

Synergistic Antitumor Activity of Sorafenib in Combination with Epidermal Growth Factor Receptor Inhibitors in Colorectal and Lung Cancer Cells

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Abstract

Purpose: Cancer cell survival, invasion, and metastasis depend on cancer cell proliferation and on tumor-induced angiogenesis. We evaluated the efficacy of the combination of sorafenib and erlotinib or cetuximab.

Experimental Design: Sorafenib, erlotinib, and cetuximab, alone or in combination, were tested *in vitro* in a panel of non-small cell lung cancer (NSCLC) and colorectal cancer cell lines and *in vivo* in H1299 tumor xenografts.

Results: Epidermal growth factor receptor (EGFR) ligand mRNAs were expressed in all NSCLC and colorectal cancer cell lines with variable levels ranging from 0.4- to 8.1-fold as compared with GEO colorectal cancer cells. Lung cancer cells had the highest levels of vascular endothelial growth factors (VEGF) A, B, and C, and of VEGF receptors as compared with colorectal cancer cells.

Combined treatments of sorafenib with erlotinib or cetuximab produced combination index values between 0.02 and 0.5, suggesting a significant synergistic activity to inhibit soft agar colony formation in all cancer cell lines, which was accompanied by a marked blockade in mitogen-activated protein kinase and AKT signals. The *in vitro* migration of H1299 cells, which expressed high levels of both VEGF ligands and receptors, was inhibited by treatment with sorafenib, and this effect was significantly increased by the combination with anti-EGFR drugs. In nude mice bearing established human H1299 xenografts, treatment with the combination of sorafenib and erlotinib or cetuximab caused a significant tumor growth delay resulting in 70 to 90 days increase in mice median overall survival as compared with single-agent sorafenib treatment.

Conclusions: Combination treatment with sorafenib and erlotinib or cetuximab has synergistic anti-tumor effects in human colorectal and lung cancer cells. *Clin Cancer Res*; 16(20): 4990–5001. ©2010 AACR.

Tumor cell growth, survival, invasion, and metastasis depend on efficient tumor cell proliferation and tumor-induced angiogenesis. Several autocrine and paracrine growth factors regulate these processes during cancer de-

velopment and progression. In this respect, key autocrine growth regulators for human epithelial cancers, including non-small cell lung cancer (NSCLC) and colorectal cancer, are the epidermal growth factor (EGF)-related growth factors and their cognate receptors (1). The EGF receptor (EGFR) is a transmembrane growth factor receptor belonging to a family of four related proteins [human epidermal growth factor receptor 2 (HER2)/ERBB2, HER3/ERBB3, and HER4/ERBB4; refs. 2, 3].

Vascular endothelial growth factors (VEGF) play a key role in cancer-induced neoangiogenesis by promoting endothelial cell proliferation, migration, and functional differentiation (4). Several ligands, including VEGF-A, VEGF-B, VEGF-C, VEGF-D, and placental growth factor (PlGF) mediate their effects through the activation of three different VEGF tyrosine kinase receptors, VEGFR-1, VEGFR-2 and VEGFR-3, on endothelial cells. The proangiogenic actions of VEGFs in endothelial cells are mediated primarily through the binding and activation of VEGFR-2 (5–8). The recent discovery of the production of different VEGF ligands and of the expression of

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Translational Relevance

In this study we provide experimental evidence that epidermal growth factor receptor (EGFR)-dependent and the vascular endothelial growth factor receptor (VEGFR)-dependent pathways are activated and functionally linked in human non-small cell lung cancer (NSCLC) and colorectal cancer cell lines. The activation of VEGF-dependent signaling could be responsible of intrinsic resistance to anti-EGFR therapies. Therefore, in addition to anti-EGFR drugs the simultaneous blockade of RAF and VEGF signaling with a multitargeted inhibitor such as sorafenib could obtain a significant antitumor inhibition of cancer cell growth. We think this study could have a relevant clinical interest because it is an extensive attempt in a large panel of selected human NSCLC and colorectal cancer cell lines to define a rational approach to combined molecular targeted therapies. The *in vitro* and *in vivo* results could be used for the design of translational research-based clinical studies with sorafenib in combination with erlotinib and cetuximab in patients with NSCLC and colorectal cancer.

VEGFR-1 and VEGFR-3 in epithelial cancer cells suggests a direct role for these ligands and receptors in the autocrine control of some biological processes in cancer cells. For example, VEGFR-1 expression has been detected in human colorectal cancer and pancreatic cancer cell lines, and the activation of this receptor by VEGF has been associated with enhanced cancer cell invasion, migration, and growth in soft agar, without direct effects on cancer cell proliferation (9, 10). Further evidence for a role of VEGFRs in cancer cell migration has been the detection of VEGF-C and of VEGFR-3 in NSCLC patient samples and what has been shown to be an active VEGF-C/VEGFR-3 autocrine pathway related to lung cancer cell migration *in vitro* and *in vivo* (11). In this respect, it was recently shown that targeting VEGFR-1 and VEGFR-3 with cediranib, a pan-VEGFR tyrosine kinase inhibitor, blocks *in vitro* migration and invasion of human pancreatic and hepatic carcinoma cell lines (12).

The activation of EGFR and related growth factor receptors by EGF, heregulin, or transforming growth factor α (TGF α) can upregulate the production of VEGF-A in human cancer cells (13, 14), suggesting that the oncogenic properties of the EGFR-driven pathway may, at least in part, be mediated by the stimulation of tumor angiogenesis by upregulating angiogenic growth factors. Thus, EGFR blockade causes inhibition of the secretion of VEGF and of other angiogenic growth factors, including basic fibroblast growth factor, interleukin 8, and TGF α (15, 16). Moreover, several studies have shown the role of VEGF-A upregulation in the acquired resistance to EGFR treatment in initially EGFR inhibitor-sensitive cancer cells (17, 18). Therefore, targeting both these pathways could provide a

better anticancer therapeutic strategy, especially for overcoming the acquired resistance of cancer cells to EGFR blockade (19, 20).

Erlotinib is a small molecule that inhibits EGFR tyrosine kinase activity, thus blocking EGFR activation by specific ligands. Erlotinib is currently available for the treatment of metastatic chemorefractory NSCLC (3). Cetuximab is a chimeric human-mouse anti-EGFR monoclonal antibody that binds to EGFR with a two-log higher affinity than the natural ligands TGF α and EGF (21). The binding of cetuximab to EGFR promotes receptor internalization and subsequent degradation without receptor phosphorylation and activation (3). It is currently used in combination with chemotherapy in the treatment of wild-type K-RAS metastatic colorectal cancer and in combination with radiotherapy or chemotherapy in locally advanced and in metastatic head and neck cancer (22, 23).

Sorafenib is a small molecule multitargeted kinase inhibitor that blocks the activation of C-RAF, B-RAF (both the wild-type and the activated V600E mutant), c-KIT, FLT-3, RET, VEGFR-2, VEGFR-3, and platelet-derived growth factor receptor β (PDGFR- β ; refs. 24, 25). Preclinical studies have shown that sorafenib is active in a broad spectrum of tumor types (26). In *in vitro* cellular assays, sorafenib inhibits the ligand-induced autophosphorylation of VEGFR-1, VEGFR-2, VEGFR-3, and PDGFR- β (27). Sorafenib is currently approved for the treatment of metastatic renal cell carcinoma and advanced hepatocellular carcinoma, and is under investigation in phase II/III trials in other malignancies, including NSCLC (25).

In this study, we evaluated the efficacy of the combination of sorafenib and erlotinib or cetuximab in a panel of 10 human NSCLC and colorectal cancer cell lines with the aim of developing models for a rational multitargeted cancer treatment approach.

Materials and Methods

Drugs

Sorafenib was kindly provided by Bayer Schering Italy. It was dissolved in sterile DMSO, and a 10 mmol/L working solution was prepared. Cetuximab was supplied by Merck-Serono Pharma Italy, and a 2 mg/mL stock solution was used. Erlotinib was kindly given by Roche Italy and was diluted in DMSO to 10 μ mol/L as stock solution.

