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 chemotherapeutics has improved treatment efficacy in advanced colorectal cancer (CRC). However, the clinical prognosis of metastatic CRC is still poor and novel drugs are currently assessed with respect to their efficacies in CRC patients. In a phase III study, the multi-kinase inhibitor regorafenib (BAY 73-4506) has recently been shown to prolong survival of CRC patients 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. Whereas a treatment for 10 days with DC101 given at a dose of 34 mg/kg every third day significantly delayed tumor growth compared to 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 contrasted enhanced magnetic resonance imaging (DCE-MRI) and confirmed by immunohistochemistry. In addition, regorafenib inhibited the angiogenic activity more strongly and induced a three times higher apoptosis rate compared with 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 to the vehicle group. Additionally, regorafenib significantly reduced the amount of infiltrating macrophages. These data demonstrate that the multi-kinase inhibitor regorafenib exerts strong anti-angiogenic, anti-tumorigenic and even anti-metastatic effects on highly aggressive colon carcinomas indicative for its high potential in the treatment of advanced CRC.
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 CRC (3, 4). However, tumor progression to a highly advanced, metastatic stage (mCRC) still decreases the overall 5-year survival to 8-10% (5). Besides different genetic mutations, enhanced epithelial growth factor receptor (EGFR)-signaling and vascular endothelial growth factor (VEGF)-mediated angiogenesis play a critical role in the progression of CRC (6). Tumor progression promoting functions of VEGF have been evidenced in various pre-clinical 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 metastatic CRC (11, 12). However, in adjuvant therapy, bevacizumab in combination with chemotherapy failed in increasing the three year disease-free survival compared with chemotherapy alone (11, 13). In this context, recent pre-clinical studies have demonstrated that anti-angiogenic 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 anti-angiogenic therapy and tumor evasion is the strong infiltration of tumor-associated macrophages (TAMs), in particular of a subset that expresses the angiopoietin (Ang) receptor tyrosine kinase with immunoglobulin and epidermal growth factor homology domain 2 (TIE2) that promote angiogenesis and tumor progression (16-19). These TIE2-expressing macrophages (TEMs) 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, in order to increase the treatment efficacy and to prevent metastasis (23, 24). A novel oral multi-kinase inhibitor, regorafenib (BAY 73-4506) (25) has been developed which targets a variety of kinases involved in angiogenic, tumor growth-promoting and tumor micro-environmental signalling pathways such as VEGFR1/2/3, platelet-derived growth factor receptor-beta (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 demonstrated potent anti-angiogenic and anti-tumorigenic effects (25-28). In order to elucidate the effects of the multi-kinase 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 colorectal cancer. 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 (DCE)-MRI and complemented by detailed immunohistochemical analyses.

Material and Methods

Cell line

The mouse colon cancer cell line CT26 (LGC Standards GmbH, Wesel, Germany) was cultivated in DMEM medium (Gibco®, Invitrogen GmbH, Darmstadt, Germany) containing 10 % fetal bovine serum (Gibco®), 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, Sulzfeld, Germany) were used for the experiment. The orthotopic implantation was performed as described by Hoffman et al. (29). A total number of 2×10⁶ CT26 cells in 100μL culture medium were injected subcutaneously into the right flank of 6-8 week old mice. 14 days after injection, these donor mice were sacrificed, the subcutaneous tumors (~500mm³) were excised, cut into 2mm sized pieces and harvested in ice-cold PBS. Necrotic areas were discarded. Mice were treated with rimadyl (Pfizer Pharma GmbH, Berlin, Germany), two hours before and after tumor implantation. For implantation, the mice were anesthetized with isofluorane (1.5%). The abdomen was sterilized with antiseptic spray (Antiseptica, Pulheim/Brauweiler, Germany). A laparotomy of 0.5cm was performed and the cecum was exposed. Subsequently, the serosa of the cecum was scraped slightly with two forceps and one tumor fragment was implanted onto the cecum directly underneath the serosa in order to prevent the risk of bowel perforation. The cecum was returned into the abdominal cavity and the abdominal wound was closed with a 6.0 suture. Finally, the wound was treated with povidon-iodine cream (B. Braun Melsungen AG, Melsungen, Germany) and wound healing spray (Beiersdorf AG Hansaplast, Hamburg, Germany) 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 post-implantation (p.i.) and were divided randomly in three groups. At that time point, the tumors had reached a size of approximately 5mm in diameter,
as determined by T2-weighted (T2w) MRI. Subsequently, the first group (n=6) was treated daily orally with multi-kinase inhibitor regorafenib (structural formula see Fig. 1A). Regorafenib was applied at a dose of 30mg/kg body weight, dissolved in polyethyleneglycol (PEG) 400, 1,2-propanediol and pluronic F68 (all from Sigma-Aldrich Chemie GmbH, Steinheim, Germany), as described in (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, Kuala Lumpur, Malaysia) in PBS at a dose of 34mg/kg body weight every third day. Therapy was continued for 10 days until day 14 p.i. (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 p.i.. On day 14 p.i., after the last MRI measurement, all animals were sacrificed. The tumors were dissected and cryoconserved for histological analysis. In addition, the lungs and livers were dissected and screened macroscopically for metastases (Fig. 1B).

