Regorafenib Inhibits Colorectal Tumor Growth through PUMA-Mediated Apoptosis

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Abstract

**Purpose:** Regorafenib, a multikinase inhibitor targeting the Ras/Raf/MEK/ERK pathway, has recently been approved for the treatment of metastatic colorectal cancer. However, the mechanisms of action of regorafenib in colorectal cancer cells have been unclear. We investigated how regorafenib suppresses colorectal cancer cell growth and potentiates effects of other chemotherapeutic drugs.

**Experimental Design:** We determined whether and how regorafenib induces the expression of PUMA, a p53 target and a critical mediator of apoptosis in colorectal cancer cells. We also investigated whether PUMA is necessary for the killing and chemosensitization effects of regorafenib in colorectal cancer cells. Furthermore, xenograft tumors were used to test if PUMA mediates the in vivo antitumor, antiangiogenic, and chemosensitization effects of regorafenib.

**Results:** We found that regorafenib treatment induces PUMA in colorectal cancer cells irrespective of p53 status through the NF-κB pathway following ERK inhibition and glycogen synthase kinase 3β activation. Upregulation of PUMA is correlated with apoptosis induction in different colorectal cancer cell lines. PUMA is necessary for regorafenib-induced apoptosis in colorectal cancer cells. Chemosensitization by regorafenib is mediated by enhanced PUMA induction through different pathways. Furthermore, deficiency in PUMA abrogates the in vivo antitumor, antiangiogenic, and chemosensitization effects of regorafenib.

**Conclusions:** Our results demonstrate a key role of PUMA in mediating the anticancer effects of regorafenib in colorectal cancer cells. They suggest that PUMA induction can be used as an indicator of regorafenib sensitivity, and also provide a rationale for manipulating the apoptotic machinery to improve the therapeutic efficacy of regorafenib and other targeted drugs. *Clin Cancer Res; 20(13): 3472–84. ©2014 AACR.*

Introduction

Colorectal cancer represents the third leading cause of cancer-related death in the United States (1). Recurrent or metastatic colorectal tumors are largely incurable with median overall survival of ~2 years (2). Conventional chemotherapy for colorectal cancer treatment involves combinations of cytotoxic drugs such as 5-fluorouracil (5-FU), oxaliplatin and irinotecan, and has limited efficacy and substantial side effects because of lack of specificity (3). The development of targeted anticancer agents has significantly improved efficacy of chemotherapy against metastatic colorectal cancer (4). For example, cetuximab, an antiepidermal growth factor receptor (EGFR) monoclonal antibody, is effective as a single agent or in combination with cytotoxic drugs for treatment of metastatic colorectal cancer (4). The inclusion of targeted therapy is expected to move oncology practice toward personalized treatment (5).

Regorafenib (Stivarga), a multikinase inhibitor that targets the Ras/Raf/MEK/ERK pathway, has recently been approved for the treatment of metastatic colorectal cancer and gastrointestinal stromal tumors (6, 7). A variety of clinical trials have also been initiated to use regorafenib to treat other malignancies, including those of lung, esophageal, and liver. Regorafenib inhibits several kinases that are aberrantly activated in tumor cells, including c-Raf, B-Raf, vascular endothelial growth factor receptors (VEGFR), platelet-derived growth factor receptor (PDGFR), c-Kit, and Tie-2 (8). The antitumor activity of regorafenib has been demonstrated in a variety of preclinical models, and is associated with suppression of cell proliferation, induction of apoptosis, and inhibition of tumor angiogenesis (8, 9). However, it is unclear whether any of these effects is essential for the antitumor activity of regorafenib.

