

Schedule-Dependent Cytotoxic Synergism of Pemetrexed and Erlotinib in Human Non – Small Cell Lung Cancer Cells

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Abstract Purpose: This study was undertaken to select the optimal combination schedule of erlotinib and pemetrexed for the treatment of relapsed non – small cell lung cancer (NSCLC) using a panel of human NSCLC lines.

Experimental Design: Human NSCLC cell lines, with variable expression of the known molecular determinants of erlotinib sensitivity, were exposed to pemetrexed and erlotinib using different schedules. Antitumor effect was measured by growth inhibition by cell count and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay, cell cycle distribution and apoptosis by flow cytometry, and expression of cell cycle mediators by immunoblots. The cytotoxic interaction between pemetrexed and erlotinib (i.e., synergistic, additive, or antagonistic) was determined by median effect analysis.

Results: When cells were exposed to concurrent pemetrexed and erlotinib or sequential pemetrexed followed by erlotinib, cytotoxic synergism was observed in both erlotinib-sensitive and erlotinib-resistant human NSCLC cell lines. This was independent of the mutation status of *epidermal growth factor receptor* or *K-Ras* genes. Synergism was associated with a combination of cell cycle effects from both agents. In contrast, exposure of cells to erlotinib followed by pemetrexed was mostly antagonistic in erlotinib-sensitive cells and additive at best in erlotinib-resistant cells. Antagonism was associated with erlotinib-induced G₁-phase blockade of erlotinib-sensitive cells, which protects cells from pemetrexed cytotoxicity. Pemetrexed induced an epidermal growth factor receptor – mediated activation of the phosphatidylinositol 3-kinase/AKT pathway, which was inhibited by erlotinib and a specific phosphatidylinositol 3-kinase inhibitor, LY294002.

Conclusions: The combination of pemetrexed and erlotinib is synergistic in NSCLC *in vitro* if exposure to erlotinib before pemetrexed is avoided, particularly in tumors that are sensitive to erlotinib. Based on these findings, a randomized phase II study comparing the progression-free survival between an intermittent combination of erlotinib and pemetrexed (experimental arm) and pemetrexed alone (control arm) in patients with relapsing NSCLC has been initiated.

Patients with relapsing non – small cell lung cancer (NSCLC) have a dismal prognosis. Until recently, the only approved agent for this indication was docetaxel as monotherapy (1, 2). No second-line combination chemotherapy has been shown to provide a survival benefit due to excessive toxicities and the rapidly declining clinical condition of these patients (1, 2). Pemetrexed and erlotinib monotherapy have different mechanisms of action and similar clinical effectiveness compared with docetaxel but their main advantage is mild toxicity and

favorable tolerability in the majority of patients with advanced NSCLC (3, 4). On this basis, both agents were approved as monotherapy for this indication by the U.S. Food and Drug Administration in 2004.

Pemetrexed (Alimta, formerly LY231514, Eli Lilly and Company) is a novel antifolate that enters tumor cells rapidly via several membrane transporters, where it is metabolized to polyglutamate derivatives that are potent inhibitors of thymidylate synthase and, to a much lesser extent, glycinamide ribonucleotide formyltransferase (5 – 7). Pemetrexed arrests cells mainly in S phase and induces apoptosis in a broad spectrum of solid tumors, including NSCLC. No biomarkers have been validated for predicting clinical response to pemetrexed (7, 8).

Erlotinib (OSI-774, Tarceva, OSI Pharmaceuticals/Genentech) is an oral, small-molecule tyrosine kinase inhibitor (TKI) that reversibly binds to the intracellular tyrosine kinase domain of epidermal growth factor receptor. This blocks autophosphorylation of EGFR with subsequent inhibition of downstream signaling pathways. Erlotinib causes cell growth arrest in G₁ phase and induces apoptosis in a variety of tumor cells *in vitro* and *in vivo* (9 – 12). Nonsmokers, patients whose NSCLC tumors contain tyrosine kinase – activating somatic mutations and/or amplifications of the *EGFR* gene, and patients who

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Table 1. Cytotoxicity of erlotinib or pemetrexed and the mutation status of *EGFR* and *K-Ras* gene in human NSCLC cell lines

Cell line	Cell type	<i>EGFR</i> gene	<i>K-Ras</i> gene	Erlotinib IC ₅₀ ($\mu\text{mol/L}$, mean \pm SD)	Pemetrexed IC ₅₀ ($\mu\text{mol/L}$, mean \pm SD)
H3255	Adenocarcinoma	L858R	WT	0.029 \pm 0.019	0.062 \pm 0.032
H322	Adenocarcinoma	WT	WT	0.82 \pm 0.30	0.53 \pm 0.11
H358	Bronchoalveolar	WT	WT	1.50 \pm 0.16	0.24 \pm 0.04
H1650	Bronchoalveolar	del exon 19	WT	10.05 \pm 3.03	0.09 \pm 0.01
H661	Large cell	WT	WT	14.10 \pm 3.38	0.58 \pm 0.13
H1975	Adenocarcinoma	L858R, T790M	WT	>20	0.24 \pm 0.18
H1299	Not specified	WT	*N-RAS (Q61K)	>20	0.70 \pm 0.21
A549	Adenocarcinoma	WT	G12S	>20	0.35 \pm 0.06
H596	Adenosquamous carcinoma	WT	WT	>20	0.55 \pm 0.05

NOTE: Characteristics of NSCLC cell lines and *in vitro* cytotoxicity of erlotinib or pemetrexed. The mutation status of the *EGFR* or *K-Ras* genes was determined by direct sequencing as described in Materials and Methods. Cytotoxicity of erlotinib and pemetrexed was determined by the cell count after a 72-h drug treatment and is expressed as the IC₅₀ as calculated by the CalcuSyn software. Each value represents the mean \pm SD from at least three independent experiments. Abbreviation: WT, wild-type.

develop significant skin rash during treatment derive the greatest survival benefit from EGFR TKI monotherapy (13–17) and probably also from an EGFR TKI in combination with standard cytotoxic agents (13, 18, 19). The presence of a *K-Ras* or an *EGFR* T790M mutation has been associated with primary and acquired resistance to EGFR TKIs (20–24).

Addition of an EGFR TKI (erlotinib or gefitinib) to first-line combination chemotherapies has failed to improve survival in unselected patients with advanced NSCLC in several large, randomized, controlled studies (18, 25–27). Because all these combination studies use continuous administration of an EGFR TKI, erlotinib-induced G₁ arrest of tumor cells could obviate the effects of subsequent pulse administration of cytotoxic agents. In support of this hypothesis, this and other groups have reported a negative interaction between erlotinib or gefitinib and the mitotic spindle inhibitors paclitaxel (28) and docetaxel (29), and the proteasome inhibitor bortezomib (30) in NSCLC cells, when exposure to erlotinib precedes these agents.