Cell lines

The human GLC82, H1299, A549, CALU3, and H460 NSCLC cell lines and the human HT29, HCT15, HCT116, SW480, and GEO colorectal cancer cell lines were obtained from the American Type Culture Collection. Cells were cultured in RPMI 1640 medium (Cellgro) supplemented with 10% fetal bovine serum (Hyclone), 1% penicillin/streptomycin (Invitrogen), and 1% nonessential

amino acid (Invitrogen). Human umbilical vein endothelial cells were obtained from Lonza Inc. and were cultured with EGM SingleQuots growth factor supplemented media (Lonza, Inc.). All cell lines were grown in a humidified incubator with 5% carbon dioxide (CO₂) at 37°C. and were routinely screened for the presence of mycoplasma (Myco Alert, Cambrex Bio Science).

Real time-PCR analysis

EGF, *TGF α* , *amphiregulin*, *ERBB1*, *ERBB2*, *ERBB3*, *ERBB4*, *VEGF-A*, *VEGF-B*, *VEGF-C*, *VEGFR-1*, *VEGFR-2*, and *VEGFR-3* genes were amplified by real time-PCR from mRNA using appropriate primers to detect their expression in colorectal cancer and in NSCLC cell lines. Real time-PCR was done on total RNA extracted with Trizol reagent (Life Technologies). First-strand cDNA was prepared from 4 μ g of total RNA according to the Pharmacia Biotech procedure and amplified by PCR. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used as a control housekeeping gene. Each sample was denatured (95°C; 3 minutes), and PCR was carried out using 40 cycles. The amplification was terminated by a final 10-minute extension step at 72°C. PCR products were separated on 1% agarose gels and visualized under UV light after ethidium bromide staining. The fragments detected were isolated and sequenced to confirm that they corresponded to the genes listed above. In controls, the reverse transcriptase was omitted.

Growth in soft agar

Cells (10⁴ cells/well) were suspended in 0.5 mL 0.3% Difco Noble agar (Difco) supplemented with complete culture medium and seeded as previously described (18). Cells were treated with different concentrations of sorafenib, erlotinib, or cetuximab, and after 14 days were stained with nitroblue tetrazolium (Sigma) and colonies >0.05 mm were counted. For the combination experiments, cancer cells were treated with different concentrations of erlotinib (range, 0.02-10 μ mol/L) plus sorafenib (range, 0.01-5 μ mol/L) and sorafenib (range, 0.01-5 μ mol/L) plus cetuximab (range, 0.02-10 μ g/mL) each day, for a total of 3 days, at the fixed drug ratio sorafenib:erlotinib or sorafenib:cetuximab of 1:2. The results of the combined treatment with sorafenib and erlotinib or cetuximab were analyzed according to the method of Chou and Talalay by using the CalcuSyn software program (Biosoft), as previously reported (28).

Evaluation of EGF- and VEGF-related growth factor secretion

The concentrations of EGF, TGF α , VEGF-A, and VEGF-C in the conditioned medium (CM) were measured using commercially available enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems Inc.) according to the manufacturer's instructions. Assays were done in triplicate. Results were normalized for the number of producing cells and reported as pg of ligands per 10⁶ cells per 72 hours. Subsequently, the secretions of VEGF-A and VEGF-C in the

CM were evaluated after stimulation for 24 hours in serum-free medium of cancer cells with two different concentrations of EGF (10 and 20 ng/mL).

Evaluation of protein expression by Western blotting

Cells were seeded into 6-multiwell plates 24 hours before treatment with each drug or drug combination as described above. The analysis was done as previous described (18). We used the following antibodies from Cell Signaling Technology: rabbit polyclonal antibody against EGFR, phosphorylated EGFR, P-AKT, AKT, signal transducers and activators of transcription 3 (STAT3), and p-STAT3 (Tyr 705); mouse monoclonal antibodies against human p44/42 mitogen activated protein kinase (MAPK) pp44/42 MAPK (Thr202/Tyr20); goat polyclonal antibodies against p44/42 MAPK; and goat polyclonal antibody against β -actin (Santa Cruz Biotechnology). Immunoreactive proteins were visualized by enhanced chemiluminescence. (Amersham International). Each experiment was done in triplicate.

Wound-healing scratch assay

Cell monolayers grown to confluence on gridded plastic dishes were wounded by scratching with a pipette tip and then cultured in the presence or absence of sorafenib (1 μ mol/L), erlotinib (1 μ mol/L), cetuximab (2.5 μ g/mL), or the combination of sorafenib and erlotinib or cetuximab for 24 hours. The wounds were photographed (10 \times objective) at 24 hours (10), and healing was quantified by measuring the distance between the edges using Adobe Photoshop (v.8.0.1; Adobe Systems, Inc.) after being washed, fixed with 90% MeOH, and stained with crystal violet. The results are presented as the percentage of the total distance of the original wound filled by cancer cells. Each experiment was done in triplicate.

Apoptosis assay and cell cycle

H1299 cells were collected in 6-well plates, treated for 72 hours, and stained with Annexin V-FITC. Apoptotic cell death was assessed by counting the numbers of cells that stained positive for Annexin V-FITC and negative for propidium iodide using an Apoptosis Annexin V-FITC Kit (Invitrogen), coupled with fluorescence-activated cell sorting analysis. Cell cycle analysis was done by using flow cytometry on cell pellets that were fixed in 70% ethanol, washed in PBS, and mixed with RNase (Invitrogen) and propidium iodide (Invitrogen).

Tumor xenografts in nude mice

Four- to six-week-old female BALB/c athymic (*nu+ / nu+*) mice were purchased from Charles River Laboratories. The research protocol was approved and the mice were maintained in accordance with the institutional guidelines of the Second University of Naples Animal Care and Use Committee. The mice were acclimatized at the Second University of Naples Medical School Animal Facility for 1 week prior to being injected with cancer cells. The mice

were injected s.c. with 10^7 H1299 cells that had been re-suspended in 200 μ L Matrigel (Becton Dickinson). After 7 days, when established tumors of approximately 0.2 to 0.3 cm^3 diameter were detected, the mice were treated with oral administration of sorafenib (50 mg/kg/day on days 1-5 of each week) and/or with oral administration of erlotinib (75 mg/kg/day on days 1-5 of each week) and/or i.p. with cetuximab (1 mg on days 2-5 of each week) for the indicated time periods. For each experiment, treatment groups comprised 10 mice. Tumor volume was measured using the formula $\pi/6 \times \text{larger diameter} \times (\text{smaller diameter})^2$.

Statistical analysis

Student's *t* test was used to evaluate the statistical significance of the results. All *P* values represent two-sided tests of statistical significance. All analyses were done with the BMDP New System statistical package version 1.0 for Microsoft Windows (BMDP Statistical Software).

Results

Expression of EGF-related growth factors and their cognate receptors

First we characterized a panel of 10 human NSCLC and colorectal cancer cell lines for the expression of the four EGF receptors (EGFR, ERBB2, ERBB3, and ERBB4) and three ligands (amphiregulin, EGF, and TGF α) that are involved in EGFR-driven autocrine growth by using quantitative real time-PCR. GEO colon cancer cells were used as a positive control. The mRNAs of the three ligands were expressed in all NSCLC and colorectal cancer cell lines with variable levels ranging between 0.4- and 8.1-fold as compared with GEO colorectal cancer cells (Fig. 1A). Of note, amphiregulin expression was higher in CALU3 and HCT116 cells, whereas EGF was more expressed in GLC82, H460, and GEO cells. TGF α was widely detected in all cancer cell lines, with the exception of A549 cells in which it was barely detectable. All cancer cell lines expressed EGFR mRNA with the highest levels in GLC-82 cells. Low levels of ERBB2 mRNA were measured in colorectal cancer cell lines and in CALU3, whereas the other four NSCLC cell lines were negative. ERBB3 mRNA was present in all cell lines, with highest levels in GEO cells. Finally, low or no detectable expression of ERBB4 mRNA was found in all cancer cell lines tested, with the exception of GLC82 cells (Fig. 1A).

