Regorafenib Inhibits Growth, Angiogenesis, and Metastasis in a Highly Aggressive, Orthotopic Colon Cancer Model

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Abstract

The combination of target-specific drugs like bevacizumab with chemotherapy has improved treatment efficacy in advanced colorectal cancer (CRC). However, the clinical prognosis of metastatic CRCs is still poor, and novel drugs are currently assessed with respect to their efficacies in patients with CRCs. In a phase III study, the multikinase inhibitor regorafenib (BAY 73-4506) has recently been shown to prolong survival of patients with CRCs after standard therapies failed. In the present study, the activity of regorafenib was investigated in comparison with the angiogenesis inhibitor DC101 in the highly aggressive, murine CT26 metastatic colon cancer model. While a treatment for 10 days with DC101 given at a dose of 34 mg/kg every third day significantly delayed tumor growth compared with vehicle-treated animals, regorafenib completely suppressed tumor growth at a daily oral dose of 30 mg/kg. Regorafenib also induced a stronger reduction in tumor vascularization, as longitudinally assessed in vivo by dynamic contrast-enhanced MRI (DCE-MRI) and confirmed by immunohistochemistry. In addition, regorafenib inhibited the angiogenic activity more strongly and induced a three times higher apoptosis rate than DC101. Even more important, regorafenib completely prevented the formation of liver metastases, whereas in DC101-treated animals, the metastatic rate was only reduced by 33% compared with the vehicle group. In addition, regorafenib significantly reduced the amount of infiltrating macrophages. These data show that the multikinase inhibitor regorafenib exerts strong antiangiogenic, antitumorigenic, and even antimetastatic effects on highly aggressive colon carcinomas indicative for its high potential in the treatment of advanced CRCs. Mol Cancer Ther; 12(7); 1322–31. ©2013 AACR.

Introduction

Colorectal cancer (CRC) is one of the leading causes of cancer-related mortality (1). Approximately one million new cases are diagnosed every year and 500,000 deaths are reported annually worldwide (2). During the last decade, screenings for early diagnosis and staging as well as improvements in surgery and therapy have increased the survival of patients with CRCs (3, 4). However, tumor progression to a highly advanced, metastatic stage (mCRC) still decreases the overall 5-year survival rate to 8% to 10% (5). Besides different genetic mutations, enhanced EGF receptor (EGFR) signaling and VEGF-mediated angiogenesis play a critical role in the progression of CRC (6). Tumor progression promoting functions of VEGF have been evidenced in various preclinical colon cancer models (7, 8), and a clear correlation was found between a high vascular density in colorectal tumors, disease recurrence, and the development of metastases (9, 10). In the meantime, the VEGF-inhibiting antibody bevacizumab has been approved in combination with chemotherapy for the treatment of mCRCs (11, 12). However, in adjuvant therapy, bevacizumab in combination with chemotherapy failed in increasing the 3-year disease-free survival compared with chemotherapy alone (11, 13). In this context, recent preclinical studies have shown that antiangiogenic treatment predominantly targeting VEGF or its receptors can induce an evasive tumor response, leading to increased vessel density and even enhanced metastasis (14, 15), raising concerns about possible unwanted effects of long-term bevacizumab treatment. One of the mechanisms described for the failure of antiangiogenic therapy and tumor evasion is the strong infiltration of tumor-associated macrophages (TAM), in particular of a subset that expresses the angiopoietin (Ang) receptor tyrosine kinase with immunoglobulin and EGF homology domain 2 (TIE2) that promote angiogenesis and tumor progression (16–19). These TIE2-expressing macrophages (TEM) have been found in various tumors including colon (20), and targeting of these TEMs by Ang2/TIE2 blockade has successfully inhibited tumor angiogenesis, progression, and metastasis (21, 22).
In consequence, novel drugs are highly desirable that target multiple pathways, including TIE2, to increase the treatment efficacy and to prevent metastasis (23, 24). A novel oral multikinase inhibitor, regorafenib (BAY 73-4506; ref. 25) has been developed that targets a variety of kinases involved in angiogenic, tumor growth-promoting, and tumor microenvironmental signaling pathways such as VEGFR1/2/3, platelet-derived growth factor receptor-β (PDGFR-β), fibroblast growth factor receptor 1 (FGFR1), the mutant oncogenic kinases KIT (CD117), RET, B-RAF, as well as TIE2. In preclinical and clinical phase I–III trials, regorafenib has shown potent antiangiogenic and antitumorogenic effects (25–28). To elucidate the effects of the multikinase inhibitor in more detail, we here compared the effects of regorafenib with the selective function blocking VEGFR2-antibody DC101 in a highly aggressive and metastatic orthotopic mouse model of CRC. The effects of regorafenib and DC101 on tumor growth, angiogenesis, macrophage infiltration, and metastasis formation were analyzed longitudinally in vivo by MRI, including dynamic contrast-enhanced MRI (DCE-MRI), and complemented by detailed immunohistochemical analyses.