The objective of the current study was to determine whether the sequence of drug exposure modulates the interaction between pemetrexed and erlotinib in a panel of human NSCLC cell lines. The results show cytotoxic synergism, independent of the presence of several molecular determinants of resistance to erlotinib, when pemetrexed is administered concurrently or before erlotinib. Pemetrexed caused a growth and survival response via EGFR-mediated activation of the phosphatidylinositol 3-kinase/AKT (PI3K/AKT) pathway, which was inhibited by erlotinib. The data suggest that inhibition of the PI3K/AKT pathway is a major determinant of synergism between these agents.

Materials and Methods

Drugs and reagents. Erlotinib (OSI 774, Tarceva) was obtained from OSI Pharmaceuticals and dissolved in DMSO as a stock solution of 10 mmol/L. Pemetrexed (Alimta) was obtained commercially from Eli Lilly and Company and dissolved in 0.9% NaCl to a final concentration of 25 mg/mL (i.e., 41.8 mmol/L). Both drugs were stored at -20°C in tightly sealed sterile tubes and diluted to the desired concentrations in PBS within 24 h of each experiment. Antibodies and

their sources were as follows: anti-EGFR rabbit polyclonal antibody, anti-phosphorylated EGFR (Tyr¹⁰⁶⁸) rabbit polyclonal antibody, anti-AKT mouse monoclonal antibody, and anti-phosphorylated AKT (Ser⁴⁷³) from Cell Signaling; anti-cyclin D1 (M-20), anti-cyclin-dependent kinase (CDK) 2 (D-12), and anti-p27^{KIP1} (F-8) from Santa Cruz Biotechnology; anti-cyclin A from Upstate Biotechnology; and anti-cyclin E (Ab-1) mouse monoclonal antibody (HE12) or anti-p21^{WAF/CIP1} (Ab-1) mouse monoclonal antibody (EA10) from Calbiochem. LY294002 (a selective PI3K inhibitor) and other chemicals were purchased from Sigma-Aldrich Co. unless otherwise indicated.

Cell lines. Human NSCLC cell lines used in this study, except H3255 (a generous gift from Dr. Pasi A. Jänne, Dana-Farber Cancer Institute), were purchased from the American Type Culture Collection. All cell lines were grown in RPMI 1640 supplemented with 10% fetal bovine serum and glutamine and maintained at 37°C in a humidified atmosphere of 95% air and 5% CO₂.

Growth inhibition assay. Exponentially growing cells were seeded in 96-well plastic plates and exposed to serial dilutions of erlotinib, pemetrexed, or the combination at a constant concentration ratio of 4:1 in triplicates for 72 h. Cell viability was assayed by cell count and the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (31). Growth inhibition was expressed as the percentage of surviving cells in drug-treated versus PBS-treated control cells (which was considered as 100% viability). The IC₅₀ value was the concentration resulting in 50% cell growth inhibition by a 72-h exposure to drug(s) compared with untreated control cells and was calculated by the CalcuSyn software (Biosoft, Inc.). Interactions between erlotinib and pemetrexed were expressed as the combination index by the CalcuSyn software: <1 represents synergistic cytotoxicity; 1 represents additive cytotoxicity; and >1 represents antagonistic cytotoxicity (32, 33).

Mutation analysis of *EGFR* and *K-Ras* genes. Genomic DNA was isolated from each cell line by the phenol/chloroform extraction method (Invitrogen). The primers that were specific for amplifying the cDNA fragments of the EGFR tyrosine kinase domain and Ras cDNA fragments containing codons 12, 13, and 61 were synthesized as described before (34, 35). After 35 cycles of PCR amplification, PCR products were purified by a PCR purification kit (Qiagen) and sequenced at the Albert Einstein Cancer Center DNA Sequencing Shared Resource.

Analysis of cell cycle distribution and apoptosis. Exponentially growing cells were seeded in six-well plates and treated with erlotinib (2 $\mu\text{mol/L}$) and pemetrexed (0.5 $\mu\text{mol/L}$) alone or in combination concurrently or sequentially for a defined interval (24, 48, or 72 h). Cells were harvested and fixed with 70% cold ethanol at 4°C overnight.

