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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Translational Relevance

Cetuximab, a blocking anti-EGFR monoclonal antibody, is effective in combination with chemotherapy or as single agent for the treatment of KRAS wild type metastatic colorectal cancer (CRC) patients. However, clinical data indicate that, in all initially responding CRC patients, 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 CRC 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 CRC 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 anti-proliferative activity in cetuximab-resistant CRC 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 CRC patients.
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 (CRC) cell lines with primary or acquired resistance to cetuximab.

Results: among the CRC cell lines, five with a KRAS mutation (LOVO, HCT116, HCT15, SW620, SW480) and one with a BRAF mutation (HT29) were resistant to the anti-proliferative 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 CRC 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 anti-proliferative and apoptotic effects with blockade in MAPK- and AKT-pathway in cells with either primary or acquired resistance to cetuximab. The synergistic anti-proliferative effects were confirmed using other two selective MEK 1/2 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, 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 CRC patients.
Introduction

CRC is a major cause of morbidity and mortality throughout the world (1). The prognosis of patients diagnosed with metastatic CRC 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, aflibercept and regorafenib (2-3).

Cetuximab and panitumumab are two blocking anti-epidermal growth factor receptor (EGFR) monoclonal antibodies (MAbs) 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 CRC (7). Resistance to anti-EGFR therapies is likely due to 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 combination with chemotherapy only in metastatic CRC patients with KRAS wild-type tumors.

In addition to KRAS gene mutations, a number of retrospective studies have provided evidence that primary resistance to EGFR inhibitors in CRC 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 CRC patients 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 CRC patient population to treat with cetuximab, all initially responding patients will
ultimately develop resistance to cetuximab (i.e., acquired resistance) (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 metastatic CRC patients could be due to the emergence of KRAS mutated cancer cell clones (13-14). Moreover, HER2 gene amplification has been found to occur in approximately 3% of unselected metastatic CRC patients 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 cetuximab-resistant metastatic CRC patients, acquired resistance is probably due to 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 MAb 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 CRC models. For this purpose, we have selected a panel of eight human CRC 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 two cancer cell lines with acquired resistance to cetuximab that we have obtained following continuous treatment with cetuximab of two cetuximab-sensitive human CRC cell lines (22-23) to further elucidate the molecular mechanisms of primary and acquired resistance to cetuximab.
Material and Methods

Drugs. Cetuximab, an anti-EGFR human-mouse chimeric monoclonal antibody (MAb) was kindly provided by Merck Serono Italy (Rome, Italy). BAY 86-9766, selumetinib and pimasertib, selective MEK1/2 tyrosine kinase inhibitors, were kindly provided by Bayer Pharma Italy (Milan, Italy), Astra Zeneca (Macclesfield, UK) and Merck-Serono (Darmstadt, Germany), respectively. All three MEK1/2 inhibitors were dissolved in sterile dimethylsulfoxide (DMSO) and a 10 mM 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 CRC cell lines were obtained from the American Type Culture Collection (ATTC) (Manassas, VA) 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) CRC 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, Cologne, Germany), supplemented with 20% fetal bovine serum (FBS) (Lonza), 1% penicillin/streptomycin (Lonzax). SW48, SW48-CR, LOVO, HCT15, HCT116 and SW480 cells were grown in RPMI-1640 (Lonza) supplemented with 10% FBS, 1% penicillin/streptomycin. SW 620 and HT29 cancer cells were grown in McCoy medium (Lonza) supplemented with 20% FBS (Lonza), 1% penicillin/streptomycin (Lonza). All cell lines were grown in a humidified incubator with 5% of carbon dioxide (CO₂) and 95% air at 37°C. All cell lines were routinely screened for the presence of mycoplasma (Mycoplasma Detection Kit, Roche Diagnostics, Monza, Italy).
**Proliferation Assay.** Cancer cell lines were seeded in 24-well plates and were treated with different concentrations of cetuximab (range, 0.001 to 20 μg/ml) alone or in combination with BAY 86-9766 (range, 0.001 to 5μM), or selumetinib (range, 0.001 to 5μM) or pimasertib (range, 0.001 to 5μM) 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 three separate experiments, each performed in quadruplicate. Results of the combination treatment were analysed according to the method of Chou and Talalay by using the CalcuSyn software programme (Biosoft, Cambridge, UK).

