Primary and Acquired Resistance of Colorectal Cancer Cells to Anti-EGFR Antibodies Converge on MEK/ERK Pathway Activation and Can Be Overcome by Combined MEK/EGFR Inhibition

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Abstract

Purpose: The EGFR-independent activation of the RAS/RAF/MEK/MAPK pathway is one of the resistance mechanisms to cetuximab.

Experimental Design: We have evaluated, in vitro and in vivo, the effects of BAY 86-9766, a selective MEK1/2 inhibitor, in a panel of human colorectal cancer cell lines with primary or acquired resistance to cetuximab.

Results: Among the colorectal cancer cell lines, five with a KRAS mutation (LOVO, HCT116, HCT15, SW620, and SW480) and one with a BRAF mutation (HT29) were resistant to the antiproliferative effects of cetuximab, whereas two cells (GEO and SW48) were highly sensitive. Treatment with BAY 86-9766 determined dose-dependent growth inhibition in all cancer cells, including two human colorectal cancer cells with acquired resistance to cetuximab (GEO-CR and SW48-CR), with the exception of HCT15 cells. Combined treatment with cetuximab and BAY 86-9766 induced a synergistic antiproliferative and apoptotic effects with blockade in the MAPK and AKT pathway in cells with either primary or acquired resistance to cetuximab. The synergistic antiproliferative effects were confirmed using other two selective MEK1/2 inhibitors, selumetinib and pimasertib, in combination with cetuximab. Moreover, inhibition of MEK expression by siRNA restored cetuximab sensitivity in resistant cells. In nude mice bearing established human HCT15, HCT116, SW48-CR, and GEO-CR xenografts, the combined treatment with cetuximab and BAY 86-9766 caused significant tumor growth inhibition and increased mice survival.

Conclusion: These results suggest that activation of MEK is involved in both primary and acquired resistance to cetuximab and the inhibition of EGFR and MEK could be a strategy for overcoming anti-EGFR resistance in patients with colorectal cancer.

Introduction

Colorectal cancer is a major cause of morbidity and mortality throughout the world (1). The prognosis of patients diagnosed with metastatic colorectal cancer has improved markedly over the last 15 years, with an increase in median overall survival from 6 months with only best supportive care to more than 2 years with the introduction of active chemotherapy drugs, such as fluopyrimidines, oxaliplatin, and irinotecan, and of molecular targeted drugs, such as bevacizumab, cetuximab, panitumumab, afiblercept, and regorafenib (2, 3).

Cetuximab and panitumumab are 2 blocking anti-epidermal growth factor receptor (EGFR) monoclonal antibodies (mAb) that inhibit the activation of the EGFR and its downstream intracellular signals, the RAS–RAF–MEK–MAPK and the PTEN–PIK3CA–AKT pathways (4–6). In particular, cetuximab is an effective treatment as single agent or in combination with standard chemotherapy regimens for a subset of patients with metastatic colorectal cancer (7). Resistance to anti-EGFR therapies is likely because of the constitutive activation in cancer cells of signaling pathways acting downstream and/or independently of EGFR. In fact, point mutations in codon 12 or 13 within the exon 2 of the KRAS gene have been found as the major negative predictor of efficacy for cetuximab (8, 9). Therefore, cetuximab is currently used in monotherapy or in
Translational Relevance

Cetuximab, a blocking anti-EGFR monoclonal antibody, is effective in combination with chemotherapy or as single agent for the treatment of patients with KRAS wild-type metastatic colorectal cancer. However, clinical data indicate that, in all initially responding patients with colorectal cancer, cancer cell acquired resistance develops leading to treatment failure. This evidence has triggered a series of studies on the molecular mechanisms of primary and acquired resistance to cetuximab. In this study, we have demonstrated that, in human colorectal cancer cells with either primary or acquired resistance to cetuximab, EGFR-independent constitutive activation of MEK signaling is observed with subsequent MAPK activation. This observation suggests that MEK activation is a convergence point for a number of different signaling pathways in EGFR inhibitor-resistant colorectal cancer cells and that direct inhibition of MEK could be of therapeutic relevance. In fact, here we show that BAY 86-9766, a selective MEK1/2 inhibitor has antiproliferative activity in cetuximab-resistant colorectal cancer cell lines and that the combined blockade of both EGFR and MEK could represent a therapeutic strategy for preventing and/or overcoming cetuximab resistance in patients with colorectal cancer.