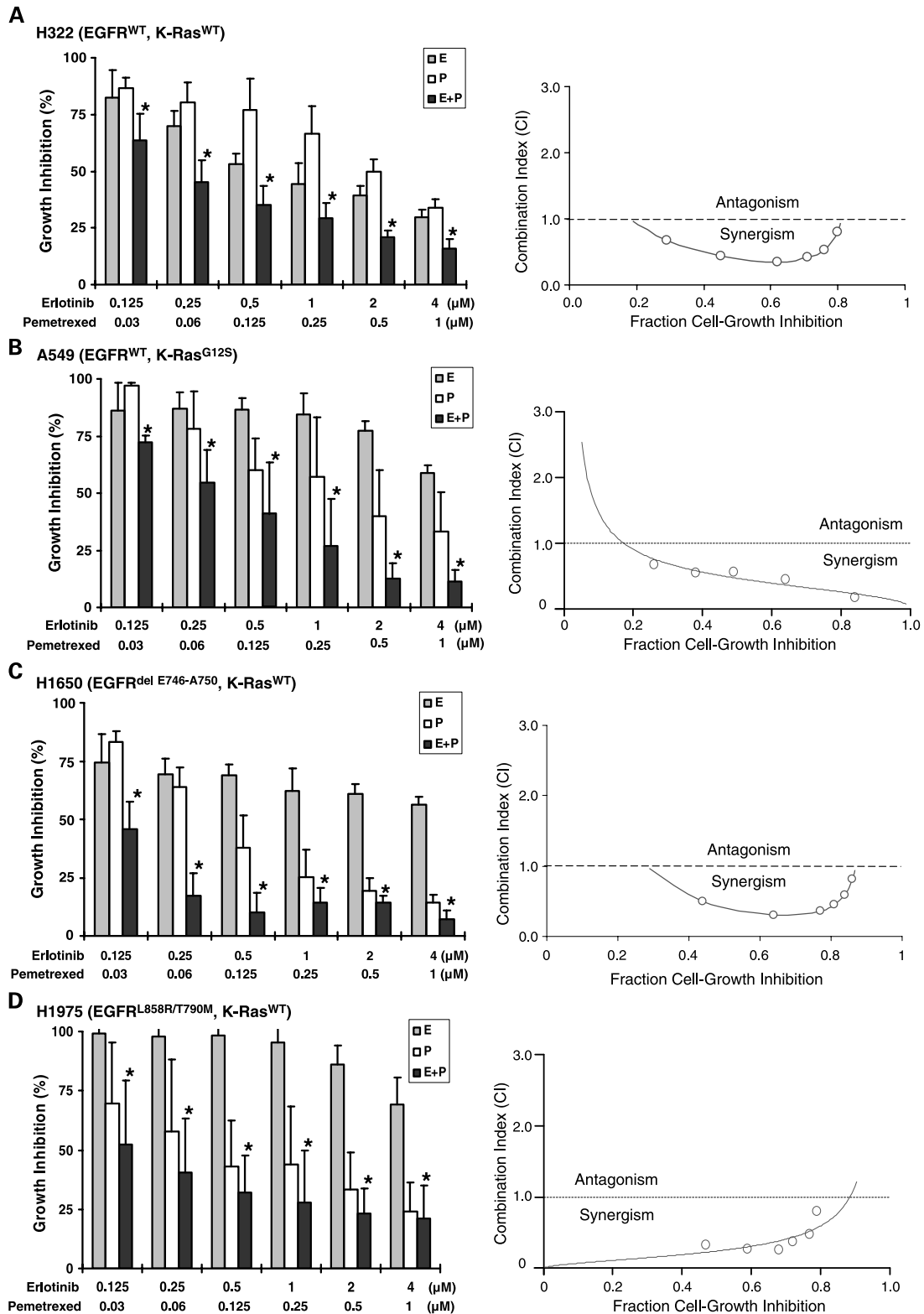


Fig. 1. Cytotoxic synergism of pemetrexed and erlotinib in human NSCLC cell lines. Erlotinib-sensitive H322 (A) and erlotinib-resistant A549 (B), H1650 (C), or H1975 (D) cells were incubated with increasing concentrations of pemetrexed (P) and erlotinib (E) alone or concurrently at a concentration ratio of 4:1 for 72 h. Left, growth inhibition was determined by direct cell count. Results are expressed as percentage surviving cells in drug-treated relative to PBS-treated control cells. Columns, mean of three independent experiments; bars, SD. *, $P < 0.05$, two-tailed Student's t test, statistically significant difference of combination versus erlotinib or pemetrexed alone. Right, the combination index defining the interaction between pemetrexed and erlotinib is plotted against the fraction of growth-inhibited cells.

The fixed cells were washed with PBS and stained with 1 µg/mL propidium iodide and 5 µg/mL RNase I at room temperature for at least 3 h. Analyses of 10,000 events were acquired on a FACSCalibur (Becton Dickinson Biosciences), and cell cycles were analyzed by ModFit DNA analysis software (Verity Software House). These analyses were done at the Albert Einstein Cancer Center Flow Cytometry Shared Resource.

Immunoblot analysis. Cell lysates were prepared and the procedures for immunoblot were done as described before (36). Equal amounts of protein (30 µg) from each sample were resolved by 10% to 15% SDS-PAGE followed by transfer to nitrocellulose membranes. The blots were probed with specific antibodies and the protein signals were visualized by an enhanced chemiluminescence reaction system, as recommended by the manufacturer (Amersham), and quantitated using ImageQuant software (Molecular Dynamics, Amersham Biosciences). Equal loading was assessed by immunoblotting for β-actin, total EGFR, or total AKT as indicated.

Statistical analysis. All data were expressed as mean ± SD obtained from at least three independent experiments. Two-tailed, paired Student's *t* test and all-pairwise, one-way ANOVA test were used to determine the differences between control and treatment groups (Analyse-It software for Microsoft Excel). All statistical tests were based on two-tailed probability. Differences at a level of *P* < 0.05 for Student's *t* test, or with Bonferroni error protection and a confidence interval of 90% for one-way ANOVA test, were considered statistically significant.

Results

Molecular characteristics and cytotoxicity of erlotinib and pemetrexed in human NSCLC cell lines. The human NSCLC cell lines used in this study were assessed for several known

molecular determinants of sensitivity and resistance to erlotinib (20–23). Table 1 summarizes the histologic and molecular characteristics and cytotoxicity of erlotinib and pemetrexed of the nine human NSCLC cell lines. Consistent with clinical observations, these cell lines exhibited a concentration-dependent but a wide-range sensitivity to erlotinib (IC₅₀, 0.03 to >20 µmol/L). Concentrations of erlotinib >20 µmol/L could not be achieved because of the low solubility of this agent in culture medium. At a dose of 150 mg daily, the plasma steady-state concentration of erlotinib in patients with advanced solid tumors is ~2 µmol/L (range, 1.35-4.23 µmol/L; 37). Accordingly, 2 µmol/L was chosen as the concentration of erlotinib for further studies. Cell lines with an IC₅₀ of <2 µmol/L were classified as erlotinib sensitive (i.e., H3255, H322, and H358) and those with an IC₅₀ of ≥2 µmol/L as erlotinib resistant (i.e., H1650, H1975, H661, H1299, A549, and H596). In contrast to a previous report (38), the H1650 cell line harboring a delE746-A750 deletion in the *EGFR* gene was resistant to erlotinib with an IC₅₀ of 10.1 µmol/L and the H1975 cell line harboring both L858R and T790M mutations in the *EGFR* gene had an IC₅₀ of >20 µmol/L. In addition, both H1650 and H1975 have *EGFR* gene amplification by fluorescence *in situ* hybridization analysis (data not shown). The small sample size of cell lines used in this study precluded assessment of the predictive value of tyrosine kinase-activating somatic mutations in the *EGFR* gene and/or high *EGFR* gene copy number as determinants of the sensitivity of NSCLC cells to erlotinib with or without pemetrexed.

