Clinical Cancer Research

Primary and Acquired Resistance of Colorectal Cancer to Anti-EGFR Monoclonal Antibody Can Be Overcome by Combined Treatment of Regorafenib with Cetuximab

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Abstract

Purpose: In colorectal cancer, the activation of the intracellular RAS–RAF and PIK3CA–AKT pathways has been implicated in the resistance to anti-EGFR mAbs. We have investigated the role of regorafenib, an oral multikinase inhibitor, in combination with cetuximab, an anti-EGFR mAb, to overcome anti-EGFR resistance.

Experimental Design: We have tested, *in vitro* and *in vivo*, the effects of regorafenib in a panel of human colorectal cancer cell lines with a *KRAS* mutation (SW480, SW620, HCT116, LOVO, and HCT15) or with a *BRAF* mutation (HT29), as models of intrinsic resistance to cetuximab treatment, and in two human colorectal cancer cell lines (GEO and SW48) that are cetuximab-sensitive, as well as in their derived cells with acquired resistance to cetuximab (GEO-CR and SW48-CR).

Results: Treatment with regorafenib determined a dose-dependent growth inhibition in all colorectal cancer cell lines. The combined treatment with cetuximab and regorafenib induced synergistic antiproliferative and apoptotic effects in cetuximab-resistant cell lines by blocking MAPK and AKT pathways. Nude mice were injected s.c. with HCT116, HCT15, GEO-CR, and SW48-CR cells. The combined treatment caused significant tumor growth inhibition. Synergistic antitumor activity of regorafenib plus cetuximab was also observed in an orthotopic colorectal cancer model of HCT116 cells. In particular, the combined treatment induced a significant tumor growth inhibition in the primary tumor site (cecum) and completely prevented metastasis formation.

Conclusions: The combined treatment with cetuximab and regorafenib could be a strategy to overcome resistance to anti-EGFR therapies in metastatic colorectal cancer patients. *Clin Cancer Res; 21(13); 2975–83. ©2015 AACR.*

Introduction

Colorectal cancer is one of the leading causes of cancer-related mortality worldwide, with more than 1.2 million new cases and 608,700 deaths estimated in 2008 (1). Despite improvements made in screening strategies, a significant number of patients are still diagnosed at late stages of the disease.

In the last decade, the introduction of targeted therapies in clinical practice, in particular of agents targeting the VEGF-related pathway (bevacizumab and aflibercept) and the EGFR (cetuximab and panitumumab) has changed the therapeutic approach to metastatic colorectal cancer patients, with a significant improvement in progression-free survival (PFS) and overall survival (OS; ref. 2). Cetuximab and panitumumab are mAbs that block the activation of the EGFR and of its downstream intracellular signals, the RAS–RAF–MEK–MAPK and the PTEN–PIK3CA–AKT pathways (3–6).These two drugs are currently approved for the treatment of metastatic colorectal cancer patients with all-*RAS* wild-type tumors. Nevertheless, prognosis remains poor for most of these patients. In fact, the use of these mAbs is limited by the presence of preexisting intrinsic resistance mechanisms or by the ability of cancer cells to acquire resistance. Possible mechanisms for primary and acquired resistance to cetuximab include mutations in the *KRAS*, *BRAF*, and *NRAS* genes, secondary mutation (S492R) in the extracellular domain of EGFR, *HER2* gene amplification, and/or increased HER2 signaling and overexpression of the MET pathway (7–10).

Recently, it has been elucidated that in the resistance to anti-EGFR therapies, different growth factors and receptors could be activated in the cancer cell to drive alternative signaling pathways that bypass the EGFR (11, 12). Molecular heterogeneity also plays an important role in the context of resistance, by limiting the success of therapies against a single target. Colorectal cancer patients can harbor different gene mutations in

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Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

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Translational Relevance

The introduction in clinical practice of mAbs against the EGFR, such as cetuximab or panitumumab, in combination with chemotherapy has demonstrated therapeutic efficacy in metastatic colorectal cancer patients with all *RAS* wild-type tumors. However, efficacy of these mAbs is limited by development of resistance mechanisms in cancer cells. Activation of alternative signaling pathways that bypass the EGFR has been implicated in the resistance to anti-EGFR therapies. Therefore, the blockade of multiple growth factor and receptor pathways could be necessary to increase the efficacy of anti-EGFR mAbs. In this study, we have demonstrated that, in human colorectal cancer cells with either primary or acquired resistance to cetuximab, the combined treatment with cetuximab and regorafenib induces synergistic antiproliferative and apoptotic effects and causes significant tumor growth inhibition. This study provides a rationale for evaluating combined treatment with cetuximab and regorafenib as a therapeutic strategy for preventing and/or overcoming cetuximab resistance in metastatic colorectal cancer patients.

distinct tumor lesions, or even within different regions of the same lesion (13). All these alterations could converge on activation of the RAS–MEK–ERK pathway (9, 10, 14, 15). Understanding the biology of such complex gene heterogeneity in tumors is necessary for developing rational combination therapies. In fact, blockade of multiple growth factor and growth factor receptor pathways could be needed to increase the efficacy of anti–EGFR-targeted therapies (16).