In addition to KRAS gene mutations, a number of retrospective studies have provided evidence that primary resistance to EGFR inhibitors in colorectal cancer could be correlated to mutations in other intracellular downstream effectors of EGFR, such as BRAF, NRAS, and PIK3CA (exon 20) genes (10). However, even among the molecularly enriched subset of patients with colorectal cancer with KRAS, BRAF, NRAS, and PIK3CA (exon 20) wild-type genes, cetuximab is not always clinically effective, suggesting that there are other undefined mechanisms of primary resistance (11). Although the evaluation of the presence of these gene mutations in the EGFR signaling pathways could identify the appropriate colorectal cancer patient population to treat with cetuximab, all initially responding patients will ultimately develop resistance to cetuximab (i.e., acquired resistance; ref. 12). The mechanisms involved in the primary resistance to anti-EGFR drugs are likely to play a role also in the acquired resistance. In this regard, it has been suggested that the onset of acquired resistance to anti-EGFR treatments in patients with metastatic colorectal cancer could be because of the emergence of KRAS-mutated cancer cell clones (13, 14). Moreover, HER2 gene amplification has been found to occur in approximately 3% of untreated patients with metastatic colorectal cancer and with a significantly higher frequency in patients with KRAS wild-type tumors that do not benefit of treatment with anti-EGFR drugs (15). In alternative to HER2 amplification, in a subset of patients with cetuximab-resistant metastatic colorectal cancer, acquired resistance is probably because of increased levels of heregulin, a ligand that binds HER3 and HER4 (16). Both HER2 gene amplification and increased heregulin production in cancer cells could cause acquired resistance to anti-EGFR MAbs treatment by leading to persistent activation of RAS–RAF–MEK–MAPK signaling (15, 16).

In this respect, the RAS–RAF–MEK–MAPK signaling pathway plays a central role in the intracellular transduction of proliferative signals from activated cell membrane growth factor receptors to the nucleus in both normal and cancer cells. RAF is a serine/threonine kinase that activates downstream signals in response to activated GTP-bound RAS by phosphorylating MEK1 and MEK2, which in turn phosphorylate and activate MAPK (or ERK1 and ERK2). MAPK phosphorylates a number of cellular substrates with key roles in cell proliferation and survival (17). Direct inhibition of MEK is a promising strategy in the development of cancer therapeutics to control tumor growth that is dependent on aberrant MEK pathway signaling. A potential advantage of targeting MEK is that the RAS/RAF/MEK/MAPK pathway is a convergence point where a number of upstream signaling pathways can be blocked with the direct inhibition of MEK. Most of the MEK inhibitors that are currently in early clinical development are selective for MEK1 and/or MEK2 and do not have off target effects on other kinases (18).

BAY 86-9766 is a highly selective, potent, orally available, small-molecule non-ATP-competitive inhibitor of MEK1/2 (19). This drug binds to an allosteric site adjacent to the ATP-binding region and then interacts with ATP, the activation loop, and other surrounding residues to prevent binding of MEK to its substrate ERK, thereby blocking ERK phosphorylation (20). BAY 86-9766 inhibited cell proliferation in human cancer cell lines, including those harboring BRAF V600E mutations, and also exhibited potent antitumor activity in human xenograft models (19). The safety profile and tolerability of BAY 86-9766 has been evaluated in a multicenter phase I clinical study. This trial showed that it was well tolerated, with good oral absorption, dose proportional pharmacokinetics, target inhibition at the maximum tolerated dose (MTD), and some evidence of clinical benefit across a range of tumor types (20, 21).

Constitutive activation of RAS–RAF–MEK–MAPK signaling, one of the key pathway downstream of EGFR, could cause primary and/or acquired resistance to anti-EGFR treatment. Based on this hypothesis, we have evaluated the efficacy of BAY 86-9766 in overcoming the resistance to cetuximab by using human colorectal cancer models. For this purpose, we have selected a panel of 8 human colorectal cancer cell lines, which we have characterized for their sensitivity to the antiproliferative effects of cetuximab and for the gene mutation profile for KRAS, BRAF, PIK3CA, and NRAS and 2 cancer cell lines with acquired resistance to cetuximab that we have obtained following continuous
treatment with cetuximab of 2 cetuximab-sensitive human colorectal cancer cell lines (22, 23) to further elucidate the molecular mechanisms of primary and acquired resistance to cetuximab.

