Molecular Mechanisms Underlying the Synergistic Interaction of Erlotinib, an Epidermal Growth Factor Receptor Tyrosine Kinase Inhibitor, with the Multitargeted Antifolate Pemetrexed in Non-Small-Cell Lung Cancer Cells

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ABSTRACT

Because the epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor erlotinib and the multitargeted antifolate pemetrexed are registered in the treatment of second-line non–small-cell lung cancer (NSCLC), empirical combinations of these drugs are being tested. This study investigated molecular mechanisms underlying their combination in six NSCLC cell lines. Cells were characterized by heterogeneous expression of pemetrexed determinants, including thymidylate synthase (TS) and dihydrofolate reductase (DHFR), and mutations potentially affecting chemosensitivity. Pharmacological interaction was studied using the combination index (CI) method, whereas cell cycle, apoptosis induction, and EGFR, extracellular signal-regulated kinases 1 and 2, and Akt phosphorylation were studied by flow cytometry, fluorescence microscopy, and enzyme-linked immunosorbent assays. Reverse-transcriptase polymerase chain reaction (RT-PCR), Western blot, and activity assays were performed to assess whether erlotinib influenced TS. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium assays demonstrated that EGFR and k-Ras mutations were related to erlotinib sensitivity, whereas TS and DHFR expression were related to pemetrexed sensitivity. Synergistic cytotoxicity was found in all cells, most pronounced with pemetrexed + erlotinib (24 h) → erlotinib (48 h) sequence (CI, 0.09–0.40), which was associated with a significant induction of apoptosis. Pemetrexed increased EGFR phosphorylation and reduced Akt phosphorylation, which was additionally reduced by drug combination (~70.6% in H1650). Erlotinib significantly reduced TS expression and activity, possibly via E2F-1 reduction, as detected by RT-PCR and Western blot, and the combination decreased TS in situ activity in all cells. Erlotinib and pemetrexed showed a strong synergism in NSCLC cells, regardless of their genetic characteristics. Induction of apoptosis, modulation of EGFR and Akt phosphorylation, and changes in the expression of critical genes involved in pemetrexed activity contribute to this synergistic interaction and support the clinical investigation of these markers.

Non–small-cell lung cancer (NSCLC) is the leading cause of cancer-related deaths in the Western world. Chemotherapy represents the backbone of treatment of advanced NSCLC, which represents more than 50% of cases diagnosed. A platinum-based doublet has become standard in first-line treatment (Pfister et al., 2004). Many patients who failed first-line treatment are in a sufficient performance status to receive second-line treatment. There are three agents presently registered for this indication: docetaxel, pemetrexed,
and erlotinib. Pemetrexed and erlotinib are distinctly less toxic than docetaxel, and their use has rapidly increased (Hanna et al., 2004; Shepherd et al., 2005). Both patient characteristics (never-smoking status, female gender, adenocarcinoma histology, East Asian race) and molecular markers are predictive of benefit to EGFR tyrosine-kinase inhibitors (TKIs), such as erlotinib and gefitinib. In particular, higher response rates to these targeted agents have been associated with the occurrence of EGFR-activating mutations (Lynch et al., 2004; Paez et al., 2004) and absence of k-Ras mutations (Eberhard et al., 2005).

Clinical benefit observed with erlotinib is not restricted to patients carrying EGFR-activating mutations (Shepherd et al., 2005), and the clinical response to EGFR-TKIs may be associated with EGFR copy number or expression (Dziadzio-uszko et al., 2006) or with molecular changes affecting several downstream EGFR intracellular signal transducers, such as the Ras/Raf/mitogen-activated protein kinase and the phosphatidylinositol-3’-kinase (PI3K)/Akt (Cappuzzo et al., 2004).

A key issue in the novel treatment modalities against NSCLC is the integration of EGFR-TKIs with chemotherapy. Targeted therapies should have limited overlapping toxicities in combination with conventional chemotherapy, and preclinical studies have shown that coadministration with EGFR-TKIs enhanced the effect of different cytotoxic agents against various tumor models (Magné et al., 2003; Morelli et al., 2005; Bianco et al., 2006), including NSCLC cells (Van Schaeybroeck et al., 2006; Li et al., 2007).

However, the combined treatment of patients with advanced NSCLC with either gefitinib or erlotinib and standard two-drug (gemcitabine/cisplatin and carboplatin/paclitaxel) chemotherapy regimens (Giaccone et al., 2004; Herbst et al., 2004, 2005; Gatzemeier et al., 2007) failed to improve survival. The lack of the expected outcome may have been caused by several factors: 1) predictive markers for response to EGFR-TKIs in combination with chemotherapy have not yet been identified, and therefore patient selection is still lacking, and 2) the choice of chemotherapeutic agents, doses, and schedules has been determined empirically or from single-agent studies (Eberhard et al., 2005). Therefore, further studies are needed to investigate new therapies combining erlotinib with cytotoxic chemotherapy and to describe molecular mechanisms and potential markers of drug combination activity.

Antimetabolites are widely used in combination regimens because of their ability to biochemically modulate the cytotoxicity of other drugs (Peters et al., 2000). Pemetrexed is an inhibitor of thymidylate synthase (TS), dihydrofolate reductase (DHFR), and glycaminide ribonucleotide formyltransferase (GARFT) (Shih et al., 1997). Resistance to pemetrexed may be related mainly to an increased TS expression (Siegmond et al., 2003), although limited uptake by the reduced folate carrier (RFC) or decreased polyglutamylation may be other important parameters (Mauritz et al., 2002). Preclinical studies demonstrated that its combination with cisplatin, taxanes, and gemcitabine produced additive or synergistic cytotoxicity (Tonkinson et al., 1999; Teicher et al., 2000; Giovannetti et al., 2005).

The ability of pemetrexed to deplete cellular ribonucleotide pools, modulate cell cycle, and induce apoptosis (Tonkinson et al., 1999) makes this drug an attractive agent for polychemotherapy regimens. In particular, pemetrexed acts on several pathways involved in DNA synthesis and cell death control, including the Akt pathway (Giovannetti et al., 2005), whereas recent studies showed that the TS inhibitor 5-fluorouracil (5-FU) increased EGFR phosphorylation, thus potentially favoring EGFR-TKI activity (Van Schaeybroeck et al., 2005). Moreover, in vitro studies demonstrated that EGFR-TKIs decreased TS expression and activity, determining synergistic interaction with 5’-deoxy-5-fluorouridine (Magné et al., 2003; Budman et al., 2006).