Regorafenib is an oral multikinase inhibitor, that could target three key oncogenic pathways, such as (i) cell growth by inhibition of *KIT*, *RET*, *RAF-1*, and *BRAF*; (ii) tumor-induced angiogenes is by targeting VEGFR1, 2, and 3, and the tyrosine kinase with immunoglobulin and EGF homology domain 2 (TIE2); and (iii) tumor microenvironment by blocking platelet-derived growth factor receptor-β (PDGR-β) and FGFR (17–19). In preclinical studies, regorafenib exhibited antitumor activity in different tumor xenografts (17). Recently, a phase III study showed that regorafenib treatment significantly improved OS and PFS in patients with metastatic colorectal cancer who failed all available therapies (20). Thus, both the FDA and the European Medicines Agency have approved regorafenib for the treatment of such metastatic colorectal cancer patients.

In the present study, we have evaluated the efficacy of regorafenib in combination with cetuximab to overcoming resistance to anti-EGFR mAbs by using different human colorectal cancer cell models. We have selected five colorectal cancer cell lines with *KRAS* mutations (SW480, SW620, HCT116, LOVO, and HCT15) one with *BRAF* mutation (HT29) and two cell lines with acquired resistance to cetuximab, that were originally obtained in our laboratory (Supplementary Table S1; refs. 10, 14, 21). We have found that combined treatment with cetuximab and regorafenib induced synergistic antiproliferative and proapoptotic effects by blocking MAPK and AKT pathways in these colorectal cancer cell lines. Moreover, a similar synergistic antitumor activity has been confirmed by *in vivo* subcutaneous and orthotopic colorectal cancer xenograft models.

Materials and Methods

Drugs

Cetuximab, an anti-EGFR human-mouse chimeric mAb, was kindly provided by Merck Serono Italy, and it was ready to use. Regorafenib was kindly provided by Bayer Pharma Italy. For *in vitro* applications, regorafenib was dissolved in sterile DMSO, and the 10 mmol/L stock solution was stored in aliquots at −20°C. Working concentrations were diluted in culture medium just before each experiment. For *in vivo* applications, regorafenib was solubilized in 0.5% Tween-80 in sterile PBS.

Cell lines

The human HT29, SW620, LOVO, and HCT15 colorectal cancer cell lines were obtained from the ATCC and have been authenticated by IRCCS "AziendaOspedalieraUniversitaria San Martino-IST IstitutoNazionale per la RicercasulCancro, Genova" Italy. The humanSW48 (catalogue number: HTL99020), SW480 (catalogue number: HTL95025), and HCT116 (catalogue number: HTL99017) colorectal cancer cell lines were obtained from IRCCS "AziendaOspedalieraUniversitaria San Martino-IST IstitutoNazionale per la RicercasulCancro, 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 (10, 14, 21). GEO and GEO-CR cell lines were grown in DMEM (Lonza), supplemented with 10% FBS (Lonza), 1% penicillin–streptomycin (Lonza). SW48, SW480, HCT116, LOVO, HCT15, and SW48-CR cells were grown in RPMI-1640 (Lonza) supplemented with 10% FBS, 1% penicillin–streptomycin. SW620 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 (CO_2) and 95% air at 37°C. All 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, 0.001–20 μg/mL) alone or in combination with regorafenib (range, 0.001–5 μmol/L) for 96 hours. Cell proliferation was measured with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). The IC_{50} value 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 analyzed according to the method of Chou and Talalay by using the CalcuSyn software program (Biosoft).

Apoptosis assay

HT29, SW480, SW620, HCT116, LOVO, HCT15, GEO-CR, and SW48-CR cells were seeded in 6-well plates, treated with cetuximab, regorafenib or their combination at different concentrations 72 hours and stained with Annexin V–FITC (Invitrogen). Apoptotic cell death was assessed by counting the numbers of cells that stained positive for Annexin V–FITC using an Apoptosis Annexin V–FITC Kit (Invitrogen), coupled with FACS analysis, by following the manufacturer's protocol.

Immunoblotting

SW480, SW620, HCT116, LOVO, HCT15, GEO-CR, and SW48-CR cells were seeded into 100 mm^3 dishes and treated with vehicle, cetuximab, regorafenib, or their combination for 24 hours at different concentration as following indicated. Fifty mg of protein lysates, estimated by a modified Bradford assay (Bio-Rad), were subjected to Western blot analysis, as previously described (22), by using the following Abs:AKT polyclonal antibody (#9272), pAKT mAb (#4060), phospho-S6 ribosomial protein (#4856), p44/42 MAPKpolyclonal antibody (#9102), phospho-p44/42MAPK mAb (#9106) were from Cell Signaling Technology. Monoclonal anti–α-tubulin antibody (T8203) was from Sigma Chemical Co. Goat anti-rabbit IgG and rabbit anti-mouse IgG secondary Abs were from Bio-Rad (Hercules). Immunoreactive proteins were visualized by enhanced chemiluminescence (ECL plus, Thermo Fisher Scientific). Each experiment was done in triplicate.