We next analyzed the secretion of two EGFR-specific ligands, EGF and TGF α , into the cell culture media. Twenty-four hours after cell seeding, culture medium was switched to serum-free medium and CM was collected after an additional 72 hours. As shown in Fig. 1B, both EGF and TGF α were secreted by all cell lines, with the highest levels of TGF α production in human CALU3, HT29, HCT116, and SW480 colorectal cancer cells. As illustrated in Fig. 1C, Western blot analysis showed the presence of EGFR in all cancer cell lines. ERBB2 was expressed in all colorectal cancer cell lines and in CALU3 NSCLC cells.

Little or no ERBB3 and ErbB4 protein expression was detected in all cancer cell lines, except for GLC-82 cells, which were positive for ERBB4 (Fig. 1C). Taken together, these findings show that a high variability in the expression of the four EGFR-related growth factor receptors and of three EGFR ligands is observed in colorectal cancer and NSCLC cell lines, suggesting that this differential expression could influence cancer cell biology and response to selected molecular targeted inhibitors.

Expression of VEGFRs and their specific ligands in NSCLC and colorectal cancer cell lines

We next evaluated the expression of VEGFR (VEGFR-1, VEGFR-2, VEGFR-3) and their ligand (VEGF-A, VEGF-B, VEGF-C) mRNAs by using quantitative real time-PCR in the panel of NSCLC and colorectal cancer cell lines. As shown in Fig. 2A, VEGF-A and VEGF-B mRNAs were detected in all NSCLC and colorectal cancer cell lines at variable levels. VEGF-C mRNA expression was absent in colorectal cancer cell lines and was detected in all NSCLC cell lines with the exception of CALU3 cancer cells. The expression of VEGFRs was more limited. In fact, VEGFR-2 mRNA was not detectable in GLC82, A549, and GEO cancer cells; it was barely measurable in HT29, HCT15, HCT116, and SW480 cancer cells; and it was expressed in H1299, CALU3, and H460 NSCLC cells (Fig. 2A). In contrast, VEGFR-1 mRNA expression was observed in all cancer cell lines with the exception of A549 cells. Finally, VEGFR-3 mRNA expression was limited to four of five NSCLC cell lines (GLC82, H1299, A549, H460) and was absent in colorectal cancer lines. As illustrated in Fig. 2B and C, we analyzed the secretion of VEGF-A and VEGF-C into the cell culture media and the levels of protein expression for VEGFR-1, VEGFR-2, and VEGFR-3. Protein expression levels confirmed the results observed for mRNAs for both ligands and receptors. In this respect, several differences could be observed between NSCLC and colorectal cancer cells. With the exception of VEGFR-1, all ligands and receptors were expressed at a higher degree in NSCLC cell lines both at mRNA and at protein level. In particular, NSCLC H1299 cells express high levels of all VEGF ligands and receptors (Fig. 2A-C).

Because it was previously shown that VEGF-A production could be induced by EGFR activation in some cancer cell lines (7, 8), we evaluated if EGFR stimulation by EGF treatment could induce VEGF-A and VEGF-C secretion. A dose-dependent upregulation in the secretion of both VEGF-A and VEGF-C into the CM following treatment with EGF was observed in all cancer cell lines (Supplementary Fig. S1). These results suggest that there is a functional cross-talk between the EGFR pathway and the production of these two ligands of the VEGF family in human NSCLC and colorectal cancer cells.

Effect of erlotinib and cetuximab on NSCLC and colorectal cancer cell growth in soft agar

To evaluate the effects of EGFR inhibition on cancer cell growth, soft agar anchorage-independent growth assays

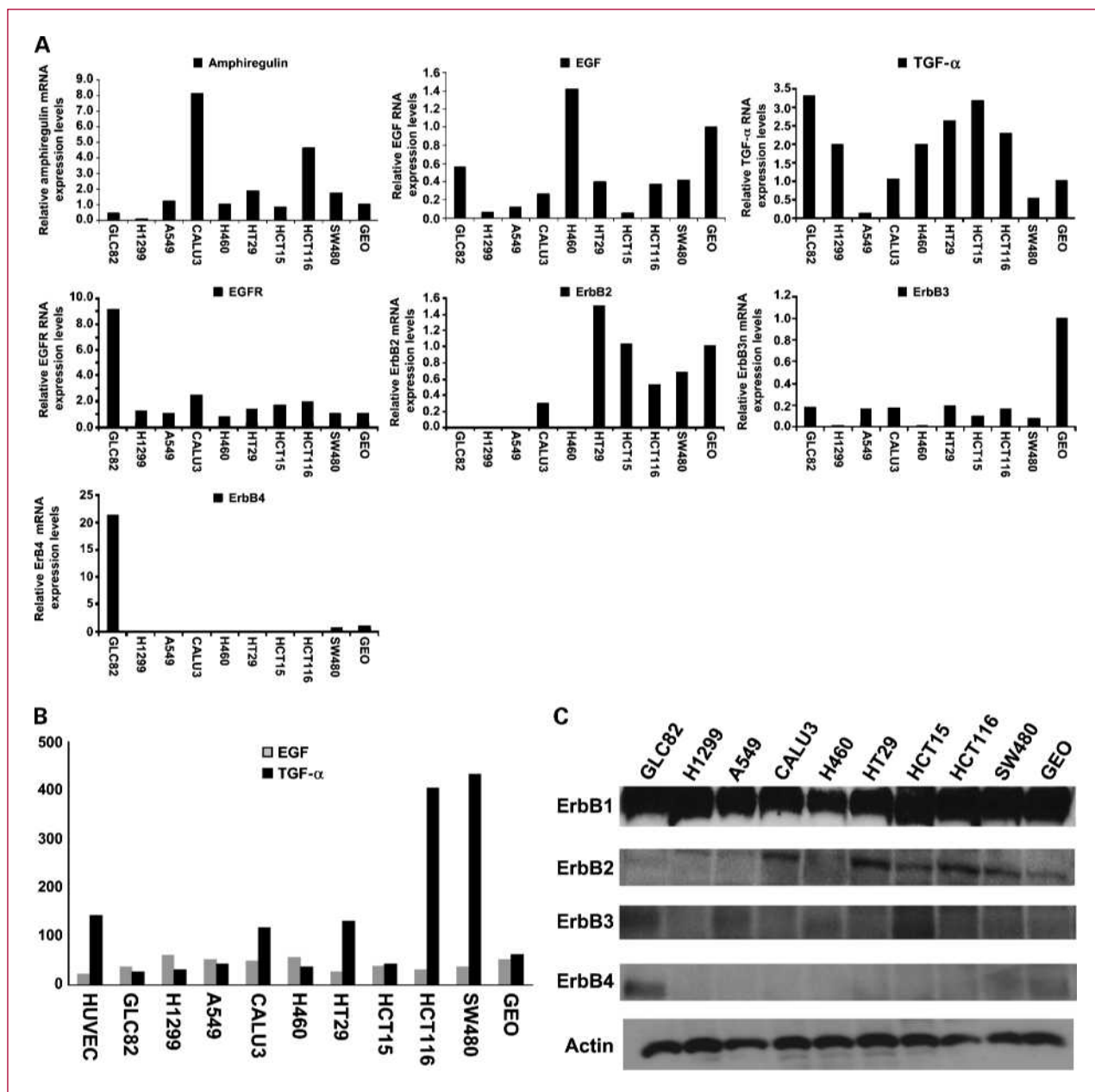


Fig. 1. Expression of EGF-related growth factors and growth factor receptors in human NSCLC and colorectal cancer cells. A, specific mRNA expression by quantitative real time-PCR: total RNA was extracted from NSCLC (GLC82, H1299, A549, CALU3, H460) and colorectal cancer (HT29, HCT15, HCT116, SW480, GEO) cell lines and quantitative real time-PCR was done to assess the expression of amphiregulin, EGF, TGF α , EGFR, ErbB2, ErbB3, and ErbB4 mRNA. B, secretion of EGF and TGF α into the conditioned medium. EGF and TGF α protein levels were measured in cell culture media by using specific ELISA as described in Materials and Methods. HUVEC, human umbilical vein endothelial cells. C, Western blotting analysis of EGFR-related growth factor receptors. Thirty micrograms of 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.

were done. Cells were treated for 72 hours with different concentrations of erlotinib or cetuximab. Colonies were counted after 10 to 14 days. A dose-dependent inhibition of anchorage-independent growth in soft agar was observed in all cancer cells lines with a different degree of sensitivity, as illustrated in Fig. 3A and B. Among the

NSCLC cell lines, CALU3 cells were the most sensitive to both erlotinib and cetuximab. On the contrary, H1299 cells were intrinsically resistant to EGFR inhibition. All colorectal cancer cell lines were sensitive to both erlotinib and cetuximab with an IC₅₀ ranging between 0.3 and 1 μ mol/L for erlotinib and 1 to 3.6 μ g/mL for cetuximab.

