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Regorafenib (BAY 73-4506): Antitumor and antimetastatic activities in preclinical models of colorectal cancer

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Regorafenib, a novel multikinase inhibitor, has recently demonstrated overall survival benefits in metastatic colorectal cancer (CRC) patients. Our study aimed to gain further insight into the molecular mechanisms of regorafenib and to assess its potential in combination therapy. Regorafenib was tested alone and in combination with irinotecan in patient-derived (PD) CRC models and a murine CRC liver metastasis model. Mechanism of action was investigated using *in vitro* functional assays, immunohistochemistry and correlation with CRC-related oncogenes. Regorafenib demonstrated significant inhibition of growth-factor-mediated vascular endothelial growth factor receptor (VEGFR) 2 and VEGFR3 autophosphorylation, and intracellular VEGFR3 signaling in human umbilical vascular endothelial cells (HuVECs) and lymphatic endothelial cells (LECs), and also blocked migration of LECs. Furthermore, regorafenib inhibited proliferation in 19 of 25 human CRC cell lines and markedly slowed tumor growth in five of seven PD xenograft models. Combination of regorafenib with irinotecan significantly delayed tumor growth after extended treatment in four xenograft models. Reduced CD31 staining indicates that the antiangiogenic effects of regorafenib contribute to its antitumor activity. Finally, regorafenib significantly delayed disease progression in a murine CRC liver metastasis model by inhibiting the growth of established liver metastases and preventing the formation of new metastases in other organs. In addition, our results suggest that regorafenib displays antimetastatic activity, which may contribute to its efficacy in patients with metastatic CRC. Combination of regorafenib and irinotecan demonstrated an increased antitumor effect and could provide a future treatment option for CRC patients.

Colorectal cancer (CRC) is one of the leading malignancies worldwide, with more than 1.2 million new cases and 600,000 deaths estimated in 2008.[1] The incidence of CRC is generally higher in economically developed countries than in developing countries.[2] With the exception of some developed countries such as the USA,[3] incidence rates are generally continuing to increase.[2–5] The overall 5-year survival rate of patients with CRC is 64%, but only 12% of patients with metastatic CRC are still alive 5 years after diagnosis,[6] highlighting the urgent need for effective treatments.

CRC is usually treated with surgery alone in early disease stages, frequently in combination with adjuvant chemotherapy

Key words: regorafenib, multikinase inhibitor, antitumorigenesis, antimetastasis, CRC

Abbreviations: ATCC: American Type Culture Collection; BSA: bovine serum albumin; CRC: colorectal cancer; DMSO: dimethyl sulfoxide; EBM: endothelial basal medium; EC_{50}: half maximal effective concentration; ECL: electrochemical luminescence; FDA: Food and Drug Administration; FGFR: fibroblast growth factor receptor; HuVECs: human umbilical vascular endothelial cells; IC_{50}: half maximal inhibitory concentration; LECs: lymphatic endothelial cells; MAPK: mitogen-activated protein kinase; MSKK: Molecular Signatures in Colorectal Cancer; PD: patient-derived; RECIST: Response Evaluation Criteria in Solid Tumors; TIE2: tyrosine kinase with immunoglobulin and epidermal growth factor homology domain 2; UICC: Union for International Cancer Control; VEGFR: vascular endothelial growth factor receptor

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What's new?

Regorafenib is a multikinase inhibitor with antiangiogenic activity recently approved in the US and in Europe for the treatment of metastatic colorectal cancer in patients who failed previous therapies. Here, a research team led by Bayer Pharma AG, the discoverer of the drug, confirms inhibition of key mediators of angiogenesis and lymphangiogenesis (VEGFR2 and VEGFR3) as the potential antiangiogenic mechanism of action of the drug. Regorafenib further inhibited growth of established and prevented formation of new liver metastases, and in combination with the chemotherapeutic drug irinotecan led to significant tumor growth delay in four patient-derived colorectal cancer xenograft models. The authors speculate that combination treatments including regorafenib may provide novel therapeutic opportunities for patients with therapy-resistant colorectal cancer.

(which may be accompanied by radiotherapy). Palliative chemotherapy, combined with targeted therapy, provides the normal treatment option as tumors progress.[6] Current chemotherapy regimens consist of fluoropyrimidine-based treatments combined with oxaliplatin or irinotecan,[7] and monoclonal antibodies targeting VEGF (bevacizumab) and, for patients without *KRAS* mutations, EGFR (cetuximab and panitumumab[8]). Until recently, there were no other approved treatments for patients in whom these therapies fail.

Advances in the molecular understanding of CRC have demonstrated that disease onset and progression involves a sequence of genetic and epigenetic events.[9] Several signaling pathways have been implicated in the disease process, including EGFR, wnt/β-catenin, RAS/RAF and PI3K/AKT.[10] EGFR expression is differentially upregulated in 60–80% of metastatic CRC cases,[11] and activating mutations of *KRAS*, an early event in tumorigenesis, have been found in 35–45% of CRC cases.[12] The mutational status of *KRAS* has been shown to be a predictive marker for successful treatment with anti-EGFR antibodies.[13]

Angiogenesis has also been demonstrated to play a key role in CRC.[14] Overexpression of VEGF and high vascular density in primary CRCs are associated with an increased risk of tumor recurrence and the formation of metastases.[15,16] Furthermore, VEGF-C and its receptor VEGFR3 are regulatory elements of lymphangiogenesis, and their expression was shown to promote the dissemination of tumor cells to regional lymph nodes in preclinical models.[17]

Regorafenib is a novel oral multikinase inhibitor that targets protein kinases involved in tumor angiogenesis [VEGFR1–3 and tyrosine kinase with immunoglobulin and epidermal growth factor homology domain 2 (TIE2)], oncogenesis (KIT, RET and RAF) and the tumor microenvironment [platelet-derived growth factor receptor-β and fibroblast growth factor receptor (FGFR)[18]]. In preclinical studies, regorafenib exhibited antitumor activity in multiple tumor xenografts.[18] Recently, regorafenib demonstrated a significant improvement in overall survival in a phase III study in patients with metastatic CRC who failed previous therapies,[19] and has subsequently become the first approved treatment for this indication by several health authorities, such as the US Food and Drug Administration (FDA) and the European Medical Agency (EMA).