The present work was aimed at evaluating molecular mechanisms underlying the synergistic cytotoxicity between erlotinib and the antimetabolite pemetrexed through the in vitro assessment of their pharmacological interaction. We observed a strong synergistic interaction between these agents against a panel of six NSCLC cell lines, regardless of their genetic signature, related to either erlotinib or pemetrexed activity. Furthermore, we characterized several factors, including apoptosis induction, modulation of EGFR and Akt phosphorylation, and expression of critical genes involved in pemetrexed activity, which may contribute to this synergistic interaction.

Materials and Methods

Drugs and Chemicals. Erlotinib [N-(3-ethynylphenyl)-6,7-bis(2-methoxyethoxy)-4-quinazolinamine] was a gift from Roche Pharmaceuticals (Mannheim, Germany), whereas pemetrexed [L-glutamic acid N-(4-[2-(2-amino-4,7-dihydro-4-oxo-1-pyrrolo[2,3-d]pyrimidin-5-yl)ethyl]benzoyl]-disodium salt, heptahydrate] and LY294002 were a gift from Eli Lilly Corporation (Indianapolis, IN). The drugs were dissolved in dimethyl sulfoxide and sterile water and diluted in culture medium before use. RPMI 1640 medium and DMEM, fetal bovine serum, penicillin (50 IU/ml), and streptomycin (50 μg/ml) were from Invitrogen (Carlsbad, CA). [5-3H]dUMP and [5-3H]deoxy-cytidine were obtained from Moravek (San Diego, CA). All other chemicals were from Sigma (St. Louis, MO).

Cell Lines. Cell lines were maintained as monolayer cultures in RPMI 1640 medium or DMEM (containing 2 mM l-glutamine) supplemented with 10% heat-inactivated fetal bovine serum, penicillin, and streptomycin. The human NSCLC cell lines NCI-H460 (H460), NCI-H1703 (H1703), NCI-H292 (H292), and NCI-H1650 (H1650) were cultured in RPMI 1640 medium, whereas A549 and SW1573 cells were cultured in DMEM. Cells were grown in 75-cm2 flasks (Costar, Cambridge, MA) at 37°C in 5% CO2/95% air and harvested with trypsin-EDTA when they were in exponential growth.

Analysis of Mutations and Polymorphisms in Determinants of Drug Activity. The NSCLC cells used in this study have been characterized previously for EGFR and k-Ras mutations (Jannata et al., 2006). Furthermore, we evaluated genetic variations that may influence pemetrexed sensitivity, such as the polymorphic tandem repeat sequence of the enhancer region of the thymidylate synthase (TS) promoter (TSER) and the methylene tetrahydrofolate reductase (MTHFR) C677T polymorphism.

Cytotoxicity Assays. To evaluate the cytotoxic activity of single drugs, the cell growth inhibitory effect of erlotinib (0.001–50 μM, 72-h exposure) and pemetrexed (0.001–100 μM, 24- and 72-h treatment) was studied using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium assay. For this purpose, cells were plated at 104 cells/well, and growth inhibition was expressed as the percentage of control (vehicle-treated cells) absorbance (corrected for absorbance before drug addition). The IC50 value was calculated by nonlinear least-squares curve-fitting (Prism; GraphPad Software Inc., San Diego, CA).

Drug Combination Studies. Several studies have shown a more efficient interaction when cytotoxic chemotherapeutic agents are
given before EGFR-TKIs (Morelli et al., 2005; Van Schaeybroeck et al., 2005). Therefore, combination studies were focused on simultaneous treatment and on treatment with an initial 24-h exposure to both pemetrexed and erlotinib, followed by a washout and an additional 48-h treatment with erlotinib alone. According to the expected biological effects and pharmacokinetics data available, pemetrexed was used for 24 or 72 h, whereas erlotinib was always used for 72 h in a continuous or a pulsatile administration. Each combination was tested in at least six different concentrations, using a constant ratio calculated with respect to drug IC50 values. The cytotoxicity of the combination was compared with the cytotoxicity of each drug alone, using the combination index (CI), where CI < 0.9, CI = 0.9 to 1.1, and CI > 1.1 indicated synergistic, additive, and antagonistic effects, respectively. Data analysis was carried out using CalcuSyn software (Biosoft, Oxford, UK). Because we considered growth inhibition lower than 50% as not relevant, CI values at fraction affected (FA) of 0.5, 0.75, and 0.9 were averaged for each experiment, and this value was used to calculate the mean between experiments (Peters et al., 2000).

Cell Cycle Analysis. Cell cycle modulation induced by treatments at IC50 values for 72 h was studied by propidium iodide staining and flow cytometry analysis using a FACScan (BD Biosciences, San Jose, CA). Data analysis was carried out with CELLQuest (BD Biosciences), whereas cell cycle distribution was determined using Modfit software (Verity Software House, Topsham, ME).

Evaluation of Apoptosis. Erlotinib, pemetrexed, and their combination were also characterized for their ability to induce apoptosis, which was detected after 72-h drug exposure. Apoptosis was measured by evaluating the sub-G1 region of the previous FACS analysis and by fluorescence microscopy analysis with bisbenzimide staining (Giovannetti et al., 2005). Two hundred cells from randomly chosen microscopic fields were counted, and the apoptotic index (AI) was calculated as the percentage ratio between the number of cells displaying apoptotic features and the number of counted cells.

EGFR, ERK1/2, and Akt Phosphorylation Assays. To study the effect of drug treatments on the activation of EGFR and of ERK1/2 and Akt, cells were exposed to IC50 values of erlotinib, pemetrexed, and erlotinib-pemetrexed combination and stimulated with EGF (10 ng/ml), as described previously (Janmaat et al., 2003). After protein extraction from cell pellets, EGFR phosphorylation at the tyrosine residue at position 1173 [EGFR (pY1173)], dual phosphorylation of ERK2 at threonine 185 and tyrosine 185 [ERK2 (pTyr185/187)] and ERK1 at threonine 202 and tyrosine 204 [ERK1 (pTyr202/204)] and Akt phosphorylation at serine residue 473 [Akt (pS473)] were evaluated with specific enzyme-linked immunosorbent assays (BioSource International, Camarillo, CA), and normalized, respectively, to the total EGFR, ERK1/2 and Akt, and protein content (Bianco et al., 2006).