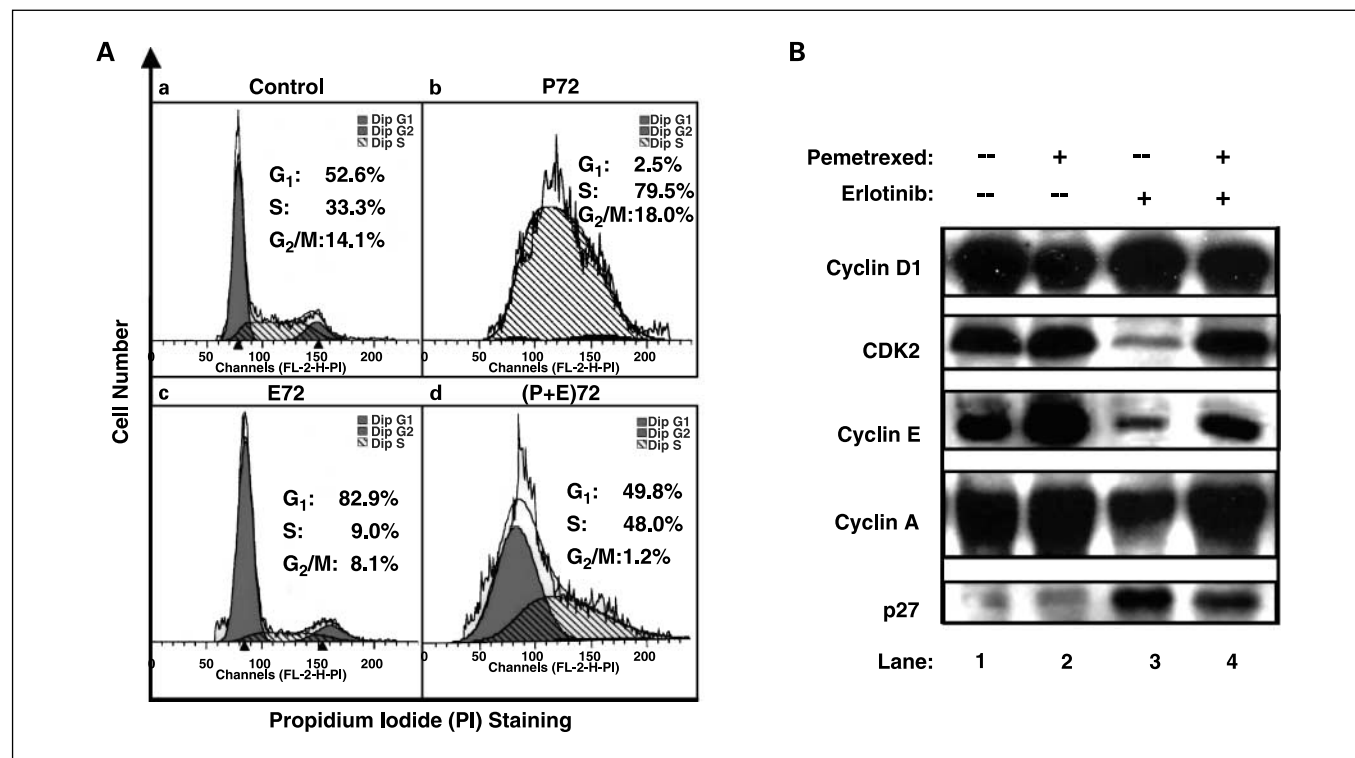


Fig. 2. Cell cycle effect of pemetrexed and erlotinib. H322 cells were incubated with pemetrexed (0.5 µmol/L) and erlotinib (2 µmol/L) alone or concurrently for 72 h. *A*, cell cycle distribution by flow cytometry. Results are expressed as the mean of percentage cells in different phases of the cell cycle in each sample (sum to 100%) from three independent experiments. *B*, Western blot analysis of cyclin, CDKs, and CDK inhibitors using corresponding antibodies in the same groups of H322 cells confirms the phase-specific cell cycle effect of pemetrexed and erlotinib alone or in combination observed in (*A*). Representative immunoblots from two independent experiments. Expression of β-actin on the same immunoblot was used for loading control (data not shown).

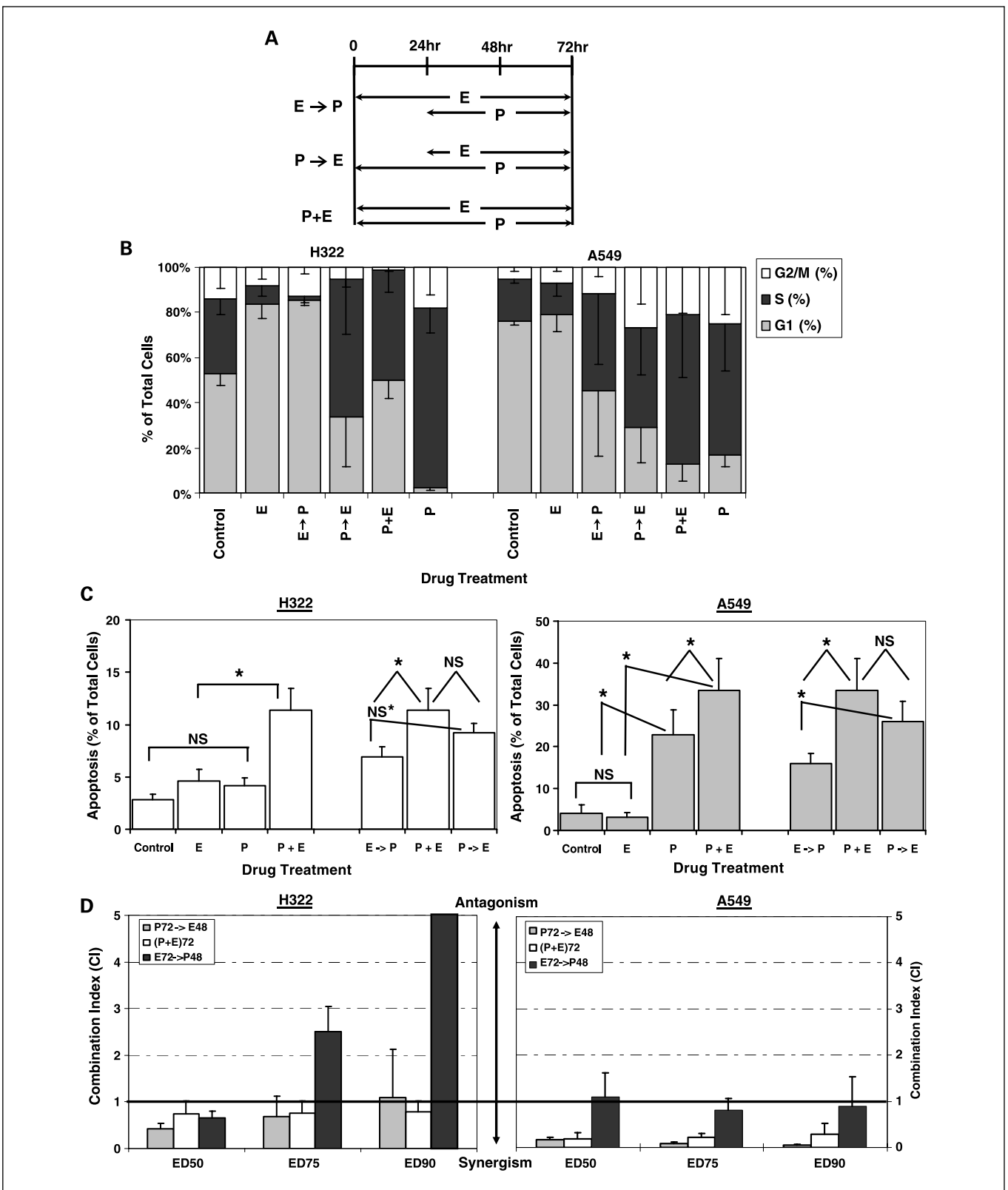


Fig. 3. Cell cycle alteration by sequence of pemetrexed and erlotinib modulates apoptosis and cytotoxicity. H322 and A549 cells were treated with different schedules of pemetrexed (0.5 $\mu\text{mol/L}$) and erlotinib (2 $\mu\text{mol/L}$) alone or in combination. Columns, mean of three independent experiments; bars, SD. **A**, schematic representation of three different schedules of pemetrexed and erlotinib. **B**, cell cycle distribution was analyzed by flow cytometry. **C**, the apoptotic effect was assessed by percentage cells in sub- G_0 - G_1 phase without debris or aggregates by flow cytometry. Asterisk, significant difference (Bonferroni 90% confidence interval) between treatment groups by the all-pairwise, one-way ANOVA and two-tailed, paired Student's t tests. NS, not significant. NS*, not significant by one-way ANOVA test but significant by the paired Student's t test. **D**, combination index values were calculated at the 50%-75%-90% of effective dose (ED).