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

**Dynamic contrast-enhanced magnetic resonance imaging (DCE-MRI) and data analysis**

MRI was performed at a clinical 3 Tesla whole-body scanner (Achieva 3.0 T, Philips, Brest, Netherlands), using a small animal solenoid receiver coil with an integrated heating system in order to keep the temperature constant at 37°C during the examination (Philips, Hamburg, Germany). For the MRI measurements, the animals were anesthetized with isofluorane (1.5
0.5 mg/kg butylscopolamine (Buscopan\textsuperscript{TM}, Boehringer Ingelheim Pharma KG, Ingelheim, Germany) was injected intraperitoneally in order 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 T\(_2\)-weighted turbo-spin echo sequence (repetition time, TR = 4781 ms, echo time, TE = 100 ms, turbo spin echo factor, TSE = 10, flip angle = 90\(^\circ\), number of signal averages, NSA = 3, field of view, FOV = 30 mm \(\times\) 30 mm \(\times\) 43.9 mm, matrix size 148\(\times\)150, slice thickness = 1 mm, voxel size = 0.2 \(\times\) 0.2 \(\times\) 1.0 mm). After defining the tumor by T\(_2\)-weighted imaging, a DCE-MRI scan was performed using a multi slice 2D T\textsubscript{1w} 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\(^\circ\), matrix = 432 \(\times\) 416, slice thickness = 3 mm, voxel size = 0.35 \(\times\) 0.35 \(\times\) 3.0 mm. 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 two minutes, 100\(\mu\)l (100\(\mu\)mol per kg body weight) of the paramagnetic contrast agent gadomer 17 (Bayer-Pharma AG, Berlin, Germany) was injected manually into the tail vein within 15 s (25).

For post-processing, the tumor was segmented semi-automatically based on the T\(_2\)-weighted MRI-images, and the tumor volume was determined using an Imalytics Research Workstation (Philips Technology GmbH Innovative Technologies, Aachen, Germany). For tracer kinetic modeling, the average signal per region was computed. The resulting signal-time curves were analyzed using the pharmacokinetic two-compartment model of Brix et al. (30, 31), providing the parameters amplitude (related to the relative distribution volume of the tumor (32) and the exchange rate constant k\(_{ep}\) (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.
Histological analyses

Frozen tumors were cut into 7 to 10μm thick slices. The following primary antibodies were used for immunofluorescent staining: a rat anti-mouse CD31 antibody (BD Biosciences, San José, CA, USA) for vessel staining, a goat anti-mouse VEGFR2-antibody (R&D Systems, Wiesbaden, Germany) and a rat anti-mouse TIE2-antibody (BioLegend, San Diego, CA, USA) for assessing the angiogenic activity. For analyzing vessel maturation, a biotinylated anti-smooth muscle actin (SMA) antibody (Progen Biotechnik GmbH, Heidelberg, Germany) was used. Apoptosis was determined using the DeadEnd™ colorimetric TUNEL assay (Roche Diagnostics, Mannheim, Germany). Macrophages were stained with a rat anti-mouse F4/80-antibody (AbD Serotec, Duesseldorf, Germany). Secondary antibodies were a donkey anti-rat-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, Hamburg, Germany). For all analyses, cell nuclei were counterstained with 0.5 μg/mL 4’,6-diamidino-2-phenylindole (DAPI) (Invitrogen, Darmstadt, Germany). For histological 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, Göttingen, Germany) and a high-resolution camera (AxioCam MRm Rev.3, Zeiss, Göttingen, Germany). Images were quantified using the AxioVision Rel 4.8 software (Zeiss, Göttingen, Germany), 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 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. In order 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’s multiple comparison post-test was performed to analyze differences between the groups using GraphPad Prism 5.0 (California, USA). 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 colorectal cancer 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 almost uniform and fast tumor growth, leading to hypervascularized tumors as well as to metastases in the liver within 14 days after implantation. We therefore used this model in order to analyze the effects of the multi-kinase inhibitor regorafenib compared with the VEGFR2-inhibitor DC101. On day 4 p.i., tumor engraftment was assessed by T2-weighted (T2w) MRI, revealing a mean tumor volume of ~ 45mm³ at the time of initiation of tumor treatment. Both, DC101 and regorafenib were well tolerated at the respective doses and no significant loss of animal weight occurred compared to the weight before treatment (Fig. 2).