Pharmacological Interaction Study with the P38K Inhibitor LY294002. To evaluate whether inhibition of the PI3K/Akt pathway may modulate the effects of the erlotinib-pemetrexed combination, we studied the pharmacological interaction of LY294002 with erlotinib, pemetrexed, and their combination. The cell growth-inhibitory effect was detected in cells treated with erlotinib (0.001–50 μM), pemetrexed (0.001–100 μM), LY294002 (30 μM), and their combinations for 72 h, whereas drug interaction with LY294002 was assessed at a nonconstant concentration ratio using the CI method.

Real-Time RT-PCR. To compare the possible influence of gene expression profile on drug sensitivity, we selected cells characterized by heterogeneous patterns of EGFR and TS expression (Janmaat et al., 2003). However, the basal expression of EGFR and TS was also assessed in this study to evaluate the effect of 72-h treatment with IC50 levels of erlotinib, pemetrexed, and their combination in all cell lines. Because previous studies have shown a strong correlation between expression levels of TS and its upstream transcriptional regulator E2F-1 (Li et al., 1995; Huang et al., 2007), and because other studies showed that EGFR-TKIs affected E2F-1 (Kobayashi et al., 2006; Suenga et al., 2006), we also evaluated the expression of E2F-1 mRNA. Moreover we studied the expression levels of DHFR, GARP, RFC, γ-glutamyl hydrolase (γGH), and folylglutamate synthetase (FPGS). RNA was extracted by the QiaAmp RNA mini-Kit (QIAGEN, San Diego, CA) and reverse-transcribed (Bianco et al., 2006). Forward and reverse primers and probes were designed with Primer Express (Applied Biosystems, Foster City, CA) on the basis of TS, DHFR, FPGS, and GARP sequence (Giovannetti et al., 2005), whereas primers and probes for E2F-1, RFC, and γ-glutamyl hydrolase were obtained from Applied Biosystems Assay-on-Demand Gene expression products (Hs00157298_m1, Hs00228858_m1, and Hs00914167_m1). Finally, EGFR basal expression was checked as reported previously (Dziadziuszko et al., 2006). Amplification data were normalized to β-actin, and quantification of gene expression was performed using standard curves obtained with dilutions of cDNA from Quantitative-PCR Human-Reference Total-RNA (Stratagene, La Jolla, CA).

Western Blot. To evaluate the possible role of breast cancer resistance protein (BCRP) and multidrug-related proteins (MRPs) expression on pemetrexed sensitivity, as suggested in a study on freshly explanted human tumor specimens (Hanauske et al., 2007), we performed Western blot analysis of BCRP, MRP1, MRP2, MRP3, MRP4, and MRP5 expression in the panel of NSCLC cells. Total lysates were prepared in buffer containing 50 mM Tris, pH 7.6, 20% (v/v) glycerol, 5 mM dithiothreitol, 0.5% (v/v) Nonidet P-40, and 4.0% (v/v) of a protease inhibitor cocktail. Lysates were sonicated and centrifuged. The protein-containing supernatant was collected, and protein content was determined using a Bradford assay. In each lane of a minigel system (Bio-Rad Laboratories, Hertfordshire, UK), 30 μg of proteins was loaded. The following monoclonal antibodies (kindly provided by Dr. G. L. Schoffer, VU University Medical Centre, Amsterdam, The Netherlands) were used: rat anti-MRP1 (MRP-1; 1:500), -MRP4 (M1-J; 1:200), -MRP5 (M1-J; 1:250), and -BCRP (BXP-53; 1:200), as well as mouse anti-MRP2 (M3-II-6; 1:500) and -MRP3 (M3-II-2; 1:500). As secondary antibodies, horseradish peroxidase-conjugated rabbit anti-rat or anti-mouse (1:2000; Dako Denmark A/S, Glostrup, Denmark) were used. As a loading control, expression of β-actin was determined using an antibody against β-actin (1:10,000; Millipore Bioscience Research Agents, Temecula, CA).

Erlotinib, pemetrexed, and their combination, at IC50 values, were also studied for their ability to modulate protein expression of possible targets or surrogate markers of drug activity, such as TS and E2F-1. After 72-h exposure, cell pellets were collected and lysed, and 20 μg of proteins was loaded and separated on a 10% SDS-PAGE gel, followed by blotting onto a nitrocellulose membrane. The membrane was preincubated in blocking buffer (0.5% milk powder, 0.5% BSA in Tris-buffered saline/Tween 20 (10 mM Tris-HCl, pH 8.0, 0.15 M NaCl, 0.5% Tween 20)) for 1 h, whereas the primary polyclonal TS (1:1000, kindly provided by Dr. G. W. Aheme, Sutton, UK) and monoclonal E2F-1 (1:100, Santa Cruz Biotechnology, Santa Cruz, CA) antibodies were added overnight at 4°C. After washing in Tris-buffered saline/Tween 20, the blots were incubated for 1 h with anti-rabbit (for TS) and anti-mouse (for E2F-1) horseradish peroxidase-labeled secondary antibodies (1:2000; Dako Denmark A/S). Antibody binding was detected using enhanced chemiluminescence. Densitometric analysis of the images captured on the VersaDoc3000 instrument (Bio-Rad) was performed with the Kontron Analysis Image software (Kontron-Electronik, Munich, Germany).

Evaluation of TS Catalytic and in Situ Activity. To evaluate the possible modulation of TS activity, pellets of A549 and SW1573 cells treated as described above in the gene expression analyses were used. The catalytic activity of TS was measured by means of the 3H release during the TS-catalyzed conversion of 1 μM [3H]dUMP to TMP. Pemetrexed is only an effective TS inhibitor.
in its polyglutamylated form. Therefore, to be able to evaluate the long-term effect of pemetrexed, which is mostly mediated by polyglutamation, we also determined the potential inhibition of TS in intact cells after 72-h drug exposure at IC_{50} values. This TS in situ activity was measured in all cell lines by exposure to [5-3H]deoxycytidine (0.3 μM final concentration; specific activity, 1.6 mCi/mmol) during the last 2 h of the assay and by processing the medium of each sample as described above for the catalytic assay (van Triest et al., 1993).