Concentration- and time-dependent pemetrexed growth inhibition was observed in all nine NSCLC cell lines, with IC_{50} values of $<1 \mu\text{mol/L}$ in all cases (Table 1). This concentration is much lower than the mean peak plasma concentration of pemetrexed achievable in patients (i.e., 120-230 $\mu\text{mol/L}$; refs. 39, 40), indicating a surprisingly high *in vitro* sensitivity of NSCLC cells to this agent. Concentrations of pemetrexed $>1 \mu\text{mol/L}$ resulted in death of $>70\%$ of cells, precluding further biochemical and other analyses (Fig. 5). H322 cells treated with 0.5 $\mu\text{mol/L}$ pemetrexed (the IC_{50}) showed intermediate cytotoxicity, and 0.5 $\mu\text{mol/L}$ pemetrexed was chosen for further combination studies. Interestingly, the H3255 cell line harboring a L858R missense mutation in the *EGFR* gene was the most sensitive to both erlotinib (IC_{50} of 0.029 $\mu\text{mol/L}$) and pemetrexed (IC_{50} of 0.062 $\mu\text{mol/L}$) among the cell lines studied.

Cytotoxic synergism of erlotinib and pemetrexed. To evaluate the interaction between erlotinib and pemetrexed, the cytotoxicity of 0.5 $\mu\text{mol/L}$ pemetrexed and 2 $\mu\text{mol/L}$ erlotinib alone or in combination was assessed in erlotinib-sensitive H322 and erlotinib-resistant A549, H1650, and H1975 cells. Compared with erlotinib or pemetrexed alone, all cells treated with erlotinib and pemetrexed concurrently exhibited increased cytotoxicity (all $P < 0.05$, two-tailed, paired Student's *t* test; Fig. 1). As shown on the right side of each figure, the combination index values

were all <1 , suggesting a synergistic interaction between erlotinib and pemetrexed in a heterogeneous group of these four NSCLC cell lines, one harboring a *K-Ras* mutation (A549), another a single activating *EGFR* mutation (H1650), a line with two *EGFR* mutations (H1975), and one line harboring neither an *EGFR* nor a *K-Ras* mutation (H322).

Cell cycle effects of pemetrexed and erlotinib. The cell cycle distribution was analyzed in cells exposed to pemetrexed or erlotinib alone or concurrently for 72 h by flow cytometric and Western blot analysis. Pemetrexed alone induced S-phase arrest in $\sim 80\%$ of H322 cells and erlotinib alone induced G_1 -phase arrest in $\sim 80\%$ H322 cells. Concurrent exposure of H322 cells to pemetrexed and erlotinib induced G_1 -phase arrest in $\sim 50\%$ of cells and S-phase arrest in the other 50% of cells (Fig. 2A). Expression of phase-specific cyclins, CDKs, and CDK inhibitors determined by immunoblotting in parallel experiments was compatible with these findings (Fig. 2B). Erlotinib inhibited the expression of CDK2, cyclin E, and cyclin A and increased the expression of p27 (Fig. 2B, lane 3) compared with control cells (Fig. 2B, lane 1), consistent with G_1 -phase arrest. Pemetrexed increased expression of CDK2, cyclin E, and cyclin A (Fig. 2B, lane 2) compared with control cells (Fig. 2B, lane 1), consistent with S-phase arrest. Concurrent administration of erlotinib and pemetrexed resulted in cell cycle distribution patterns indicative of overlapping effects from both agents (Fig. 2B, lane 4), which