Tumor xenografts in nude mice

Four- to 6-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. Animal care was in compliance with Italian (Decree 116/92) and European Community (E.C. L358/1 18/12/86) guidelines on the use and protection of laboratory animals. 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 five under controlled conditions (12–12 hours light-dark cycle; room temperature $20 \pm 22°C$; humidity 55%–60%). A total number of 3.5×10^6 GEO-CR, SW48-CR cells, and 2×10^6 HCT116, HCT15 cells in 200 μL of Matrigel (BD Biosciences):PBS (1:1) were s.c. injected to the dorsal flank of mice. When the mean values of tumors were between 200 and 300 mm^3, mice were randomly assigned to one of the following groups (10 mice/group). Group 1, vehicles administrated orally and i.p. Group 2, cetuximab injected twice a week i.p. at the dose of 1 mg for 3 weeks. Group 3, regorafenib administered by daily oral gavage at the dose of 10 mg/kg for 3 weeks. Group 4, combination of regorafenib and cetuximab. Monitoring of tumor growth was performed until tumors reached approximately 2,000 mm^3, when mice were euthanized. Tumor size was evaluated twice a week by calliper measurements using the following formula: $\pi/6 \times$ larger diameter $\times$ (smaller diameter)^2. The Student *t* test was used to evaluate the statistical significance of the results.

Orthotopic colorectal cancer model

Four- to 6-week-old female balb/c athymic (nu^+/nu^+) mice purchased from Charles River Laboratories were used. The orthotopic implantation was performed as described by Hoffman and colleagues (23). In brief, subcutaneous tumors derived from HCT116 cells were obtained. When tumors reached a mean volume of 500 mm^3, animals were euthanized, the tumors were removed using sterile techniques, divided into 2- to 3-mm sized pieces, and harvested in PBS on ice. Mice were treated with antibiotics, ticarcillin (50mg/kg i.v.) 2 hours before and after tumor implantation. Animals were anesthetized with 2,2,2-tribomoethanol 97% TBE, Avertin (Sigma-Aldrich). TBE solution was prepared fresh daily by mixing 0.625 g of 97% crystalline TBE powder with 25 mL sterile 0.9% saline and then injected i.p. at 0.01 mL/g body mass (250 mg/kg). The abdomen was prepped with betadine solution and the surgical site was isolated in a sterile fashion. A laparotomy of 0.5 cm was conducted; the cecum was exteriorized and isolated using pre-cut, sterile gauze. A warm saline solution was used to keep the cecum wet. Subsequently, the cecum wall was slightly damaged, and a single tumor fragment from HCT-116 subcutaneous tumors was sutured to the mesenteric border of the cecum wall using 6.0 nylon surgical sutures. Upon completion, the cecum was placed into the abdominal cavity and the abdominal wound was sutured using a 6.0 Ethicon absorbable stitches (Ethicon Inc.). Fourteen days after the injection, mice were randomly assigned to four groups (7 mice for each group) to receive one of the following treatments. Group 1, daily administration of PBS/0.5% Tween 80 by oral gavage for 5 days a week and i.p. injection of PBS twice a week (control group). Group 2, daily administration of diluent for 5 days a week and i.p. injection of cetuximab 1 mg twice a week. Group 3, daily administration of regorafenib 10 mg/kg by oral gavage for 5 days a week and i.p. injection of PBS twice a week. Group 4, combination of oral regorafenib and i.p. cetuximab. Treatment was continued for 3 weeks, and the mice were euthanized 1 week later. The body weights were monitored daily. Primary tumors in the cecum were excised and weighed. The final tumor was measured with a caliper and the volume was calculated by the following formula: $\pi/6 \times$ larger diameter $\times$ (smaller diameter)^2. The presence of metastasis was evaluated in the peritoneum, liver, intestines, lungs, rectum, and spleen and confirmed by histologic review. The tumor excised from each mouse was divided into three parts. One piece was formalin-fixed; the other two pieces were frozen at −80°C in RNA later. Hematoxylin and eosin staining confirmed the presence of tumors in each sample.

Results

Sensitivity to cetuximab and regorafenib treatment in a panel of human colorectal cancer cell lines

We first tested *in vitro* the activity of cetuximab and regorafenib, as single agents, in a panel of human colorectal cancer cell lines to characterize their spectrum of activity. We selected eight human colorectal cancer (GEO, SW48, HT29, SW480, SW620, HCT116, LOVO, and HCT15) cell lines, having different mutation profiles in *KRAS*, *NRAS*, *BRAF*, and *PIK3CA* genes (Supplementary Table S1). Cancer cells were treated with cetuximab at concentrations ranging from 0.01 to 20 μg/mL and with regorafenib at concentrations ranging from 0.05 to 5 μg/mL for 96 hours. The drug concentrations required to inhibit cell growth by 50% (IC_{50}) were determined by interpolation from the dose–response curves.

Two colorectal cancer cell lines were sensitive to cetuximab: SW48, a cell line "quadruple wild type" for *KRAS*, *BRAF*, *NRAS*, and *PIK3CA* genes, and GEO cells with a *KRAS* codon 12 mutation, with a IC_{50} value of 0.5 and 0.1 μg/mL, respectively. Despite GEO cells harbor a *KRAS* gene mutation, previous studies from different laboratories, including our own, demonstrated that this colorectal cancer cell line is one of the most sensitive to the *in vitro* and *in vivo* antitumor activity of cetuximab treatment (14, 21, 24–26). HT29, SW480, SW620, HCT116, LOVO, and HCT15 were primarily resistant to cetuximab, as shown in Fig. 1. These cells have an activating *KRAS* gene mutation in either codon 12 or 13 within exon 2, except HT29 cells that have a *BRAF* mutation (V600E). Cetuximab was also not effective in GEO-CR and SW48-CR cells, two models of cetuximab-acquired resistance, previously