Materials and Methods

Cell line

The mouse colon cancer cell line CT26 (LGC Standards GmbH) was cultivated in Dulbecco’s Modified Eagle Medium (DMEM; Gibco, Invitrogen GmbH) containing 10% FBS (Gibco) and 1% penicillin/streptomycin (Gibco). The cell line CT26 was authenticated at ATCC/LGC Standards GmbH and was assayed for mycoplasma by Hoechst stain, PCR, and the standard culture test. The cells were passaged for less than 6 months in our laboratory after receipt or resuscitation.

Orthotopic murine colorectal cancer model (CT26)

All experiments were approved by the Governmental Review Committee on Animal Care. Athymic female CD1-nude mice (Charles River) were used for the experiment. The orthotopic implantation was conducted as described by Hoffman and colleagues (29). A total number of 2 × 10⁶ CT26 cells in 100 μL culture medium was injected subcutaneously into the right flank of 6- to 8-week-old mice. Fourteen days after injection, these donor mice were sacrificed, the subcutaneous tumors (~500 mm³) were excised, cut into 2-mm-sized pieces, and harvested in ice-cold PBS. Necrotic areas were discarded. Mice were treated with rimadyl (Pfizer Pharma GmbH), 2 hours before and after tumor implantation. For implantation, the mice were anesthetized with isofluorane (1.5%). The abdomen was sterilized with antiseptic spray (Antiseptics spray (Beiersdorf AG Hansaplast) until complete wound closure. Finally, the wound was treated with povidon-iodine cream (B. Braun Melsungen AG) and wound-healing spray (Beiersdorf AG Hansaplast) until complete wound closure.

Study design and therapy with regorafenib and DC101

For the longitudinal analysis, a total of 18 mice carrying an orthotopically implanted tumor were scanned by MRI on day 4 postimplantation and were divided randomly in 3 groups. At that time point, the tumors had reached a size of approximately 5 mm in diameter, as determined by T2-weighted (T2w) MRI. Subsequently, the first group (n = 6) was treated daily orally with multikinase inhibitor regorafenib (structural formula, see Fig. 1A). Regorafenib was applied at a dose of 30 mg/kg body weight, dissolved in polyethyleneglycol (PEG) 400, 1,2-propanediol and pluronic F68 (all from Sigma-Aldrich Chemie GmbH), as described (25). The second group (n = 6) received a daily oral administration of the vehicle (PEG400, 1,2-propanediol, and pluronic F68). The third group (n = 6) was treated intraperitoneally with the VEGFR2-blocking monoclonal antibody DC101 (Fig. 1A; Bio-XCell) in PBS at a dose of 34 mg/kg body weight every third day. Therapy was continued for 10 days until day 14 postimplantation (Fig. 1B). The animal weight was measured daily. Tumor volumes were determined by MRI measurements on day 4 (before drug administration), 7, 11, and 14 postimplantation. On day 1323

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14 postimplantation, after the last MRI measurement, all animals were sacrificed. The tumors were dissected and cryoconserved for histologic analysis. In addition, the lungs and livers were dissected and screened macroscopically for metastases (Fig. 1B).

For additional histologic validation, another 28 animals received an orthotopic tumor implantation and 4 animals were sacrificed before therapy start on day 4 postimplantation. The remaining animals were randomly divided into 3 groups and treated as described above. Four animals per group (vehicle, DC101, regorafenib) were sacrificed on day 7 and day 11 postimplantation, respectively, and were analyzed as described below.