**Apoptosis assay.** HCT15, HCT116, SW48-CR and GEO-CR cells were seeded in six-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, CA, USA), coupled with fluorescence-activated cell sorting (FACS) analysis.

**Immunoblotting.** HCT15, HCT116, SW48-CR and GEO-CR cells were seeded into 100 mm³ dishes and treated with vehicle, cetuximab, BAY 86-9766, or their combination for 24 hours. Protein lysates containing comparable amounts of proteins, estimated by a modified Bradford assay (Bio-Rad, Munich, Germany), were subjected to Western blot, as previously described (24). Immuno-complexes were detected with the enhanced chemiluminescence kit (Pierce Biotechnology Inc, Rockford, USA). 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), Anti-PARP polyclonal antibody (#9542) were from Cell Signaling (Beverly, MA, USA). Caspase-3 monoclonal antibody (sc-65496) was from Santa Cruz Biotechnology, (Santa Cruz, CA, USA). Monoclonal anti-α-tubulin antibody (T8203) was from Sigma Chemical Co. (St. Louis, MO, USA). The following secondary antibodies from Bio-rad (Hercules, CA, USA) were used: goat anti-rabbit IgG
and rabbit anti-mouse IgG. Immunoreactive proteins were visualized by enhanced chemiluminescence. (ECL plus, Thermo Fisher Scientific, Rockford, IL, USA). 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 (Lafayette, CO, USA). The siCONTROL Non-targeting Pool (#D-001206-13-05) was used as a negative (scrambled) control. Cells were transfected with 100 nM siRNAs using Hiperfect reagent (Qiagen, Monza, Italy) 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. 48 hours after transfection, cells treated with MEK1/2 siRNA received 5 µg/ml of cetuximab and cell proliferation and Western blot 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 (Milan, Italy). 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 prior to being injected with cancer cells and then caged in groups of five. Mice were injected s.c. with 2.5 x 10^6 HCT15, HCT116, GEO-CR and SW48-CR cells that had been resuspended in 200 µl of matrigel (BD Biosciences, Milan, IT). When the mean values of tumors were between 200-300 mm^3, mice were randomly divided in four 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 mm$^3$, when mice were sacrificed. Tumor size was evaluated twice per week by calliper measurements using the following formula: $\pi/6 \times \text{larger diameter} \times (\text{smaller diameter})^2$. Student's t test was used to evaluate the statistical significance of the results.

**Statistical analysis.** The statistical analyses of *in vitro* and *in vivo* data were carried out using Prism version 4.02 (GraphPad Software, Inc). The Student’s t test was used to evaluate the statistical significance of the results. All p values represent two-sided tests of statistical significance with p value $< 0.05$. 
Results

Effects of cetuximab treatment on cell proliferation and on EGFR-dependent intracellular signalling in human CRC cell lines. We first evaluated the sensitivity to the cell growth inhibiting effects of the anti-EGFR MAb cetuximab in a panel of eight human CRC (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 2). Cancer cells were treated with cetuximab at concentrations ranging from 0.01 to 20 µg/ml 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 Figure 1A and Supplementary Table 1A, there was a differential sensitivity to cetuximab-induced cell growth inhibition. In fact, six CRC cell lines (HT29, LOVO, HCT116, HCT15, SW620 and SW480) were primarily 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 two cetuximab-sensitive CRC 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 CRC cell line is one of the most sensitive CRC cell lines to the in vitro and in vivo antitumor activity of cetuximab treatment (23;25-27). Furthermore, Figure 1A and Supplementary Table 1A show that cetuximab was also not effective in GEO-CR and SW48-CR cells, two 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 two 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 to the parental cell lines (data not shown). As shown in Figure 1B, cetuximab treatment suppressed EGFR-induced intracellular signaling only in the two CRC 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 CRC cell lines.