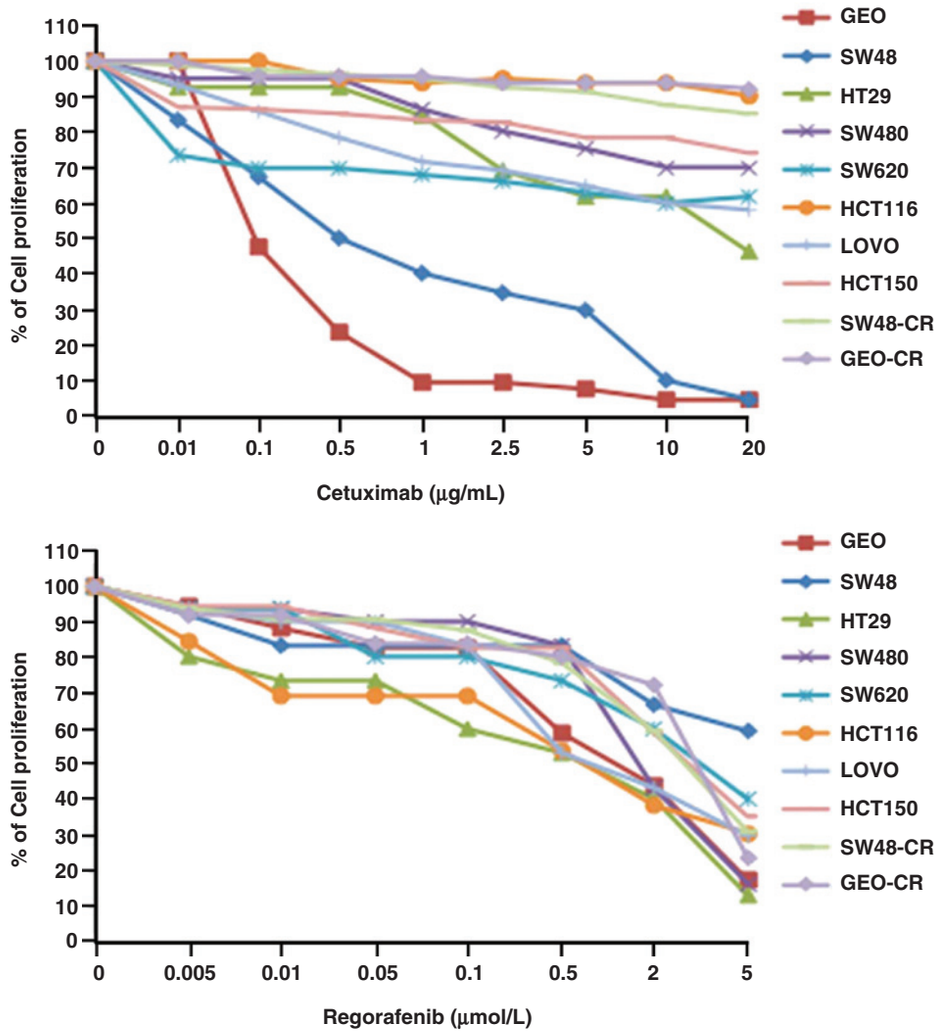

Figure 1.
Effects of cetuximab and regorafenib treatment on cell proliferation in a panel of human colorectal cancer cell lines. Cells were treated with different concentrations of cetuximab (range, 0.01–20 μg/mL) and regorafenib (range, 0.05–5 μg/mL) for 96 hours and evaluated for proliferation by MTT staining, as described in Materials and Methods. The IC_{50} value was determined by interpolation from the dose–response curves. Results represent the median of three separate experiments, each performed in quadruplicate.

obtained in our laboratory (10, 14, 21). As shown in Fig. 1, regorafenib shows a different proliferation inhibitory effect in these human colorectal cancer cell lines, with IC_{50} values ranging between 0.5 μmol/L (HCT116, HT29, and LOVO), 1 μmol/L (GEO), 2 μmol/L (SW480 and HCT15), and >2 μmol/L (SW48, SW620, SW48-CR, and GEO-CR). No significantly differences in regorafenib efficacy were observed among colorectal cancer cell lines harboring *KRAS*, *NRAS*, *BRAF*, *PIK3CA* mutations, indicating that its antitumor activity seems to be independent of the molecular profile of colorectal cancer cell lines tested.

Effects of cetuximab in combination with regorafenib in a panel of human colorectal cancer cell lines with primary and acquired resistance to anti-EGFR drugs *in vitro*

We evaluated the antiproliferative activity of cetuximab and regorafenib in combination in the panel of human colorectal cancer cell lines (Supplementary Figs. S1 and S2). Combination index (CI) values were calculated according to the Chou and Talalay mathematical model for drug interactions using the Calcusyn software, as previously described (10, 14, 24, 27). A synergistic growth inhibitory effect was observed in human colorectal cancer cell lines with both primary and acquired resistance to 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; Supplementary Figs. S1 and S2). In contrast, an antagonistic effect of the combined treatment was observed in sensitive colorectal cancer cell lines (GEO and SW48) with CI values significantly >1.0 (data not shown).