Here, we provide new preclinical *in vitro* and *in vivo* data on the activity of regorafenib when given alone or in combination with irinotecan in CRC cell lines, patient-derived (PD) CRC xenografts and a murine CRC liver metastasis model.

Material and Methods

Cell lines and reagents

Human umbilical vascular endothelial cells (HuVECs) and human lymphatic endothelial cells (LECs) were purchased from Lonza (Wakersville, MD). Human CRC cell lines were provided by Eurofins Panlabs (Bothell, WA) or purchased from the American Type Culture Collection (ATCC). The murine MC38 colonic adenocarcinoma cell line was originally derived from C57BL/6 mice treated with the carcinogen 1,2-dimethylhydrazine[20] and was purchased from ATCC (Rockville, MD). Cell lines were maintained in culture for no longer than 6 months and their identities confirmed.[21]

Recombinant human VEGF-A and VEGF-C were purchased from R+D (Minneapolis, MN). Antibodies against pAKT (#3787; pS^{473}), pERK1/2 (#9106; pT^{202}/pY^{204}), ERK1/2 (#4695), pVEGFR2 (#2478; pY^{1175}) and VEGFR2 (#2479) were from Cell Signaling Technologies (Danvers, MA); pVEGFR3 (CB5793; pY^{1063}/pY^{1068}) was from Cell Applications (San Diego, CA); VEGFR3 (MAB3757) was from Millipore (Billerica, MA); biotinylated anti-mouse CD31 (#553371) and IgG2a (#553928) were from BD Pharmingen (Heidelberg, Germany) and the ExtrAvidin peroxidase (#E2886) for their detection was from Sigma-Aldrich (Hamburg, Germany).

Oxaliplatin (Sanofi-Aventis, Paris, France) and irinotecan (Fresenius Kabi, Bad Homburg, Germany) were purchased from a local pharmacy as solutions in saline. Regorafenib was dissolved in polypropylene glycol/PEG400/Pluronic F68 (42.5/42.5/15 + 20% aqua) or Transcutol/Cremophor/sodium chloride (1:1:8) for *in vivo* applications and in 100% dimethyl sulfoxide (DMSO) for *in vitro* applications.

Endothelial cell VEGFR2 and VEGFR3 assays

HuVECs and LECs were grown in endothelial basal medium (EBM-2) supplemented with growth factors (Lonza). After serum starvation for 6 hr in EBM-2 media containing 0.1% bovine serum albumin (BSA), 2×10^5 cells were treated with various concentrations of regorafenib for 1 hr before stimulation with VEGF-A (50 ng/mL) or VEGF-C (200 ng/mL) for 10 min. Cells were lysed, and total cell lysates were analyzed for inhibition of phosphorylation by Western blotting using

antibodies against total and phosphorylated VEGFR2 and VEGFR3, pERK1/2 and pAKT. Signals were detected by electrochemical luminescence (ECL; GE Healthcare Biosciences; Pittsburgh, PA).

For the migration inhibition assay, 2–3 $\times$ 10^5 LECs per well were grown overnight on a gelatin-coated six-well plate, serum starved for 6 hr in EBM-2 media containing 0.1% BSA and treated with 100 nmol/L regorafenib for 1 hr before the addition of VEGF-C to a final concentration of 200 ng/mL. Cells were scratched using a sterile pipette tip, and images were taken after continued incubation for 40 hr.

CRC cell assays

Cell proliferation was assessed using a panel of 25 tumor cell lines derived from human colon cancers. Cells were grown in RPMI1640 with 10% fetal bovine serum or a custom medium, in a humidified atmosphere of 5% carbon dioxide at 37°C. Cells were seeded into 384-well plates and incubated for 24 hr, at which point regorafenib was added. The compound was serially diluted 3.16-fold and assayed over ten concentrations in a final assay concentration of 0.1% DMSO. After a 72-hr incubation period, cells were fixed and stained with fluorescently labeled antibodies and nuclear dye to allow visualization of nuclei, apoptotic cells and mitotic cells. Apoptotic cells were detected using an anti-active caspase-3 antibody. Mitotic cells were detected using an anti-phosphohistone-3 antibody. Twelve-bit tiff images were acquired using the InCell Analyzer 1000 3.2 and analyzed with Developer Toolbox 1.6 software. Half maximal effective/inhibitory concentration (EC_{50}/IC_{50}) values were calculated using nonlinear regression to fit data to a sigmoidal four-point, four-parameter one-site dose-response model. Inhibition of ERK activation was performed as previously described.[22]

PD CRC xenografts

Mouse experiments were approved by the relevant regulatory agency of the federal state of Berlin (Landesamt für Gesundheit und Soziales Berlin). Human CRC xenografts were either developed at EPO GmbH (Berlin, Germany)[23] or derived from the multicenter Molecular Signatures in Colorectal Cancer (MSKK) prospective study. A total of 239 fresh tumor tissue samples from patients of all four Union for International Cancer Control (UICC) stages were collected over a period of 2 years by a collaborating network of four clinics, using a standardized procedure. All patients gave written informed consent before surgery. The MSKK study was approved by the relevant ethics committees.[24] Xenografts with a weak response to oxaliplatin and bevacizumab treatment in preclinical studies were grown subcutaneously in male NMRI nu/nu mice to a palpable size of ~60–140 mm^3. Mice were then randomly assigned to one of five treatments: (*i*) oral regorafenib 10 mg/kg per day for at least 22 days; (*ii*) intraperitoneal (i.p.) oxaliplatin 15 mg/kg daily for 5 days; (*iii*) i.p. irinotecan 5 mg/kg daily for 5 days; (*iv*) a combination of regorafenib and irinotecan or (*v*) vehicle. Oxaliplatin and irinotecan, which is metabolized to the active drug SN38 in mice,[25] were given at close to the dose maximally tolerated in mice.[23] Groups were terminated after tumors had reached the ethically allowed limits. Tumor volume was determined using caliper measurements, and the volume was calculated using the formula $(D^*d^2)/2$, with d defined as the minor axis and D as the major axis of the measurement.