Effects of treatment with BAY 86-9766, a selective MEK1/2 inhibitor, on cell proliferation of human CRC 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 CRC cell lines. Cancer cells were treated with BAY 86-9766 at concentrations ranging from 0.01 to 5 μM for 96 hours. As illustrated in Figure 2A and Supplementary Table 1B, BAY 86-9766 treatment caused a dose-dependent cell growth inhibition in all CRC cell lines, with IC_{50} values ranging between 0.05 μM (HCT116) and 2 μM (SW48-CR), except for HCT15 cells. The same effects on cell growth inhibition were obtained by using other two different MEK1/2 inhibitors such as selumetinib and pimasertib (Figure 2B, C and Supplementary Table 1B).

Effects of cetuximab in combination with the selective MEK1/2 inhibitor BAY 86-9766 in CRC cells growth in vitro. All CRC cell lines were treated with different concentrations of cetuximab, of BAY 86-9766, or with the combination of the two drugs (Figure 3; Supplementary Figure 1A and 1B; Supplementary Table 3). 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 CRC cell lines with primary resistance to cetuximab (HT29, LOVO, HCT116, HCT15, SW620, SW480) and in the CRC 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) (Figure 3; Supplementary Figure 1A
and 1B; Supplementary Table 3). 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 CRC cell lines the combined treatment with cetuximab and the MEK1/2 inhibitor could negatively interfere on cell growth inhibition (Supplementary Figure 1A and 1B; Supplementary Table 3). To confirm the synergistic growth inhibitory effects of cetuximab in combination with BAY 86-9766, we have used other two selective MEK1/2 inhibitors, such as selumetinib and pimasertib (24; 28). As depicted in Supplementary Table 1B the combined treatment of cetuximab with selumetinib or pimasertib induced a clearly synergistic antitumor activity only in the CRC 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 3).

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 CRC cells. We next selected for further experiments two CRC cell lines with primary resistance to cetuximab (HCT15 and HCT116) and the two CRC cell lines with acquired resistance to cetuximab (GEO-CR and SW48-CR) to determine whether the synergistic growth inhibition which was obtained by the combined treatment with the selective MEK1/2 inhibitor BAY 86-9766 and cetuximab was due to 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 (Figure 4A). While 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 CRC cells with primary resistance to cetuximab and CRC cells with acquired
resistance to cetuximab (Figure 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 (Figure 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 (Figure 4C and D; Supplementary Figure 2).

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 Figure 5A. As illustrated in Figures 5B, MEK1/2 siRNA treatment slightly reduced cell growth in HCT15 and HCT116. Whereas 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 down-regulation of phospho-MAPK levels. (Figure 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 three weeks with cetuximab, with BAY 86-9766 or with the combination of the two drugs. As shown in Figure 6, treatment with cetuximab had little or
no effect on tumor growth in all tumor xenografts, whereas BAY 86-9766 treatment caused approximately 40-50% reduction in tumor growth in tumor xenograft models of both primary and acquired resistance to cetuximab. In contrast, the combined treatment with the two drugs suppressed almost completely HCT15, HCT116, GEO-CR or SW48-CR tumor growth at the end of the three weeks of therapy (Figure 6). Furthermore, the combined treatment with cetuximab and BAY 86-9766 determined a significant increase in mice survival as compared to 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 Figure 3).
Discussion

Elucidating the mechanisms of cancer cell resistance to anti-cancer 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 chemorefractory metastatic CRC patient as a result of EGFR-independent intracellular downstream signaling activation (8; 30-34).