Effects of cetuximab in combination with regorafenib on intracellular signaling pathways in a panel of human colorectal cancer cell lines with primary and acquired resistance to anti-EGFR drugs

To examine the mechanism by which the combined treatment contributes to inhibition of proliferation in colorectal cancer cell lines with primary or acquired resistance to anti-EGFR inhibitor, the activation of EGFR downstream signaling molecules was evaluated. SW480, SW620, HCT116, LOVO, HCT15, SW48-CR, and GEO-CR cells were treated with cetuximab, regorafenib and/or their combination. The activation of PIK3CA–AKT and RAS–MAPK pathways was analyzed by Western blotting. The combined treatment with cetuximab and regorafenib substantially inhibited

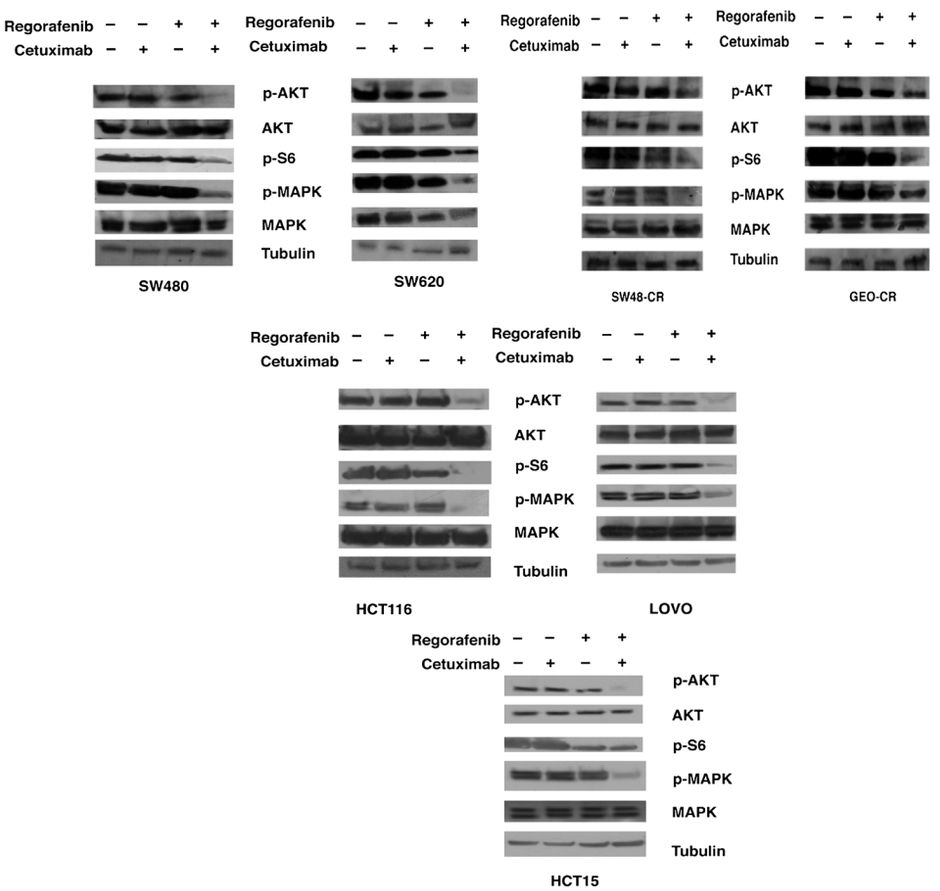

Figure 2.
Effects of cetuximab in combination with regorafenib on intracellular signaling pathways in a panel of colorectal cancer cell lines with primary and acquired resistance to anti-EGFR inhibitor. Cells were treated with cetuximab at a dose of 1 mg/mL, with regorafenib at a dose of 1 μmol/L, or with their combination for 24 hours. Total cell protein extracts (50 μg) were subjected to immunoblotting with the indicated Abs, as described in Materials and Methods. Antitubulin antibody was used for normalization of protein extract content. Experiments were repeated three times.

phosphorylation of both AKT and MAPK after 24 hours of treatment compared with single-agent treatments (Fig. 2). A strong reduction of phosphorylated S6 ribosomal protein (pS6) levels, the major downstream effect or of AKT/m-TOR signaling, was observed in the combination treatment (Fig. 2). These findings suggested that cetuximab in combination with regorafenib could overcome resistance to anti-EGFR treatment by inhibiting PIK3CA–AKT and MAPK pathways.

Proapoptotic effect of cetuximab in combination with regorafenib in colorectal cancer cell lines with primary and acquired resistance to anti-EGFR drugs

We measured the ability of cetuximab and regorafenib as single agents or in combination, to induce apoptosis in colorectal cancer cell lines by the Annexin V–FITC assay (Table 1 and Supplementary Fig. S3). Compared with single agent, the combined treatment induced significantly early and late apoptosis in the whole

Table 1. Proapoptotic effects of cetuximab in combination with regorafenib in colorectal cancer cell lines with primary and acquired resistance to anti-EGFR inhibitor

Cell line	Treatment	Apoptotic cells (24 h), %	Cell line	Treatment	Apoptotic cells (24 h), %
HT29	CTR	14%	LOVO	CTR	12%
	Cetuximab	15%		Cetuximab	14%
	Regorafenib	24%		Regorafenib	18%
	Combination	46%		Combination	41%
SW480	CTR	11%	HCT15	CTR	11%
	Cetuximab	16%		Cetuximab	13%
	Regorafenib	22%		Regorafenib	19%
	Combination	45%		Combination	44%
SW620	CTR	14%	SW48-CR	CTR	13%
	Cetuximab	21%		Cetuximab	15%
	Regorafenib	27%		Regorafenib	22%
	Combination	48%		Combination	64%
HCT116	CTR	10%	GEO-CR	CTR	15%
	Cetuximab	18%		Cetuximab	17%
	Regorafenib	20%		Regorafenib	17%
	Combination	42%		Combination	52%

NOTE: The rate of apoptosis was expressed as a percentage of the total cells counted.