**DCE-MRI and data analysis**

MRI was conducted at a clinical 3-Tesla whole-body scanner (Achieva 3.0 T, Philips), using a small-animal solenoid receiver coil with an integrated heating system to keep the temperature constant at 37°C during the examination (Philips). For the MRI measurements, the animals were anesthetized with isofluorane (1.5%). 0.5 mg/kg butylscopolamine (Buscapan, Boehringer Ingelheim Pharma KG) was injected intraperitoneally to reduce the peristaltic movement of the colon. For detecting the tumors and assessing the tumor volume, morphologic MR images were acquired using a transversal T2-weighted turbo-spin echo sequence (repetition time, TR; echo time, TE; flip angle; number of signal averages, NSA; field of view; FOV; slice thickness; voxel size; number of signal averages, NSA; field of view; FOV; slice thickness; voxel size)3; matrix size = 148 × 150; slice thickness = 1 mm; voxel size = 0.2 × 0.2 × 1.0 mm3). After defining the tumor by T2-weighted imaging, a DCE-MRI scan was conducted using a multi slice 2-dimensional (2D) T1w saturation recovery gradient echo sequence (saturation recovery turbo fast low angle shot), TR = 9.94 ms, TE = 4.296 ms, NSA = 1 flip angle = 25°, matrix = 432 × 416, slice thickness = 3 mm, voxel size = 0.35 × 0.35 × 3.0 mm3. In total, 300 sequential images were acquired per slice with a temporal resolution of 2.2 seconds, resulting in a total scan time of 11 minutes. After the acquisition of baseline images over approximately 2 minutes, 100 µL (100 µmol/kg body weight) of the paramagnetic contrast agent gadomer 17 (Bayer-Pharma AG) was injected manually into the tail vein within 15 seconds (25).

For postprocessing, the tumor was segmented semiautomatically based on the T2-weighted MRI images, and the tumor volume was determined using an Imalytics Research Workstation (Philips Technology GmbH Innovative Technologies). For tracer kinetic modeling, the average signal per region was computed. The resulting signal–time curves were analyzed using the pharmacokinetic 2-compartment model of Brix and colleagues (30, 31), providing the parameters amplitude (related to the relative distribution volume of the tumor; ref. 32) and the exchange rate constant k_{ex} (a marker for perfusion and blood vessel permeability). The linearity between applied contrast agent concentration and signal intensity in the measured range was proven by phantom experiments.

**Histologic analyses**

Frozen tumors were cut into 7- to 10-µm-thick slices. The following primary antibodies were used for immunofluorescent staining: a rat anti-mouse CD31 antibody (BD Biosciences) for vessel staining, a goat anti-mouse VEGFR2-antibody (R&D Systems) and a rat anti-mouse TIE2-antibody (BioLegend) for assessing the angiogenic activity. For analyzing vessel maturation, a biotinylated anti-smooth muscle actin (SMA) antibody (Progen Biotechnik GmbH) was used. Apoptosis was determined using the DeadEnd colorimetric TUNEL assay (Roche Diagnostics). Macrophages were stained with a rat anti-mouse F4/80-antibody (AbD Serotec). Secondary antibodies were a donkey anti-rat-fluorescein isothiocyanate (FITC) antibody, a donkey anti-rat-Cy3 antibody, a conjugated streptavidin-Cy3 antibody, a donkey anti-goat-Cy3 antibody, and a donkey anti-rabbit-Cy3 antibody (all from Dianova). For all analyses, cell nuclei were counterstained with 0.5 µg/mL 4’,6-diamidino-2-phenylindole (DAPI; Invitrogen). For histologic examination of liver metastases, fixed liver tissues were sectioned at 4 μm and stained with hematoxylin and eosin (H&E). All stained sections were examined using an epifluorescence microscope (Axio Imager.M2, Zeiss) and a high-resolution camera (AxioCam MRm Rev.3, Zeiss). Images were quantified using the AxioVision Rel 4.8 software (Zeiss), covering the whole tumor area. Microvessel area (MVA) was analyzed by determining the area fraction of CD31. The angiogenic activity was determined by the ratio of the VEGFR2-positive area to the CD31-positive area fraction. Apoptosis was assessed by determining the terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL)-positive area fraction. SMA- and TIE2-positive -and negative vessels were counted manually. SMA+ and TIE2+ vessels were divided by the total vessel number, respectively.