Materials and Methods

Drugs

Cetuximab, an anti-EGFR human-mouse chimeric mAb was kindly provided by Merck Serono Italy. BAY 86-9766, selumetinib and pimasertib, selective MEK1/2 tyrosine kinase inhibitors, were kindly provided by Bayer Pharma Italy, Astra Zeneca, and Merck-Serono, respectively. All 3 MEK1/2 inhibitors were dissolved in sterile dimethylsulfoxide (DMSO) and a 10 mmol/L working solution was prepared and stored in aliquots at −20°C. Working concentrations were diluted in culture medium just before each experiment.

Cell lines

The human HT29, LOVO, HCT15, SW620 colorectal cancer cell lines were obtained from the American Type Culture Collection (ATCC) and have been authenticated by IRCCS "Azienda Ospedaliera Universitaria San Martino-IST Istituto Nazionale per la Ricerca sul Cancro, Genova," Italy. The human SW48 (catalogue number: HTL99020), HCT116 (catalogue number: HTL95025), SW480 (catalogue number: HTL99017) dimethylsulfoxide cell lines were obtained from IRCCS "Azienda Ospedaliera Universitaria San Martino-IST Istituto Nazionale per la Ricerca sul Cancro, Genova," Italy. The human GEO colon cancer cell line was kindly provided by Dr. N. Normanno (National Cancer Institute, Naples, Italy), GEO-CR and SW48-CR cells were established as previously described (22, 23). GEO and GEO-CR cell lines were grown in DMEM (Lonza), supplemented with 20% fetal bovine serum (FBS; Lonza) and 1% penicillin/streptomycin (Lonza). SW48, SW48-CR, LOVO, HCT15, HCT116, and SW480 cells were grown in RPMI-1640 (Lonza) supplemented with 10% FBS and 1% penicillin/streptomycin (Lonza). SW48, SW48-CR, LOVO, HCT15, HCT116, and SW480 cells were grown in RPMI-1640 (Lonza) supplemented with 10% FBS and 1% penicillin/streptomycin (Lonza). SW48, SW48-CR, LOVO, HCT15, HCT116, and SW480 cells were grown in RPMI-1640 (Lonza) supplemented with 10% FBS and 1% penicillin/streptomycin (Lonza). SW48, SW48-CR, LOVO, HCT15, HCT116, and SW480 cells were grown in RPMI-1640 (Lonza) supplemented with 10% FBS and 1% penicillin/streptomycin (Lonza). Cell lines were grown in McCoy medium (Lonza) supplemented with 20% FBS and 1% penicillin/streptomycin (Lonza). SW48, SW48-CR, LOVO, HCT15, HCT116, and SW480 cells were grown in RPMI-1640 (Lonza) supplemented with 10% FBS and 1% penicillin/streptomycin (Lonza). Cell lines were routinely screened for the presence of mycoplasma (Mycoplasma Detection Kit; Roche Diagnostics).

Proliferation assay

Cancer cell lines were seeded in 24-well plates and were treated with different concentrations of cetuximab (range, 0.01–20 μg/mL) alone or in combination with BAY 86-9766 (range, 0.01–5 μmol/L), or selumetinib (range, 0.01–5 μmol/L) or pimasertib (range, 0.01–5 μmol/L) for 96 hours. Cell proliferation was measured with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). The IC50 was determined by interpolation from the dose–response curves. Results represent the median of 3 separate experiments, each performed in quadruplicate. Results of the combination treatment were analyzed according to the method of Chou and Talalay by using the CalcuSyn software programme (Biosoft).

Apoptosis assay

HCT15, HCT116, SW48-CR, and GEO-CR cells were seeded in 6-well plates, treated for 72 hours and stained with Annexin V-fluorescein isothiocyanate (FITC). Apoptotic cell death was assessed by counting the numbers of cells that stained positive for Annexin V-FITC and negative for propidium iodide (PI) using an Apoptosis Annexin V-FITC Kit (Invitrogen), coupled with fluorescence-activated cell sorting (FACS) analysis.