Induction of apoptosis in tumor cells has emerged as a key mechanism of targeted therapies (10). p53-upregulated modulator of apoptosis (PUMA), a BH3-only Bcl-2 family member, functions as a critical regulator of apoptosis in colorectal cancer cells (11). PUMA is transcriptionally activated by p53 and initiates apoptotic response to DNA damage (12). It can also be induced in a p53-independent manner by rapamycin and in p53-deficient cells (13). Our findings provide a rationale for manipulating the apoptotic machinery to improve the therapeutic efficacy of regorafenib and other targeted drugs.
Regorafenib Kills Colon Cancer Cells through PUMA

Translational Relevance
Regorafenib, a multikinase inhibitor targeting the Ras/Raf/MEK/ERK pathway, has recently been approved for the treatment of metastatic colorectal cancer and gastrointestinal stromal tumors. It is currently in many clinical trials in combination with other chemotherapeutic agents, such as the cytotoxic drugs 5-fluorouracil, oxaliplatin and irinotecan, and the antiepidermal growth factor receptor antibodies cetuximab and panitumumab, for colorectal cancer treatment. However, the anti-cancer mechanism of regorafenib in colorectal cancer cells has remained unclear. No effective biomarker has been described for predicting response of colorectal cancer to regorafenib. We demonstrate that the pro-apoptotic Bcl-2 family protein PUMA is critical for the antitumor, antiangiogenic and chemosensitization effects of regorafenib against colorectal cancer. Our results suggest that PUMA induction can be used as an indicator of responsiveness to regorafenib, and for developing more effective combination therapies involving regorafenib.

for genotypes, drug response, morphology, and absence of mycoplasma in October 2012. All colorectal cancer cell lines were cultured in McCoy’s 5A modified media (Invitrogen). Immortalized wild-type (WT), p65-KO, and PUMA-KO mouse embryonic fibroblast (MEF) cells were previously described (19), and were cultured in DMEM media (Invitrogen). Cells were maintained in a 37°C incubator at 5% CO2. Cell culture media were supplemented with 10% defined FBS (HyClone), 100 units/ml penicillin, and 100 µg/ml streptomycin (Invitrogen).

Cells were plated and treated by drugs at 40% to 50% density in 12-well plates. The anticancer agents and chemicals used include regorafenib (Active Biochem), BAY 11-7082, PD98059 (Merck Chemicals), 5-FU, UCN-01, cisplatin, oxaliplatin (Sigma), gefitinib (AstraZeneca), and cetuximab (ImClone). All agents were diluted with DMSO (Sigma), except for cisplatin, which was dissolved in 0.9% NaCl. For NF-κB inhibition, cells were pretreated with BAY 11-7082 for 1 hour before regorafenib treatment.

Real-time reverse transcriptase PCR
Total RNA was isolated from regorafenib-treated cells using Mini RNA Isolation II Kit (Zymo Research) according to the manufacturer’s protocol. One µg of total RNA was used to generate cDNA using SuperScript II reverse transcriptase (Invitrogen). Real-time PCR was performed for PUMA and β-actin using previously described primers and conditions (13).

Western blotting
Western blotting was performed as previously described (16), with antibodies for PUMA (18), Akt, phospho-Akt (S473), Bid, active caspase-3, caspase-8, caspase-9, ERK, phospho-ERK (T202/Y204), IκB, phospho-IκB (S22/23), p65, phospho-p65 (S336, S276, and S468), phospho-Bax (T32), STAT1, phospho-STAT1 (Y701), glycogen synthase kinase 3β (GSK3β), cytochrome c (S9; Cell Signaling Technology), Bak, FoxO3A (Millipore), cytochrome oxidase subunit IV (Invitrogen), Mcl-1, IκB, cytochrome c, lamin A/C (Santa Cruz Biotechnology), β-actin (Sigma), Bim, α-tubulin (EMD Biosciences), and Bcl-XL (BD Transduction).

Apoptosis assays
Nuclear staining with Hoechst 33258 (Invitrogen) was performed as previously described (13). Annexin V/propidium iodide (PI) staining was performed using annexin-Alexa Fluor 488 (Invitrogen) and PI as described (21). Cytochrome c release was analyzed by cytochrome c Western blotting of mitochondrial and cytosolic fractions isolated by differential centrifugation (17). Colony formation was assayed by plating the treated cells in 12-well plates at appropriate dilutions, and allowing for cell growth for 10 to 14 days, followed by crystal violet (Sigma) staining of cell colonies. Mitochondrial membrane potential changes were detected by flow cytometry of treated cells stained with MitoTracker Red CMXRos (Invitrogen) at room temperature for 15 minutes.