Effects of sorafenib on NSCLC and colorectal cancer cell growth in soft agar

We next tested the antiproliferative effects of sorafenib. A dose-dependent inhibition of anchorage-independent growth in semisolid medium was observed in all cancer cell lines treated with sorafenib with the IC_{50} ranging from 0.2 to 2.8 $\mu\text{mol/L}$ (Fig. 3C).

Mutations within the *K-RAS* that occur in approximately 40% of colorectal cancer and in 15% to 25% of NSCLC (22) have been correlated to intrinsic resistance to EGFR inhibitors (29). An activating *B-RAF* gene mutation (V600E) is less frequent and generally mutually exclusive with *K-RAS* mutations (30). We tested the panel of 10 NSCLC and colorectal cancer cell lines for the presence of such mutations. Eight of 10 cancer cell lines harbored an activating *K-RAS* gene mutation, whereas *B-RAF*

was mutated only in HT29 colorectal cancer and H1299 NSCLC cells. No significant correlation between sensitivity to sorafenib or to anti-EGFR drugs and *K-RAS* or *B-RAF* gene status was observed (Supplementary Fig. S2).

Effects of sorafenib in combination with EGFR inhibitors on NSCLC and colorectal cancer cell growth in soft agar

To evaluate the interaction between sorafenib and erlotinib or cetuximab, combination analyses were done. Cancer cells were treated with different concentrations of erlotinib (range, 0.02-10 $\mu\text{mol/L}$) and sorafenib (range, 0.01-5 $\mu\text{mol/L}$) or sorafenib (range, 0.01-5 $\mu\text{mol/L}$) and cetuximab (range, 0.02-10 $\mu\text{g/mL}$) each day, for a total of 3 days at a fixed drug ratio sorafenib:erlotinib or sorafenib:cetuximab of 1:2. Colonies were counted after 14 days.

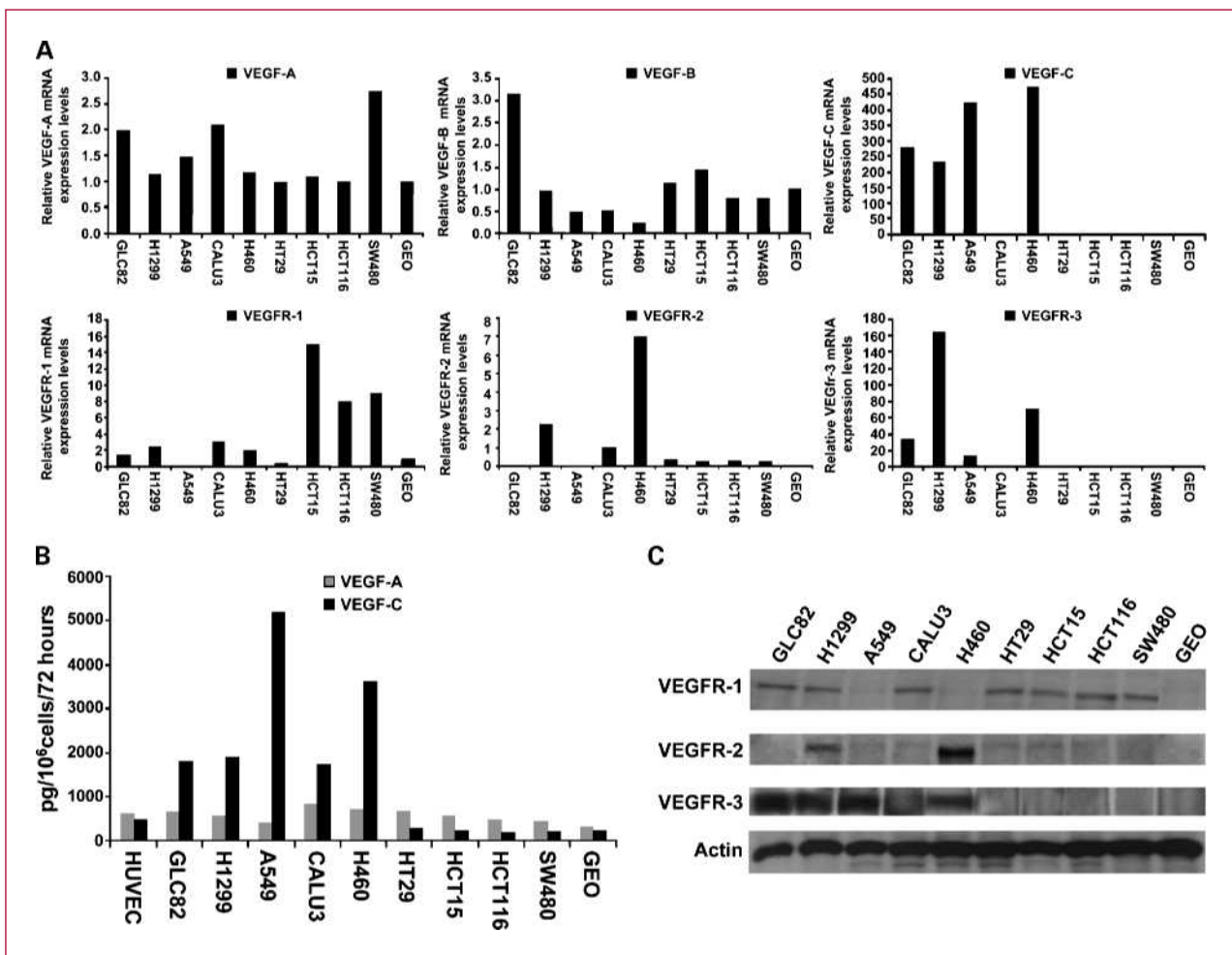


Fig. 2. Expression of VEGF-related growth factors and growth factor receptors in human NSCLC and colorectal cells. A, specific mRNA expression by quantitative real time-PCR. Total RNA was extracted from NSCLC (GLC82, H1299, A549, CALU3, H460) and colorectal cancer (HT29, HCT15, HCT116, SW480, GEO) cell lines, and quantitative real time-PCR was done to assess the expression of VEGF-A, VEGF-B, VEGF-C, VEGFR-1, VEGFR-2, and VEGFR-3 mRNA. B, secretion of VEGF-A and VEGF-C into the conditioned medium. VEGF-A and VEGF-C protein levels were measured in cell culture media by using specific ELISA as described in Materials and Methods. C, Western blotting analysis of VEGFR-related growth factor receptors. Thirty micrograms of 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.