Immunoblotting

HCT15, HCT116, SW48-CR, and GEO-CR cells were seeded into 100 mm dishes and treated with cetuximab, BAY 86-9766, or their combination for 24 hours. Protein lysates containing comparable amounts of proteins, estimated by a modified Bradford assay (BioRad), were subjected to Western blot analysis, as previously described (24). Immuno-complexes were detected with the Enhanced Chemiluminescence Kit (Pierce Biotechnology Inc.). EGFR monoclonal antibody (#4267), phospho-EGFR monoclonal antibody (#3777), p44/42 MAPK polyclonal antibody (#9102), phospho-p44/42MAPK monoclonal antibody (#9106), anti-AKT polyclonal antibody (#9272), pAKT monoclonal antibody (#4060), and anti-PARP polyclonal antibody (#9542) were from Cell Signaling (Beverly). Caspase-3 monoclonal antibody (sc-65496) was from Santa Cruz Biotechnology. Monoclonal anti-α-tubulin antibody (T8023) was from Sigma Chemical Co. The following secondary antibodies from Bio-Rad were used: goat anti-rabbit IgG, and rabbit anti-mouse IgG. Immunoreactive proteins were visualized by enhanced chemiluminescence (ECL plus; Thermo Fisher Scientific). Each experiment was done in triplicate.

RNA interference and PCR analysis

The small inhibitor RNAs (siRNA) ON-target plus SMARTpool MEK1/2 (human: #L-003571-00/L-003573-00) were from Dharmacon. The siCONTROL Non-targeting Pool (#D-001206-13-05) was used as a negative (scrambled) control. Cells were transfected with 100 nmol/L siRNAs using Hiperfect reagent (Qiagen) following manufacturer’s instructions. The day before transfection, the cells were plated in 35 mm dishes at 40% of confluence in medium supplemented with 5% FBS without antibiotics. Cells were harvested at different time points (24, 48, 72, and 96 hours) after transfection. Western blot analysis for MEK1/2 expression was done. The siRNA effects on cell proliferation and on cell signaling were evaluated by MTT and Western blot analysis as previously described. Briefly, cells were seeded into 24-multiwell cluster dishes and transfected with MEK1/2 siRNA. Forty-eight hours after transfection, the cells treated with MEK1/2 siRNA received 5 μg/mL of cetuximab and cell proliferation and Western blot analysis were determined 24 hours later (after 72 hours of transfection).
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 mice were maintained in accordance with the institutional guidelines of the Second University of Naples Animal Care and Use Committee. Mice were acclimatized at the Second University of Naples Medical School Animal Facility for 1 week before being injected with cancer cells and then caged in groups of 5. Mice were injected subcutaneously with 2.5 × 10⁶ HCT15, HCT116, GEO-CR, and SW48-CR cells that had been resuspended in 200 μL of matrigel (BD Biosciences). When the mean values of tumors were between 200 and 300 mm³, mice were randomly divided in 4 groups (10 mice per group). BAY 86-9766 was prepared in vehicle 1 (10% cremohor EL in saline) and was administered through oral gavage daily (5 mg/kg) for 3 weeks. Cetuximab at the dose of 1 mg was injected intraperitoneally twice a week for 3 weeks. Monitoring of tumor growth was continued until tumors reached approximately 2,000 mm³ when mice were sacrificed. Tumor size was evaluated twice per week by caliper measurements using the following formula: π/6 × larger diameter × (smaller diameter)². Student t test was used to evaluate the statistical significance of the results.

Statistical analysis

The statistical analyses of in vivo and in vitro data were carried out using Prism version 4.02 (GraphPad Software, Inc.). The Student t test was used to evaluate the statistical significance of the results. All P values represent 2-sided tests of statistical significance with P value < 0.05.

