E7080 (Lenvatinib), a Multi-Targeted Tyrosine Kinase Inhibitor, Demonstrates Antitumor Activities Against Colorectal Cancer Xenografts

Armin Wiegering*,†, Doreen Korb‡, Andreas Thalheimer*†, Ulrike Kämmerer§, Jan Allmanritter†, Niels Matthes*, Michael Linnebacher*, Nicolas Schlegel*, Ingo Klein*, Süleyman Ergün¶, Christoph-Thomas Germer* and Christoph Otto*‡

*Department of General, Visceral, Vascular and Pediatric Surgery, University Hospital of Würzburg, Oberdürrbacher Str. 6, D-97080, Würzburg, Germany; †Department of Biochemistry and Molecular Biology, Theodor-Boveri-Institute, Biocenter, University of Würzburg, D-97070, Würzburg, Germany; ‡Experimental Surgery, Department of General, Visceral, Vascular, and Pediatric Surgery, University Hospital of Würzburg, Oberdürrbacher Str. 6, D-97080, Würzburg, Germany; §Department of Obstetrics and Gynaecology, University Hospital of Würzburg, Josef-Schneider-Str. 4, D-97080, Würzburg, Germany; ¶Institute of Anatomy and Cell Biology, University of Würzburg, Koellikerstr. 6, 97070, Würzburg, Germany; #Molecular Oncology and Immune Therapy, Department of General, Thoracic, Vascular and Transplantation Surgery, University of Rostock, Schillingallee 35, D-18055, Rostock, Germany

Abstract

Clinical prognosis of metastasized colorectal carcinoma (CRC) is still not at desired levels and novel drugs are needed. Here, we focused on the multi-tyrosine kinase inhibitor E7080 (Lenvatinib) and assessed its therapeutic efficacy against human CRC cell lines in vitro and human CRC xenografts in vivo. The effect of E7080 on cell viability was examined on 10 human CRC cell lines and human endothelial cells (HUVEC). The inhibitory effect of E7080 on VEGF-induced angiogenesis was studied in an ex vivo mouse aortic ring angiogenesis assay. In addition, the efficacy of E7080 against xenografts derived from CRC cell lines and CRC patient resection specimens with mutated KRAS was investigated in vivo. A relatively low cytotoxic effect of E7080 on CRC cell viability was observed in vitro. Endothelial cells (HUVEC) were more susceptible to the incubation with E7080. This is in line with the observation that E7080 demonstrated an anti-angiogenic effect in a three-dimensional ex vivo mouse aortic ring angiogenesis assay. E7080 effectively disrupted CRC cell-mediated VEGF-stimulated growth of HUVEC in vitro. Daily in vivo treatment with E7080 (5 mg/kg) significantly delayed the growth of KRAS mutated CRC xenografts with decreased density of tumor-associated vessel formations and without tumor regression. This observation is in line with results that E7080 did not significantly reduce the number of Ki67-positive cells in CRC xenografts. The results suggest antiangiogenic activity of E7080 at a dosage that was well tolerated by nude mice. E7080 may provide therapeutic benefits in the treatment of CRC with mutated KRAS.
Introduction

Colorectal carcinoma (CRC) is the most common malignancy of the gastrointestinal tract and constitutes approximately 15% of all cases of cancer. Despite multiple advances in diagnosis and treatment of CRC, approximately 45% of patients with CRC experience local recurrence and/or metastases with a consequent dramatic decline in prognosis. In the industrialized West, CRC is therefore, the third most common cause of death from cancer [1].

Metastases of CRC are localized in the liver in 40% to 80% of patients. The principal curative treatment option is surgical resection, although only one fourth of patients with colorectal liver metastases are primary operable [2]. Due to this fact, in daily clinical situations, patients are stratified into three groups: patients with resectable liver metastases who are treated by curative surgery, patients with resectable liver metastases after a neoadjuvant therapy undergoing surgical resection at a later date, and patients with wide-spread and unresectable metastases even after downsizing chemotherapy. In recent years marked improvements have been made in the medical treatment of patients with CRC metastasis. Angiogenesis is essential for solid tumor growth and anti-angiogenic therapy may offer an additional treatment option at this stage [3]. New cytostatic agents and antibodies targeting epidermal growth factor receptor (EGFR) on the cancer cell surface and vascular epithelial growth factor (VEGF) released by cancer cells [4] have increased median survival of patients with advanced CRC to more than 2 years, almost doubling the survival time of the 5-Fluorouracil (5-FU) era [5,6].