In agreement with these findings, in the present study we have found that among the panel of human CRC 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 CRC 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 et al. have developed cetuximab-resistant clones of GEO cells, in which activation of ERBB2 signaling, either through ERBB2 amplification or through heregulin protein up-regulation, leads to persistent, constitutive ERK1/2 activation (16). Moreover, Normanno et al. 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 activation of MEK/MAPK signaling (35). In this respect, we have recently developed human cancer cell lines with acquired resistance to erlotinib and gefitinib,
two selective EGFR-tyrosine kinase inhibitors (TKIs), following chronic exposure to these drugs of CALU-3 lung adenocarcinoma and HCT116 CRC cells (36). We have found that EGFR inhibitor acquired resistance in these cells was predominantly driven by the constitutive activation of the RAS/RAF/MEK/MAPK pathway and it could be overcome by treatment with sorafenib, which blocked RAF signaling (36). Furthermore, 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 CRC cell lines. Here we show a synergistic growth inhibition of cetuximab-resistant CRC cell lines following combined treatment with the anti-EGFR monoclonal antibody and selective MEK1/2 inhibitor. The synergistic antiproliferative 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 CRC 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 the present 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 CRC 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 ten selective MEK inhibitors are currently in clinical development (17; 38-43). So far, clinical efficacy of MEK inhibitors as single agents has been demonstrated in metastatic melanoma patients with BRAF mutated cancers (44), whereas it has been rarely observed in unselected chemorefractory metastatic patients with other cancer types (45-46). A recent phase I study of selumetinib in combination with cetuximab in chemorefractory solid tumors, including KRAS mutant metastatic CRC, has been reported. The combination has been well tolerated, with preliminary evidence of antitumor activity in metastatic CRC patients with two partial responses and two disease stabilization (47). Finally, a phase Ib/II clinical trial is currently ongoing in patients with BRAF mutant metastatic CRC and it evaluates the combination of LGX818, a MEK inhibitor, with cetuximab or with BYL719, a PIK3CA inhibitor (48).

Finally to our knowledge, the present study provides the first experimental evidence of the synergistic antitumor activity of the combination of cetuximab and a selective MEK inhibitor in...
cetuximab-resistant CRC 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 CRC 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 metastatic CRC patients. 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.
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Figure Legends

Figure 1. Effects of cetuximab on cell proliferation and on EGFR-dependent intracellular signalling in a panel of human CRC cell lines. A Cells were treated with different concentrations of cetuximab (range, 0.01 to 20 μg/ml) for 96 hours and evaluated for proliferation by MTT staining, as described in Materials and Methods. The IC50 was determined by interpolation from the dose-response curves. Results represent the median of three separate experiments, each performed in quadruplicate. B, Analysis of intracellular signalling pathways by Western blotting in cancer cells treated with cetuximab at the indicated dose for 24 hrs. Total cell protein extracts (50μg) were subjected to immunoblotting with the indicated antibodies, as described in Materials and Methods.

Figure 2. Effects of MEK1/2 inhibitors, on cell proliferation in a panel of human CRC cell lines. A-C Cells were treated with different concentrations of BAY 86-9766, Selumetinib and Pimasertib (range, 0.01 to 5 μM for all three drugs) for 96 hours and evaluated for proliferation by MTT staining, as described in Materials and Methods.

Figure 3. Effects of cetuximab in combination with the selective MEK1/2 inhibitor BAY 86-9766 in HCT15, HCT116, GEO-CR and SW48-CR CRC cells growth in vitro. The indicated CRC cell lines were treated every day with different concentrations of cetuximab (range, 0.01 to 20 μg/ml) and/or BAY-86-9766 (range, 0.01 to 5 μM) for 96 hours at a fixed drug ratio of 1:1 and cell proliferation was evaluated by MTT assay. Combination Index (CI) values were calculated according to the Chou and Talalay mathematical model for drug interactions using the CalcuSyn software, as described in Materials and Methods. For each cell line, it is shown in the left panel the percent of cell growth inhibition determined by treatment with cetuximab, with BAY 86-9766 or with their combination. For each cell line, in the right panel it is shown the combination index for the combination of cetuximab plus BAY 86 9766. Results represent the median of three separate experiments, each performed in quadruplicate.
Figure 4. Effects of cetuximab in combination with the selective MEK1/2 inhibitor BAY 86-9766 on induction of apoptosis and on intracellular signaling pathways in HCT15, HCT116, GEO-CR and SW48-CR CRC cells. A, Apoptosis was evaluated with Annexin V staining, as described in Materials and Methods. Cells were treated with cetuximab, with BAY 86-9766 or with their combination with the indicated doses for 72 hrs. The rate of apoptosis was expressed as a percentage of the total cells counted. Columns are the means of three independent experiments. B, Cells were treated with cetuximab, with BAY 86-9766 or with their combination at the indicated doses for 24 hrs. Expression of caspase 3 and of the cleaved form of PARP were evaluated by immunoblotting, as described in Materials and Methods. Anti-tubulin antibody was used for normalization of protein extract content. C-D, Analysis of intracellular signaling pathways by Western blotting in the indicated CRC cell lines treated with cetuximab, with BAY 86-9766 or with their combination at the indicated doses for 24 hrs. Total cell protein extracts (50 μg) were subjected to immunoblotting with the indicated antibodies, as described in Materials and Methods. Anti-tubulin antibody was used for normalization of protein extract content.