Immunohistochemistry

Frozen sections (5 μM) of xenografts from models Co5896 and Co8541, taken at study termination of regorafenib or vehicle treatment, were stained immunohistochemically with antibodies against the endothelial cell marker protein CD31. Sections were fixed with freshly prepared 4% paraformaldehyde for 20 min at 4°C in the dark, blocked against unspecific protein binding and peroxidase activity and sequentially incubated at room temperature with biotinylated CD31 or IgG2a antibodies for 60 min and with ExtrAvidin-peroxidase for detection for 30 min. Sections were developed with diaminobenzidine and finally counterstained with hematoxylin. For quantification, one entire tumor section of each of four randomly selected xenografts was scanned using ARIOL version 3.2 (Applied Imaging, San Jose, CA) and analyzed using the software package Angiosight.

Syngeneic MC38 CRC liver metastasis model

Female C57/Bl6 mice (Charles River, Sulzfeld, Germany) were housed and maintained in accordance with Bayer institutional animal care and the regulatory requirements of the federal state of Berlin, and received food and water *ad libitum*. For induction of syngeneic liver metastases, 1 $\times$ 10^5 murine MC38 CRC cells were injected in 50 μL medium, without additives, into the spleens, under total body anesthesia. The injection site was sealed with tissue glue. The spleens were resected three days later, and the mice were randomly divided into vehicle and treatment groups, each containing 16 animals. Treatment was started nine days after tumor cell injection, at which time the animals showed initial signs of metastatic spread (determined by visual inspection of resected satellite mice). Animals were treated orally with regorafenib at a dose of 10 mg/kg per day or the corresponding vehicle.

After the first animal in the vehicle group was sacrificed, six mice from each group were euthanized and dissected. Details of liver weight plus metastatic spread to the liver, mesenterium, diaphragm, stomach, lungs and kidneys were recorded. Treatment was continued in the remaining ten mice of each group until the disease had progressed to predefined criteria, at which point the animals were sacrificed. The general health status of the mice was monitored daily.

Statistics

Statistical analyses were performed using GraphPad Prism 5®, SAS® 9.2 and R 2.15.0. To describe the change of tumor volume for the sample, data were graphically presented as arithmetic means with standard deviations. The statistical

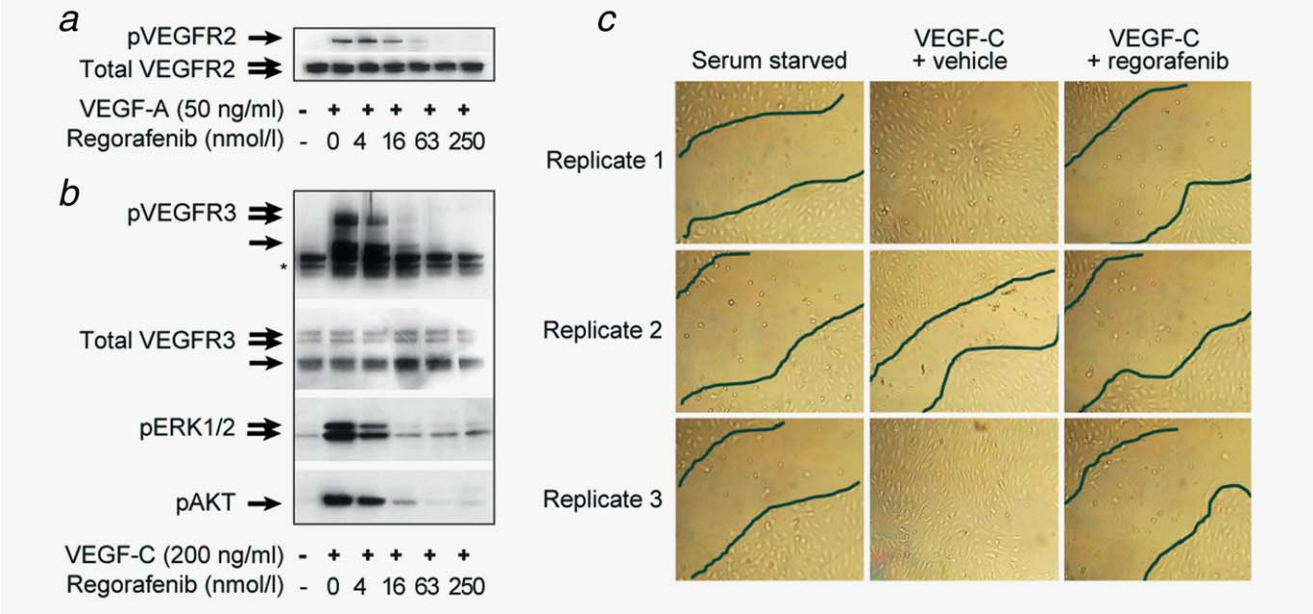

Figure 1. Regorafenib inhibits growth-factor-stimulated VEGFR2 and VEGFR3 autophosphorylation in human umbilical vascular endothelial cells (HuVECs) and intracellular signaling and migration in lymphatic endothelial cells (LECs). Western blot analysis of (*a*) VEGFR2 and (*b*) VEGFR3, ERK1/2 and AKT from total cell lysates from (*a*) HuVECs and (*b*) LECs. All cells were treated with the indicated concentrations of regorafenib and subsequently stimulated with (*a*) VEGF-A or (*b*) VEGF-C. (*c*) Migration inhibition analyzed by scratch assay in LECs. Images are taken after 40 hr of incubation with 200 ng/mL VEGF-C. Boundaries of cell growth are marked by black lines. * indicates nonspecific signals.

tests that were used to draw conclusions from the sample were based on geometric means and ratios of geometric means, as tumor volume is considered to be lognormally distributed.

Tumor growth inhibition was defined as the ratio of geometric mean tumor volume of treated compared with vehicle animals (T/C) and determined from tumor volumes 22 days after the start of treatment for all models and on the day of the vehicle group termination for models followed for more than 22 days. Tumor growth delay, which was defined as the difference in median time until a defined tumor volume was reached, was assessed using a survival type analysis.

Tumor volumes were logarithmically transformed (base = 10) and compared across groups at specified time points using a linear model with group as independent variable. Two-sided comparisons were made between all pairs of vehicle, regorafenib, irinotecan and the combination of irinotecan and regorafenib. The significance level was set to 0.05, and Šidák correction for multiplicity was used per time point. Results were transformed back to the original scale, presented as geometric means and corresponding 95% confidence intervals for each group and ratios of geometric means with 95% confidence intervals for comparisons.