In addition, macrophages were quantified by determining the F4/80+ area fractions. To determine the amount of TIE2-positive macrophages, TIE2-positive vessels were segmented and excluded from analysis. The fraction of TIE2-positive macrophages was determined by dividing the remaining area fractions of TIE2+ by F4/80+.

**Statistical analysis**

For statistical analysis, a one-way ANOVA combined with a Bonferroni multiple comparison posttest was conducted to analyze differences between the groups using GraphPad Prism 5.0. P < 0.05 was considered as significant, P < 0.01 and P < 0.001 as more significant.

**Results**

**Regorafenib inhibits growth of orthotopic CRC xenografts**

The murine CT26 model is described as hypervascular and metastatic CRC model in syngeneic BALB/c mice after subcutaneous, orthotopic, or intraportal injection of the tumor cells (33). The implantation of CT26 tumor pieces into the cecum of CD1 nude mice induced an
Regorafenib inhibits angiogenesis and induces apoptosis

To longitudinally analyze the effects of the drugs on angiogenesis and tumor vascularization in vivo, DCE-MRI was conducted because it has been shown as very suitable technique for the longitudinal assessment of angiogenesis and antiangiogenic therapy response (32, 34). In regorafenib-treated mice, the relative distribution volume of the contrast agent (RDV) in the tumor indicated by the amplitude significantly dropped after a delay of 3 days between day 7 and 11 postimplantation, whereas it remained stable until day 14 postimplantation in the vehicle control group (Fig. 4A; \( P < 0.05 \) on day 11 and \( P < 0.01 \) on day 14 postimplantation). The RDV decreased also in the DC101-treated group between day 7 and 11 postimplantation, but the decrease was less strong than in the regorafenib-treated group and not significant compared with the vehicle control group (Fig. 4A). On day 14, the RDV in DC101-treated tumors was significantly higher than in regorafenib-treated tumors (\( P < 0.05 \), \( P \) value not shown in the graph). The blood vessel permeability, as monitored by the exchange rate constant \( k_{ep} \), was not significantly different between all groups during the entire observation period (Fig. 4B).

Immunohistochemical analyses of the MVA revealed similar results as the in vivo MRI measurements of the RDV (amplitude). The MVA was not remarkably different for all treatment groups on day 7 postimplantation. However, while an increase in MVA was observed in the vehicle control group between day 7 and 14 postimplantation, it decreased in both, regorafenib and DC101-treated animals, to significantly lower levels compared with the controls on day 11 postimplantation (\( P < 0.05 \) for both) and on day 14 postimplantation (\( P < 0.01 \) for both; Fig. 4C, exemplary images of day 14 are shown in E). However, regorafenib induced stronger decrease in MVA compared with DC101, reaching a significantly lower MVA than DC101 on day 14 (Fig. 4C, \( P < 0.05 \)). When analyzing vessel maturation by determining the fraction of CD31-staining vessels that are SMA-positive, a trend to a higher number of mature vessels was detected in regorafenib compared with...
DC101 or vehicle-treated animals on day 14 postimplantation, although the differences were not significant ($P = 0.394$ for regorafenib vs. control; $P = 0.132$ for regorafenib vs. DC101; Fig. 4D, exemplary images are shown in E).

Further analysis of angiogenesis by determining the ratio of VEGFR2-positive vessels (VEGFR2\(^+\)/CD31 area fraction) revealed a similar angiogenic activity in all groups on day 7 postimplantation (Fig. 5A). The angiogenic activity was significantly decreased in regorafenib-treated tumors on day 11 postimplantation ($P < 0.05$) and day 14 postimplantation ($P < 0.01$) compared with the vehicle controls (Fig. 5A, exemplary images of day 14 are provided in Fig. 5B). The effects of DC101 on the angiogenic activity were less pronounced than regorafenib. A significant reduction compared with the vehicle controls was only reached on day 14 postimplantation ($P < 0.01$). Additional analysis of TIE2 expression on the vasculature revealed a significant decrease in TIE2-positive vessels in regorafenib-treated tumors compared with the vehicle-treated tumors on day 14 postimplantation ($P < 0.01$). DC101 did not significantly reduce the ratio of TIE2-positive vessels (Fig. 5C, exemplary images of day 14 are provided in Fig. 5D).