Figure 5. Inhibition of MEK1/2 expression restores cetuximab sensitivity in HCT15, HCT116, CRC cells. A, HCT15, HCT116, cells were transfected with either specific siRNA targeting MEK1/2 or with a control (scrambled) RNA sequence and harvested at 24, 48, 72 and 96 hours after transfection. Western blot analysis for MEK1/2 expression was done as described in Materials and Methods. B, HCT15, HCT116, cells were transfected with a specific siRNA targeting MEK1/2. 48 hours after transfection, cells were treated with cetuximab, 5 μg/ml. Viable cells were counted after 24 hours of treatment (after 72 hours of transfection) and plotted relative to untreated control. The results are average ± SD of three independent experiments each done in duplicate. C, Western blot analysis of cell signalling proteins in HCT15, HCT116, GEO-CR and SW48-CR cells transfected with a specific siRNA targeting MEK1/2 or with a scrambled, control siRNA for 48 hours and subsequently treated with the indicated dose of cetuximab for 24 hrs (after 72 hours of
transfection). Total cell protein extracts were subjected to immunoblotting with the indicated antibodies, as described in Materials and Methods.

**Figure 6. Effects of cetuximab in combination with the selective MEK1/2 inhibitor BAY 86-9766 on HCT15, HCT116, GEO-CR and SW48-CR tumor xenografts.** The indicated cancer cell lines were grown as subcutaneous tumor xenografts in nude mice. After tumor establishment (200-300 mm³), mice were treated with cetuximab (1 mg/dose twice a week intraperitoneally) and/or BAY 86-9766 (5 mg/kg/daily oral gavage) for 3 weeks. Animals were sacrificed when tumors achieved 2,000 mm³ in size. Each group consisted of 10 mice. For each CRC cell lines, the left graph shows tumor xenograft growth and the right graph shows mice survival. *** p < 0.0001.
Fig. 2

A

% of proliferation (BAY 86-9766 (µM))

B

% of proliferation (Selumetinib (µM))

C

% of proliferation (Pimarsertib (µM))

Legend:
- GEO
- SW48
- HT29
- LOVO
- HCT110
- HCT15
- SW620
- SW400
- GEO-CR
- SW46-CR
Fig. 3

HCT15

HCT116

GEO-CR

SW48-CR

- combination
- cetuximab
- BAY 86-9766
**Fig. 4**

(A) Bar graph showing the percentage of apoptotic cells in different cell lines: Control, Cetuximab 0.01 μg/ml, BAY 86-9766 0.01 μM, and Combination.

(B) Western blot analysis showing PARP, pro-Caspase3, and Tubulin expression in HCT15 and HCT116 cell lines.

(C) Western blot analysis showing p-EGFR, EGFR, p-MEK, MEK, p-AKT, AKT, p-MAPK, MAPK, and Tubulin expression in HCT15 and HCT116 cell lines.

(D) Western blot analysis showing p-EGFR, EGFR, p-MEK, MEK, p-AKT, AKT, p-MAPK, MAPK, and Tubulin expression in GEO-CR and SW48-CR cell lines.
Fig. 6

HCT15

Mean Tumor Volume (mm$^3$) vs. Days of dosing

Control, Cetuximab, BAY 86-9766, Combination

HCT116

Mean Tumor Volume (mm$^3$) vs. Time (Days)

Control, Cetuximab, BAY 86-9766, Combination

SW48-CR

Mean Tumor Volume (mm$^3$) vs. Days of dosing

Control, Cetuximab, BAY 86-9766, Combination

GEO-CR

Mean Tumor Volume (mm$^3$) vs. Time (Days)

Control, Cetuximab, BAY 86-9766, Combination
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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