For models Co8183, Co8213, Co8496 and Co8541 the time from start of treatment until the tumor reached a defined size limit for the first time (tumor growth delay) was analyzed using time-to-event analysis. The maximum possible time to event was either end of treatment or end of regular treatment regimen. Animals whose tumor had not exceeded the threshold at the maximum time point contributed as a censored observation. The times to event were plotted as Kaplan–Meier curves, and groups were compared using the log-rank test using a significance level of 0.05. Because of the low number of events, no correction for multiplicity was applied. Median times to event were estimated from the Kaplan–Meier model. If more than 50% of animals showed tumor volumes below the limit, the median time to event was indicated as "> maximum possible time point."

In the MC38 liver metastasis model, time to event was defined as the time until the animals in the treatment group had to be sacrificed based on predefined criteria. Results were plotted as Kaplan–Meier curves and differences were assessed using the log-rank test (significance level = 0.05).

For the comparison of immunohistochemistry results, the number of CD31-positive vessels per mm^2 between vehicle and regorafenib was analyzed using the Mood median test (significance level = 0.05). The proportion of area with CD31-positive vessels was analyzed using the logistic regression methodology. To test if there were significant differences between the two groups, a Wald test was carried out for the respective regression coefficient from the logistic regression model on a significance level of 0.05.

Results

Regorafenib potently inhibits growth-factor-stimulated VEGFR2 and VEGFR3 in human endothelial cells

The effect of regorafenib on growth-factor-stimulated VEGFR2 and VEGFR3 activation was assessed in endothelial cell models. Regorafenib inhibited VEGFR2 autophosphorylation in

serum-deprived HuVECs and VEGFR3 autophosphorylation in serum-deprived LECs after stimulation with VEGF-A or VEGF-C, respectively. The IC_{50} for both receptors was between 4 and 16 nmol/L as measured by Western blotting using antibodies against pY^{1175} (pVEGFR2) and $pY^{1063/1068}$ (pVEGFR3; Figs. 1*a* and 1*b*). Total amounts of VEGFR2 and VEGFR3 were unchanged.

Regorafenib inhibited activation (phosphorylation) of ERK and AKT kinases with a similar potency to that of VEGFR3, as measured by Western blotting with antibodies against pT^{202}/pY^{204} (ERK) and pS^{473} (AKT; Fig. 1*b*).

The effect of regorafenib on VEGF-C-induced migration of LECs was assessed in a functional assay. Regorafenib inhibited cell migration, as determined by the closure of a scratch in a confluent layer of cells, after an incubation period of 40 hr using VEGF-C at a concentration of 200 ng/mL (Fig. 1*c*). No apoptotic cells were detected during the assay despite the serum-deprived conditions and potent inhibition of the AKT pathway.

Regorafenib is a weak inhibitor of proliferation in human colon cancer cell lines

To characterize the antitumor activity of regorafenib in CRC, we performed an *in vitro* proliferation assay in a panel of 25 tumor cell lines derived from human colon cancers. Regorafenib inhibited the proliferation of 19 cell lines, with IC_{50} values ranging from 2.6 to 10 μmol/L (Fig. 2*a*), which reflects the C_{max} observed in plasma from patients.[26] Signs of apoptosis induction and/or cell cycle inhibition were observed (data not shown). Six cell lines did not respond to regorafenib at any concentration. No cell lines were available from the PD CRC models, precluding their comparative *in vitro* testing.

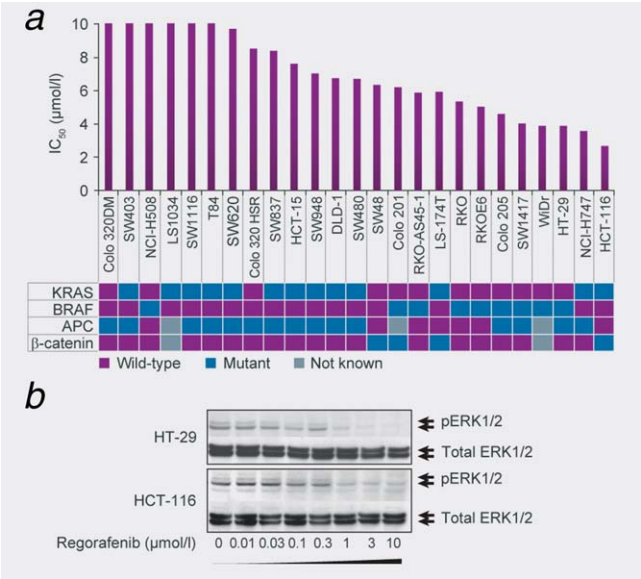

Figure 2. Inhibition of proliferation and intracellular signaling by regorafenib in human colorectal cancer (CRC) cell lines. (*a*) Dose-dependent regorafenib-mediated inhibition of proliferation in 25 human CRC cell lines and mutational status of key oncogenic CRC driver genes taken from the COSMIC database of the Wellcome Trust Sanger Institute. (*b*) Western blot analyses of phosphorylated ERK1/2 from total cell lysates treated with the indicated concentrations of regorafenib.

No correlation was seen between the regorafenib-mediated antiproliferative effects and mutational status of the cell lines, several of which carried mutations in the CRC-related oncogenes *KRAS*, *BRAF*, *APC* and *β-catenin* (Fig. 2*a*).

Inhibition of the mitogen-activated protein kinase (MAPK) signaling pathway was investigated by Western blot analysis of pERK. Regorafenib reduced pERK levels by ~50% at concentrations of 0.5–1 μmol/L in HT-29, HCT-116 and Colo-205 cell lines (Fig. 2*b* and data not shown). No effect on the total amount of ERK was observed in these analyses. Similar to Koyama *et al.*,[27] we did not detect constitutive pERK in the Colo320DM cell line (data not shown). This finding might help to explain the lack of inhibition of proliferation upon treatment with regorafenib in some of the cell lines, if there is also a lack of induction of the CDK inhibitor p15 upon MAPK pathway inhibition by regorafenib.