The effects of the treatments on apoptosis were investigated using a TUNEL staining assay. On day 14 postimplantation, the strongest rate of apoptosis was observed in tumors from animals treated with regorafenib. Regorafenib significantly increased apoptosis by 18.4- and 4-fold compared with vehicle and DC101-treated animals, respectively ($P < 0.01$ for both; Fig. 5C; exemplary images are provided in Fig. 5F). The proapoptotic effects of DC101 were less pronounced compared with regorafenib, showing only a 5.3-fold increase in apoptosis compared with the
controls (Fig. 5E). Taken together, regorafenib exerted strong antiangiogenic and anti-tumorigenic effects, clearly exceeding the effects of the selective VEGFR2 blockade by DC101 at the given doses.

**Regorafenib inhibits the formation of liver metastasis**

To determine whether administration of regorafenib had an effect on the metastatic spread of the CT26 tumor cells, we examined the livers and lungs of all mice in the different treatment groups on day 14 postimplantation in vivo by T2w MRI and subsequently by visual macroscopic and histologic inspection. Remarkably, no liver metastases were detected by MRI and by macroscopic screening after treatment with regorafenib, whereas liver metastases were found in 4 and 5 of 6 animals in DC101- or vehicle-treated animals, respectively (Fig. 6).

No lung metastases were detected macroscopically in all groups. H&E staining of livers and lungs confirmed the occurrence of metastases in the livers of DC101- and vehicle-treated mice, whereas the lungs were metastasis-free in all groups (Fig. 6A). These data show that besides interfering with angiogenesis and tumor growth, regorafenib also inhibited the metastatic dissemination of the colon cancer cells to the liver.

**Regorafenib decreases macrophage accumulation**

As TAMs are discussed to be crucially involved in angiogenesis and metastatic spreading (35), we further analyzed the effects of regorafenib and DC101 on...
macrophage infiltration by determining the F4/80-positive area fraction. On day 14 postimplantation, a significantly decreased macrophage infiltration was found in regorafenib- and DC101-treated tumors compared with the vehicle-treated controls (3.9-fold reduction for regorafenib and 2.1-fold reduction for DC101, both \( P < 0.001 \)). However, the effect of regorafenib was more pronounced, as obvious by the significant reduction in TAMs (1.9-fold, \( P < 0.05 \)) compared with DC101-treated tumors (Fig. 7A and B). When further analyzing the TEMs, a strongly enhanced ratio of TIE2/ F4/80+ macrophages was found in DC101-treated tumors on day 14 postimplantation, whereas the ratio of TEMs in regorafenib- and vehicle-treated tumors was low (Fig. 7C and D). These data clearly show that besides its strong antiangiogenic and antitumorigenic effects, regorafenib additionally reduced the amount of infiltrating macrophages.

Discussion

First-line therapy in mCRCs combines chemotherapeutic drugs with targeted therapy, either directed against VEGF or EGFR (6, 11, 36). Major limitations for an anti-EGFR antibody therapy (e.g., by cetuximab or panitumumab) are activating mutations in downstream signaling mediators like K-RAS or B-RAF that confer treatment resistance and the K-RAS gene status is currently assessed in clinical diagnosis as predictive marker for the treatment efficacy (37). However, a considerable number of patients with wild-type K-RAS still fail to anti-EGFR antibody therapy (6, 37). Treatment schemes combining bevacizumab with chemotherapeutics were not successful in preventing cancer recurrence when used as long-term adjuvant therapy (11, 13, 37, 38). In this context, a preclinical study has revealed that antiangiogenic treatment, which solely blocks VEGF-signaling, can lead to tumor evasion (14). Even the combination of chemotherapeutics with EGFR antibodies and bevacizumab did not exert beneficial effects, as this treatment scheme increased the cytotoxicity without improving the efficacy (11, 39). These data show that novel drugs are highly desirable that target various and alternative pathways to improve the treatment efficiency in advanced CRCs.