One promising mechanism to inhibit tumor growth or induce tumor shrinking in combination with classical chemotherapy is the use of VEGF signaling inhibitors. The recombinant humanized monoclonal antibody bevacizumab is directed against the pro-angiogenic VEGF subgroup A. It has been approved, in combination with chemotherapy, for the treatment of colorectal liver metastasis. Bevacizumab is currently in a phase 2 study with KIT5B-RET-positive adenocarcinoma of the lung (Clinical Trials.gov; NCT01877083). E7080 shows potent antitumor effects in xenograft models of various cancer types, such as breast and lung cancer, pleura mesothelioma or sarcoma [18–20].

The aim of the present study was to evaluate a) the efficacy of E7080 (Lenvatinib) on a broad range of human CRC cell lines and primary human endothelial cells (HUVEC) in vitro, b) the anti-angiogenic effect of E7080 in a three-dimensional ex vivo mouse aortic ring angiogenesis assay, and c) the therapeutic efficacy of E7080 against xenografts derived from CRC cell lines and CRC patient resection specimens with mutated KRAS in vivo.

Materials and Methods

Cell Lines

Human CRC cells (Table 1) were routinely cultured in their recommended media supplemented with 10% (v/v) heat-inactivated fetal calf serum (FCS), 2 mmol/l glutamine (Invitrogen), 100 U/ml penicillin, and 100 μg/ml streptomycin. Cancer cell lines were obtained from the American Type Culture Collection (www.atcc.org) with exception of HCT116 and HT29 (Leibniz Institute, German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany), SW620 (European Collection of Cell Cultures, Salisbury, UK) and WiDr (Cell Lines Service, Epphenheim, Germany). Human umbilical vein endothelial cells (HUVEC) were obtained from PromoCell (Heidelberg, Germany) and cultured according to the manufacturer’s recommendations in endothelial growth medium (PromoCell).

Human CRC Xenografts from Patient Primary Resection Specimens

Low-passage CRC xenografts HROC71 (T2M1) were established from primary resection specimens of a patient with Lynch syndrome [21]. For this, tumor samples were subcutaneously transplanted into

| Cell lines | KRAS status | IC50 (μmol/l) | 95% CI (μmol/l) |
|------------|-------------|---------------|-----------------|
| CaCo2      | wt          | >1000         | 973-2311        |
| Colo741    | wt          | >1000         | 299-620         |
| SW620      | mut         | 548           | 296-538         |
| HT29       | mut         | 431           | 276             |
| HCT116     | mut         | 399           | 265             |
| T84        | mut         | 276           | 219             |
| SW480      | mut         | 240           | 313             |
| Colo678    | wt          | 222           | 175             |
| LS174T     | mut         | 144           | 96-215          |
| HCT15      | mut         | 120           | 91-158          |
| HUVEC      | wt          | 22            | 14-36           |
nude mice. Established xenografts were removed, cut in small pieces (3 x 3 x 3 mm) and stored in liquid nitrogen.

**Reagents**

Lenvatinib (E7080) was obtained from Selleckchem (Boston, USA). Stock solutions for *in vitro* (1 mmol/l) and *in vivo* (5.9 mmol/l) studies were prepared with phosphate buffered saline (PBS; Gibco, Life Technologies GmbH, Darmstadt, Germany) with 10% DMSO (final concentration; Sigma Aldrich) and aliquots were stored at -20 °C. The stock solution was further diluted for *in vitro* and *in vivo* studies. The final concentration of DMSO was ≤1% in all assays, with and without E7080.