Regorafenib potently inhibits growth of PD oxaliplatin-refractory CRC xenografts alone and in combination with irinotecan

The antitumor effects of regorafenib were evaluated in seven PD CRC xenografts (supporting information (SI) Table 1). Regorafenib alone significantly inhibited the growth of xenografts in two tumor models (Co8183 and Co8435) and moderately in three models (Co5896, Co8213 and Co8469), demonstrating 54–63% mean tumor growth inhibition compared with the vehicle control group on the day when the control group was terminated (Fig. 3, SI Fig. 1, SI Tables 2 and 3). Weak or no inhibition was observed in the remaining two models (Co8434 and Co8541), with 34 and 1% tumor growth inhibition, respectively, compared with the vehicle group (SI Fig. 1, SI Tables 2 and 3). Regorafenib markedly delayed median tumor growth by 20 days or more compared with vehicle in three of the four models that received treatment or vehicle for more than 22 days (Co8183, Co8213 and Co8469; Fig. 3, SI Fig. 1, SI Tables 4 and 5).

At the initial observation point at 22 days of treatment, irinotecan alone significantly inhibited all but one (Co8469) of the xenografts, with mean tumor growth inhibition of 52–96% compared with vehicle (Fig. 3, SI Fig. 1, SI Tables 2 and 3). In the four models that were followed beyond 22 days, tumors progressed, but three of these models (Co8213, Co8469 and Co8541) responded to further cycles of irinotecan with transient tumor regression and delayed tumor growth beyond at least 10 days compared with vehicle (Fig. 3, SI Fig. 1, SI Tables 4 and 5). One xenograft model (Co8183) showed no response to additional irinotecan treatment, while two models (Co8213 and Co8541) also eventually became refractory to further cycles of irinotecan (Fig. 3*a*, SI Fig. 1). In these models, animals were crossed over to receive regorafenib treatment to test whether tumor growth could still be affected, and a transient delay in tumor growth was observed (Fig. 3, SI Fig. 1).

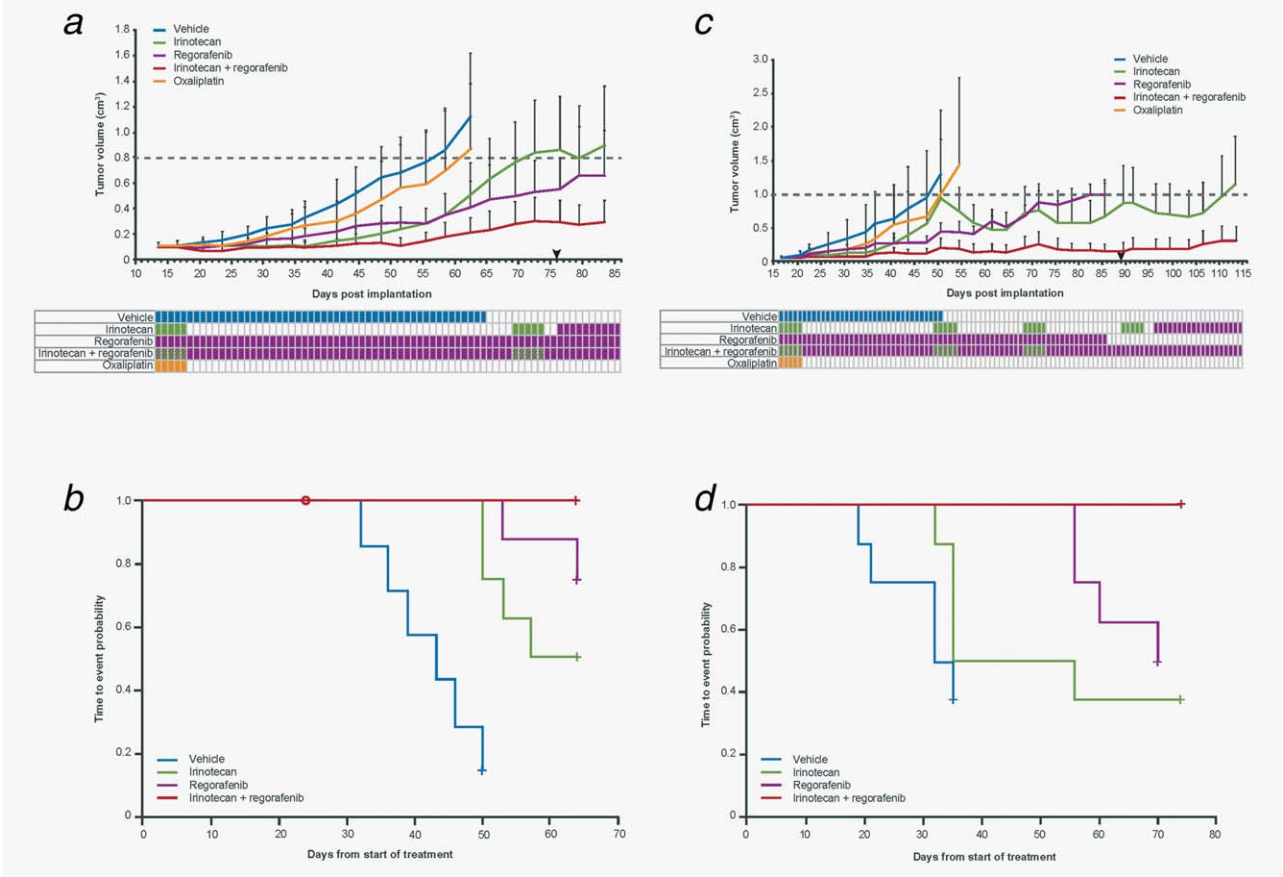

Figure 3. Antitumor activity of regorafenib alone and in combination with irinotecan in oxaliplatin- and bevacizumab-refractory patient-derived colorectal cancer xenografts. Tumor growth curves (*a*, *c*) and time-to-event probability curves (*b*, *d*) of xenograft Co8183 (*a*, *b*) and xenograft Co8469 (*c*, *d*) are shown. Respective data for xenografts Co8541 and Co8213 are provided in Supporting Information Fig. 1. For tumor growth curves, data show arithmetic mean (±standard deviation) tumor volumes after treatment with oral vehicle or regorafenib, or intraperitoneal oxaliplatin or irinotecan according to the schedule depicted in *a* and *c*. Hatched areas indicate combination treatments. $n = 8$ animals/group. Arrows in *a* and *c* indicate the endpoints and dotted horizontal lines the defined tumor volume (event) used for the time-to-event probability calculations. In figures *b* and *d* (+) indicates censored events and (o) indicates nondrug-related death.