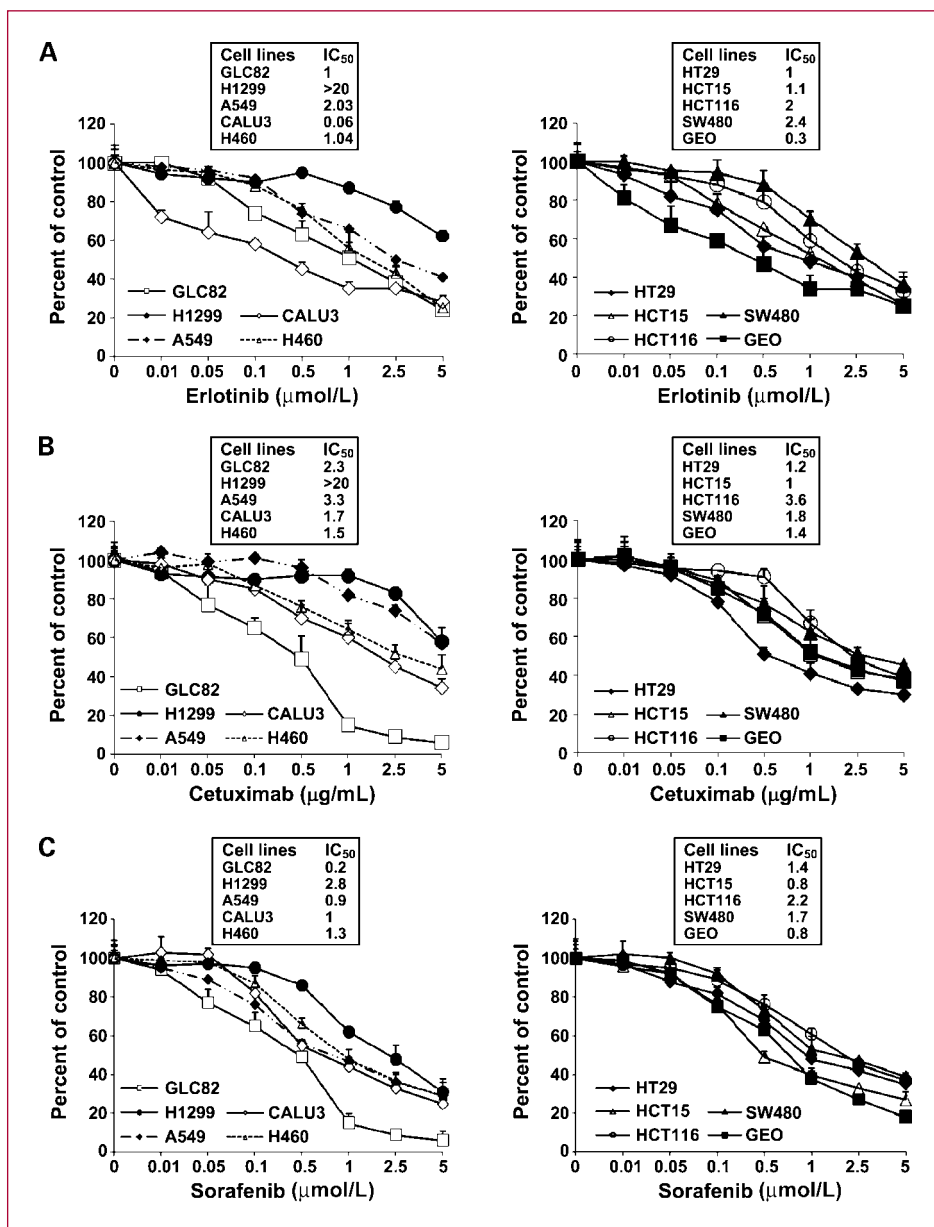


Fig. 3. Evaluation of the inhibitory effects on soft agar colony formation of erlotinib, cetuximab, or sorafenib given as single agent in human NSCLC and colorectal cancer cells. As described in Materials and Methods, cancer cells were treated for 72 hours with erlotinib (0.01-5 μmol/L), cetuximab (0.01-5 μg/mL), or sorafenib (0.01-5 μmol/L). Colonies were counted after 10 to 14 days. The results are average ± SD of three independent experiments each done in triplicate.

Experiments were done in triplicate. Combination index (CI) values were calculated according to the Chou and Talalay mathematical model for drug interactions using the Calcsyn software (28). In all 10 cancer cell lines tested, treatment with the multitargeted inhibitor sorafenib caused synergistic growth inhibitory effects in combination with each EGFR inhibitor (Fig. 4A and B). In fact, the CI values for the combined treatments ranged between 0.02 and 0.5, suggesting a significant synergism in the ability to inhibit soft agar colony formation in all cancer cell lines. These results were comparable for both combination treatments. Similar results in anchorage-dependent growth were obtained (data not shown).

Effects of sorafenib in combination with EGFR inhibitors on apoptosis and cell cycle in H1299 cancer cells

Seventy-two-hour treatment with sorafenib in combination with either erlotinib or cetuximab induced a marked increase in apoptosis as compared with control or single-agent treatments for both combinations (Supplementary Fig. S3A). To further explore this synergistic antiproliferative activity, we investigated the effects on cell cycle distribution. As compared with erlotinib or cetuximab, treatment with sorafenib induced an increase in the G₀-G₁ phase of the cell cycle. In particular, the combination of sorafenib plus erlotinib caused an almost complete

arrest of cells in the G₀-G₁ phase with further reduction in the G₂-M and S phases (Supplementary Fig. S3B).

Effects of sorafenib in combination with EGFR inhibitors on intracellular mitogenic signaling in H1299 cancer cells

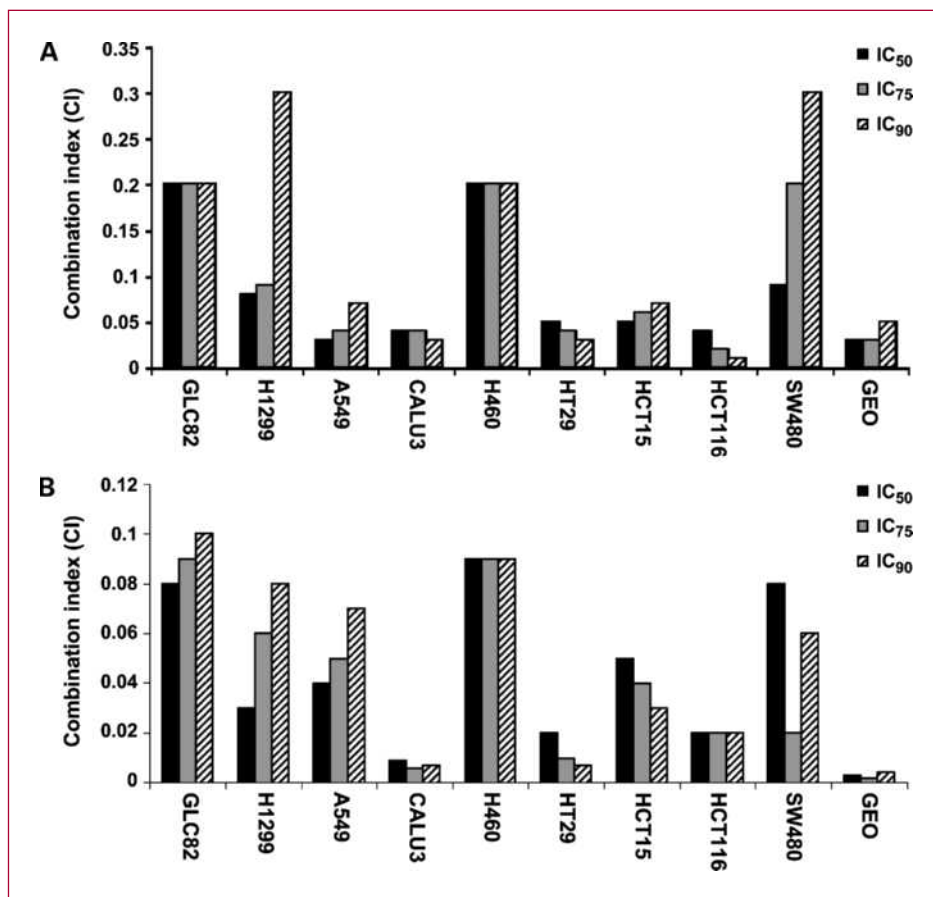
To determine whether the synergistic growth inhibition obtained by the combination of sorafenib and EGFR inhibitors was due to a more effective inhibition of EGFR activation and the intracellular signaling through MAPK and/or AKT, Western blots were done on protein extracts from H1299 NSCLC cells that were treated for different periods of time with sorafenib, cetuximab, or erlotinib, or with combinations of sorafenib and an anti-EGFR drug. We selected these cells because they are intrinsically resistant to treatment with anti-EGFR drugs as single agent, harbor a V600E B-RAF mutation, and express high levels of both VEGF ligands and receptors. Figure 5 illustrates that cetuximab or erlotinib treatment, although causing a decrease on EGFR phosphorylation, has little effect in inhibiting downstream mitogenic and prosurvival signals. Treatment with sorafenib in combination with erlotinib or cetuximab resulted in a more pronounced decrease in the levels of examined proteins phosphorylation (P-EGFR, P-MAPK, P-AKT) after 60 minutes of treatment, which was

maintained up to 120 minutes of treatment. The combined treatment also affected P-AKT-dependent downstream signaling (mTOR) as suggested by the sustained inhibition of the phosphorylation of the translational repressor protein eukaryotic initiation factor 4E binding protein 1 (P4E-BP1) and of the ribosomal protein S6 kinase (pp70S6K). Furthermore, total levels of STAT-3 protein were not substantially changed by treatments. However, within 30 minutes a marked reduction in the phosphorylated form of STAT-3 was observed in H1299 cancer cells treated with sorafenib in combination with anti-EGFR drugs (Supplementary Fig. S3C).