Regorafenib exerted the strongest antitumor effect and completely suppressed tumor growth (Fig. 3A). Its tumor growth inhibition was statistically significant (p<0.01) from day 7 p.i. onwards compared to the vehicle group, in which tumors had continuously grown to a mean
tumor volume of 449.20 mm³ ±88.46 by day 14 p.i. (Fig. 3A,B). DC101 also significantly inhibited tumor growth (p<0.01) when compared to the vehicle group, but less efficient than regorafenib (Fig. 3A). Tumor growth inhibition by regorafenib was also significantly better from day 7 p.i. onwards when compared to DC101 (p<0.01, p-value not shown in the graph) (Fig. 3A). The differences in tumor volume between DC101-treated and control tumors were only significantly on day 14 post-implantation (p<0.01).

**Regorafenib inhibits angiogenesis and induces apoptosis**

In order to longitudinally analyze the effects of the drugs on angiogenesis and tumor vascularization *in vivo*, DCE-MRI was performed because it has been demonstrated as very suitable technique for the longitudinal assessment of angiogenesis and anti-angiogenic 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 three days between day 7 and 11 p.i. whereas it remained stable until day 14 p.i. in the vehicle control group (Fig. 4A; p<0.05 on day 11 and p<0.01 on day 14 p.i.). The RDV decreased also in the DC101-treated group between day 7 and 11 p.i., 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 compared with regorafenib-treated tumors (p<0.05, p-value not shown in the graph). The blood vessel permeability, as monitored by the exchange rate constant kₚ, 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 p.i.. However, whereas an increase in MVA was observed in the vehicle control group between day 7 and 14 p.i., it decreased in both, regorafenib and DC101-treated animals to significantly lower levels compared with the controls on day 11 p.i. (p<0.05.
for both) and on day 14 p.i. (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 compared with DC101 on day 14 (Fig. 4C, p<0.05). When analyzing vessel maturation by determining the fraction of CD31 staining vessels which are SMA-positive, a trend to a higher number of mature vessels was detected in regorafenib compared to DC101 or vehicle-treated animals on day 14 p.i., 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 p.i. (Fig. 5A). The angiogenic activity was significantly decreased in regorafenib-treated tumors on day 11 p.i. (p<0.05) and day 14 p.i. (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 compared with regorafenib. A significant reduction compared with the vehicle controls was only reached on day 14 p.i. (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 p.i. (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 p.i., the strongest rate of apoptosis was observed in tumors from animals treated with regorafenib. Regorafenib significantly increased apoptosis by 18.4-fold and 4-fold compared to vehicle and DC101-treated animals, respectively (p<0.01 for both) (Fig. 5C; exemplary images are provided in Fig. 5F). The pro-apoptotic 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 anti-
angiogenic 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 p.i. in vivo by T2w MRI and subsequently by visual macroscopic and histological inspection. Remarkably, no liver metastases were detected by MRI and by macroscopic screening after treatment with regorafenib, whereas liver metastases were found in four and five out of six 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 metastases-free in all groups (Fig. 6A). These data demonstrate 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 p.i., a significantly decreased macrophage infiltration was found in regorafenib- and DC101-treated tumors compared to 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, B). When further analyzing the TIE2-expressing macrophages, a strongly enhanced ratio of TIE2+/ F4/80+ macrophages was found in DC101-treated tumors on day 14 p.i., whereas
the ratio of TEMs in regorafenib- and vehicle-treated tumors was low (Fig. 7C, D). These data clearly show that besides its strong anti-angiogenic and anti-tumorigenic effects, regorafenib additionally reduced the amount of infiltrating macrophages.

Discussion

First line therapy in mCRC 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 signalling 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 anti-angiogenic 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, since this treatment scheme increased the cytotoxicity without improving the efficacy (11, 39). These data demonstrate that novel drugs are highly desirable that target various and alternative pathways in order to improve the treatment efficiency in advanced CRC.