Statistical Analysis. All experiments were performed in triplicate and repeated at least twice. Data were expressed as mean values ± S.E. and analyzed by Student’s t test or analysis of variance followed by the Tukey’s multiple comparison test. The Pearson/ Spearman correlation and regression analysis were used to demonstrate the relationship between gene expression profile and chemosensitivity, and between erlotinib and gefitinib cytotoxic activity, TS mRNA and protein expression, and modulation of E2F-1 and TS protein expression; the level of significance was P < 0.05.

**Results**

**Genetic Background of the NSCLC Cell Lines Regarding Determinants of Drug Activity.** DNA extracted from the panel of NSCLC cells was used to detect mutations and polymorphisms in genes potentially affecting erlotinib and pemetrexed activity, as reported in Table 1.

**Cytotoxicity of Pemetrexed and Erlotinib and Correlation with Genetic Background.** Erlotinib caused a concentration-dependent inhibition of proliferation in all cell lines, with IC_{50} values ranging from 0.20 to 18.17 μM, in H1650 and H460 cells, respectively (Table 1). A concentration-dependent inhibition of cell growth was also observed after 72-h exposure of H460 cells, respectively (Table 1). A concentration-dependent inhibition of cell growth was also observed after 72-h exposure to pemetrexed, with IC_{50} values ranging from 0.08 (SW1573) to 1.05 μM (H460). Higher IC_{50} values were obtained after 24-h exposure to pemetrexed followed by 48-h incubation in drug-free medium. In contrast, erlotinib exposure for 24 h, followed by washout and addition of new medium with erlotinib for the ensuing 48 h, slightly decreased IC_{50} values observed after continuous 72-h incubation. H1650 cells, carrying the EGFR-activating mutation Del746–750, were the most sensitive cells, whereas H460 cells, harboring the G61H mutation in k-Ras, were the least sensitive cells to both erlotinib and pemetrexed, after all of the different exposures. In contrast, no relationship was found between tandem repeat sequence of the enhancer region of the TSER promoter or MTHFR polymorphisms and pemetrexed chemosensitivity. In this panel of NSCLC cells, a significant correlation was found between erlotinib and gefitinib sensitivity (R^2 = 0.92, P = 0.017).

**Correlation between Gene Expression and Chemosensitivity.** The expression of genes involved in the action of erlotinib and pemetrexed showed a large heterogeneity, suggesting that the different sensitivity to drug treatment may be related to variable cellular gene expression profiles (Table 1). No correlation was found between EGFR mRNA gene expression and erlotinib sensitivity in this panel of NSCLC cells. However, a clear correlation was found between the IC_{50} values of pemetrexed and the expression of its target enzymes TS (R^2 = 0.72, P = 0.030) and DHFR (R^2 = 0.97, P = 0.003); the lower chemosensitivity of the H460 cells seemed mostly dependent on the higher expression levels of the genes encoding TS and DHFR with respect to the other cells. In addition, this cell line has a low expression of RFC, which may be associated with a low uptake of pemetrexed despite a high expression of FPGS. In contrast, the sensitive H1650 cells had a high RFC expression.

**Correlation between Protein Expression and Chemosensitivity.** TS protein expression was measured with Western blot, and the analysis of band density demonstrated a significant link between TS mRNA and protein expression in control cells (R^2 = 0.92, P = 0.002). A significant correlation was also found between TS protein expression and pemetrexed sensitivity (R^2 = 0.73, P = 0.03; data not shown). In contrast, no correlation was found between the protein expression of BCRP and MRPs and pemetrexed sensitivity (Fig. 1).

**Pharmacological Interaction.** Because the CI method recommends a ratio of concentrations at which drugs are equipotent, the combination studies were performed using

<table>
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<tr>
<th>IC_{50}</th>
<th>Pemetrexed (24 h) (μM)</th>
<th>0.83 ± 0.04</th>
<th>0.33 ± 0.14</th>
<th>1.78 ± 0.68</th>
<th>0.16 ± 0.01</th>
<th>0.28 ± 0.04</th>
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<td>0.08 ± 0.02</td>
<td>1.05 ± 0.34</td>
<td>0.10 ± 0.01</td>
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<td>Erlotinib (24 + 48 h) (μM)</td>
<td>2.16 ± 0.28</td>
<td>6.29 ± 0.37</td>
<td>15.31 ± 0.74</td>
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<td>Erlotinib (72 h) (μM)</td>
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<td>18.17 ± 0.79</td>
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<td>Gefitinib (72 h) (μM)^b</td>
<td>3.55 ± 1.02</td>
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**Mutations**

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**Polymorphisms**

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**Gene expression**

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^a IC_{50} values for gefitinib and mutational status (Wt, wild type; Mut, mutated) for EGFR and k-Ras have been partially reported previously (Janmaat et al., 2006).

^b Mean values calculated in comparison with standard curves and with respect to the respective expression values of the housekeeping gene β-actin; S.E. values are less than 20%. Data were reported with two decimals, given the accuracy of the method used.
fixed ratios with IC<sub>50</sub> values calculated from the previous cytotoxicity analysis for the different drug treatments in each cell line. Both combination schedules reduced the IC<sub>50</sub> values of pemetrexed in all cell lines. Representative growth inhibition curves for SW1573 cells are shown in Fig. 2, A and B. The multiple drug-effect analysis revealed additive-synergistic effects in the simultaneous treatment and strong synergism in the 24-h pemetrexed + erlotinib followed by 48-h erlotinib treatment. For example, the CI plots (Fig. 2C) in SW1573 cells showed a clear synergistic interaction at the more relevant FA values (≥0.9). In all of our experiments, we obtained growth-inhibition curves still decreasing at the highest concentration values toward the d = 0 absorption values, which for the combination were well below those for the single drugs. Therefore, the computer-generated CI values at FA = 0.9 are correct, and we included this value for calculation of the mean of CI values. The average CI values for erlotinib-pemetrexed combinations in all of the NSCLC cell lines are summarized in Fig. 2, D and E. Simultaneous drug administration resulted in a synergistic-additive effect in A549 cells (mean CI, 0.92), whereas a synergistic interaction was observed in all of the other NSCLC cell lines, with CI values ranging from 0.46 (H460 cells) to 0.66 (H292) (Fig. 2D). The pemetrexed + erlotinib (24 h) → erlotinib (48 h) schedule proved to be the most effective combination in all cell lines (Fig. 2E), displaying the strongest synergistic effect in H460 (mean CI, 0.09). To evaluate the mechanisms underlying the additive-synergistic (in A549 cells) and the synergistic interaction (in all of the other NSCLC cells), several biochemical analyses were performed with the simultaneous combination, as detailed below.