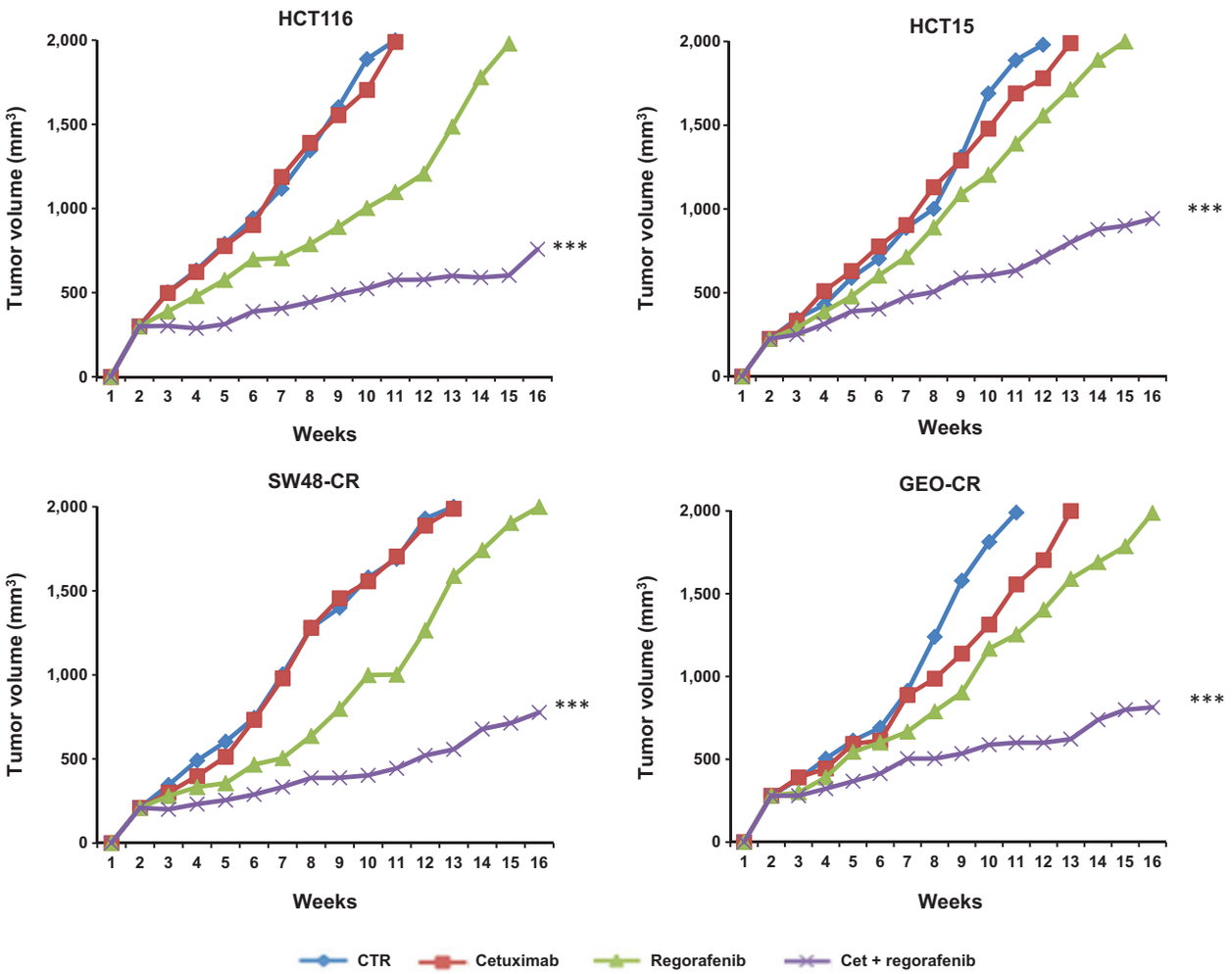

Figure 3.
Effects of cetuximab in combination with regorafenib on HCT15, HCT116, GEO-CR, and SW48-CR tumor xenografts. Mice bearing xenografts of the human colorectal cancer cell line HCT15, the human colorectal cancer cell line HCT116, the human colorectal cancer cell line GEO-CR, or the human colorectal cancer cell line SW48-CR were treated with cetuximab (1 mg/dose twice a week i.p.) and/or regorafenib (10 mg/kg/daily oral gavage) for 3 weeks. Animals were euthanized when tumors achieved 2,000 mm^3 in size. Each group consisted of 10 mice; ***, $P < 0.0005$ (combination vs. control).

panel of human colorectal cancer cell lines with primary or acquired resistance to cetuximab.