Effects of sorafenib in combination with EGFR inhibitors on *in vitro* migratory capabilities of H1299 cancer cells

We assessed if the migratory potential of H1299 NSCLC cells could be affected by treatment with the multitargeted agent sorafenib or by EGFR blockade by treatment with either erlotinib or cetuximab. We selected H1299 cancer cells because they express high levels of VEGF-A, VEGF-B, and VEGF-C, as well as high levels of VEGFR-1, VEGFR-2 and VEGFR-3. As determined by *in vitro* scratch motility assays, the 24-hour migration ability of H1299 cancer cells was not affected by single-agent treatment with either

Fig. 4. Evaluation of the inhibitory effects on soft agar colony formation of sorafenib in combination with erlotinib or with cetuximab in human NSCLC and colorectal cancer cells. Cancer cells were treated with different concentrations of sorafenib plus erlotinib (A) or of sorafenib plus cetuximab (B), as described in Materials and Methods. Colonies were counted after 14 days. Experiments were done in triplicate. CI values were calculated according to the Chou and Talalay model for drug interactions using the Calcsyn software for different fractions affected (fa), as described in Materials and Methods.



erlotinib or cetuximab, whereas sorafenib treatment determined a partial (30-50%) inhibition of migration. Moreover, the combined treatment with sorafenib and cetuximab or erlotinib induced an almost complete (80-90%) suppression on H1299 migratory behavior, which was probably due to the simultaneous inhibition of both VEGF- and EGF-dependent pathways in these cells (Supplementary Fig. S4).

Antitumor activity of sorafenib in combination with EGFR inhibitors on H1299 tumor xenografts

We next investigated the *in vivo* antitumor activity of sorafenib and/or cetuximab or erlotinib in nude mice bearing H1299 lung adenocarcinoma s.c. xenografts. After 7 days, when established H1299 tumors of approximately 0.3 cm³ were detectable, groups of 10 mice were treated for 4 weeks with each agent alone or in combination. Cetuximab or erlotinib as single agents were unable to affect tumor growth, whereas a 70% to 80% inhibition of H1299 tumor growth was caused by sorafenib single-agent treatment at the end of the 4 weeks of therapy. However, this effect was reversible upon cessation of treatment because H1299 tumors resumed a growth rate similar to that observed in control untreated mice within 3 to 4 weeks. In contrast, a prolonged tumor growth inhibition resulted

from the combination of sorafenib and cetuximab or of sorafenib and erlotinib. As a surrogate of survival, mice were sacrificed when tumor burden was not compatible with normal life (approximately 2.5 cm³; Supplementary Fig. S4). In Table 1 and in Supplementary Fig. S4, H1299 tumors in mice treated with sorafenib in combination with erlotinib for 4 weeks survived for an average period of 145 (SD, ± 5) days as compared with control, untreated mice (35 \pm 2 days), erlotinib-treated mice (37 \pm 2 days), or sorafenib-treated mice (75 \pm 5 days). A similar increase in survival was observed in H1299 xenograft-bearing mice treated with the combination of sorafenib and cetuximab (Table 1 and Supplementary Fig. S5). Combined treatments with sorafenib and cetuximab or erlotinib at the dose and schedule tested for the 4-week treatment protocol were well tolerated by mice, with no weight loss or other signs of acute or delayed toxicity (data not shown).

Discussion

In the present study we investigated the expression of ligands and receptors of the EGF and VEGF families in a panel of colorectal cancer and NSCLC cell lines, to assess the potential functional role of these ligands and receptors. Whereas EGF-related growth factors and their cognate

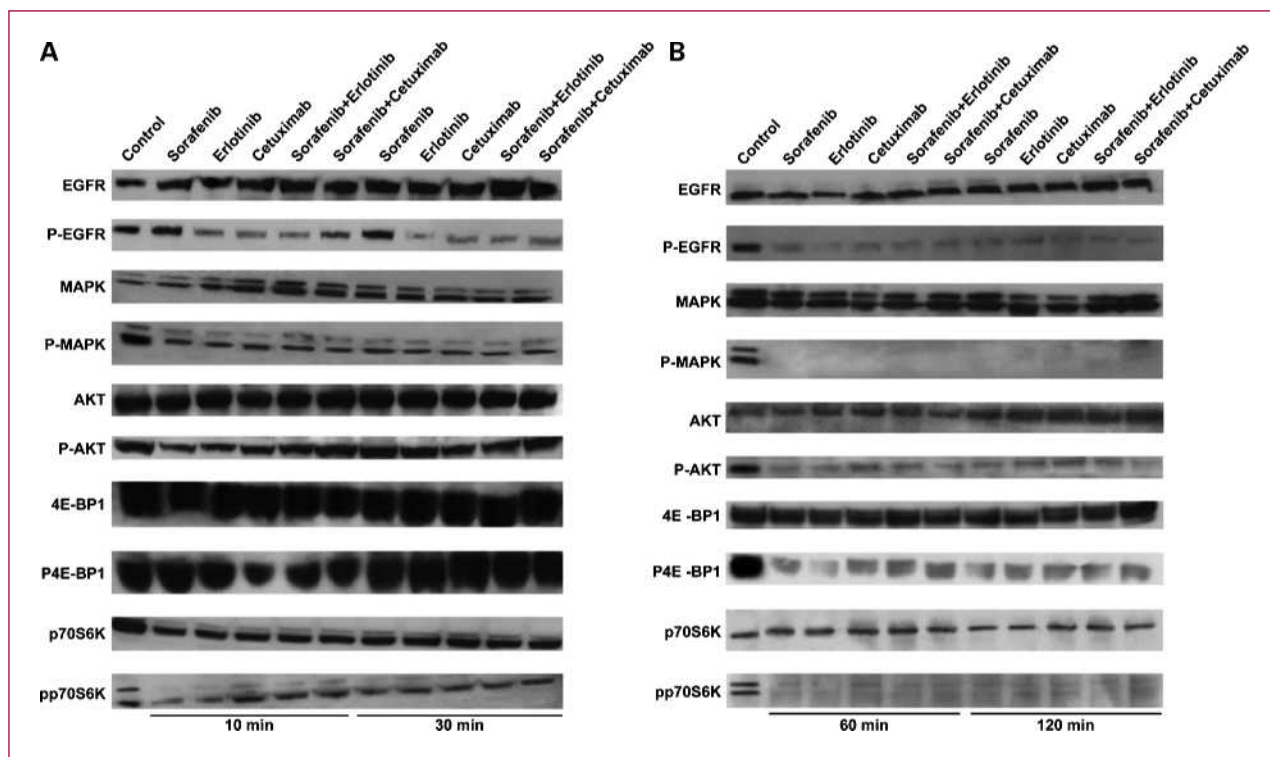


Fig. 5. Western blot analysis of protein expression following treatment with sorafenib, erlotinib, cetuximab, alone or in combination in H1299 NSCLC cells. Analysis of intracellular signaling pathways was done by Western blotting in H1299 cancer cells treated with 2.5 μ g/mL cetuximab, 2 μ mol/L erlotinib, 1 μ mol/L sorafenib, alone or with the indicated combinations for the indicated time periods. 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.