Recently, the novel multi-kinase inhibitor regorafenib, which targets various signaling pathways, has been shown to efficiently inhibit tumor growth and angiogenesis in pre-clinical and clinical phase I-III studies (25-28, 40). In order 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 colorectal cancer 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 (11, 42). Treatment with the respective drugs
(DC101, regorafenib) was started on day 4 p.i., where no liver metastases were visible by
MRI and histology. DC101 was applied at a dose of 34mg/kg body weight which has been
shown to efficiently inhibit angiogenesis and tumor growth in various pre-clinical studies (8,
41, 43). Regorafenib (30mg/kg body weight) was given at a dose which leads to exposures
similar to those reached in man at the MTD of 160mg/d (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 not only influenced by vessel permeability but also by tumor
perfusion (32, 34). A higher number of mature vessels can result from pro-apoptotic 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 anti-angiogenic and pro-apoptotic effects,
regorafenib completely inhibited metastasis. Whereas 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 anti-tumorigenic effects of regorafenib, including metastasis inhibition. Regorafenib
blocks various angiogenesis-related tyrosine kinase receptors, whereas DC101 only blocks
VEGFR-2. In a study on metastasizing breast cancer xenografts, the multi-kinase 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 p.i. (Fig 5C, 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 (Ang-2) are currently discussed as mediators of metastatic spreading. Inhibition of Ang-2 with monoclonal antibodies did not only inhibit angiogenesis and tumor growth, but additionally prevented the formation of metastases, even in models that developed resistance towards anti-VEGF/VEGFR therapy (22, 46-48). In addition, the combined blockade of VEGFR2 and TIE2 signaling increased the therapeutic efficacy in a pre-clinical angiosarcoma and melanoma cancer model (49). Thus, these data suggest that the additional blockade of TIE2 increased the anti-angiogenic effects and contributed to metastasis inhibition. In this context, the enhanced vessel maturation observed in regorafenib-treated tumors, most probably due to its broad anti-angiogenic effects, can additionally have hampered metastases formation since mature, stable and tight vessels covered by pericytes impede tumor cell intravasation and metastatic spreading (50).

Besides on endothelial cells, TIE2-expression has been detected on a subset of tumor-infiltration macrophages which are currently suggested as strong pro-angiogenic inflammatory cells that may additionally facilitate tumor cell intravasation as further crucial step in the process of metastasis (19). A recent study has revealed that targeting these TEMs by 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 p.i.. 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 demonstrating an enhanced TEM infiltration after therapy with a vascular-disrupting agent (51). This remarkable increase in pro-angiogenic and pro-metastatic 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 mCRC 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 mCRC (52).

With respect to tumor growth and metastasis supporting mechanisms that are rather related to tumor cell proliferation, the downstream signalling mediators K-RAS and B-RAF are frequently constitutively activated in mCRC (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 tumor and the metastases (57). Interestingly, in clinical trials of advanced CRC, no differences in the treatment efficiencies of regorafenib were observed between patients harboring K-RAS wildtype or mutations, indicating that regorafenib might have potential in the therapy of mCRC patients with mutant K-RAS (25, 27, 40, 58, 59). Future studies will further investigate the potential of regorafenib in advanced CRC and elucidate its mechanisms of action in more detail.

In conclusion, our data demonstrate 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 in order to improve the clinical treatment efficiency in patients with advanced CRC.

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References


Figure Legends

Figure 1

Structures of the drugs and study plan. A, Structures of regorafenib (left) and DC101 (right). The chemical name of regorafenib is 4-([4-chloro-3-(trifluoromethyl)phenyl]carbamoyl)amino)-3-fluorophenoxy]-N-methylpyridine-2-carboxamide. Regorafenib is a multi-kinase inhibitor which targets VEGFR1/2/3, PDGFR-β, FGFR1, KIT, RET, B-RAF and TIE2. DC101 is a monoclonal antibody that binds to murine VEGFR2 with high affinity and blocks ligand-induced receptor activation. B, Study plan and overview of the therapy and measuring schedules.

Figure 2

Regorafenib is well tolerated. Median weights of animals (n=6; ± standard error) recorded during the therapy period of 10 days.

Figure 3

Regorafenib inhibits tumor growth. A, Growth curves of vehicle, DC101 and regorafenib-treated tumors, as assessed by T2w MRI (p**<0.01; n=6). B, Representative T2w MRI images (left and middle panels) and images of the dissected tumors (right panels) demonstrate the smallest tumors in the regorafenib-treated group, bar=5mm, white arrows depict the tumors. Data are presented as median ± interquartile range.