Materials and Methods
Cell culture and drug treatment
Human colorectal cancer cell lines were purchased from American Type Culture Collection or obtained from Sidney Kimmel Comprehensive Cancer Center at Johns Hopkins. Isogenic p53-knockout (KO) and PUMA-KO HCT116, and PUMA-KO DLD1 cell lines were previously described (18, 20). The cell lines were last tested and authenticated on July 14, 2017. © 2014 American Association for Cancer Research.
Transfection and siRNA knockdown

Cells were transfected with Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. The knockdown experiments were performed 24 hours before regorafenib treatment using 200 pmol of siRNA. The control scrambled siRNA and siRNA for human p65 (15), c-Raf (AAGCGCCGTACATGGAGAAT-dTdT), PDGFβR (GCCACUUUCAGGGCUUAA-dTdT), B-Raf (ACAGAGACCCUAAAGAGAA-UU), and c-Kit (GGCCGCAGAAAAAGGAGAUCUU-dTdT) were from Dharmacon. The siRNA for GSK3β (sc-35527) and VEGFR2 siRNA (sc-29318) were generated by Santa Cruz Biotechnology. A nondegradable IκBα super repressor mutant (S32A/S36A; IκBαM) was previously described (15).

Analysis of NF-κB nuclear translocation

HCT116 cells were pretreated with BAY11-7082 or transfected with GSK3β siRNA, and then subjected to regorafenib treatment for 3 hours. Nuclear fractionation and immunofluorescence were used to analyze NF-κB nuclear translocation. For nuclear fractionation, nuclear extracts were isolated from cells treated in 75-cm² flasks using the NE-PER Nuclear/Cytoplasmic Extraction Kit (Thermo Fisher) according to the manufacturer’s instructions, and analyzed by p65 Western blot analysis. For immunofluorescence, cells treated in chamber slides were stained with anti-p65 (Cell Signaling) overnight at 4°C followed by secondary staining with the anti-rabbit Alexa Fluor 488–conjugated secondary antibody (Invitrogen) for 1 hour at room temperature, as previously described (22). Images were acquired with an Olympus IX71 microscope (Olympus Imaging America, Inc.).

Luciferase assays

Luciferase reporter constructs containing wild-type or mutant PUMA promoter sequence in pB-V-Luc vector were previously described (13, 23). To measure reporter activities, cells were transfected with the wild-type or mutant PUMA reporters along with the transfection control β-galactosidase reporter pCMVB (Promega). Luciferase activities were measured as previously described (22), and normalized to samples similarly transfected but without drug treatment. All reporter experiments were performed in triplicate and repeated 3 times.

Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) with p65 antibody (Santa Cruz) was performed using the Chromatin Immunoprecipitation Assay Kit (Millipore) as previously described (20). The precipitates were analyzed by PCR using primers 5'-GTCGGTCTGTGACGCTAGC-3' and 5'-CCCGTGTTACGCTAGGCCC-3' to amplify a PUMA promoter fragment containing putative κB sites.

Animal tumor experiments

All animal experiments were approved by the University of Pittsburgh Institutional Animal Care and Use Committee. Female 5- to 6-week-old Nu/Nu mice (Charles River) were housed in a sterile environment with micro isolator cages and allowed access to water and chow ad libitum. Mice were injected subcutaneously in both flanks with 4 × 10⁶ wild-type or PUMA-KO HCT116 cells. After tumor growth for 7 days, mice were treated daily with regorafenib at 30 mg/kg by oral gavage for 10 consecutive days. For combination experiments, mice were treated with 15 mg/kg regorafenib daily by oral gavage, 25 mg/kg 5-FU (APP Pharmaceuticals) every other day by i.p. injection, or their combination for 10 consecutive days. Regorafenib was dissolved in Cremophor EL/95% ethanol (50:50) as a 4× stock solution (24), and 5-FU was supplied as a stock solution. Both drugs were diluted to the final concentration with sterile water before use. Tumor growth was monitored by calipers, and tumor volumes were calculated according to the formula 0.5 × length × width². Mice were euthanized when tumors reached ~1.0 cm³ in size. Tumors were dissected and fixed in 10% formalin and embedded in paraffin. Terminal deoxynucleotidyl transferase mediated dUTP Nick End Labeling (TUNEL; Millipore), active caspase-3 (Cell Signaling), CD31 (Spring Bioscience), and carbonic anhydrase 9 (CA9; Santa Cruz) immunostaining was performed on 5 μm/L paraffin-embedded tumor sections as previously described (25), by using an Alexa Fluor 488– or Alexa Fluor 594–conjugated secondary antibody (Invitrogen) for detection.