Cetuximab plus regorafenib combination exhibits antitumor activity in subcutaneous colorectal cancer xenograft models

We evaluated the *in vivo* activity of cetuximab alone or in combination with regorafenib in nude mice s.c. injected with cetuximab-resistant HCT15, HCT116, GEO-CR, or SW48-CR cell lines. Mice were randomly assigned to receive vehicle, cetuximab, regorafenib, or their combination and were treated for 3 weeks. As shown in Fig. 3, treatment with cetuximab had little or no effect on tumor growth in all tumor xenografts. Similar results were obtained in the groups treated with regorafenib alone. On the contrary, the combined treatment significantly inhibited tumor growth compared both with the control group and to single-agent treatments in all tumor xenografts (Fig. 3). Single-agent and combination treatment protocols were well tolerated by mice and were not accompanied by any major side effect or treatment-related weight loss. No cellular abnormalities were observed in the examined organs, including heart, lung, liver, kidney, and spleen, derived from all xenograft mouse models (data not shown).

Cetuximab plus regorafenib combination inhibits tumor growth in an orthotopic human colorectal cancer xenograft

An orthotopic colorectal cancer model with HCT 116 colorectal cancer cells was established, as described in Materials and Methods. Both cetuximab and regorafenib were well tolerated, and no significant loss of animal weight was observed in the group of combined treatment, whereas a significant weight loss occurred in the single-agent treatment groups, compared with the mice weight before treatment. The observed weight loss in these groups was probably caused by the presence of growing tumors and peritoneal metastases (Supplementary Fig. S4). Of interest, combined

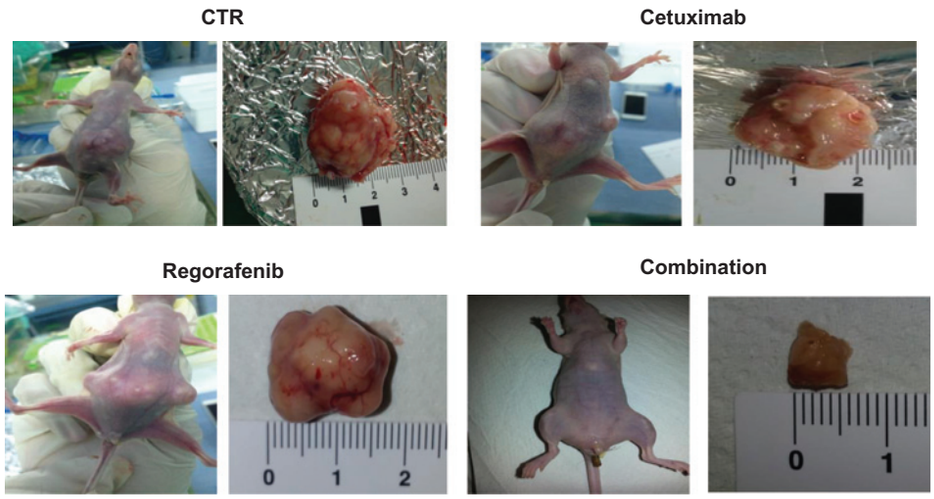

Figure 4.
Cetuximab plus regorafenib combination inhibits growth of orthotopic HCT116 colorectal cancer xenografts. HCT116 cells were injected into the cecal wall of nude mice. Two weeks later, the mice were randomly assigned (7 mice each group) to receive daily administration of PBS/0.5% Tween 80 by oral gavage for 5 days a week and i.p. injection of PBS twice a week (control); daily administration of diluent for 5 days a week and i.p. injection of cetuximab 1 mg twice a week; daily administration of regorafenib 10 mg/kg by oral gavage for 5 days a week and i.p. injection of PBS twice a week; or combination of oral regorafenib and i.p. cetuximab. The treatment continued for 3 weeks, and 1 week later mice were killed and necropsied.

treatment showed a significant antitumor effect compared with vehicle, cetuximab, or regorafenib single-agent groups (Fig. 4 and Table 2). Mice treated with vehicle had large tumors in the cecum and peritoneum with 100% incidence of regional (mesenteric) lymph node metastases. Mice receiving cetuximab or regorafenib alone had large tumors with 80% and 70%, respectively, incidence of lymph node metastases. The combined treatment strongly inhibited the tumor growth in the cecum and no peritoneum metastases were observed (Fig. 4 and Table 2). The combined treatment was also evident on tumor vascularization. In fact, tumors in mice treated with vehicle, cetuximab or regorafenib were large and highly vascularized, whereas cetuximab plus regorafenib-treated mice developed small tumors without evidence of neovascularization (Fig. 4 and Table 2). No liver or lung metastases were detected macroscopically in all groups (data not shown).