Figure 4

Regorafenib reduces tumor vascularization. A, Tracer kinetic analysis of longitudinal DCE-MRI measurements of control-, DC101- and regorafenib-treated animals demonstrate a significantly lower amplitude in regorafenib-treated animals on day 11 (p*<0.05; n=6) and
day 14 p.i. (p**<0.01; n=6) compared with the controls. DC101 has a weaker effect on the
tumor vascularization, showing a higher median value than in regorafenib-treated tumors on
day 14 p.i.; n=6. B, No significant differences are recorded for the kep values in all groups;
n=6. C, Quantification of the CD31+ area fraction confirms the strongest decrease in the
MVA in the regorafenib-treated tumors over time. Regorafenib-treated tumors reveal
significant differences compared with the controls and DC101-treated tumors on day 14 p.i.
(Regorafenib vs vehicle p**<0.01, regorafenib vs DC101 p*<0.05; n=6). DC101 and
regorafenib lead to a significantly lower vascularization than the controls on day 11 (p*<0.05;
n=4) and day 14 p.i. (p**<0.01; n=6). D, The quantification of SMA+/CD31 reveals a slightly
higher amount of mature vessels in regorafenib-treated tumors compared with the control and
DC101 group, though the differences are not significant (n=6). E, Representative
immunostainings of tumors on day 14 p.i.; CD31 (green), SMA (red), nuclei counterstained
(blue) by DAPI, bar=50μm. Data are presented as median ± interquartile range.

Figure 5

Regorafenib inhibits angiogenesis and induces apoptosis. A, Quantification of
VEGFR2+/CD31 reveals the strongest decrease in the angiogenic activity in the regorafenib-
treated tumors, showing a significant decrease on day 11 (p*<0.05; n=4) and 14 p.i.
(p**<0.01; n=6) compared with the vehicle-treated animals. DC101 significantly reduces the
angiogenic activity compared with the controls on day 14 p.i. (p**<0.01; n=6).
B, Representative immunostainings of tumors on day 14 p.i.; CD31 (green), VEGFR2 (red),
nuclei (blue), bar=50μm. C, Further quantification of TIE2+/CD31 demonstrates the lowest
ratio in regorafenib-treated tumors on day 14 p.i.. D, Representative immunostainings of
tumors on day 14 p.i.; CD31 (green), TIE2 (red), nuclei (blue), bar=50μm. E, Quantification
of the TUNEL-positive area fraction demonstrates the highest increase in apoptosis in the
regorafenib-treated tumors on day 14 p.i. (p**<0.01). F, Representative immunostainings of
tumors on day 14 p.i.; CD31 (green), TUNEL (red), nuclei (blue), bar=50μm. N=
neptic/apoptotic areas. Data are presented as median ± interquartile range.

Figure 6

Regorafenib inhibits metastasis. A, T_{2w} MRI images of day 14 p.i. (left panels) show metastases only in the livers of control and DC101-treated animals (arrows show the metastases; bar=2 mm). After dissection, metastatic nodules were detected macroscopically in control and DC101-treated animals in contrast to regorafenib-treated mice (middle left panels). H&E staining confirms liver metastases in control and DC101-treated tumors, whereas the liver of the regorafenib-treated animals lacks metastases (middle right panels). No lung metastases (right panels) were observed in all groups (right panels), bar=200μm.

B, Quantification showing the number of liver metastases per animal (n=6).

Figure 7

Regorafenib reduces macrophage infiltration. A, Tumors of DC101- and regorafenib-treated animals show a significantly decrease in F4/80+ macrophages compared with the vehicle-treated animals on day 14 p.i. (p***<0.001; n=6). Notably, regorafenib has also a significantly stronger effect compared to DC101 (p*<0.05) B, Representative immunostainings of tumors on day 14 p.i.; CD31 (green), F4/80 (red), nuclei counterstained by DAPI (blue), bar=50μm. C, Quantification of TIE2+/F4/80+ macrophages reveals a remarkably higher amount of TEMs in DC101-treated animals compared to vehicle and regorafenib-treated animals on day 14 p.i. (p***<0.001; n=6). D, Representative immunostainings of tumors on day 14 p.i.; F4/80 (green), TIE2 (red), nuclei counterstained by DAPI (blue), bar=50μm. Data are presented as median ± interquartile range.
Figure 1
Figure 2

- Vehicle
- DC101
- Regorafenib

Animal weight (g)

Days after tumor implantation

15 20 25 30 35

4 6 8 10 12 14
Figure 3

A

Tumor volume by MRI (mm³)

Vehicle
DC101
Regorafenib

Days after tumor implantation

B

Day 4
Day 14

Vehicle
DC101
Regorafenib

T2w MRI
Ex vivo
Figure 4
Figure 6
Figure 7
Molecular Cancer Therapeutics

Regorafenib inhibits growth, angiogenesis and metastasis in a highly aggressive, orthotopic colon cancer model

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