**Western Blotting**

Cultured cells were rinsed three times with ice-cold PBS, harvested, and lysed directly in RIPA buffer (Pierce, Thermo Scientific, Rockford, USA) for immunoblot analysis. Cell debris was removed by centrifugation at 15,000 g for 10 minutes at 4°C. The supernatant was used as total protein lysate. For each sample, 10 μg of total protein lysate was subjected to 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS page), followed by immunoblot analysis. Immunoblots were probed with antibodies against MYC (Y69, #ab32072; Abcam, Cambridge, UK), GAPDH (#G9295, Sigma Aldrich GmbH, München, Germany), Vinculin (hVIN-1, #9131, Sigma Aldrich GmbH), pERK1/2 (T202/T204, #9101, Cell Signaling Technology, Danvers, MA, USA), and ERK1/2 (C14, #SC-154, Santa Cruz Biotechnology, USA). All antibodies were used according to the manufacturer’s instructions. The results were visualized with secondary antibodies (GE Healthcare Life Sciences Europe, Freiburg, Germany) against mouse (NA9310) or rabbit (NA9340) primary antibodies combined with enhanced chemiluminescence (ECL) western blotting substrates (Pierce, Thermo Scientific).

**Immunostaining**

Following excision, tumors were snap frozen in liquid nitrogen and subsequently stored at −80°C. Sections were used for staining towards CD31, CD34, Ki67 and CAIX using standard procedures [22]. The following antibodies, diluted in a commercial antibody diluent (DAKO, Hamburg, Germany), were used: anti-Ki-67 antigen (clone MIB-1, DAKO), final dilution 1:25; anti-CD31 (clone SZ31, DAKO), final dilution 1:100; anti-CD34 (clone MEC14.7, Linaris, Dossenheim, Germany), final dilution 1:200 and anti-carbonic anhydrase 9 (CA9, CAIX, clone GT12, GeneTex, Irvine, USA), final dilution 1:500. After incubation with the primary antibody for 1 hour at room temperature, the slides were washed in PBS and incubated with the following secondary antibodies: goat anti-mouse-HRP (DAKO), final dilution 1:100 or rabbit anti-rat-HRP (DAKO), final dilution 1:100. After development in 5% 3,3′-diaminobenzidine (Dako) and counterstaining with haematoxin, the sections were dehydrated in graded ethanol and embedded in Vitro Clud (Langenbrinck, Emmendingen, Germany). Stained slides were photographed at 20x magnification with a Keyence Biorevo BZ-9000 Microscope (Keyence Corporation, Osaka, Japan) applying Z-stack technology to improve the quality of images. The number of stained cells per section was quantified by using measurement module BZ-H3C (Hybrid Cell Count Vers.1.1, Keyence).

**Aortic Ring Assay**

The thoracic aortae from C57BL/6 mice (8 weeks) were isolated and subsequently cut into 1 mm thick rings after removal of adventitial fat. In two steps the aortic rings were then embedded in 48-well plates between two layers of 200 μl collagen gel, containing collagen type 1 (Purecol, CellSystems, Germany), sodium hydroxide, L-glutamine, sodium pyruvate, sodium bicarbonate, MEM (10×), penicillin-streptomycin and dH2O. First, the aortic rings were set on the first layer after polymerization of the gel. Then, the rings were covered with the second gel layer and after polymerization cultivated in 300 μl DMEM (10% FCS, 1% penicillin-streptomycin) at 37°C and 5% CO2. Some of the rings were treated with VEGF (50 ng/ml) or E7080 (1 μmol/l and 10 μmol/l), or with a combination of both. The medium was changed every third day. A phase contrast microscope (Leica, Germany) equipped with a digital camera (Leica, Germany) was used for evaluation and documentation of the aortic rings.

**CRC Xenograft Studies**

To generate CRC xenografts, 2.5 × 106 HCT116 cells, or LS174T cells resuspended in 100 μl phosphate-buffered saline were injected subcutaneously into the left hind flank of athymic female NMRI-Foxn1nu (nude) mice aged 8 to 10 weeks with 24 to 27 g body weight (Janvier, Le Genest-Saint-Isle, France). Subcutaneous CRC xenograft implantation was performed as described previously [23]. Tumors were measured using calipers and tumor volume VT (mm3) was calculated using the ellipsoid formula A2 x B x π/6, where A represents the smaller diameter [22]. Endpoint for the experiments was attainment of a tumour volume between 600 and 700 mm3 (target tumor volume) or within 6 weeks, when arrest of tumor growth was observed. When tumors reached a volume of 40 to 60 mm3, mice were randomized into therapy and control groups. Mice in therapy groups were treated daily with 5 mg/kg E7080 by oral gavage until tumors of control group reached target tumor volume. Tumour size and body weight of all animals were measured every day. All animal experiments were authorized by the local ethics committee, and mice were treated according to institutional and European Union guidelines.