Discussion

The development of targeted therapies has provided new options for the personalized management of patients with advanced solid tumors. mAbs directed against the EGFR, such as cetuximab and panitumumab, have emerged as important therapeutic agents in the treatment of metastatic colorectal cancer patients. However, their use is substantially limited by intrinsic and acquired cancer cell resistance. Several hypotheses have been developed to explain why resistant cancer cell arises and how it is possible to overcome it. One possibility is cancer intrinsic genetic heterogeneity, which could be more prominent in the metastatic setting (28, 29). Heterogeneous genetic alterations in genes involved in the EGFR pathways have been hypothesized to play a role in resistance to anti-EGFR drugs in colorectal cancer, including activating mutations in *KRAS*, *NRAS*, *B-RAF*, and *PIK3CA*, and loss of expression of *PTEN* (13). The overall scenario is complicated by presence of additional genetic mechanisms able to activate the RAS pathway in the absence of molecular alterations affecting RAS or its immediate downstream effectors (30–37). One strategy to overcome the limitations of targeting an individual growth factor receptor such as the EGFR is to combine different drugs that target different growth controlling pathways. In fact, the use of mAbs blocking an individual pathway has been largely limited by the presence of a compensatory feedback loop in other pathways. In our study, to circumvent this compensatory feedback, we have tested cetuximab in combination with regorafenib in human colorectal cancer cell lines with primary or with acquired resistance to the anti-EGFR mAb cetuximab. The combined treatment with cetuximab plus regorafenib shows a synergistic antitumor effect both *in vitro* and *in vivo*, providing the rational for the clinical development of this combination. These results are consistent with previous reports, which showed that combined inhibition of different growth controlling pathways might potentially exhibit a better therapeutic efficacy compared with inhibition of a single pathway (38–40). In this respect, regorafenib inhibits multiple cell membrane tyrosine kinase receptors that are involved in key processes of cancer development and progression, including angiogenesis (17). Furthermore, regorafenib antitumor activity could be also due in part by its ability to inhibit RAF serine/threonine kinase (41–43).

We have previously shown that a mechanism of acquired resistance to EGFR inhibitors could be the increased secretion of VEGF, suggesting a key role for tumor-induced angiogenesis in the development of anti-EGFR resistance (21). Moreover, treatment with vandetanib, a dual inhibitor of EGFR and VEGFRs, of human EGFR inhibitor-sensitive colorectal cancer cells could delay the onset of cancer cell resistance (21). Bianco and colleagues (44) have shown that human EGFR inhibitor–resistant cancer cells, secrete VEGF and placental growth factor, and express VEGFR-1. Treatment with vandetanib significantly inhibits VEGFR-1 activation, cell proliferation, and migration in these EGFR inhibitor–resistant human cancer cell lines. Martinelli and colleagues (24) have investigated the role of combined treatment with selective

Table 2. Cetuximab plus regorafenib combination inhibits growth of orthotopic HCT116 colorectal cancer xenografts

Treatment group	Tumor volume (mm^3; %)	Cecal tumor weight (g; %)	Incidence of lymph node metastasis
CTR	15,300 (100%)	5.1 (100%)	10/10
Cetuximab	13,500 (88%)	4.9 (82%)	8/10
Regorafenib	10,900 (71%)	4.2 (82%)	7/10
Combination	750 (4.9%)	1 (19.6%)	0/10

anti-EGFR drugs, such as erlotinib or cetuximab, and sorafenib, another multitargeted inhibitor of C-RAF and B-RAF and of all three VEGFRs (3). Also in this study, the combined treatment determined significant antiproliferative and antimigratory effects *in vitro* and antitumor activity *in vivo* in xenografts models of human cancer cell lines (24).

In the clinical setting, several studies have explored the possibility of combining anti-EGFR drugs such as cetuximab, panitumumab, or erlotinib, with different antiangiogenic drugs, including bevacizumab or sorafenib. The results in unselected non–small cell lung cancer or colorectal cancer patients have been contradictory (45–49). However, the results of a randomized phase II study in 154 advanced non–small cell lung cancer patients that were selected for the presence of activating *EGFR* gene mutations have recently demonstrated a statistically and clinically relevant increase in the efficacy of the combined treatment with erlotinib plus bevacizumab compared with single-agent standard therapy with erlotinib. Median PFS was significantly longer in the combination arm (16 months) compared with single-agent erlotinib arm (9.7 months; ref. 50).

A difficult question to answer is whether combining anti-VEGF and anti-EGFR mAbs, at least in combination with cytotoxic drug, has definitively proven to be detrimental, or at least not effective in the first line treatment of metastatic colorectal cancer. Two large randomize phase III studies have evaluated the efficacy of adding an anti-EGFR mAb such as cetuximab (CAIRO-2) or panitumumab (PACCE), to an oxaliplatin-containing chemotherapy doublet plus bevacizumab (48, 49). Both studies have shown that the addition of the anti-EGFR mAbs does not improve efficacy. The possibility of a negative interaction between bevacizumab and anti-EGFR Abs or of a negative interaction when the two Abs and chemotherapy are combined cannot be ruled out, although no mechanisms behind such potential interactions are known. Although these studies have demonstrated a detrimental effect of the combine treatment of cetuximab with bevacizumab in addition to chemotherapy in metastatic colorectal cancer, in our study we have explored the antitumor activity of cetuximab in combination with a different antiangiogenic drug such as regorafenib. Although bevacizumab is an mAb directed against VEGFA, regorafenib has a broader spectrum of activity blocking different tyrosine kinase receptors that are potentially involved in the mechanisms of resistance to cetuximab. This may explain the synergistic effect that we have found in this study.

In summary, the present study provides experimental evidence that the combined treatment with anti-EGFR drugs, such as cetuximab, and with a multiple signaling pathway inhibitor, such as regorafenib, could be a potential therapeutic strategy to investigate in a clinical setting for overcoming intrinsic or acquired resistance to EGFR inhibitors in colorectal cancer patients.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): B. Rinaldi, E. Martinelli, M. Donniacuo, G. Barra, R. De Palma, F. Merolla, F. Ciardiello, T. Troiani
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Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): F. Ciardiello
Study supervision: E. Martinelli, L. Berrino, F. Ciardiello

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