Statistical analysis

Statistical analyses were carried out using GraphPad Prism IV software (GraphPad Software, Inc.). P values were calculated using the Student t test and were considered significant if P < 0.05. The means ± 1 SD were displayed in the figures.

Results

Upregulation of PUMA by regorafenib correlates with apoptosis induction in colorectal cancer cells

The recent approval of regorafenib for colorectal cancer treatment prompted us to investigate its mechanisms of action in colorectal cancer cells (7). Treating p53-WT HCT116 colon cancer cells with regorafenib markedly induced PUMA protein and mRNA expression in a dose- and time-dependent manner (Fig. 1A and B). The peaks of PUMA protein and mRNA induction were detected at 24 hours following 40 μmol/L regorafenib treatment (Fig. 1B). Regorafenib also induced PUMA protein and mRNA expression in p53-KO HCT116 cells (Fig. 1A–C). In contrast, regorafenib treatment did not upregulate other proapoptotic Bcl-2 family members, including Bim, Bid, and Bak, but reduced the expression of the antiapoptotic proteins Bcl-XL and McI-1 (Fig. 1E). PUMA was also induced by regorafenib in other colorectal cancer cell lines, including p53-WT Lim2405, LoVo, Lim1215, SW48, and RKO cells, and p53-mutant SW837, SW1463, SW480, Vaco432, Vaco400, DLD1, and HT29 cells (Fig. 1E). Analysis of 13 colorectal cancer cell lines revealed a correlation between the induction of PUMA and apoptosis by regorafenib (Fig. 1E and F). Following regorafenib treatment, cell lines with low...
Figure 1. Upregulation of PUMA expression by regorafenib correlates with apoptosis induction in colorectal cancer cells. A, WT and p53-knockout (p53-KO) HCT116 colon cancer cells were treated with regorafenib at indicated concentrations for 24 hours. Left, PUMA mRNA induction by regorafenib was analyzed by real-time reverse transcriptase (RT)-PCR, with β-actin as a control. Right, PUMA and β-actin expression was analyzed by Western blotting. B, WT and p53-KO HCT116 cells were treated with 40 μmol/L regorafenib and analyzed at different time points after treatment. Left, time course of PUMA mRNA induction was determined by real-time RT-PCR, with β-actin as a control. Right, time course of PUMA protein induction was analyzed by Western blotting. C, WT and p53-KO HCT116 cells were treated with 40 μmol/L regorafenib for 24 hours. PUMA expression was analyzed by Western blotting. D, the expression of indicated Bcl-2 family members was analyzed by Western blotting in HCT116 cells treated with 40 μmol/L regorafenib at indicated time points. E, Western blot analysis of PUMA expression in indicated colorectal cancer cell lines treated with 40 μmol/L regorafenib for 24 hours. Relative PUMA expression, which was quantified by Image J program and normalized to that of β-actin, is indicated, with that in untreated cells arbitrarily set as 1.0. F, indicated colorectal cancer cell lines were treated with 40 μmol/L regorafenib for 48 hours. Apoptosis was quantified by counting condensed and fragmented nuclei after nuclear staining with Hoechst 33258, and plotted against PUMA induction from E. The results represent means ± SD of 3 independent experiments.
Figure 2. PUMA mediates the apoptotic and anticancer effects of regorafenib through the mitochondrial pathway. A, WT, p53-KO, and PUMA-KO HCT116 cells were treated with regorafenib at indicated concentrations for 48 hours. Apoptosis was analyzed by counting condensed and fragmented nuclei after nuclear staining with Hoechst 33258. B, apoptosis in cells treated with 40 μmol/L regorafenib for 48 hours was analyzed by annexin V/PI staining followed by flow cytometry. The percentages of annexin-positive apoptotic cells are indicated in the 2 right quadrants. C, comparison of apoptosis in WT and PUMA-KO DLD1 colon cancer cells (left), WT and PUMA-KO MEFs (middle), and WT and p65-KO MEFs (right) following treatment with 40 μmol/L regorafenib for 48 hours. Apoptosis was analyzed by nuclear staining as in A. D, Western blot analysis of active caspase-3, active caspase-8, and active caspase-9 (indicated by arrow heads) in WT and PUMA-KO HCT116 cells with or without regorafenib (40 μmol/L) treatment for 24 hours. E, after treatment of WT and PUMA-KO HCT116 cells with 40 μmol/L regorafenib for 36 hours, mitochondrial membrane potential was analyzed by flow cytometry following MitoTracker Red CMXRos staining. (Continued on the following page.)
endogenous expression but strong induction of PUMA had relatively high levels apoptosis, whereas those with low or no PUMA induction, such as SW837, Lim1215, and SW1463, had low or barely detectable levels of apoptosis, with the exception of Lim2045 (Fig. 1F). These results suggest that selective induction of PUMA by regorafenib contributes to apoptosis induction in colorectal cancer cells.