Results

Effects of cetuximab treatment on cell proliferation and on EGFR-dependent intracellular signaling in human colorectal cancer cell lines

We first evaluated the sensitivity to the cell growth inhibiting effects of the anti-EGFR MAb cetuximab in a panel of 8 human colorectal cancer (GEO, SW48, HT29, LOVO, HCT116, HCT15, SW620, and SW480) cell lines, that were selected for having different mutation profiles in KRAS, NRAS, BRAF, PIK3CA, and EGFR genes (Supplementary Table S2). Cancer cells were treated with cetuximab at concentrations ranging from 0.01 to 2 μmol/L for 96 hours. The drug concentrations required to inhibit cell growth by 50% (IC₅₀) were determined by interpolation from the dose–response curves. As shown in Fig. 1A and Supplementary Table S1A, there was a differential sensitivity to cetuximab-induced cell growth inhibition. In fact, 6 colorectal cancer cell lines (HT29, LOVO, HCT116, HCT15, SW620, and SW480) were similarly resistant to cetuximab. LOVO, HCT116, HCT15, SW620, and SW480 cells have an activating KRAS gene mutation in either codon 12 or 13 within exon 2, whereas HT29 cells have a BRAF gene mutation (V600E). Between the 2 cetuximab-sensitive colorectal cancer cell lines, SW48 is “quadruple wild type” for KRAS, BRAF, NRAS, and PIK3CA genes, whereas GEO cells has a KRAS gene codon 12 mutation. In particular, although GEO cells harbor a KRAS gene mutation, previous studies from different laboratories, including our own, have demonstrated that this colorectal cancer cell line is one of the most sensitive colorectal cancer cell lines to the in vitro and in vivo antitumor activity of cetuximab treatment (23, 25–27). Furthermore, Fig. 1A and Supplementary Table S1A show that cetuximab was also not effective in GEO-CR and SW48-CR cells, 2 cell lines models of acquired resistance, that we have recently obtained following continuous cetuximab treatment in vivo of nude mice bearing GEO tumor xenografts or following continuous in vitro exposure to cetuximab of SW48 cells (22, 23). These 2 cell lines have been previously evaluated for the presence of mutations in both the KRAS and the NRAS genes by next generation sequencing using the Ion AmpliSeq cancer panel (22). No additional mutations were observed in KRAS and NRAS genes in GEO-CR and SW48-CR cells compared with the parental cell lines (data not shown). As shown in Fig. 1B, cetuximab treatment suppressed EGFR-induced intracellular signaling only in the 2 colorectal cancer sensitive cell lines GEO and SW48, with a significant inhibition in the expression of active, phosphorylated MAPK and AKT proteins, whereas it had little or no effect on the levels of active, phosphorylated MAPK and AKT proteins in cetuximab-resistant colorectal cancer cell lines.

Effects of treatment with BAY 86-9766, a selective MEK1/2 inhibitor, on cell proliferation of human colorectal cancer cell lines

We next evaluated the cell growth inhibiting effects of the selective MEK1/2 inhibitor BAY 86-9766 in the same panel of human colorectal cancer cell lines. Cancer cells were treated with BAY 86-9766 at concentrations ranging from 0.01 to 5 μmol/L for 96 hours. As illustrated in Fig. 2A and Supplementary Table S1B, BAY 86-9766 treatment caused a dose-dependent cell growth inhibition in all colorectal cancer cell lines, with IC₅₀ values ranging between 0.05 μmol/L (HCT116) and 2 μmol/L (SW48-CR), except for HCT15 cells. The same effects on cell growth inhibition were obtained by using other 2 different MEK1/2 inhibitors such as selumetinib and pimasertib (Fig. 2B and C and Supplementary Table S1B).

Effects of cetuximab in combination with the selective MEK1/2 inhibitor BAY 86-9766 in colorectal cancer cells growth in vitro