Recently, the novel multikinase inhibitor regorafenib, which targets various signaling pathways, has been shown to efficiently inhibit tumor growth and angiogenesis in preclinical and clinical phase I–III studies (25–28, 40). To analyze the effects of regorafenib in more detail, we directly compared its effects with the classical angiogenesis inhibitor DC101 in a highly aggressive orthotopic CRC model (8, 41). After implantation of subcutaneous CT26 tumor pieces onto the cecum of nude mice, the tumors grew fast, were highly vascularized, and metastasized to the liver within 14 days, thus reliably reflecting an advanced, metastatic tumor stage.

Figure 7. Regorafenib reduces macrophage infiltration. A, tumors of DC101- and regorafenib-treated animals show a significant decrease in F4/80+ macrophages compared with the vehicle-treated animals on day 14 postimplantation (**, \( P < 0.001 \); n = 6). Notably, regorafenib has also a significantly stronger effect compared with DC101 (*, \( P < 0.05 \)). B, representative immunostainings of tumors on day 14 postimplantation; CD31 (green), F4/80 (red), nuclei counterstained by DAPI (blue); bar, 50 \( \mu \)m. C, quantification of TIE2+/F4/80+ macrophages reveals a remarkably higher amount of TEMs in DC101-treated animals compared with vehicle and regorafenib-treated animals on day 14 postimplantation (***, \( P < 0.001 \); n = 6). D, representative immunostainings of tumors on day 14 postimplantation; F4/80 (green), TIE2 (red), nuclei counterstained by DAPI (blue); bar, 50 \( \mu \)m. Data are presented as median ± interquartile range.
(11, 42). Treatment with the respective drugs (DC101, regorafenib) was started on day 4 postimplantation, where no liver metastases were visible by MRI and histology. DC101 was applied at a dose of 34 mg/kg body weight, which has been shown to efficiently inhibit angiogenesis and tumor growth in various preclinical studies (8, 41, 43). Regorafenib (30 mg/kg body weight) was given at a dose that leads to exposures similar to those reached in man at the MTD of 160 mg/day (27).

Clearly, at the given doses, regorafenib was therapeutically more efficient than DC101, as obvious by its complete tumor growth suppression (Fig. 3A), the significantly stronger reduction in angiogenesis and vascularization and by the significantly enhanced induction of apoptosis. In addition, a slightly higher number of SMA-positive mature vessels were detected by immunohistology after 10 days of treatment with regorafenib, whereas the degree of maturation in DC101-treated tumors was similar to the vehicle group. The similar $k_{ep}$ values of regorafenib- and DC101-treated mice measured by DCE-MRI in vivo can be explained by the fact that $k_{ep}$ is influenced not only by vessel permeability but also by tumor perfusion (32, 34). A higher number of mature vessels can result from proapoptotic effects of the drug that predominantly affect the immature, angiogenic vessels that are dominant in this highly aggressive tumor model (44).