The combination of regorafenib and irinotecan demonstrated significant inhibition of tumor growth in all xenograft models after 22 days of treatment compared with vehicle (Fig. 3, SI Fig. 1, SI Tables 2 and 3). Levels of inhibition were similar to those observed with irinotecan alone apart from model Co8469, which demonstrated increased inhibition with the drug combination. In the four models that were treated with drug combination for an extended period, significantly increased tumor inhibition was observed with regorafenib (in two of four models) or irinotecan treatment (in three of four models) and a marked delay in median tumor growth beyond 21–41.5 days, relative to vehicle (Fig. 3, SI Fig. 1, SI Tables 4 and 5). In xenografts Co8213 and Co8469, the combination of regorafenib and irinotecan led to a statistically significant delay in tumor growth compared with irinotecan alone at the end of the regular treatment schedule (SI Table 4). In model Co8469, the combination treatment resulted in stable disease over a treatment period of 63 days, whereas in Co8183 and Co8213, slow increases in tumor volumes were observed towards the end of the study (Fig. 3, SI Fig. 1).

Oxaliplatin alone displayed weak activity in all models, with tumor growth inhibition below 50% and no delay in tumor growth relative to vehicle (Fig. 3, SI Fig. 1; not statistically tested).

Regorafenib exerts antiangiogenic effects in PD CRC models

To evaluate the mode of action of regorafenib in the PD CRC models, we performed immunohistochemical analyses on frozen sections from tumor xenografts after treatment for 22 days with regorafenib at a dose of 10 mg/kg per day. Significantly reduced tumor vessel area ($p = 0.0028$) and number of tumor vessels ($p = 0.0047$; data not shown), as determined by CD31 staining, were observed in Co5896 xenografts from mice treated with regorafenib compared with the vehicle group (Fig. 4). However, no such differences were observed in Co8541 xenografts; this model was refractory to treatment with regorafenib (SI Table 3), and the average CD31-positive tumor area was 2.8%, irrespective of treatment received. As previously shown for xenografts derived from CRC cell

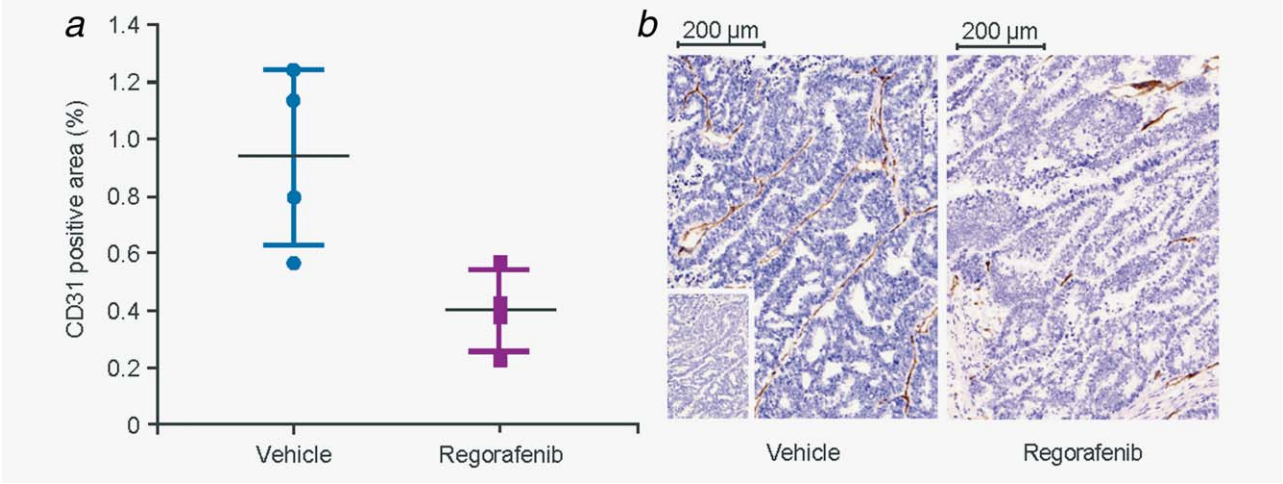

Figure 4. Tumor growth inhibition of patient-derived colorectal cancer (PD CRC) xenografts by regorafenib is mediated by antiangiogenic effects. Frozen sections of xenografts of model Co5896 grown in mice and treated orally with vehicle or regorafenib at a dose of 10 mg/kg per day for 22 days were stained immunohistochemically with antibodies against the endothelial cell marker protein CD31. (*a*) The percentage of CD31-positive area was determined and statistically evaluated as described in Material and Methods ($n = 4$; $p = 0.0028$). (*b*) Representative images of CD31 staining. The inset depicts an isotype IgG2a control image.

lines,[18] these results suggest that antiangiogenic effects of regorafenib also contribute to the growth inhibition of PD CRC xenografts.

Regorafenib exhibits antimetastatic activity in the murine MC38 CRC liver metastasis model

The antimetastatic effect of regorafenib was tested in a syngeneic murine model of CRC liver metastasis. Regorafenib treatment, after the development of liver metastases, significantly prolonged median time to event by 14 days compared with vehicle (42 *vs.* 28 days, respectively; $p = 0.0009$; Fig. 5*a*).

In a satellite study, the livers of six animals per group were resected as soon as the first animal of the vehicle group was sacrificed, and their weights were measured as an indicator of metastatic burden. The mean liver weight of regorafenib-treated animals was markedly lower than that of vehicle-treated mice (1.2 ± 0.33 g *vs.* 1.6 ± 0.49 g; Fig. 5*b*), indicating that regorafenib inhibited the growth of metastases in the livers. For comparison, the mean liver weight of healthy control animals that received no treatment was 1.1 ± 0.14 g.

All mice showed hepatic tumor nodules; however, liver metastasis was more pronounced in the vehicle group than in the regorafenib group. The animals were also examined for metastases outside the liver. Three of six vehicle animals had two or more metastases in other organs, such as the diaphragm, kidney, stomach and mesenterium (Fig. 5*c*), whereas no metastases outside the liver were found in regorafenib-treated animals. No tumors had formed at the splenectomy site suggesting that no tumor cells had drained from the injection site.

For the duration of the study, regorafenib was well tolerated and did not cause significant weight loss or overt clinical symptoms.