**Regorafenib is dependent on PUMA to induce apoptosis in colorectal cancer cells**

We then investigated the role of PUMA in regorafenib-induced apoptosis using isogenic HCT116 cell lines. Apoptosis induced by 10 to 40 μmol/L regorafenib was significantly reduced in PUMA-KO cells, but unaffected in p53-KO cells in comparison with wild-type HCT116 cells (Fig. 2A). Annexin V/PI staining confirmed the reduction of regorafenib-induced apoptosis in PUMA-KO cells (Fig. 2B). PUMA-dependent apoptotic response to regorafenib was also observed in DLD1 and MEF cells (Fig. 2C). The absence of PUMA in HCT116 cells abrogated regorafenib-induced mitochondrial events, including activation of caspase-3, -8, and -9 (Fig. 2D), mitochondrial membrane permeabilization (Fig. 2E), and cytochrome c release (Fig. 2F). Furthermore, PUMA-KO cells had improved long-term survival compared with wild-type HCT116 cells following regorafenib treatment (Fig. 2G). Therefore, PUMA is necessary for the apoptotic effect of regorafenib in colorectal cancer cells.

**PUMA activation by regorafenib is mediated by NF-kB**

We then analyzed the mechanism of p53-independent PUMA induction by regorafenib in colorectal cancer cells. Knockdown of several known targets of regorafenib, including B-Raf, VEGFR2, PDGFR-B, and c-Kit, by siRNA did not affect PUMA expression (Supplementary Fig. S1A and S1B). In contrast, depletion of c-Raf by siRNA led to increased levels of PUMA (Fig. 3A), suggesting that PUMA induction by regorafenib is because of c-Raf inhibition. Several transcription factors that can mediate PUMA upregulation in p53-deficient cells were examined (11). FoxO3a is not involved because of unchanged inhibitory phosphorylation following regorafenib treatment (Supplementary Fig. S1C; ref. 26), and lack of an effect of FoxO3a knockdown on PUMA induction (Supplementary Fig. S1D). p73, a p53 family member (23), and STAT1, which mediates the effects of sorafenib in pancreatic cancer cells (27), were also ruled out because of lack of induction or a change in phosphorylation (Supplementary Fig. S1C).

The p65 subunit of NF-kB was recently identified as a transcriptional activator of PUMA in response to TNFα or sorafenib treatment (15, 19). Activation of NF-kB signaling is characterized by phosphorylation of p65 on several residues and its subsequent translocation to the nucleus, where it activates transcription of target genes (28). We found that regorafenib treatment induced phosphorylation of S536, the major regulatory site of p65 (29), in a time- and dosage-dependent manner in HCT116 cells (Fig. 3B and Supplementary Fig. S2A). Phosphorylation of S276, another site associated with p65 activation (29), was also increased after regorafenib treatment, whereas that of the controversial S468 site was not detected (Supplementary Fig. S2B). Knockdown of p65 by siRNA abrogated PUMA induction by regorafenib in both wild-type and p53-KO HCT116 cells (Fig. 3C), as well as in DLD1 cells (Supplementary Fig. S2C). The induction of PUMA and apoptosis by regorafenib was also suppressed in p65-KO MEFs (Fig. 2C and Supplementary Fig. S2D). Furthermore, regorafenib treatment led to nuclear translocation of p65 as detected by immunofluorescence (Fig. 3D) and Western blot analysis (Fig. 3E, left).