**Cell Cycle Modulation and Induction of Apoptosis.** DNA flow cytometry studies were performed to evaluate the effect of erlotinib, pemetrexed, and their combination on the cell cycle distribution and to determine whether their cell cycle-modulating activity might provide clues to optimize drug scheduling. Both agents were able to affect the cell cycle of NSCLC cells (Table 2). Erlotinib caused a 1.2- to 1.4-fold increase in the population of cells in the G<sub>1</sub> phase. In contrast, a 72-h treatment with pemetrexed resulted in a 1.3- to 2.0-fold increase in the percentage of cells in the S phase. The increment in the S phase cell population was most pronounced in A549 and H1650 cells (1.7- and 2-fold, respectively), whereas H292 cells showed only a 1.3-fold enhancement. A marked increase in the S phase population with respect to controls was also observed for the simultaneous combination in all NSCLC cells. In the 24-h pemetrexed + erlotinib followed by 48-h erlotinib schedule, however, the increase in the S phase population was lower, whereas more cells accumulated in the G<sub>M</sub> phase.

All treatments induced cell death, as shown by the presence of a cell population with sub-G<sub>1</sub> DNA content in the FACS analysis, which was confirmed by fluorescence microscopy analysis after bisbenzimide staining to determine the AI values (Table 2). Cells exposed to erlotinib, pemetrexed, and their combination, at the IC<sub>50</sub> values, presented typical apoptotic morphology with cell shrinkage, nuclear condensation, and fragmentation, and rupture into apoptotic bodies. Erlotinib slightly increased AI with respect to controls in A549, SW1573, H460, and H1703 cells, whereas higher AI values were observed in the more sensitive cell lines (8.0 and 6.5% in H1650 and H292 cells, respectively). Pemetrexed induced more apoptosis than erlotinib, with AI values ranging from 6.2 to 13.7% in H460 and H292 cells, respectively. The combination of the two drugs additionally increased the AI, up to 21.2% in H292 and H1650 cells, and the 24-h pemetrexed + erlotinib followed by 48-h erlotinib schedule was slightly more active and caused a significant induction of apoptosis compared with both controls and pemetrexed-treated cells in all cell lines.

**Modulation of EGFR, ERK1/2, and Akt Phosphorylation.** As expected, erlotinib induced a significant suppression of EGF-induced phosphorylation of EGFR at the tyrosine residue pY1173 in all of the NSCLC cell lines, with percentages of reduction of EGFR-phosphorylated protein ranging from −39.6 to −65.5% with respect to controls in A549 and H1650 cells, respectively. Conversely, pemetrexed significantly enhanced pY1173-EGFR levels, varying from +33.3 to +74.4% in all cell lines, whereas drug combinations reduced the phosphorylation status of EGFR but generally to a lower extent than erlotinib alone (Fig. 3A). All of these molecular changes, which occurred before 24 h, might influence the activity of erlotinib when this agent is added to the cells for 48 h after 24-h exposure to pemetrexed + erlotinib.

Because EGFR signaling is transduced mainly through the Akt and ERK1/2 kinase pathways, we investigated the phosphorylation status of Akt and ERK1/2 to determine their activity after drug treatment. Erlotinib resulted in an inhibition of pERK1/2 and pAkt in all of the NSCLC cells. In particular, pERK1/2 levels were potently (>50%) down-regulated by erlotinib in H460, H1650, and H292, whereas a lower degree of inhibition (approximately 25%) was detected in A549, SW1573, and H1703 cells. Pemetrexed exposure hardly affected ERK1/2 phosphorylation. A slight reduction of pERK1/2, less pronounced than the one observed with erlotinib alone, was detected after drug combination in all cells (Fig. 3B). Akt phosphorylation at the serine residue pS473 was also significantly decreased (>50%) by erlotinib in A549, SW1573, H292, and H1650, whereas the inhibition was less efficient (approximately 30%) in H460 and H1703 cells and also by pemetrexed. Akt phosphorylation was addi-
tionally reduced by the simultaneous combination of erlotinib and pemetrexed, with a degree of inhibition up to −70.6% in H1650 cells (Fig. 3C).

**Synergistic Cytotoxic Effects of Erlotinib and Pemetrexed with LY294002.** To determine whether the effects of erlotinib and pemetrexed on EGFR phosphorylation and the activity of downstream pathways are responsible for their synergistic interaction, we investigated the effect of the specific PI3K inhibitor LY294002 at an effective concentration (Janmaat et al., 2006). LY294002 enhanced the growth inhibition of erlotinib in all NSCLC cell lines tested, with IC_{50} values ranging from 0.05 to 8.01 μM in H1650 and H460 cells, respectively. Similar effects were observed in the combination with pemetrexed and with the erlotinib-pemetrexed simultaneous combination. The mean CI values showed an additive-synergistic interaction in all of these combinations (Fig. 4), indicating that additional inhibition of PI3K by LY294002 led to a somewhat enhanced effect.

**Modulation of TS and DHFR mRNA and Protein Expression by Erlotinib, Pemetrexed, and Their Combination.** To gain further insight into the mechanisms involved in regulating the interaction between erlotinib and pemetrexed, we examined changes in TS and DHFR mRNA expression in treated cells. In comparison with the respective controls, all of the NSCLC cells treated with erlotinib presented a 1.3- to 1.9-fold decrease (statistically significant reduction was observed in five of six cell lines) in TS expression. Similar results were detected after erlotinib-pemetrexed simultaneous combination, whereas pemetrexed significantly increased TS mRNA expression in four of six cell lines (Fig. 5A). Erlotinib-treated cells were also characterized by a significant reduction in DHFR mRNA levels. In particular, DHFR expression was markedly down-regulated by erlotinib in A549 (−63.2%), SW1573 (−70.3%), H460 (−79.4%), H292 (−59.6%), and H1650 (−86.5%), whereas a lower degree of inhibition (−25.0%) was detected in H1703 cells. Furthermore, a 1.4- to 1.9-fold reduction in DHFR mRNA levels in A549, H1703 cells exposed to the drug combination, whereas pemetrexed treatment resulted in a −28.9% reduction in A549, a 1.4-fold increase in H292 and no significant modulation in the other NSCLC cells.