**Blood Count and Clinical Chemistry**

Blood counts (erythrocytes, leukocytes, thrombocytes), the amount of hemoglobin, and the activity of liver enzymes alanine transaminase (ALT) and aspartate transaminase (AST) were measured at the central laboratory of the University Hospital of Würzburg.

**Detection of VEGF Production**

One million CRC cells and HUVEC were cultured in 6-well plates with serum-reduced (1%) RPMI 1640 medium for 48 hours. Afterwards, supernatant were harvested, centrifuged and stored at -80 °C. VEGF concentration was assayed by ELISA according to manufacturer’s instructions (R&D Systems GmbH, Germany). All samples were run in triplicate and each assay was conducted 2 times independently.

**Proliferation Assay**

WST-8 is reduced by cellular dehydrogenases to an orange formazan product that is soluble in tissue culture medium. The amount of formazan produced is directly proportional to the number of living cells. Cells (CRC cell lines and HUVEC) were seeded in 96-well flat-bottom plates at 5 x 103 cells per well with 100 μl RPMI 1640 medium supplemented with 1% (v/v) FCS, 2 mmol/l glutamine (Invitrogen, Darmstadt, Germany) and antibiotics (100 IU/ml penicillin, and 1 μg/ml streptomycin). The assays were...
cultured at 37 °C in a humidified atmosphere of 5% CO2 in air. The cells were cultured to reach the 50%-60% confluence on the second day when treated with serial dilutions of E7080 as indicated or supernatant. The assays were cultured at 37 °C in a humidified atmosphere of 5% CO2 in air for 3 days. Subsequently, cell viability was determined by WST-8 assay following manufacturer’s instructions (PromoCell GmbH, Heidelberg, Germany).

**Statistical Analysis**

All graphs and statistical analyses were made using Prism 5 statistical software (GraphPad Software, Inc, La Jolla, CA, USA). IC50 values were calculated with nonlinear regression fit to a sigmoidal dose-response curve (variable slope). Values for tumor volume and body weight were compared with two-way ANOVA with Bonferroni post hoc tests. One-way ANOVA was performed for comparison between different groups. Differences were considered statistically significant at a P value of less than 0.05.

**Results**

**Human Endothelial Cells are More Susceptible to E7080 than CRC Cells**

The effect of E7080 on cell viability was examined on 10 human CRC cell lines harboring wildtype or mutated KRAS (Table 1) and human umbilical endothelial cells (HUVEC) using the cell viability test WST8. Dose response analysis showed that VEGF stimulated HUVEC were 5 times more susceptible to the incubation with E7080 (IC50: 22 μmol/l) than CRC cell line HCT15 (KRAS mutated) with IC50 value of 120 μmol/l, the lowest of the tested CRC cell lines (Figure 1A, Table 1). E7080 had less effect on cell viability of CaCo2 (KRAS wildtype), Colo741 (KRAS wildtype), SW620 (KRAS mutated) and HT29 (KRAS mutated) cells. The IC50 values ranged between >1000 μmol/l for CaCo2 and Colo741 and 431 μmol/l for HT29. Colo678 with wildtype KRAS was more susceptible to E7080 (IC50: 222 μmol/l) than CaCo2 and Colo741 (both with wildtype KRAS). It seems that E7080 did not demonstrate a strong cytotoxic effect on human CRC cells in comparison to HUVEC.