Table 1. Antitumor activity of sorafenib in combination with erlotinib or cetuximab in H1299 human cancer xenografts

Treatment	Average tumor volume (\pm SD) on day 35 after tumor cell injection, cm ³	Average survival (\pm SD), days
Control	2.5 (\pm 0.2)	35 (\pm 2)
Erlotinib	2.5 (\pm 0.3)	37 (\pm 4)
Sorafenib	0.56 (\pm 0.01)	75 (\pm 5)
Cetuximab	2.55 (\pm 0.2)	34 (\pm 5)
Erlotinib plus sorafenib	0.15 (\pm 0.04)	145 (\pm 5)
Cetuximab plus sorafenib	0.10 (\pm 0.03)	165 (\pm 10)

NOTE: Each treatment (erlotinib, sorafenib, cetuximab, erlotinib plus sorafenib, or cetuximab plus sorafenib) was started on day 7 following H1299 tumor cell s.c. injection when the average tumor volume was 0.30 (\pm 0.05) cm³.

Erlotinib treatment: 75 mg/kg/dose orally 5 days/week starting on the day 7 following tumor cell injection.

Sorafenib treatment: 50 mg/kg/dose orally 5 days/week starting on the day 7 following tumor cell injection.

Cetuximab treatment: 1 mg/dose i.p. twice weekly starting on the day 7 following tumor cell injection.

Treatment was done for 4 weeks. Each group consisted of 10 mice.

Mice were sacrificed when tumor volume reached 2.5 cm³ (approximately 10% of a nude mouse body weight).

receptors are widely recognized as key autocrine regulators in the control of cancer cell proliferation, invasion, and metastasis (1), the role of VEGFR expression and function in epithelial cancer cells is more controversial (9). In this respect, although VEGFRs are expressed in a wide variety of cancer cell lines as well as in tumor specimens from cancer patients, little is known on the significance of their expression on cancer cell functions (8).

The results of the present study show the expression of EGFR and three EGFR-specific ligands, i.e., EGF, amphiregulin, and TGF α , in a panel of 10 NSCLC and colorectal cancer cell lines, suggesting that in all these cancer cell lines a potential EGFR-driven autocrine growth pathway is active. On the contrary, expression of ERBB2, ERBB3, and ERBB4 is more restricted, with ERBB2 being expressed mostly in colorectal cancer cells. We also showed that NSCLC and colorectal cancer cell lines express different levels of mRNA and proteins of the VEGF families. In particular, NSCLC cells had the highest levels of VEGF-A, VEGF-B, and VEGF-C, and of VEGFR-1, VEGFR-2, and VEGFR-3 as compared with colorectal cancer cells. The presence of active VEGF/VEGFR signaling pathways in human cancer cells is an important issue because this pathway plays a critical role in angiogenesis by promoting the endothelial cell proliferation, invasion, and migration required for neovascularization (5). Ellis and colleagues (8) first reported the expression of VEGFR-1 in colorectal cancer cell lines and suggested that VEGFR-1 activation by VEGF-A and VEGF-B could increase the migration and the metastatic capabilities of these cells. In fact, expression of VEGFR-1 and VEGFR-2 and their ligands may sustain an autocrine loop in some human cancers, as has been suggested in melanoma, mesothelioma, and leukemia cells, in which exogenous VEGF stimulation induces cancer cell proliferation and migration by activating

VEGFR-2 (31–33). It was also shown recently that ligand-stimulated activation of VEGFR-1 and VEGFR-3 does not affect cell proliferation but induces *in vitro* migration and invasion of human gastrointestinal cancer cell lines (9). Furthermore, overexpression of VEGFR-1 may contribute to the acquisition of anti-EGFR drug resistance in human cancer cells. In this respect, *VEGFR-1* gene silencing with inhibition of VEGFR-1 protein expression or blockade of VEGFR-1 autophosphorylation and signaling by treatment with vandetanib restored the sensitivity to anti-EGFR drugs and impaired migration abilities in human cancer cells with acquired resistance to gefitinib or to cetuximab (34).

The results of the present study also suggest a functional cross-talk between the EGFR-dependent autocrine pathway and the control of angiogenesis because a dose-dependent increase in VEGF-A and VEGF-C release in the conditioned media of all cancer cell lines was observed following treatment with EGF.

Therefore, to obtain a simultaneous blockade of the EGFR-dependent autocrine pathway and of VEGFR-dependent signaling in cancer cells, we evaluated the *in vitro* and *in vivo* antitumor activity of the combined treatment with selective anti-EGFR drugs, such as erlotinib, cetuximab, and sorafenib, a multitargeted inhibitor of C-RAF and B-RAF and of all three VEGFRs (3, 24). Both NSCLC and colorectal cancer cell lines were sensitive to the antiproliferative effects of sorafenib *in vitro*. This effect does not correlate with gene mutations within the RAS-RAF signaling pathway, because no significant correlation between sensitivity to sorafenib or to anti-EGFR drugs and *K-RAS* or *B-RAF* gene status was observed. To evaluate the degree of interaction between sorafenib and erlotinib or cetuximab, combination treatments were analyzed according to the Chou and Talalay method. A synergistic inhibition of colony formation in

soft agar was observed in all cancer cell lines following the combination with sorafenib plus anti-EGFR drugs; moreover, antiproliferative effects with the combination were observed, although maximally inhibitory single-agent dose was used.

We selected H1299 NSCLC cells for further experiments because they are intrinsically resistant to treatment with anti-EGFR drugs as single agent (35, 36), harbor a V600E activating B-RAF mutation, and have the lowest expression of amphiregulin and EGF and high levels of TGF α , as ligands for EGFR (37); indeed they present high levels of both VEGF ligands and VEGFR-1, VEGFR-2, and VEGFR-3. This expression may sustain an autocrine loop that could be related with cell migration. In fact, among all the NSCLC and colorectal cancer cell lines that have been tested, H1299 cells exhibit the highest level of spontaneous migration. The migratory activity is a crucial step for a cancer cell in the development of metastasis. Combined treatment with sorafenib plus erlotinib or with sorafenib plus cetuximab significantly inhibited H1299 cancer cell migration, probably for the simultaneous inhibition of both the VEGF and the EGF signaling pathways in these cells. The antiproliferative and antimigratory effects of the combination of anti-EGFR drugs and sorafenib in H1299 cancer cells were accompanied by a significant inhibition of the MAPK and AKT pathways. The sustained inhibition of both the AKT and the MAPK pathways is therapeutically relevant because the EGFR-independent activation of these growth factor receptor downstream signaling molecules may represent one of the causes of resistance to EGFR-targeted therapies in human cancer (18, 34, 38, 39).

In vivo experiments in nude mice bearing established EGFR inhibitor-resistant H1299 lung adenocarcinoma

xenografts showed a significant cooperative antitumor activity when sorafenib and cetuximab or erlotinib were used in combination. In this respect, a 4-week treatment with the two agents was well tolerated and caused a significant tumor growth delay which determined an approximately 70- to 90-day increase in mice median overall survival as compared with single-agent sorafenib treatment.

In conclusion, the results of the current study show that both the EGFR-dependent and the VEGFR-dependent pathways are activated and functionally linked in a majority of human NSCLC and colorectal cell lines. The activation of VEGF-dependent signaling could drive an intrinsic resistance to anti-EGFR therapies, as shown in H1299 NSCLC cells. Therefore, in addition to anti-EGFR drugs the simultaneous blockade of RAF and VEGF signaling with a multitargeted inhibitor such as sorafenib could obtain a sustained and significant antitumor inhibition of cancer cell growth *in vitro* and *in vivo*, supporting the concept that molecular targeting of distinct but functionally linked cancer cell pathways could represent a suitable strategy in the treatment of cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References