TS expression was also studied at the protein level, using Western blotting and densitometry. A representative example is shown in Fig. 5C. This analysis revealed that erlotinib and pemetrexed affected TS protein expression in most
NSCLC cells. In particular, a marked induction was detected in H292, H460, and H1703 pemetrexed-treated cells, whereas erlotinib repressed TS expression, and the faintest bands were observed in the extracts of H1650 and H292 cells. A reduction of TS expression was also detected in NSCLC cells treated with the erlotinib-pemetrexed combination.

Modulation of E2F-1 mRNA and Protein Expression by Erlotinib, Pemetrexed, and Their Combination. To further investigate the causes of the decrease in TS and DHFR mRNA after erlotinib exposure, we also evaluated E2F-1 mRNA. Both erlotinib and the drug combination significantly reduced E2F-1 mRNA levels in SW1573, H292, H460, H1650, and H1703 cells, as shown in Fig. 5B. Similar results were observed in the blots of E2F-1, showing a slight increase in E2F-1 protein expression in pemetrexed-treated cells, whereas a reduction was observed after both erlotinib and erlotinib-pemetrexed exposure in most NSCLC cells. Finally, the modifications in TS and E2F-1 expression levels after erlotinib treatment were positively correlated (data not shown).

Modulation of TS Catalytic Activity in Cell Extracts and in Situ Activity. Given the changes in TS levels described above, we measured the activity of this enzyme using both a catalytic assay in cell extracts and an in situ assay in intact cells. The catalytic assay demonstrated a significant increase in TS activity in pellets from cells treated with pemetrexed for 72 h, in line with the results of TS mRNA and protein expression analysis, whereas erlotinib significantly decreased TS activity (by 35 and 60% in A549 and SW1573 cells, respectively). No clear differences with respect to controls were detected in samples exposed to the erlotinib-pemetrexed simultaneous combination (Fig. 5D).

Because in the extracts the drug is washed away, we also determined the activity of the enzyme in intact cells. In contrast to the results of the catalytic assay, we found that pemetrexed significantly inhibited TS in all cell lines when measured using the in situ assay. In addition, erlotinib inhibited the TS in situ activity (Fig. 5E). It is most interesting that the combination almost completely inhibited the TS in situ, and statistical analysis revealed significant reductions with respect to those observed after pemetrexed exposure.

**Discussion**

The present study demonstrates that the combination of two targeted agents, the biological agent erlotinib against EGFR-TK and the cytotoxic compound pemetrexed against TS, was strongly synergistic in a panel of NSCLC cell lines characterized by different molecular properties. In particular, these cells have either favorable characteristics for sen-