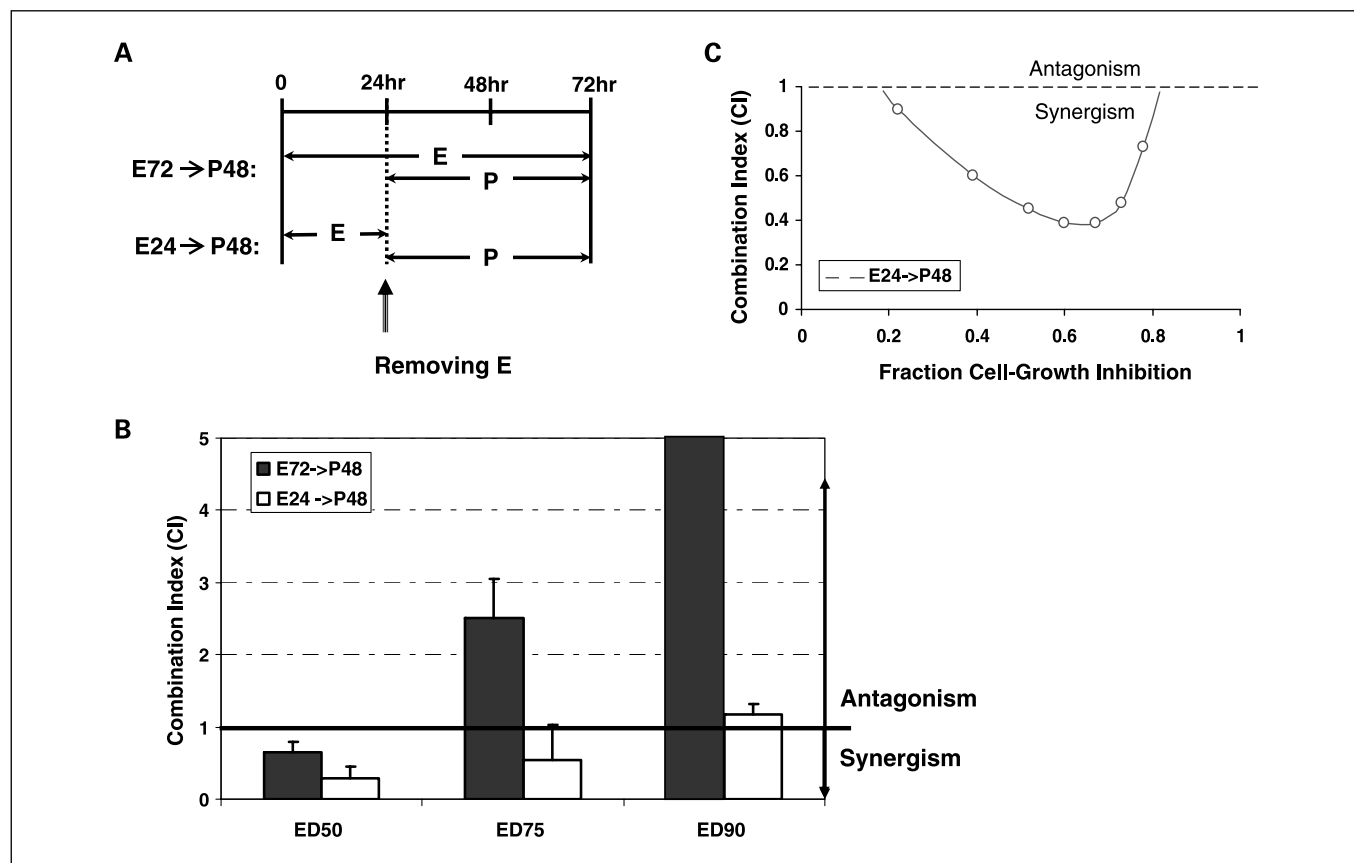
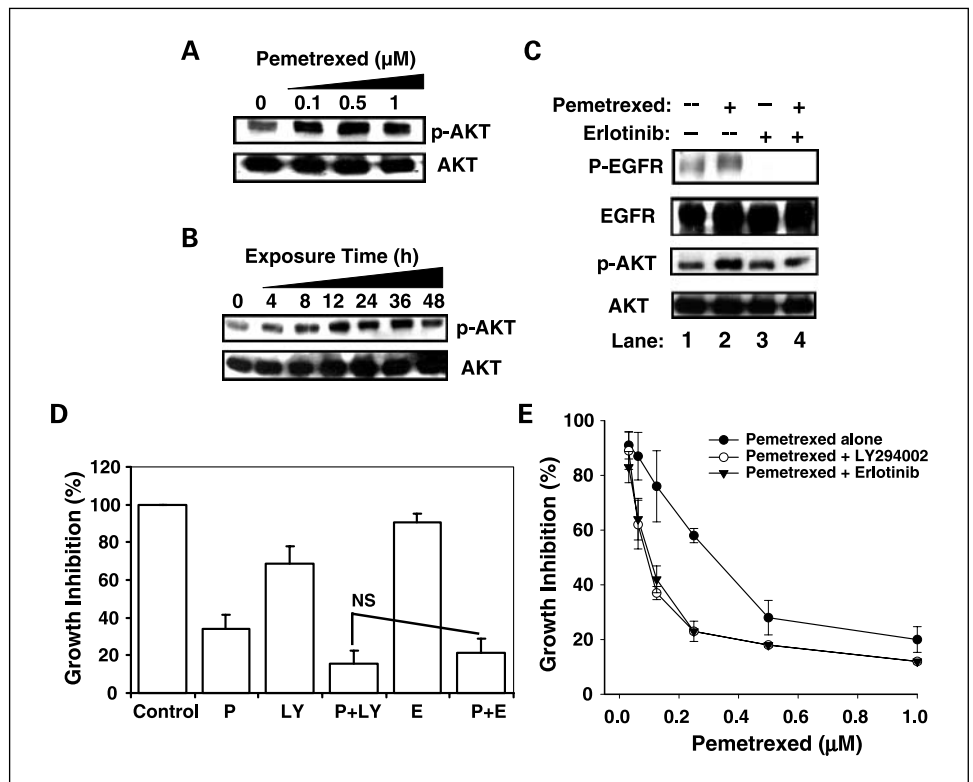


Fig. 4. Reversibility of the antagonistic effect of erlotinib on pemetrexed. *A*, erlotinib-sensitive H322 cells were incubated with erlotinib (2 $\mu\text{mol/L}$) for 24 h following which the culture medium was changed to include pemetrexed (0.5 $\mu\text{mol/L}$) without erlotinib. Schematic representation of two schedules with continuous and discontinuous exposure to erlotinib followed by pemetrexed. *B*, growth inhibition was determined by the cell count method, and the interaction between pemetrexed and erlotinib was expressed by the combination index values. Columns, mean of three independent experiments; bars, SD.

Fig. 5. Effect of pemetrexed and erlotinib on the activity of EGFR/PI3K/AKT pathway. Western blot analysis of phosphorylated AKT (*p*-AKT) expression in erlotinib-resistant A549 cells treated with increasing concentration (A) or increasing exposure (B) to pemetrexed. C, Western blot analysis of phosphorylated EGFR (*P*-EGFR) and *p*-AKT expression in A549 cells treated with 0.5 μ mol/L pemetrexed alone (lane 2) or in combination with 2 μ mol/L erlotinib (lane 4) compared with PBS-treated control cells (lane 1) or cells treated with 2 μ mol/L erlotinib (lane 3). Representative immunoblots from two independent experiments. D, growth inhibition of A549 cells by pemetrexed (0.5 μ mol/L) and erlotinib (2 μ mol/L) was compared with inhibition by pemetrexed and a PI3K inhibitor LY294002 (LY; 10 μ mol/L) by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. E, growth inhibition of A549 cells treated with increasing concentrations of pemetrexed and a fixed concentration of erlotinib (2 μ mol/L) or LY294002 (10 μ mol/L) by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Results are expressed as percentage cell number in drug-treated, relative to PBS-treated control, cells. Points, mean of three independent experiments; bars, SD.



might affect the cytotoxicity of pemetrexed and erlotinib in these cells.

Sequence of drug exposure modulates the cytotoxic synergism of erlotinib and pemetrexed. The effect of different exposure schedules of pemetrexed and erlotinib on cell cycle in H322 and A549 cells was assessed. Figure 3A illustrates the three exposure schedules tested that mimic possible clinical scenarios: (a) erlotinib for 24 h followed by addition of pemetrexed for a total of 72 h (E→P), (b) pemetrexed for 24 h followed by addition of erlotinib for a total of 72 h (P→E), and (c) erlotinib and pemetrexed concurrently for 72 h (E+P). The cell cycle effects of these different combinations compared with control untreated cells, and cells treated with erlotinib or pemetrexed alone for 72 h, are shown in Fig. 3B: erlotinib-sensitive H322 cells treated with erlotinib followed by pemetrexed (E→P) resulted in a similar cell cycle distribution pattern as observed with erlotinib alone (mainly G₁-phase arrest), which prevented the S-phase arrest effect of subsequent pemetrexed. Erlotinib-resistant A549 cells treated with erlotinib followed by pemetrexed (E→P) resulted in altered cell cycle distribution, although cells treated with erlotinib alone did not produce any additional G₁-phase arrest. Collectively, these data suggest that exposing erlotinib before pemetrexed abrogates the S-phase arrest by the latter agent to a much greater extent in erlotinib-sensitive than erlotinib-resistant cells.