Discussion

Regorafenib, a novel multikinase inhibitor, mediated tumor growth inhibition and delayed time to tumor growth in PD models of CRC when administered at a dose comparable to the human therapeutic dose, alone and in combination with irinotecan (the latter at close to the murine maximum tolerated dose). Additionally, in a murine model of CRC liver metastasis, regorafenib displayed antimetastatic potential. Antiangiogenic effects were observed that are presumed to contribute to tumor growth retardation, probably through concerted kinase inhibition by regorafenib. In particular, regorafenib inhibits VEGFR2 and VEGFR3, both of which are important mediators of angiogenesis and lymphangiogenesis in CRC growth and metastasis.

Autophosphorylation of VEGFR2 and VEGFR3 was inhibited by regorafenib in human vascular and lymphatic cells, respectively. For VEGFR2, the IC_{50} range was similar to recent data from biochemical and cellular kinase assays and a VEGF-A-stimulated HuVEC proliferation assay.[18] The IC_{50} values for regorafenib were similar between studies, despite the use of three different phosphotyrosine-specific VEGFR2 antibodies. In our study, we used antibodies against pY^{1175} of VEGFR2, which was previously demonstrated to be phosphorylated by src kinase.[28] As src kinase is not inhibited by regorafenib at the concentrations used, inhibition of Y^{1175} phosphorylation is indirectly mediated by inhibition of $Y^{1054/1059}$, which prevents src binding rather than direct src inhibition.[18] We previously reported regorafenib-mediated

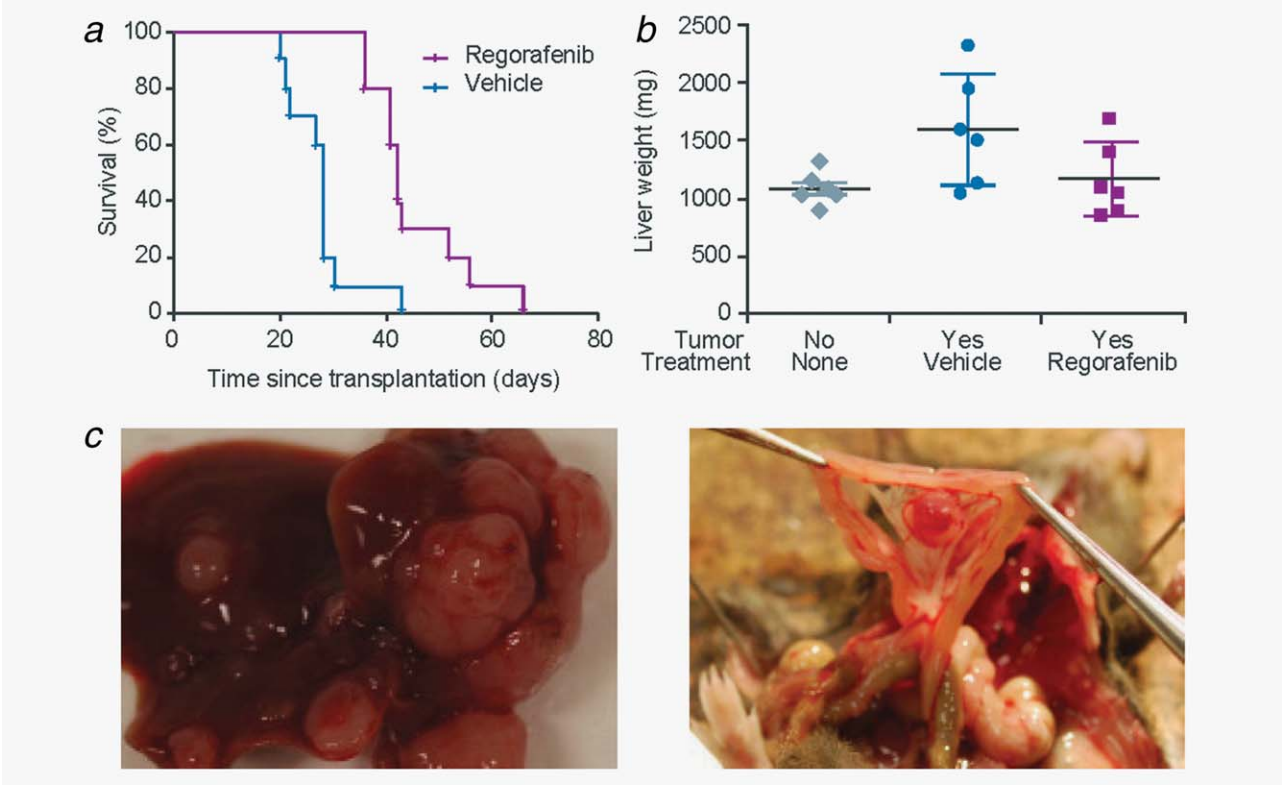

Figure 5. Antimetastatic activity of regorafenib in a murine CRC liver metastasis model. (*a*) Kaplan–Meier plot showing animal survival after treatment with vehicle or regorafenib ($n = 10$; $p = 0.009$). (*b*) Liver weights of mice killed at the time when the first animal in the vehicle group was sacrificed ($n = 6$; $p < 0.05$). (*c*) Representative images of metastases of the (left) liver and (right) mesenterium of vehicle-treated animals.

inhibition of VEGFR3 autophosphorylation at IC_{50} values of 46 and 135 nmol/L in biochemical and cellular kinase assays, respectively.[18] In these studies, murine VEGFR3 was used either directly in a biochemical assay or in a surrogate system after expression in human HEK293 cells. This may explain the differences between our current and previous findings.

To investigate the potential for regorafenib in a combination therapy setting, antitumor activity was assessed in combination with irinotecan in several PD CRC xenograft mouse models. To further mimic the clinical scenario, regorafenib was administered at a dose that leads to a similar exposure to CRC patients receiving 160 mg/day.[29] Heterogeneous responses were observed for regorafenib, irinotecan and their combination in different xenograft models. This probably reflects the individuality of each of the tumors, which show differences in histopathological staging and mutational status for CRC oncogenes, although no clear correlation between mutational status and efficacy was observed, which may be attributable to the small sample size (SI Table 1).

