as single agents, can induce NSCLC cell apoptosis, although their treatment induces specific intracellular signaling pathways with an increase in pAkt, pERK1/2, and Bcl-xL, suggesting that the surviving NSCLC cancer cells can activate as an escape mechanism compensatory prosurvival responses. In general, Raf/MEK/ERK1/2 pathway activation is coupled with cell proliferation, although a prolonged activation may exert a proapoptotic influence depending on the cellular context (47–49). Our observations suggest that the hyperactivation state of the Raf/MEK/ERK1/2 pathway observed in gemcitabine- and pemetrexed-treated cancer cells may contribute to growth proliferation being a sort of mechanism that cancer cells use to escape to the treatment-induced death. In the present study, basal activation of Akt was higher than that of ERK1/2, suggesting a predominant role of the PI3K/Akt pathway in the tumor growth of these lung cancer cell lines in basal conditions. However, in stress conditions such as cytotoxic treatments, the increase in Akt activation was relatively low, with a stronger activation of ERK1/2 as prosurvival response, indicating a relevant role of this pathway in response to stress. Consistent with this observation, the synergistic sequence of treatment (chemotherapy followed by enzastaurin) shows, in addition to a significant inhibition of Akt activity, also the strongest down-regulation of ERK1/2 activation.

The observation that enzastaurin treatment increases the proportion of cells in the G_1 phase of the cell cycle may explain, in part, the synergy and the antagonism observed depending on sequences of treatment. Induction of G_1 arrest by treatment with enzastaurin before gemcitabine or pemetrexed may limit the activity of these two phase-specific cytotoxic agents. Indeed, enzastaurin caused a decrease in the proportion of cancer cells in S phase. In contrast, the sequential treatment with enzastaurin following cancer cell exposure for 24 h to gemcitabine or pemetrexed caused a significant increase in apoptosis. Furthermore, this sequence schedule induced a maximum accumulation of cancer cells in the S phase of the cell cycle and it is conceivable that it significantly limited the ability of cancer cells to repair damaged DNA and restore normal progression through the cell cycle.

Collectively, our study suggests that a more effective approach to combine chemotherapy with enzastaurin is a sequence in which cytotoxic drug treatment is followed by this biological agent. The results of our study also suggest that, when multiple cycles of therapy are given, this sequence should be recycled with a relatively short-term and intermittent exposure to the PKC antagonist after chemotherapy to avoid negative cancer cell kinetic interactions.

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