**p65 binds to the PUMA promoter to directly activate its transcription following regorafenib treatment**

To investigate how NF-kB activates PUMA transcription in response to regorafenib treatment, cells were pretreated with BAY 11-7082, an NF-kB inhibitor that suppresses p65 nuclear translocation (Fig. 3E, left). BAY 11-7082 treatment impeded PUMA expression and p65 phosphorylation following regorafenib treatment (Fig. 3F). Furthermore, p65 binds to the PUMA promoter to directly activate its transcription following regorafenib treatment.

(Continued.) F, cytosolic fractions isolated from WT and PUMA-KO HCT116 cells treated with 40 μmol/L regorafenib for 36 hours were probed for cytochrome c by Western blotting. α-Tubulin and cytochrome oxidase subunit IV (Cox IV), which are expressed in cytoplasm and mitochondria, respectively, were analyzed as the control for loading and fractionation. G, colony formation of WT and PUMA-KO HCT116 cells treated with 40 μmol/L regorafenib for 48 hours at 14 days following crystal violet staining of attached cells. Left, representative pictures of colonies; right, quantification of colony numbers. Results in A, C, and G were expressed as means ± SD of 3 independent experiments. *, P < 0.001; **, P < 0.01; *, P < 0.05.
The canonical pathway of p65 activation is mediated by IκB phosphorylation and degradation (28). However, IκB phosphorylation or degradation was unaffected by regorafenib treatment (Supplementary Fig. S2G). Transfecting cells with IκBαM, a nondegradable mutant of IκB (15), also did not affect regorafenib-induced PUMA expression (Fig. 4A), suggesting that the canonical NF-κB pathway is not involved in p65 activation or PUMA induction by regorafenib. Further analysis of other kinases known to activate NF-κB revealed that GSK3β is involved in regorafenib-induced p65 activation. Knockdown of GSK3β by siRNA suppressed regorafenib-induced p65 nuclear translocation (Fig. 4B), as well as PUMA expression in both HCT116 and RKO colon cancer cells (Fig. 4C). Furthermore, regorafenib treatment suppressed Ser9 phosphorylation of GSK3β, which inhibits its kinase activity (30), in both wild-type and p53-KO HCT116 cells (Fig. 4D). It has been shown that the ERK kinase can prime the inhibitory GSK3β Ser9 phosphorylation (31). We found that regorafenib treatment strongly suppressed phosphorylation of ERK1/2 (T202/Y204; Fig. 4E). Treatment of the ERK inhibitor PD98059 phenocopied regorafenib treatment in blocking GSK3β Ser9 phosphorylation, and promoting p65 phosphorylation, PUMA expression, and apoptosis induction (Fig. 4F and G), suggesting that ERK inhibition mediates the activation of GSK3β, p65 and PUMA by regorafenib. MEK inhibitors were also shown to suppress in vivo growth of colorectal cancer cells including HCT116 cells (32). Together, these results demonstrate that PUMA induction by regorafenib is mediated by ERK inhibition, relief of GSK3β inhibition, and subsequent p65 activation.
Regorafenib is necessary for the in vivo antitumor and antiangiogenic activities of regorafenib

Regorafenib suppresses growth of colorectal cancer xenograft tumors effectively (8, 9). To determine if PUMA mediates tumor suppression by regorafenib, wild-type and PUMA-KO HCT116 cells were injected subcutaneously into nude mice to establish xenograft tumors. Mice were then treated with 30 mg/kg regorafenib or the control vehicle by oral gavage for 10 consecutive days, as previously described (8, 9). Wild-type and PUMA-KO tumors were not significantly different in growth in the control group (Fig. 5A and B). In line with previous studies (8, 9), regorafenib treatment suppressed the growth of wild-type tumors by 70% to 90% (Fig. 5A). In contrast, PUMA-KO tumors were significantly less sensitive to regorafenib treatment compared with wild-type tumors (Fig. 5A and B), indicating that absence of PUMA abrogated the antitumor activity of regorafenib. Consistent with observations from cultured cells, p65 phosphorylation and PUMA expression were markedly increased in regorafenib-treated xenograft tumors (Fig. 5C). TUNEL staining revealed significant apoptosis induction in wild-type tumor tissues from the regorafenib-treated mice, but not the control mice. In contrast, apoptosis was largely reduced in the PUMA-KO tumors (Fig. 5D). Active caspase-3 staining confirmed regorafenib-induced and PUMA-dependent apoptosis in tumors (Fig. 5E). Analysis of tumor vasculature by CD31 staining showed that the antiangiogenic effect of regorafenib was reduced in the PUMA-KO tumors (Fig. 5F). Suppression of tumor hypoxia, analyzed by CA9 staining, by regorafenib was also decreased in the PUMA-KO tumors compared with wild-type tumors (Fig. 5G). Therefore, the antitumor, apoptotic, and antiangiogenic activities of regorafenib in vivo are largely dependent on PUMA.