### TABLE 2

<table>
<thead>
<tr>
<th>Cells and Treatment</th>
<th>G1 Phase</th>
<th>S Phase</th>
<th>G2/M Phase</th>
<th>Apoptotic Index</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>%</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>A549</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>59.9 ± 2.4</td>
<td>21.1 ± 1.4</td>
<td>18.9 ± 1.4</td>
<td>1.2 ± 0.2</td>
</tr>
<tr>
<td>Erlotinib (E)</td>
<td>69.2 ± 2.0</td>
<td>18.2 ± 1.5</td>
<td>12.5 ± 2.3</td>
<td>4.5 ± 0.5*</td>
</tr>
<tr>
<td>Pemetrexed (P)</td>
<td>40.5 ± 5.0</td>
<td>37.0 ± 2.2</td>
<td>22.5 ± 1.4</td>
<td>9.2 ± 0.2*</td>
</tr>
<tr>
<td>E + P (72 h)</td>
<td>40.9 ± 1.7</td>
<td>39.1 ± 1.8</td>
<td>19.9 ± 1.7</td>
<td>11.8 ± 0.6*</td>
</tr>
<tr>
<td>E + P (24 h)–E (48 h)</td>
<td>34.0 ± 1.2</td>
<td>37.4 ± 3.2</td>
<td>28.6 ± 2.0</td>
<td>16.7 ± 2.2**</td>
</tr>
<tr>
<td>SW1573</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>47.2 ± 4.0</td>
<td>24.6 ± 2.3</td>
<td>28.2 ± 1.9</td>
<td>0.8 ± 0.2</td>
</tr>
<tr>
<td>Erlotinib (E)</td>
<td>55.0 ± 6.6</td>
<td>23.1 ± 9.0</td>
<td>21.9 ± 2.2</td>
<td>4.8 ± 1.0*</td>
</tr>
<tr>
<td>Pemetrexed (P)</td>
<td>32.6 ± 1.8</td>
<td>39.1 ± 2.1</td>
<td>28.2 ± 0.2</td>
<td>10.8 ± 0.6*</td>
</tr>
<tr>
<td>E + P (72 h)</td>
<td>34.1 ± 5.7</td>
<td>36.6 ± 4.0</td>
<td>29.3 ± 1.8</td>
<td>13.2 ± 1.9*</td>
</tr>
<tr>
<td>E + P (24 h)–E (48 h)</td>
<td>33.8 ± 3.7</td>
<td>33.2 ± 0.1</td>
<td>33.0 ± 3.6</td>
<td>15.8 ± 1.9**</td>
</tr>
<tr>
<td><strong>H460</strong></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>41.8 ± 3.0</td>
<td>25.0 ± 2.0</td>
<td>33.2 ± 1.3</td>
<td>1.3 ± 0.3</td>
</tr>
<tr>
<td>Erlotinib (E)</td>
<td>57.7 ± 3.2</td>
<td>19.2 ± 3.7</td>
<td>23.1 ± 3.7</td>
<td>3.5 ± 1.0*</td>
</tr>
<tr>
<td>Pemetrexed (P)</td>
<td>28.0 ± 3.9</td>
<td>34.6 ± 2.9</td>
<td>37.4 ± 2.2</td>
<td>6.2 ± 0.8*</td>
</tr>
<tr>
<td>E + P (72 h)</td>
<td>30.1 ± 3.5</td>
<td>35.3 ± 2.8</td>
<td>34.6 ± 1.8</td>
<td>9.7 ± 0.9**</td>
</tr>
<tr>
<td>E + P (24 h)–E (48 h)</td>
<td>27.6 ± 0.1</td>
<td>34.8 ± 0.5</td>
<td>37.6 ± 0.5</td>
<td>16.2 ± 2.8**</td>
</tr>
<tr>
<td><strong>H292</strong></td>
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<td></td>
<td></td>
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</tr>
<tr>
<td>Control</td>
<td>46.8 ± 2.2</td>
<td>24.7 ± 2.2</td>
<td>28.5 ± 4.4</td>
<td>1.0 ± 0.3</td>
</tr>
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<td>Erlotinib (E)</td>
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<td>19.7 ± 1.9</td>
<td>17.6 ± 5.0</td>
<td>6.5 ± 1.0*</td>
</tr>
<tr>
<td>Pemetrexed (P)</td>
<td>34.7 ± 2.0</td>
<td>33.1 ± 1.6</td>
<td>32.1 ± 3.5</td>
<td>13.7 ± 1.0**</td>
</tr>
<tr>
<td>E + P (72 h)</td>
<td>35.0 ± 1.5</td>
<td>37.8 ± 1.3</td>
<td>27.2 ± 0.4</td>
<td>18.5 ± 0.3**</td>
</tr>
<tr>
<td>E + P (24 h)–E (48 h)</td>
<td>33.8 ± 2.6</td>
<td>32.7 ± 3.7</td>
<td>33.5 ± 6.3</td>
<td>21.2 ± 2.2**</td>
</tr>
<tr>
<td><strong>H1650</strong></td>
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<tr>
<td>Control</td>
<td>60.1 ± 2.1</td>
<td>24.2 ± 0.8</td>
<td>15.7 ± 1.3</td>
<td>1.2 ± 0.2</td>
</tr>
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<td>Erlotinib (E)</td>
<td>73.1 ± 1.4</td>
<td>18.9 ± 0.2</td>
<td>7.9 ± 1.3</td>
<td>8.0 ± 0.1*</td>
</tr>
<tr>
<td>Pemetrexed (P)</td>
<td>34.6 ± 5.1</td>
<td>47.5 ± 1.6</td>
<td>17.9 ± 3.5</td>
<td>10.7 ± 0.6*</td>
</tr>
<tr>
<td>E + P (72 h)</td>
<td>37.3 ± 6.5</td>
<td>51.3 ± 1.5</td>
<td>11.4 ± 5.0</td>
<td>19.3 ± 0.4**</td>
</tr>
<tr>
<td>E + P (24 h)–E (48 h)</td>
<td>43.5 ± 5.1</td>
<td>35.5 ± 7.5</td>
<td>21.0 ± 2.4</td>
<td>21.2 ± 0.4**</td>
</tr>
<tr>
<td><strong>H1703</strong></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Control</td>
<td>47.5 ± 2.1</td>
<td>38.7 ± 2.8</td>
<td>13.8 ± 0.7</td>
<td>1.3 ± 0.2</td>
</tr>
<tr>
<td>Erlotinib (E)</td>
<td>58.8 ± 1.6</td>
<td>32.2 ± 1.1</td>
<td>9.0 ± 0.5</td>
<td>4.2 ± 0.6*</td>
</tr>
<tr>
<td>Pemetrexed (P)</td>
<td>40.2 ± 0.2</td>
<td>55.3 ± 0.7</td>
<td>4.4 ± 0.7</td>
<td>8.0 ± 0.5*</td>
</tr>
<tr>
<td>E + P (72 h)</td>
<td>31.6 ± 2.0</td>
<td>56.2 ± 3.3</td>
<td>12.2 ± 1.2</td>
<td>10.5 ± 1.3*</td>
</tr>
<tr>
<td>E + P (24 h)–E (48 h)</td>
<td>38.6 ± 1.7</td>
<td>46.1 ± 7.8</td>
<td>15.4 ± 6.0</td>
<td>13.2 ± 0.7**</td>
</tr>
</tbody>
</table>

* P < 0.05 with respect to control cells.
** P < 0.05 with respect to pemetrexed-treated cells.
** Cells were treated for 72 h to IC50 concentrations of the single drugs and of the combinations.
itivity to either erlotinib or pemetrexed, such as the occurrence of the activating EGFR mutations and a low TS activity or unfavorable properties, such as k-Ras mutations or a high TS activity. However, no predictive biomarkers for clinical outcome to combinations of EGFR-TKIs with chemotherapy have been identified until now, and the molecular mechanisms underlying pharmacological interaction in the preclinical setting were rarely explored.

In a recent article, schedule-dependent synergism of pemetrexed and erlotinib was found, associated with pemetrexed-induced EGFR phosphorylation (Li et al., 2007). Similar effects were observed for other cytotoxic drugs in NSCLC cells displaying a synergistic interaction with gefitinib (Van Schaeybroeck et al., 2006) and with 5-FU in colorectal cancer cells (Van Schaeybroeck et al., 2005). However, our findings are novel because they show that the synergistic interaction seems to be mediated by several mechanisms, which enhanced both the sensitivity to erlotinib and to pemetrexed (Table 3). In addition to the effect of pemetrexed on EGFR phosphorylation, downstream mediators of the EGFR signal transduction pathway were affected not only by erlotinib but also by pemetrexed, which reduced Akt phosphorylation.

Previous work has shown controversial results regarding the modulation of phospho-Akt by pemetrexed (Giovannetti et al., 2005; Li et al., 2007), which may be related to the discrepancy between drug exposure conditions and different sensitivity of experimental methods. Our findings are in agreement with several studies demonstrating a reduced Akt phosphorylation after exposure to the antimetabolite gemcitabine (Giovannetti et al., 2005; Bianco et al., 2006; Chun et al., 2006; Mey et al., 2006; Feng et al., 2007). In particular, Feng et al. (2007) proposed a model of gemcitabine-induced apoptosis via EGFR degradation, explaining the possible mechanism by which a cytotoxic compound may affect EGFR and Akt signaling pathways. Drug treatment, through c-Src activation, leads to EGFR phosphorylation, which promotes ubiquitination of the receptor. EGFR is then targeted to proteosomes and lysosomes for degradation, resulting in phospho-Akt down-regulation and induction of apoptosis (Feng et al., 2007).