All colorectal cancer cell lines were treated with different concentrations of cetuximab, of BAY 86-9766, or with the combination of the 2 drugs (Fig. 3 and Supplementary Fig. S1A and S1B and Table S3). Combination index (CI) values were calculated according to the Chou and Talalay mathematical model for drug interactions using the CalcuSyn software, as previously described (25, 28). Both in the colorectal cancer cell lines with primary resistance to cetuximab (HT29, LOVO, HCT116, HCT15, SW620, and SW480) and in the colorectal cancer cell lines with acquired resistance to cetuximab (GEO-CR and SW48-CR), treatment
with the selective MEK1/2 inhibitor BAY 86-9766 determined synergistic growth inhibitory effects in combination with cetuximab. In fact, the CI values for the combined treatments were significantly <1.0 for all the drug doses tested (CI values ranging between 0.0001 and 0.7; Fig. 3 and Supplementary Fig. S1A and S1B and Table S3). In contrast, in cetuximab-sensitive GEO and SW48 cells, both cetuximab and BAY 86-9766 were highly effective as single-agent treatments in determining cell growth inhibition, whereas their combination was clearly antagonistic (CI values significantly >1.0), suggesting that in EGFR inhibitor-sensitive colorectal cancer cell lines the combined treatment with cetuximab and the MEK1/2 inhibitor could negatively interfere on cell growth inhibition (Supplementary Fig. S1A and S1B and Table S3). To confirm the synergistic growth inhibitory effects of cetuximab in combination with BAY 86-9766, we have used other 2 selective MEK1/2 inhibitors, such as selumetinib and pimasertib (24, 28). As depicted in Supplementary Table S1B, the combined treatment of cetuximab...
with selumetinib or pimasertib induced a clearly synergistic antitumor activity only in the colorectal cancer cell lines with either primary or acquired resistance to the anti-EGFR inhibitor. In contrast, the combined treatment was antagonistic on cetuximab sensitive cell lines, GEO and SW48 in accord to the results with BAY 86-9766 and cetuximab combinations (Supplementary Table S3).

**Effects of cetuximab in combination with the selective MEK1/2 inhibitor BAY 86-9766 on induction of apoptosis and on EGFR-dependent intracellular signaling pathways in HCT15, HCT116, GEO-CR, and SW48-CR colorectal cancer cells**

We next selected for further experiments 2 colorectal cancer cell lines with primary resistance to cetuximab (HCT15 and HCT116) and the 2 colorectal cancer cell lines with acquired resistance to cetuximab (GEO-CR and SW48-CR) to determine whether the synergistic growth inhibition that was obtained by the combined treatment with the selective MEK1/2 inhibitor BAY 86-9766 and cetuximab was because of a more effective inhibition of key intracellular signals for cell survival and proliferation. We, therefore, measured the ability of these drugs (as single agents or in combination) to induce apoptosis by using Annexin V-FITC (Fig. 4A). Although cetuximab or BAY 86-9766 single-agent treatments resulted in little or no induction of apoptosis in all cancer cell lines, the combined treatment determined a significant induction of apoptosis in both colorectal cancer cells with primary resistance to cetuximab and colorectal cancer cells with acquired resistance to cetuximab (Fig. 4A). The combined treatment resulted also in the activation of specific intracellular mediators of apoptosis, such as induction of cleaved PARP protein in all cancer cell lines (Fig. 4B). To determine whether the combined inhibition of EGFR and MEK1/2 could be effective in blocking EGFR-activated intracellular pathways, Western blots were performed on protein extracts from HCT15, HCT116, GEO-CR, and SW48-CR cells, which were treated for 24 hours with cetuximab or BAY 86-9766 as single agents or in combination. The combined treatment resulted in effective inhibition of active, phosphorylated MAPK and AKT proteins in all cancer cell lines (Fig. 4C and D and Supplementary Fig. S2).

**Inhibition of MEK1/2 expression by siRNA restores sensitivity to cetuximab**

To determine if MEK1/2 expression could be involved in the acquisition of primary and acquired cetuximab resistance, we investigated whether inhibition of MEK1/2 expression could restore cetuximab sensitivity in HCT15 and HCT116. Transfection with a specific MEK1/2 siRNA for 72 hours significantly reduced MEK1/2 protein expression in these cells, as shown in Fig. 5A. As illustrated in Fig. 5B, MEK1/2 siRNA treatment slightly reduced cell growth in HCT15 and HCT116. Although single-agent cetuximab treatment did not affect cell proliferation, cetuximab treatment in combination with MET silencing determined cell growth inhibition in HCT15 and HCT116. MEK silencing also restored the ability of cetuximab to inhibit MAPK activation in both cell lines as shown by downregulation of phospho-MAPK levels (Fig. 5C).