---

**Figure 1.** Effects of E7080 on cell viability and cell signalling. (A) Dose-response effects of E7080 on cell viability of human colorectal carcinoma (CRC) cells and endothelial cells (HUVEC). IC50 values were determined in serum reduced (1%) RPMI 1640 medium following treatment with different concentrations of E7080 for 72 hours. The final concentration of DMSO (vehicle) was ≤1%. Results are expressed as the mean ± S.E.M. of at least three independent proliferation assays with triplicates. IC50 values were determined using GraphPad Prism software. See also Table 1. (B) E7080 inhibits VEGF-induced HUVEC proliferation. HUVEC (1.5 × 104 cells) were seeded in experiment with triplicates. *E7080 as indicated. Cell viability was determined with WST-8 assay after 48 hours. Shown are the results of one representative experiment. Cell viability was determined with WST-8 assay after 48 hours. Shown are the results of one representative experiment. (C) E7080 inhibits VEGF-induced phosphorylation of ERK1/2. Serum starved HUVEC were incubated with indicated amounts of E7080 for 4 hours and then stimulated with VEGF (20 ng/ml) for 5 minutes. Cells were harvested immediately on ice and immunoblotted with indicated antibodies against ERK1/2 and pERK1/2 as described in Materials and Methods. (D) VEGF-stimulated MYC expression is suppressed by E7080. HUVEC were incubated in serum reduced (1%) RPMI 1640 medium with E7080 (1 μmol/l) for 4 hours and afterwards with VEGF (20 ng/ml) until indicated time points; subsequently cells were harvested for western blot analysis of MYC. Detection of MYC and GAPDH was performed using enzyme-linked chemiluminescence. GAPDH was used as loading control. The intensity of the signals corresponding to MYC and GAPDH was quantified by densitometry using ImageJ software (National Institutes of Health). The level of MYC protein is expressed as the ratio of the MYC band to that of GAPDH of a representative experiment.
HUVEC are very responsive to stimulation by VEGF. Since E7080 is supposed to inhibit VEGF signaling, we analyzed the effect of E7080 on cell viability of VEGF-stimulated HUVEC. For this, serum starved HUVEC were stimulated with VEGF (20 ng/ml) and treated with increased levels of E7080 (0.001-100 μmol/l). VEGF-induced cell growth was prevented already at 0.001 μmol/l E7080 and cytotoxic effects were observed at levels of 1.0 μmol/l E7080 (Figure 1B).

It has been hypothesized that the multi-kinase inhibitor E7080 inhibits several receptor tyrosine kinases (RTK) such as VEGF-, PDGF receptors and cKIT. One of the major downstream signaling pathways of these RTK is supposed to be the mitogen-activated protein kinase (MAPK) pathway. HUVEC were serum starved overnight and then stimulated with VEGF (20 ng/ml) in combination with different concentrations of E7080 (0.1 to 100 μmol/l). Addition of VEGF to starved HUVEC led to a pronounced induction of MAPK activity after 5 minutes, shown by induced phosphorylation of extracellular signal regulated kinase (ERK1/2). E7080 inhibits VEGF-induced stimulation of MAPK signaling pathway (Figure 1C). Since MAPK up-regulates the cell proliferation promoting transcription factor MYC, we analyzed the effect of E7080 on MYC expression in VEGF-stimulated HUVEC. For this, we stimulated serum starved HUVEC with VEGF for 8, 16, 24 and 36 hours in the presence or absence of E7080. VEGF leads to an up-regulation of MYC after 8 hours, which was nearly completely abrogated by addition of E7080 (Figure 1D).

**E7080 Inhibits Micro Vessel Sprouting in a Three-Dimensional Ex Vivo Mouse Aortic Ring Angiogenesis Assay**

The inhibitory effect of E7080 on VEGF-induced angiogenesis was studied in a three-dimensional ex vivo mouse aortic ring angiogenesis assay. The micro vessel sprouting from aortic rings embedded in collagen was stimulated by VEGF (50 ng/ml), leading to a network of vessels around the aortic rings after 11 days. In contrast, the presence of E7080 (1 μmol/l and 10 μmol/l) reduced the VEGF-induced sprouting in a dose-dependent manner (Figure 2).

**CRC Cells Produce High Levels of VEGF and Express VEGF Receptors**

VEGF receptor (VEGFR) expression of CRC cell lines and HUVEC were proved on both mRNA and protein levels. HUVEC and CRC cell lines expressed mRNA of all three VEGFR (Supplementary Figure 1A). In addition, VEGFR 1 and 3 protein expression in all CRC cell lines and HUVEC were demonstrated by immunohistochemical staining (Supplementary Figure 1B). VEGF protein expression was determined in supernatant of CRC cell lines cultured for 24 hours. All CRC cells secreted large amounts of VEGF without any stimulation. In comparison to CRC cells, no VEGF protein could be determined in supernatant of HUVEC cultures (Figure 3A). To check if E7080 could interrupt the VEGF-mediated interaction between CRC cells and HUVEC, serum starved HUVEC were stimulated with CRC cell supernatant, either with or without E7080 (1 μmol/l) for 24 hours. CRC cell supernatant stimulated HUVEC proliferation, whereas the presence of E7080 prevented the growth induction of HUVEC (Figure 3B, Supplementary Figure 2).