These different exposure schedules altered the induction of apoptosis (Fig. 3C) and *in vitro* cytotoxicity (Fig. 3D). Compared with control untreated cells, simultaneous exposure to pemetrexed and erlotinib resulted in increased induction of apoptosis in both erlotinib-sensitive H322 and erlotinib-resistant A549 cells (Fig. 3D, left). However, exposure to pemetrexed after erlotinib (E→P) resulted in a decrease in

induction of apoptosis compared with concurrent exposure or exposure to pemetrexed before erlotinib (P+E or P→E) in both H322 and A549 cells (all-pairwise, one-way ANOVA test). These results were confirmed by the two-tailed, paired Student's *t* test. No difference in induction of apoptosis between the latter two schedules (P+E or P→E) was observed in either H322 or A549 cells (*P* > 0.05, two-tailed, paired Student's *t* test).

The interaction of pemetrexed and erlotinib in H322 and A549 cells was assessed by the median effect/combination index method (32, 41). Figure 3D illustrates that, in erlotinib-sensitive H322 cells, exposure to pemetrexed after erlotinib (E→P) resulted in a profound dose-dependent, antagonistic interaction as shown by the higher combination index values compared with pemetrexed given concurrently or before erlotinib (P+E or P→E). In contrast, in erlotinib-resistant A549 cells, concurrent exposure to pemetrexed and erlotinib (P+E), or exposure to pemetrexed before erlotinib (P→E), resulted in a synergistic interaction, whereas exposure to pemetrexed after erlotinib (E→P) resulted in only an additive interaction (combination index close to 1). These data confirm that exposure to erlotinib before pemetrexed results in a negative interaction in erlotinib-sensitive cells and an additive interaction in erlotinib-resistant NSCLC cells.

Reversal of the antagonistic interaction between erlotinib and pemetrexed in erlotinib-sensitive H322 cells. Studies from this laboratory have shown that optimal G₁-phase arrest and cytotoxicity can be achieved after 24-h exposure to erlotinib. Cytotoxicity is, however, obviated by 8 h after removal of erlotinib from the cell culture medium in erlotinib-sensitive H322 cells (42). To avoid the potential negative interaction between erlotinib and pemetrexed in patients, the effects of

removing erlotinib from the cell culture medium on the antagonistic effect between these agents compared with cells exposed to erlotinib before pemetrexed (E→P) were assessed (Fig. 4A). Results are shown in Fig. 4B and C. The antagonistic effect of erlotinib on pemetrexed-induced cytotoxicity could be reversed after erlotinib was removed from the cell culture medium, and this reversal was inversely related to the drug concentration level (i.e., combination index values were 0.29, 0.55, and 1.17 at the ED₅₀, ED₇₅, and ED₉₀ concentration levels, respectively). These data suggest that the negative interaction between erlotinib and pemetrexed in erlotinib-sensitive tumors may be avoided by discontinuing erlotinib in patients receiving the combination, for an interval equal to four to five half-lives, to ensure negligible erlotinib plasma levels at the time pemetrexed is administered.

Pemetrexed activates the PI3K/AKT signaling pathway in erlotinib-resistant human NSCLC cells. To gain insights into the mechanism(s) underlying the cytotoxic synergism between pemetrexed and erlotinib, the effect of pemetrexed on the EGFR pathway in erlotinib-resistant A549 cells was determined by Western blot analysis. Pemetrexed alone induced phosphorylated AKT expression among several EGFR-mediated downstream signaling molecules in both concentration-dependent (Fig. 5A) and time-dependent (Fig. 5B) manner, and this was associated with increased phosphorylated EGFR expression (Fig. 5C). Although erlotinib alone did not inhibit phosphorylated AKT expression as expected in the erlotinib-resistant cells (Fig. 5C, lane 3), erlotinib blocked the pemetrexed-induced activation of phosphorylated EGFR and phosphorylated AKT (Fig. 5C, lane 4). These results suggest that cells exposed to pemetrexed may respond to the cytotoxic insult with an EGFR-

mediated survival response, which sensitizes the cells to erlotinib.

The role of the PI3K/AKT pathway in mediating synergistic growth inhibition of pemetrexed and erlotinib was further confirmed in A549 cells using a potent PI3K inhibitor LY294002 by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (Fig. 5D). Erlotinib (2 μmol/L) or LY294002 (10 μmol/L) alone induced growth inhibition of A549 cells by 93% and 69%, respectively. Pemetrexed (0.5 μmol/L) alone resulted in growth inhibition of 34%, and the addition of erlotinib or LY294002 to pemetrexed led to a comparable increase in growth inhibition of 21% and 16%, respectively (*P* > 0.05, paired Student's *t* test). Figure 5E illustrates that A549 cells treated with increasing concentrations of pemetrexed in the presence of a constant concentration of erlotinib (2 μmol/L) or LY294002 (10 μmol/L) exhibited comparable growth inhibition measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (*P* > 0.05, paired Student's *t* test). Collectively, these data indicate that at least one of the mechanisms of erlotinib potentiation of pemetrexed cytotoxicity is related to its inhibition of the pemetrexed-activated, EGFR-dependent PI3K/AKT pathway.

Discussion

Four large, randomized phase III randomized trials of continuous daily administration of erlotinib or gefitinib in combination with conventional chemotherapy doublets as frontline therapy have failed to improve survival in patients with advanced NSCLC (18, 25–27). This has led to the conclusion that an EGFR TKI cannot be combined with