**Effects of cetuximab in combination with the selective MEK1/2 inhibitor BAY 86-9766 on HCT15, HCT116, GEO-CR, and SW48-CR tumor xenografts**

We finally investigated the *in vivo* antitumor activity of cetuximab alone or in combination with BAY 86-9766 in nude mice bearing cetuximab-resistant HCT15, HCT116, GEO-CR, or SW48-CR cells grown subcutaneously as tumor xenografts. After 10 days, when established HCT15, HCT116, GEO-CR, or SW48-CR tumors were detectable, mice were treated for 3 weeks with cetuximab, with BAY 86-9766 or with the combination of the 2 drugs. As shown in Fig. 6, treatment with cetuximab had little or no effect on tumor growth in all tumor xenografts, whereas BAY 86-9766 treatment caused approximately 40% to 50% reduction in tumor growth in tumor xenograft models of both primary and acquired resistance to cetuximab. In contrast, the combined treatment with the 2 drugs suppressed almost completely HCT15, HCT116, GEO-CR, or SW48-CR tumor growth at the end of the 3 weeks of therapy (Fig. 6). Furthermore, the combined treatment with cetuximab and
BAY 86-9766 determined a significant increase in mice survival as compared with control and to single-agent treatments in all tumor xenografts. Single-agent and combination treatment protocols were well tolerated by mice, with no weight loss or other signs of acute or delayed toxicity (Supplementary Fig. S3).

**Discussion**

Elucidating the mechanisms of cancer cell resistance to anticancer drugs is critical for the development of more effective therapies. An extensive effort has been made to understand cancer cell resistance mechanism(s) to EGFR inhibitors (6, 22, 29). Retrospective studies have revealed that mutations of KRAS in hotspot regions of exons 2, 3, 4, BRAF, NRAS, and PIK3CA exon 20 genes are predictors of lack of efficacy of either cetuximab or panitumumab in patients with chemorefractory metastatic colorectal cancer as a result of EGFR-independent intracellular downstream signaling activation (8, 30–34).

In agreement with these findings, in this study we have found that among the panel of human colorectal cancer cell lines, those with primary resistance to cetuximab have mutations in the RAS/RAF/MEK/MAPK pathway. In
particular, LOVO, HCT116, HCT15, SW620, and SW480 cells have an activating KRAS gene mutation in either codon 12 or 13 within exon 2, whereas HT29 cells have a BRAF gene mutation (V600E).

Moreover, MAPK activation, a major downstream effector of EGFR pathway, is one of the potential mechanisms of resistance to anti-EGFR therapies. In fact, cetuximab treatment was not effective in primary and acquired (GEO-CR and SW48-CR) cetuximab-resistant colorectal cancer cell lines in which activation of MAPK and AKT was not blocked, despite EGFR inhibition. The lack of phosphorylated MAPK protein inhibition as a molecular finding of acquired resistance to anti-EGFR therapies is in agreement with other recent reports. In particular, Yonesaka and colleagues have developed cetuximab-resistant clones of GEO cells, in which activation of ERBB2 signaling, either through ERBB2 amplification or through heregulin protein upregulation, leads to persistent, constitutive ERK1/2 activation (16). Moreover, Normanno and colleagues have shown, in human breast cancer cell lines models of acquired resistance to the small molecule EGFR tyrosine kinase inhibitor (TKI) gefitinib, a role for constitutive...
sorafenib, which blocked RAF signaling (36). Furthermore, by the constitutive activation of the RAS/RAF/MEK/MAPK acquired resistance in these cells was predominantly driven to cancer cells (36). We have found that EGFR inhibitor CALU-3 lung adenocarcinoma and HCT116 colorectal EGFR TKIs, following chronic exposure to these drugs of have recently developed human cancer cell lines with activation of MEK/MAPK signaling (35). In this respect, we have demonstrated that a common phenotype correlated to acquired resistance to different TKIs is represented by a MEK-dependent transition from an epithelial to mesenchimal phenotype and could be overcome by using selective MEK inhibitors (37). Moreover, a clone of GEO cells with acquired resistance to cetuximab were sensitive to the growth inhibitory effects of the MEK1/2 inhibitor selumetinib (16). Taken together, these findings suggest that MEK inhibition might be a potentially effective therapeutic strategy for preventing and/or overcoming cancer cell resistance to different molecular targeted agents that block growth factor receptor–driven intracellular signaling.