**E7080 Delays Growth of CRC Xenografts**

The antitumor effect of E7080 was analyzed for human CRC xenografts in nude mice. Subcutaneous implantation of 2.5 × 10⁶ HCT116 or LS174T cells into nude mice has been shown to induce reproducible tumor development within 7 to 10 days. Mice were randomized into a control group treated with saline and an intervention group treated with E7080 when tumors reached a volume of 40-60 mm³. The intervention group received a daily dosage of 5 mg/kg E7080. After 8 to 10 days, tumors of E7080 treated animals demonstrated a significant delay in tumor growth (Figure 4, A and B). E7080 slowed tumor growth but did not cause tumor regression. The experiments were stopped when tumors of control mice reached a tumor volume of 600-800 mm³. Then, tumors were explanted for in vitro evaluation. The volume of E7080 treated HCT116 and LS174T tumors at the end of experiment...
The E7080 induced growth reduction at the end of experiment was 62% (Figure 4C).

The daily application of 5 mg/kg E7080 was well tolerated by the animals without treatment-associated lethality and adverse events such as loss of body weight (Figure 4, D–F). In addition, no differences were observed in liver enzyme activity (Figure 4G), white and red blood count, thrombocytes, and hemoglobin (Figure 4H).

E7080 induces hypoxic conditions in CRC tumors by inhibition of angiogenesis without reduction of Ki67-positive tumor cells

Control and E7080 treated tumors showed central necrosis areas without significant differences in size (data not shown). There was no reduction in the number of Ki67-positive cells in the viable part of tumors in both E7080 treated mice and untreated mice (Figure 5A). E7080 is supposed to inhibit VEGF signaling between tumor cells and endothelial cells within stromal microenvironment and therefore, we evaluated the effect of E7080 on angiogenesis. The CD31 and CD34 staining results showed that E7080 significantly decreased micro vessel density (Figure 5, B and C). A qualitative reduction in vascularity was also observed in primary tumor samples of E7080 treated mice (Supplementary Figure 3). The decreased micro vessel density leads to an insufficient supply with oxygen and, increasing hypoxic conditions within the tumor. Staining with the hypoxia marker carbonic anhydrase 9 (CA9, CAIX) revealed increased areas of hypoxia in tumors of E7080 treated mice (Figure 5, B and C; Supplementary Figure 3). These results indicate that E7080 effectively inhibits angiogenesis in vivo and therefore, delays the growth of CRC tumors.

Discussion

In this study we show that the multiple kinase inhibitor, E7080, effectively delays the growth of human CRC xenografts from both HCT116 and LS174T cells (KRAS mutated), respectively, and from primary resection specimens with mutated KRAS in nude mice. The presented in vivo and in vitro data suggest that E7080 suppresses capillary sprouting and inhibits endothelial cell proliferation. The anti-angiogenic effect of E7080 was also observed in a three-dimensional ex vivo mouse aortic ring angiogenesis assay. In addition, the therapy with E7080 in vivo decreased the density of tumor-associated vessel formations that leads to an increase of hypoxic areas within CRC xenografts. Conclusively, these data suggest that the major mechanism by which E7080 interferes with solid tumor growth is apparently the inhibition of angiogenesis, essential for supplying the tumor with oxygen and nutrients.

In the present study we also show that E7080 demonstrates different cytotoxic effects on human CRC cells in vitro. The IC50 values for the 10 CRC cell lines tested ranged between 120 μmol/l und > 1000 μmol/l (Table 1). It is important to emphasize that in vitro assays to determine IC50 values depend to a significant degree on a number of factors, including cell viability, cell proliferation rates, cell confluency at the time of drug exposure and method used to determine cell viability (type of assay). This may explain why IC50 values for certain tumor cell lines can differ [24,25]. Nevertheless, with this panel of CRC cell lines we demonstrate a relatively low cytotoxic effect of E7080 on CRC cell.
viability in vitro. In addition, a direct influence of E7080 on the viability of endothelial cells described here was also shown by others [26].