PUMA mediates the chemosensitization effects of regorafenib

Regorafenib has been used in combination with other chemotherapeutic agents for colorectal cancer treatment (33). It is possible that the chemosensitization effects of regorafenib are mediated by simultaneous PUMA induction by regorafenib and other agents through different pathways. We found that regorafenib combined with 5-FU, oxaliplatin, or cisplatin induced higher levels of PUMA, compared with single agent alone (Fig. 6A and B and Supplementary Fig. S3A). This is consistent with concurrent PUMA induction through both p53-dependent and -independent mechanisms by DNA damage and regorafenib, respectively.
Accordingly, the level of apoptosis was also significantly enhanced in wild-type HCT116 cells, but not in PUMA-KO cells following the combination treatment (Fig. 6A and B and Supplementary Fig. S3B). Similarly, regorafenib combined with cetuximab, which induces PUMA through AKT inhibition (14), enhanced PUMA and apoptosis induction relative to regorafenib or cetuximab alone, and the enhanced apoptosis was PUMA-dependent (Fig. 6C). The PUMA-dependent sensitization effect was also observed in cells treated with regorafenib combined with the EGFR TKI gefitinib (Supplementary Fig. S3C), or the broad-range kinase inhibitor UCN01 (Supplementary Fig. S3D).

To determine if PUMA mediates chemosensitization by regorafenib in vivo, nude mice with wild-type and PUMA-KO HCT116 xenograft tumors were treated with 15 mg/kg regorafenib, 25 mg/kg 5-FU, or their combination. The combination treatment more effectively suppressed the growth of wild-type tumors, compared with either regorafenib or 5-FU alone (Fig. 6D). However, the enhanced tumor suppression by the combination treatment was
largely abolished in the PUMA-KO tumors (Fig. 6D and Supplementary Fig. S4A), which was also associated with decreased apoptosis detected by TUNEL staining (Fig. 6E and Supplementary Fig. S4B), and active caspase-3 staining (Fig. 6F and Supplementary Fig. S4C). These findings suggest that PUMA mediates the chemosensitization effects of regorafenib in vitro and in vivo.
regorafenib in vitro and in vivo, and manipulating PUMA-mediated apoptosis can improve the therapeutic efficacy of regorafenib.

Discussion

The Ras/Raf/MEK/ERK pathway is aberrantly activated in a large fraction of colorectal tumors because of Ras and Raf mutations (34). Regorafenib is the first small molecule inhibitor of this pathway that has been approved by the U.S. Food and Drug Administration (FDA) for the treatment of metastatic colorectal cancer. Regorafenib differs from its analog sorafenib by single fluorine, which leads to a similar, yet distinct biochemical profile compared with sorafenib (24, 35). Although sorafenib has been extensively characterized, little is known about how regorafenib inhibits growth of colorectal cancer cells. The antiangiogenic activity of regorafenib is thought to largely contribute to its anti-tumor effects (35). Our results demonstrate for the first time that tumor suppression by regorafenib is dependent on the cell autonomous process of apoptosis induction, progressing from ERK inhibition, GSK3β activation, and p65 nuclear translocation, leading to PUMA induction and onset of mitochondria-mediated apoptosis. In initial clinical studies, regorafenib seems to be primarily cytostatic, which is unlike many chemotherapeutic drugs or targeted agents that are cytotoxic. Long-lasting tumor stabilization was observed in the majority of treated colorectal cancer patients (7). However, retrospective analysis revealed that a subset of treated patients had decreased radiologic tumor density as well as cavitation of lung metastases during the course of treatment (36), which may be related to the proapoptotic activity of regorafenib. Changes in tumor microenvironment have recently emerged as an important mechanism of differential response and innate resistance to targeted therapies (37). Our findings suggest that both cell autonomous and microenvironmental effects of regorafenib contribute to tumor suppression, and they can affect each other through PUMA-mediated apoptosis. A systematic dissection of interactions between tumors and their microenvironment is necessary for understanding how regorafenib and other targeted agents inhibit tumor growth.