Even more importantly than the observed antiangiogenic and proapoptotic effects, regorafenib completely inhibited metastasis. While the liver metastasis rate was only reduced by about 33% after 10 days of therapy with DC101, no metastases at all were detected in the regorafenib-treated mice (Fig. 6A). Different mechanisms can explain the stronger antitumorigenic effects of regorafenib, including metastasis inhibition. Regorafenib blocks various angiogenesis-related tyrosine kinase receptors, whereas DC101 only blocks VEGFR2. In a study on metastasizing breast cancer xenografts, the multikinase inhibitor E7080 targeting different angiogenesis related tyrosine kinases was also superior to the anti-VEGF antibody bevacizumab and efficiently suppressed metastases formation (45). Besides strong inhibitory effects on the VEGF receptors, regorafenib inhibits TIE2. The TIE2-inhibitory effects on the vasculature became obvious in our study by the significantly reduced ratio of TIE2-positive vessels in regorafenib-treated tumors on day 14 postimplantation (Fig. 5C and D). Thus, the combined inhibition of key regulators in angiogenesis can explain the stronger reduction in vascularization, which also affects metastatic spreading. TIE2 and its ligand angiopoietin-2 (Ang2) are currently discussed as mediators of metastatic spreading. Inhibition of Ang2 with monoclonal antibodies did not only inhibit angiogenesis and tumor growth but also prevented the formation of metastases, even in models that developed resistance toward anti-VEGF/VEGFR therapy (22, 46–48). In addition, the combined blockade of VEGFR2 and TIE2 signaling increased the therapeutic efficacy in a preclinical angiosarcoma and melanoma cancer model (49). Thus, these data suggest that the additional blockade of TIE2 increased the antiangiogenic effects and contributed to metastasis inhibition. In this context, the enhanced vessel maturation observed in regorafenib-treated tumors, most probably due to its broad antiangiogenic effects, can additionally have hampered metastases formation as mature, stable, and tight vessels covered by pericytes impede tumor cell intravasation and metastatic spreading (50).

Apart from endothelial cells, TIE2 expression has also been detected on a subset of tumor-infiltrating macrophages that are currently suggested as strong proangiogenic inflammatory cells that may additionally facilitate tumor cell intravasation as a further crucial step in the process of metastasis (19). A recent study has revealed that targeting these TEMs with ANG2/TIE2 blockade does not only inhibit tumor growth but also efficiently prevents metastasis (22). Interestingly, the strongest reduction in macrophage accumulation was observed in regorafenib-treated tumors on day 14 postimplantation. In addition, the amount of TEMs was low after 10 days of treatment with regorafenib. In DC101-treated tumors, macrophage infiltration was significantly higher at day 14 and a significantly enhanced ratio of TEMs was detected. These observations are in agreement with a previous study showing an enhanced TEM infiltration after therapy with a vascular-disrupting agent (51). This remarkable increase in proangiogenic and prometastatic TEMs in DC101-treated tumors is suggested as a possible mechanism for the occurrence of liver metastases, whereas TEMs are obviously efficiently inhibited by regorafenib. This hypothesis is sustained by a recent clinical study on mCRCs revealing a shorter median progression-free and a reduced mean overall survival in patients with a high number of TEMs in the blood. In this context, circulating TEMs in the blood were discussed as biomarker for patient stratification with respect to additional bevacizumab treatment to conventional chemotherapy in mCRCs (52).

With respect to tumor growth and metastasis supporting mechanisms that are rather related to tumor cell proliferation, the downstream signaling mediators K-RAS and B-RAF are frequently constitutively activated in mCRCs (6, 36, 37), conferring resistance to anti-EGFR antibody therapies (53). K-RAS and B-RAF mutations seem to occur independently but are both associated with a poor prognosis (54–56). Regorafenib strongly inhibits B-RAF (27), suggesting that this blockade can also be responsible for growth inhibition of the primary tumors and the metastases (57). Interestingly, in clinical trials of advanced CRCs, no differences in the treatment efficiencies of regorafenib were observed between patients harboring K-RAS wild-type or mutations, indicating that regorafenib might have potential in the therapy of patients with mCRCs with mutant K-RAS (25, 27, 40, 58, 59). Future studies will further investigate the potential of regorafenib in advanced CRCs and elucidate its mechanisms of action in more detail.

In conclusion, our data show that regorafenib efficiently inhibits tumor growth, angiogenesis, macrophage...
infiltration, and metastasis in highly aggressive murine orthotopic colorectal tumors. Its inhibitory effects are clearly going beyond the effects of VEGFR2 blockade. These results further corroborate regorafenib as promising candidate to improve the clinical treatment efficiency in patients with advanced CRCs.

Disclosure of Potential Conflicts of Interest

F. Gremse has a commercial research grant from Philips Research. No potential conflicts of interest were disclosed by the authors.

Authors’ Contributions

Conception and design: L. Abou-Elkacem, D. Zopf, F. Kiessling, W. Lederle
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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): L. Abou-Elkacem, S. Arns, G. Brix, F. Gremse, F. Kiessling, W. Lederle

References

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