These data also fit with the concept that an additional inhibition may be required to prevent the inhibition of one pathway by an EGFR antagonist and may lead to optimal use of alternative signaling pathways (Janmaat et al., 2006). Furthermore, the combination of pemetrexed and erlotinib with the PI3K inhibitor LY294002 resulted in a synergistic interaction, suggesting that combinations of specific signal transduction inhibitors targeting different steps of EGFR-PI3K pathways may be a successful strategy against NSCLC.
Because phospho-Akt regulates antiapoptotic mechanisms and previous in vitro studies showed that its down-regulation by pemetrexed correlated with the enhancement of gemcitabine-induced apoptosis and antitumor activity in lung and bladder cancer cells (Giovannetti et al., 2005; Mey et al., 2006), the reduction of pS-473Akt may explain the increased apoptosis found in the pemetrexed-erlotinib combination.

This increased induction in apoptosis may be related to cell cycle modulation, which was reported to be important for the efficacy of the combination of EGFR-TKIs with cytotoxic compounds (Morelli et al., 2005; Bianco et al., 2006). Cellular damage induced by chemotherapy can convert EGFR ligands from growth factors into survival factors for cancer cells that express functional EGFR (Mendelsohn and Baselga, 2000).

**Fig. 5.** Modulation of TS expression and activity. Top and bottom, left, modulation of TS (A) and E2F-1 (B) mRNA as determined by real-time RT-PCR. Protein expression as determined by Western blots; one representative blot (B) out of at least three independent Western blots is shown for TS and E2F-1 protein expression. Bottom, right, modulation of TS catalytic and TS in situ activity. The TS in situ activity values were calculated as percentages of values obtained in control cells (215, 321, 344, 251, and 311 pmol/h/10^6 cells in A549, SW1573, H460, H292, H1650, and H1703 cells, respectively). All effects were studied in NSCLC cells treated for 72 h with erlotinib, pemetrexed, and their simultaneous combination at IC_{50} concentrations. *, significantly different from controls (P < 0.05). **, significantly different from cells treated with pemetrexed (P < 0.05).

**TABLE 3**

Proposed molecular mechanisms involved in the additive-synergistic interaction of erlotinib and pemetrexed combination in NSCLC cells

<table>
<thead>
<tr>
<th>Mechanism</th>
<th>A549</th>
<th>SW1573</th>
<th>H460</th>
<th>H292</th>
<th>H1650</th>
<th>H1703</th>
</tr>
</thead>
<tbody>
<tr>
<td>Increase of EGFR phosphorylation by pemetrexed^a</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<td>+</td>
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<tr>
<td>Induction of apoptosis^b^c</td>
<td>+</td>
<td>+</td>
<td>+/−</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Reduction of Akt phosphorylation^d^e</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Reduction of E2F-1 mRNA expression^f^g</td>
<td>+/−</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+/−</td>
<td>+/−</td>
</tr>
<tr>
<td>Reduction of DHFR mRNA expression^h^i</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+/−</td>
<td>+/−</td>
</tr>
<tr>
<td>Reduction of TS mRNA expression^j^k</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Reduction of TS in situ activity^l^m</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

^a The modulation was classified as +/−, +, or ++ if the increase or reduction with respect to control values were <25%, between 25 and 50%, or >50%, respectively.
^b The apoptosis induction was evaluated by AI values, as described under Materials and Methods, and was classified as +/−, +, or ++ if AI values were <10%, between 10 and 15%, or >15%, respectively.
^c Cells were treated for 72 h to IC_{50} concentrations of the simultaneous erlotinib-pemetrexed combination.
In this context, the blockade of EGFR signaling after the exposure to cytotoxic drugs could cause irreparable cell damage leading to apoptosis. Therefore, the 24-h pemetrexed + erlotinib combination followed by 48-h erlotinib exposure may favor this process, because treatment with erlotinib after pemetrexed may prevent repair of cell damage and induce apoptosis.

In addition to the effects of pemetrexed on signaling pathways, the present study also shows that erlotinib interfered with the cytotoxic activity of pemetrexed. Indeed, erlotinib significantly inhibited the activity of TS, whose expression is significantly correlated with pemetrexed sensitivity both in the preclinical and in the clinical setting (Giovannetti et al., 2005; Gomez et al., 2006). These data are in agreement with previous observations that not only antibiotics and 5-FU modulate TS. Vinorelbine suppressed TS expression in the PC14 NSCLC cells, favoring the activity of 5-FU (Matsumoto et al., 2004), whereas other drugs such as platinum derivatives and irinotecan can down-regulate TS levels as well (van der Wilt et al., 1992; Guichard et al., 1998).

For instance, TS, as an RNA binding protein, also regulates its own synthesis by impairing the translation of its mRNA, and the binding to a specific inhibitor leads to up-regulation of TS protein (Chu et al., 1991). In agreement with this hypothesis and with the observed increase in TS mRNA expression, as detected previously with pemetrexed and 5-FU (Peters et al., 2002; Mauritz et al., 2007), TS protein expression and catalytic activity in cell extracts were enhanced after pemetrexed exposure.

Recent studies reported that EGFR-TKIs decreased TS expression and activity in breast and colorectal cancer cells, showing synergistic interaction with 5-deoxy-5-fluorouridine (Magne et al., 2003; Budman et al., 2006). In the latter study, it was postulated that TS down-regulation was related to a decrease in S phase and increase of the G1 phase. However, the marginal increase in G1 phase observed in the present study does not fit with this hypothesis, and the effects on mRNA and protein expression suggest that TS modulations are caused by mechanisms involving transcriptional control.

In particular, our data show a reduction of E2F-1 mRNA and protein expression after both erlotinib and erlotinib-pemetrexed exposure. These results may be related to the nuclear effects of EGFR, which influence the activity of some cell cycle proteins and transcription factors, including E2F-1 (Lo and Hung, 2006). For instance, TS, as an RNA binding protein, also regulates its own synthesis by impairing the translation of its mRNA, and the binding to a specific inhibitor leads to up-regulation of TS protein (Chu et al., 1991). In agreement with this hypothesis and with the observed increase in TS mRNA expression, as detected previously with pemetrexed and 5-FU (Peters et al., 2002; Mauritz et al., 2007), TS protein expression and catalytic activity in cell extracts were enhanced after pemetrexed exposure.

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