We performed most in vitro experiments using 40 μmol/L regorafenib to substantially induce PUMA and apoptosis in colorectal cancer cells. Importantly, the results of these experiments were confirmed by those from xenograft tumor experiments (Figs. 5 and 6). Phase I studies showed that the plasma concentrations of regorafenib can reach 5 to 10 μmol/L in patients (38, 39). The biologically active concentrations of regorafenib are likely to be higher than those in plasma because of binding to plasma proteins (35). Colorectal cells may also be topically exposed to orally administrated regorafenib at higher concentrations than those in circulation. Furthermore, regorafenib at a lower dose of 10 μmol/L was sufficient to induce PUMA expression (Fig. 1A). These observations suggest that induction of PUMA is likely to be involved in the effects of regorafenib at clinically relevant doses.

PUMA induction plays a role in apoptosis induced by a variety of chemotherapeutic agents, and may be a useful indicator of chemosensitivity. We previously showed that PUMA induction correlates with differential sensitivity to EGFR TKIs in head and neck cancer cells, and lack of PUMA induction is associated with resistance to EGFR TKIs (14). Increased PUMA expression was associated with better prognosis in patients receiving 5-FU-based therapy in stage II and stage III colorectal cancer (40). Furthermore, a recent study demonstrated that response of isolated mitochondria from tumor cells to PUMA Bcl-2 homology 3 (BH3) peptide correlates with chemotherapy response in patients with cancer (41). The results in this study suggest that PUMA induction may be useful as a surrogate biomarker for response of colorectal cancer to regorafenib. Although it is often difficult to obtain biopsies from colorectal tumors treated with chemotherapy after surgery, recent studies showed that biomarkers of therapeutic response can be analyzed using circulating tumor DNA (42, 43), or circulating tumor cells (44). It might be possible to determine PUMA induction by using such noninvasive approaches.

A number of clinical trials have been initiated to test combinations of regorafenib with conventional cytotoxic agents in patients with colorectal cancer, such as 5-FU, oxaliplatin, and irinotecan (33, 45). Our data indicate that enhanced PUMA induction mediates the in vitro and in vivo chemosensitization effects of regorafenib (Fig. 6). Regorafenib has also been combined with other targeted agents, such as the anti-VEGF antibody bevacizumab and the anti-EGFR antibodies cetuximab and panitumumab in ongoing clinical trials (33, 45). Incorporation of regorafenib is expected to sensitize colorectal cancer containing the G12 mutations in K-Ras or the V600E mutation in B-Raf, which compromise the effects of EGFR-targeted therapies (46). Although regorafenib inhibits ERK and activates p65 to induce PUMA, EGFR inhibitors suppress AKT and induce p73 to activate PUMA (14). Concurrent PUMA induction through 2 distinct mechanisms can account for improved therapeutic efficacy of combining regorafenib and EGFR antibodies. In addition, p53-independent and/or p53-dependent PUMA induction also mediates apoptotic response to other targeted agents, such as the c-MET/ALK inhibitor crizotinib (47), the multikinase inhibitor drug sunitinib (48), and the HSP90 inhibitors (49). PUMA induction may serve as a useful indicator of effective drug combinations for developing more efficacious combination regimens with reduced doses and nonoverlapping toxicities.

Drug resistance represents a major limitation of chemotherapy, and even more so for targeted therapies. Exploring PUMA-mediated apoptosis can be used to discover more effective anticancer agents to overcome chemoresistance. For this purpose, we have recently developed a high-throughput assay for detecting PUMA induction (unpublished data), which can be used for screening compound libraries to identify novel PUMA-inducing small molecules. A number of apoptosis-targeting agents have recently
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