Although regorafenib treatment alone showed a median delayed tumor growth of up to 37.5 days in some xenografts relative to vehicle (SI Table 5), disease stabilization or tumor regression was not achieved according to clinical Response Evaluation Criteria in Solid Tumors (RECIST). For irinotecan, transient tumor regressions were observed after each cycle, but no sustained remissions occurred, and all tumors became less responsive to irinotecan treatment over time. In a phase II clinical trial, irinotecan treatment alone resulted in a response rate of ~10% in metastatic CRC.[30] In mice with irinotecan-refractory xenografts that were crossed over to receive daily regorafenib, responses were observed in two of four models. This result indicates that regorafenib may be effective in tumors that are refractory to irinotecan. Overall, these results are consistent with recent clinical trial evidence that showed regorafenib-mediated improvement in overall survival in metastatic CRC patients that were refractory to fluoropyrimidine-based therapy. This was demonstrated primarily by disease stabilization and not by objective responses.[19]

A clear beneficial effect was observed when regorafenib was combined with irinotecan, which led to significant tumor growth delay in all four xenograft models that were treated for an extended period. Regorafenib was well tolerated in these studies even when combined with irinotecan. There were no drug-related animal deaths, and a very transient weight loss, reaching a maximum of 17% shortly after irinotecan application, was observed in one model only. Similar tolerability was previously observed with other targeted therapies used in combination treatments, such as the MEK inhibitor BAY86–9766,[31] indicating that regorafenib may be appropriate for combination therapy. Indeed, acceptable

tolerability was observed in a clinical phase Ib study where regorafenib was scheduled following a regimen containing irinotecan in combination with 5-fluorouracil and folinic acid.[32]

Mechanistically, the combined effects of regorafenib and irinotecan may be explained by regorafenib-mediated normalization of the tumor vasculature, which could improve accessibility of irinotecan to the tumor.[33] A preclinical study showed an antiangiogenic effect of metronomic dosing of irinotecan and synergism with the VEGFR2 inhibitor, semaxanib, in a CRC model.[34] Reduced microvessel density and increased expression of the antiangiogenic factor thrombospondin-1 was observed in that study. Other potential explanations include differential effects on tumor perfusion and induction of hypoxia.[35] Indeed, regorafenib, as shown by dynamic contrast-enhanced magnetic resonance imaging, exerts a rapid and prolonged decrease in vascular hyperpermeability in rat glioblastoma tumors.[18]

The models used in our study were refractory to bevacizumab treatment,[24] suggesting that they may have developed other proangiogenic mechanisms, for example, involving fibroblast growth factor.[36] Regorafenib inhibits various angiogenic kinases, including VEGFR3, FGFR and TIE2, which may explain its activity in these models; further investigation is required.

In addition to providing models for studying potential treatment, PD CRCs also offer the opportunity to identify biomarkers for predicting the efficacy of a particular drug. Our *in vitro* proliferation assays and *in vivo* xenograft studies demonstrate that regorafenib acts independently of the mutational status of *KRAS* and *BRAF*. This is in contrast to anti-EGFR-based antibody therapies, where the mutational status of *KRAS* and *BRAF* predicts responsiveness.[13,24] Indeed, the xenograft models used in our study that expressed either mutated *KRAS* or *BRAF* were refractory to cetuximab treatment.[24] We did not see a correlation between the effects of regorafenib and the mutational status of *β-catenin* and *APC*. However, there may be other genes worth considering and further research is required.

Of note, no antiangiogenic effect and only poor antitumor activity were seen in model Co8541 when animals were treated with regorafenib alone. Preliminary attempts to identify markers to help explain this finding involved the comparison of the expression profiles of the PD models investigated in our study by hierarchical clustering and principle component analyses. Co8541 was clearly discriminated from the other models and significantly raised concentrations of COX-2 and mucin-2 transcripts (consistent with the histotype) were detected (SI Table 1 and data not shown). Elevated COX-2 is a known promoter of tumor growth in colon cancer[37] and may explain the lack of effect of regorafenib in this model. Further investigations are required. Interestingly, the Co8541 model is derived from a mucinous CRC (SI Table 1); it has been observed, in a clinical study, that this particular tumor subtype responds poorly to fluoro-based therapy,[38] indicating that, in this instance, additional factors may contribute to therapy resistance. The combination of regorafenib with irinotecan inhibited the growth of this model and may provide a treatment option for such tumors.

Regorafenib demonstrated a potent growth-inhibitory effect on established liver metastases in the orthotopic, syngeneic MC38 model. This suggests an additional potential benefit of regorafenib because previous studies only showed effects in inhibiting metastases formation.[31] We hypothesize that metastases are inhibited in the same way that regorafenib exerts its antitumor activity, *via* antiangiogenic and antiproliferative mechanisms.[18] Inhibition of VEGFR2 signaling by regorafenib may induce a reduction of microvessels and increased apoptosis in metastatic tumor cells and endothelium, similar to the effects observed through treatment with antibodies against VEGF[39] or VEGFR2[40] in preclinical models of CRC liver metastasis. In addition, VEGFR3 inhibition may contribute to the antiangiogenic effect by suppression of endothelial sprouting and vascular network formation[41] and may affect metastatic spread by blockade of tumor lymphangiogenesis.[42] Indeed, no metastases were detected outside of the liver in regorafenib-treated mice, whereas these were detected in half of the vehicle-treated animals. This fits well with recent findings that regorafenib prevents liver metastasis formation in an orthotopic murine CT26 CRC model and in lungs and lymph nodes in orthotopic murine 4T1 and human MDA MB 231 breast cancer models.[31,43] Interestingly, no metastases were detected in the lungs of any animals, despite the lungs being a metastatic site in 10–20% of patients with advanced CRC.[44] This suggests that this model is not suitable for studying CRC lung metastasis. *In vitro*, MC38 cell proliferation was inhibited by regorafenib with an IC_{50} of ~5 μmol/L (data not shown), suggesting that the antimetastatic effect is not directly mediated by a potent inhibition of proliferation.

In summary, the results of these studies demonstrate the potential of regorafenib to target CRC and provide an antimetastatic effect that may contribute to the overall survival benefit observed in the phase III study of patients with metastatic CRC.[19] The use of PD CRC xenografts has provided some rationale for future clinical development strategies. Detailed genetic characterization of these models will give further insight into the mechanism of action of regorafenib and other potential CRC therapies.

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