- Salomon DS, Brandt R, Ciardiello F, Normanno N. Epidermal growth factor-related peptides and their receptors in human malignancies. *Crit Rev Oncol Hematol* 1995;19:183-232.
- Mendelsohn J, Baselga J. The EGF receptor family as targets for cancer therapy. *Oncogene* 2000;19:6550-65.
- Ciardiello F, Tortora G. EGFR antagonists in cancer treatment. *N Engl J Med* 2008;358:1160-74.
- Ferrara N. The role of vascular endothelial growth factor in pathological angiogenesis. *Breast Cancer Res Treat* 1995;36:127-37.
- Hicklin DJ, Ellis LM. Role of the vascular endothelial growth factor pathway in tumor growth and angiogenesis. *J Clin Oncol* 2005;23:1011-27.
- Zetter BR. Angiogenesis and tumor metastasis. *Annu Rev Med* 1998;49:407-24.
- Wey JS, Stoeltzing O, Ellis LM. Vascular endothelial growth factor receptors: expression and function in solid tumors. *Clin Adv Hematol Oncol* 2004;2:37-45.
- Ellis LM, Takahashi Y, Liu W, Shaheen RM. Vascular endothelial growth factor in human colon cancer: biology and therapeutic implications. *Oncologist* 2000;5 Suppl 1:11-5.
- Fan F, Wey JS, McCarty MF. Expression and function of vascular endothelial growth factor receptor-1 on human colorectal cancer cells. *Oncogene* 2005;24:2647-53.
- Lesslie DP, Summy JM, Parikh NU, et al. Vascular endothelial growth factor receptor-1 mediates migration of human colorectal carcinoma cells by activation of Src family kinases. *Br J Cancer* 2006;94:1710-7.
- Su JL, Yang PC, Shih JY. The VEGF-C/Fit-4 axis promotes invasion and metastasis of cancer cells. *Cancer Cell* 2006;9:209-23.
- Morelli MP, Brown AM, Pitts TM. Targeting vascular endothelial growth factor receptor-1 and -3 with cediranib (AZD2171): effects on migration and invasion of gastrointestinal cancer cell lines. *Mol Cancer Ther* 2009;8:2546-58.
- Gill J, Swerlick RA, Caughman SW. Transforming growth factor - induced transcriptional activation of the vascular permeability factor (VPF/VEGF) gene requires AP2-dependent DNA binding and transactivation. *EMBO J* 1997;16:750-9.
- Goldman CK, Kim J, Wong WL, King V, Brock T, Gillespie GY. Epidermal growth factor stimulates vascular endothelial growth factor production by human malignant glioma cells: a model of glioblastoma multiforme pathophysiology. *Mol Biol Cell* 1993;4:121-33.
- Bruns CJ, Solorzano CC, Harbison MT, et al. Blockade of the epidermal growth factor receptor signaling by a novel tyrosine kinase inhibitor leads to apoptosis of endothelial cells and therapy of human pancreatic carcinoma. *Cancer Res* 2000;60:2926-35.
- Hirata A, Ogawa S, Kometani T. ZD1839 (Iressa) induces antiangiogenic effects through inhibition of epidermal growth factor receptor tyrosine kinase. *Cancer Res* 2002;62:2554-60.
- Viloria-Petit A, Crombet T, Jothy S, et al. Acquired resistance to the antitumor effect of epidermal growth factor receptor-blocking

- antibodies *in vivo*: a role for altered tumor angiogenesis. *Cancer Res* 2001;61:5090–101.
18. Ciardiello F, Bianco R, Caputo R, et al. Antitumor activity of ZD6474, a vascular endothelial growth factor receptor tyrosine kinase inhibitor, in human cancer cells with acquired resistance to anti-epidermal growth factor receptor therapy. *Clin Cancer Res* 2004;10:784–93.
 19. Tortora G, Ciardiello F, Gasparini G. Combined targeting of EGFR-dependent and VEGF-dependent pathways: rationale, preclinical studies and clinical applications. *Nat Clin Pract Oncol* 2008;5:521–30.
 20. Naumov GN, Nilsson MB, Cascone T, et al. Combined vascular endothelial growth factor receptor and epidermal growth factor receptor (EGFR) blockade inhibits tumor growth in xenograft models of EGFR inhibitor resistance. *Clin Cancer Res* 2009;15:3484–94.
 21. Kim ES, Khuri FR, Herbst RS. Epidermal growth factor receptor biology (IMC-C225). *Curr Opin Oncol* 2001;13:506–13.
 22. Normanno N, Tejpar S, Morgillo F, De Luca A, Van Cutsem E, Ciardiello F. Implications for KRAS status and EGFR-targeted therapies in metastatic CRC. *Nat Rev Clin Oncol* 2009;6:519–27.
 23. Martinelli E, De Palma R, Orditura M, De Vita F, Ciardiello F. Anti-epidermal growth factor receptor monoclonal antibodies in cancer therapy. *Clin Exp Immunol* 2009;158:1–9.
 24. Wilhelm SM, Adnane L, Newell P, Villanueva A, Llovet JM, Lynch M. Preclinical overview of sorafenib, a multikinase inhibitor that targets both Raf and VEGF and PDGF receptor tyrosine kinase signaling. *Mol Cancer Ther* 2008;7:3129–40.
 25. Takimoto CH, Awada A. Safety and anti-tumor activity of sorafenib (Nexavar®) in combination with other anti-cancer agents: a review of clinical trials. *Cancer Chemother Pharmacol* 2008;61:535–48.
 26. Lyons JF, Wilhelm S, Hibner B, Bollag G. Discovery of a novel Raf kinase inhibitor. *Endocr Relat Cancer* 2001;8:219–25.
 27. Wilhelm SM, Carter C, Tang L, et al. BAY 43–9006 exhibits broad spectrum oral antitumor activity and targets the RAF/MEK/ERK pathway and receptor tyrosine kinases involved in tumor progression and angiogenesis. *Cancer Res* 2004;64:7099–109.
 28. Troiani T, Lockerbie O, Morrow M, Ciardiello F, Eckhardt SG. Sequence-dependent inhibition of human colon cancer cell growth and of prosurvival pathways by oxaliplatin in combination with ZD6474 (Zactima), an inhibitor of VEGFR and EGFR tyrosine kinases. *Mol Cancer Ther* 2006;5:1883–94.
 29. Bardelli A, Siena S. Molecular mechanisms of resistance to cetuximab and panitumumab in colorectal cancer. *J Clin Oncol* 2010;28:1254–61.
 30. Rajagopalan H, Bardelli A, Lengauer C, Kinzler KW, Vogelstein B, Velculescu VE. Tumorigenesis: RAF/RAS oncogenes and mismatch-repair status. *Nature* 2002;418:934.
 31. Byzova TV, Goldman CK, Pampori N, et al. A mechanism for modulation of cellular responses to VEGF: activation of the integrins. *Mol Cell* 2000;6:851–60.
 32. Dias S, Shmelkov SV, Lam G, Rafii S. VEGF (165) promotes survival of leukemic cells by Hsp90-mediated induction of Bcl-2 expression and apoptosis inhibition. *Blood* 2002;99:2532–40.
 33. Strizzi L, Catalano A, Vianale G. Vascular endothelial growth factor is an autocrine growth factor in human malignant mesothelioma. *J Pathol* 2001;193:468–75.
 34. Bianco R, Rosa R, Damiano V, et al. Vascular endothelial growth factor receptor-1 contributes to resistance to anti-epidermal growth factor receptor drugs in human cancer cells. *Clin Cancer Res* 2008;14:5069–80.
 35. Morgillo F, Woo JK, Kim ES, Hong WK, Lee HY. Heterodimerization of insulin-like growth factor receptor/epidermal growth factor receptor and induction of survivin expression counteract the antitumor action of erlotinib. *Cancer Res* 2006;66:10100–11.
 36. Thomson S, Buck E, Petti F, et al. Epithelial to mesenchymal transition is a determinant of sensitivity of non-small lung carcinoma cell lines and xenograft to epidermal growth factor receptor inhibition. *Cancer Res* 2005;65:9455–62.
 37. Tabernero J, Cervantes A, Rivera F, et al. Pharmacogenomic and pharmacoproteomic studies of cetuximab in metastatic colorectal cancer: biomarker analysis of a phase I dose-escalation study. *J Clin Oncol* 2010;28:1181–9.
 38. Janmaat ML, Kruyt FA, Rodriguez JA, Giaccone G. Response to epidermal growth factor receptor inhibitors in nonsmall cell lung cancer cells: limited antiproliferative effects and absence of apoptosis associated with persistent activity of extracellular signal-regulated kinase or Akt kinase pathways. *Clin Cancer Res* 2003;9:2316–26.
 39. Magne N, Fischel JL, Dubreuil A, et al. Influence of epidermal growth factor receptor (EGFR), p53 and intrinsic MAP kinase pathway status of tumour cells on the antiproliferative effect of ZD1839 (“Iressa”). *Br J Cancer* 2002;86:1518–23.

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