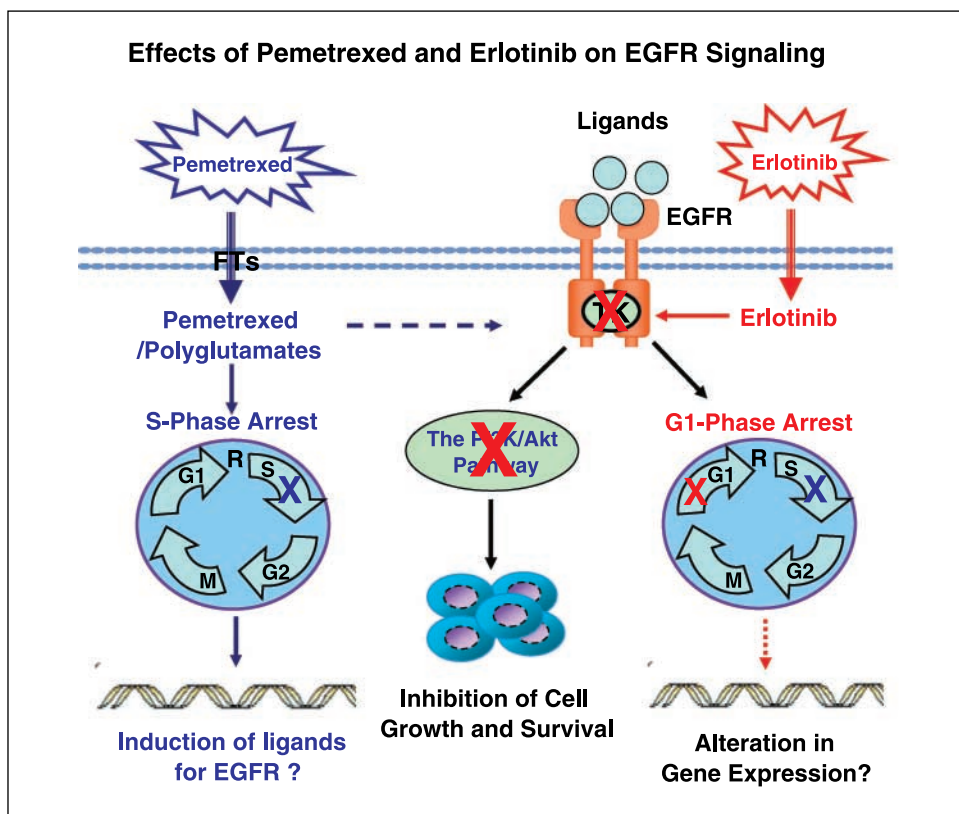


Fig. 6. Mechanisms of pemetrexed and erlotinib synergism. The schema illustrates proposed mechanisms of pemetrexed and erlotinib synergism in NSCLC cells. FTs, folate transporters; TK, tyrosine kinase; R, restriction point of cell cycle.

cytotoxic therapies in NSCLC and little interest in pursuing such a strategy. Nevertheless, a recent randomized, placebo-controlled, phase III study showed a significant improvement in overall and progression-free survival in patients with advanced pancreatic cancer receiving continuous daily erlotinib in addition to gemcitabine compared with gemcitabine alone (43). The current study provides a plausible explanation for these discordant results. Because cytotoxic agents and EGFR TKIs have definite activity as monotherapy and different mechanisms of action, the initial assumption was that at least an additive antitumor effect would be expected if both agents could be tolerated at full dose. However, the antitumor activity could be compromised if there was a negative interaction between the two agents or both acted redundantly on the same population of tumor cells (28, 44). Given the survival benefit observed with erlotinib monotherapy in chemotherapy-refractory NSCLC patients in the second- and third-line advanced disease setting, it seems likely that EGFR TKIs and cytotoxic agents act on different populations of tumor cells. Furthermore, there is growing laboratory evidence of a possible sequence-dependent antagonism between EGFR TKIs and cytotoxic agents as a result of the well-known G₁-phase arrest of tumor cells by EGFR TKIs, which protect tumor cells from the cell cycle-specific cytotoxic agents (28–30). This is consistent with the observation that tamoxifen, which blocks breast cancer cells in G₁, is most effective when administered sequentially rather than concurrently with systemic chemotherapy in patients with early and advanced breast cancer (45, 46).

The current studies indicate that exposure of NSCLC cells to erlotinib before pemetrexed confers a negative interaction that is much more pronounced in erlotinib-sensitive than in erlotinib-resistant cells. This is a result of an erlotinib-induced G₁-phase arrest that protects these cells from the cytotoxicity of subsequent exposure to pemetrexed. This negative interaction can be avoided by removing erlotinib from the cell culture medium for a sufficient interval (≥ 8 h) before exposure to pemetrexed. The data suggest that interrupting erlotinib before the subsequent administration of pemetrexed treatment should be the most effective sequence for combined treatment with these agents. Further, the synergism observed was independent of the mutation status of the *EGFR* and *K-Ras* genes or the intrinsic sensitivity to erlotinib. Thus, this schedule-modulated administration of pemetrexed and erlotinib could potentially benefit patient subgroups that have little benefit from EGFR TKI monotherapy. This could be particularly important for the 90%

of NSCLC patients with wild-type *EGFR* gene in the United States who respond poorly to EGFR TKI monotherapy (4, 14, 19). Extrapolating to the PA.3 study, these observations provide a potential mechanism for the observed synergism of continuous daily erlotinib and gemcitabine in patients with pancreatic cancer, in whom 90% of the tumors harbor *K-Ras* mutations, despite the lack of clinical activity of erlotinib monotherapy (43). Because erlotinib cannot induce G₁-phase arrest in tumors harboring *K-Ras* mutations, continuous daily erlotinib should have a minimal negative effect on the cytotoxicity of gemcitabine, another S-phase inhibitor that might induce an EGFR-dependent survival response similar to that of pemetrexed. It will be interesting to test whether clinical efficacy can be improved further by stopping erlotinib before the administration of gemcitabine.

The dependency of NSCLC tumors on the EGFR pathway for growth and survival is an important determinant of sensitivity to EGFR TKI monotherapy (9). Previous reports showed that inhibition of PI3K/AKT activity correlates with the sensitivity to EGFR TKIs in NSCLC cells harboring both a wild-type and a tyrosine kinase-activating mutant *EGFR* gene (38, 47, 48). Consistent with this, we found that pemetrexed induces an EGFR/PI3K/AKT-mediated growth and survival response in erlotinib-resistant A549 cells harboring the wild-type *EGFR* gene, thus rendering the cells more dependent on the EGFR pathway and more susceptible to the inhibition by erlotinib as summarized in Fig. 6. Likewise, a recent report showed that the mammalian target of rapamycin inhibitor rapamycin activates the PI3K/AKT pathway and enhances cytotoxicity of a PI3K/AKT inhibitor, LY294002 (49). Collectively, these observations suggest that the combination of erlotinib or a PI3K/AKT inhibitor with pemetrexed or a mammalian target of rapamycin inhibitor should be explored in NSCLC. Additionally, in patients with advanced cancer and large tumor burdens, pemetrexed and erlotinib may act on different population of tumor cells simultaneously. The antiangiogenesis property of erlotinib might be also of benefit in this setting (50, 51) along with enhanced transport of pemetrexed into tumor cells in the hypoxic, acidic regions of solid tumors mediated by a proton-coupled folate transporter that has high affinity for this agent (7, 52, 53). Based on the findings in this report, a randomized phase II study comparing the progression-free survival between an intermittent combination of erlotinib and pemetrexed (experimental arm) and pemetrexed alone (control arm) in patients with relapsing NSCLC has been initiated.

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