Based on these findings, we have explored whether the use of MEK1/2 inhibitors could be able to overcome intrinsic and acquired resistance to the anti-EGFR inhibitor. For this reason, we investigated the combination of cetuximab and MEK1/2 inhibitors (either BAY 86-9766, selumetinib and pimasertib) in a panel of human colorectal cancer cell lines. Here we show a synergistic growth inhibition of cetuximab-resistant colorectal cancer cell lines following combined treatment with the anti-EGFR monoclonal antibody and selective MEK1/2 inhibitor. The synergistic anti-proliferative effects were observed only in cetuximab-resistant cell lines, whereas in cetuximab-sensitive GEO and SW48 cells, the combined treatment induced a clearly antagonistic activity. These data may suggest that in anti-EGFR sensitive colorectal cancer cell lines the combined treatment with cetuximab and a MEK1/2 inhibitor could negatively interfere on cell growth inhibition, although this hypothesis needs further studies.

The results of this study demonstrate that the synergistic anti-proliferative effect is correlated with an inhibition of activated pMAPK and pAKT, that leads to increased apoptosis, as shown by increased levels of cleaved PARP and caspase-3 activation. Collectively, these results suggest that RAS/RAF/MEK/MAPK pathway activation may play a relevant role in determining resistance to cetuximab. To further validate this hypothesis and verify that the effect of resistance to cetuximab is correlated to MEK activation, we have used selective MEK1/2 siRNA. Inhibition of MEK1/2 correlated with a partially restored sensitivity to cetuximab in colorectal cancer cells resistant to anti-EGFR inhibitors. In fact, MEK silencing restores cetuximab ability to inhibit MAPK and cell proliferation.

Finally, the in vivo experiments on nude mice bearing tumor xenograft models of both primary and acquired resistance to cetuximab with the combined treatment with cetuximab and BAY 86-9766 resulted in tumor growth inhibition with consequent increase in mice survival.

More than 10 selective MEK inhibitors are currently in clinical development (17, 20, 38–42). So far, clinical efficacy of MEK inhibitors as single agents has been demonstrated in patients with metastatic melanoma with BRAF-mutated cancers (43), whereas it has been rarely observed in patients with unselected chemorefractory
metastatic with other cancer types (44, 45). A recent phase I study of selumetinib in combination with cetuximab in chemoresistant solid tumors, including KRAS-mutant metastatic colorectal cancer, has been reported. The combination has been well tolerated, with preliminary evidence of antitumor activity in patients with metastatic colorectal cancer with 2 partial responses and 2 disease stabilization (46). Finally, a phase Ib/II clinical trial is currently ongoing in patients with BRAF-mutant metastatic colorectal cancer and it evaluates the combination of LGX818, a MEK inhibitor, with cetuximab or with BYL719, a PIK3CA inhibitor (47).

Finally, to our knowledge, this study provides the first experimental evidence of the synergistic antitumor activity of the combination of cetuximab and a selective MEK inhibitor in cetuximab-resistant colorectal cancer cells with either primary or acquired resistance in which cetuximab single-agent treatment fails to inhibit RAS-RAF-MEK-MAPK intracellular signaling. On the contrary, selective MEK inhibition by treatment with BAY 86-9766 significantly blocks MAPK activation, inhibits cell proliferation, and induces apoptosis in almost all cetuximab-resistant colorectal cancer cell lines. Finally, treatment of these cells with BAY 86-9766 restores cetuximab sensitivity, suggesting that the combined inhibition of both EGFR and MEK could represent a rational therapeutic strategy for preventing and/or overcoming cetuximab resistance in patients with metastatic colorectal cancer. In this respect, it could be clinically relevant to target the MEK pathway in patients whose tumors develop resistance to anti-EGFR therapies or, eventually, to combine MEK1/2 inhibitors and anti-EGFR drugs such as cetuximab to delay the emergence of resistance.

Disclosure of Potential Conflicts of Interest
F. Ciardiello is a consultant/advisory board member for Bayer and Merck Serono. No potential conflicts of interest were disclosed by the other authors.

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