The ability to induce and sustain tumor vascularization by angiogenesis is a hallmark of cancer [27] that correlates with advanced-stage disease and poor prognosis [28]. Solid tumors go through a prolonged state of avascularity in which they are supplied with oxygen and nutrients by simple passive diffusion up to a size of 2 to 4 mm in diameter [29]. Starting from this size, growth and survival of solid tumors require vascularization through angiogenesis and postnatal vasculogenesis [30]. The new blood vessels secure the supply of oxygen and nutrition [29,31]. Tumor cells secrete numerous growth factors such as VEGF, which is known to be one of the major pro-angiogenic factors [4,32]. VEGF stimulates both the proliferation and migration of endothelial cells leading to new vessel formation [33]. In tumor cells, VEGF signalling affects tumor function independently of angiogenesis [4]. Our data show that the cells lines used here produce VEGF, which induces the growth of human endothelial cells including HUVEC. The VEGF-induced growth of HUVEC was inhibited by low dose E7080 (0.1 μmol/l). CRC cell lines were relatively resistant to the treatment with E7080 in vitro and the E7080 concentration, which was toxic for HUVEC (IC50: 22 μmol/l), did not influence CRC cell viability. HUVEC treated with low dose E7080 (10 μmol/l) demonstrated a dramatic inhibition of cell viability by blocking the induction of VEGF-mediated downstream MAPK signalling. In this respect, our results are in line with previous reports [26]. Furthermore, our data show a dramatic reduction of MYC up-regulation in HUVEC by E7080 after VEGF stimulation. MYC is a helix-loop-helix leucine zipper transcription factor and is one of the major downstream targets of the active MAPK-signaling pathway. VEGF has been demonstrated to induce MYC expression in human endothelial cells [34,35] and up-regulation of MYC in turn has been shown to lead to cell proliferation [36].

The present results of CD31 and CD34 staining show that E7080 significantly decreases the tumor micro vessel density, which might prevent exponential tumor growth by limiting the tumor supply with essential oxygen and nutrients. The undersupply with oxygen in turn increases hypoxic conditions within the tumor. Staining with the hypoxia marker carbonic anhydrase 9 [37] revealed an increase in hypoxic areas within solid tumors grown in E7080-treated mice. Carbonic anhydrase 9 is a hypoxia-inducible protein that regulates cellular pH to promote cancer cell survival and invasion in hypoxic microenvironments. It is also a biomarker of poor prognosis for breast cancer metastasis and patient survival [38]. It is well known, that...
tumor cells perfectly adapt to hypoxic conditions by constitutive up-
regulation of glycolysis with excessive oxygen-independent glucose
degradation[39,40]. The basis of this adaption is the up-regulation
of glycolytic enzymes mediated by hypoxia-inducible factor 1α (HIF-
1α) and proto-oncogenes like KRAS and MYC or tumor suppressor
genes like p53[39,41].

CRC is one of the most common cancers and accounts for about
500,000 deaths worldwide every year[1]. Whereas a five-year survival
rate of 90% for local limited CRC without lymph node metastasis is
relatively good, prognosis drops to less than 10% in the case of distant
metastasis. A combination of chemotherapy and a targeted therapy
directed against VEGF or EGFR is presently used for metastatic
CRC, either as palliative or neoadjuvant therapy[42]. Currently,
KRAS mutation status of CRC is used to predict the outcome for
treatment with monoclonal antibodies. Patients with CRC harboring
wild-type KRAS may benefit from anti-EGFR antibody therapy with
Cetuximab[43], whereas patients with KRAS mutated CRC are
currently treated with the anti-VEGF antibody Bevacizumab in
combination with chemotherapy. Nevertheless, the therapeutic
options for patients with KRAS mutated CRC are limited.

First, the treatment duration for classic chemotherapeutics is
restricted due to cumulative dosage toxicity. Second, addition of
Bevacizumab to classic chemotherapy has only a small positive effect.
Third, Bevacizumab has to be administered intravenously, making it
impractical for maintenance therapy. Therefore, new therapeutic
agents are needed to improve the survival of this patient group.

E7080 is currently under evaluation in several clinical trials
[16,44–46]. Recently, the FDA approved the multi-kinase receptor
inhibitor Regorafenib for metastatic colorectal cancer[14]. In
comparison to Regorafenib, E7080 shows a more selective inhibition
profile for VEGFR2 and VEGFR3, whereas Regorafenib also inhibits
several members of the MAPK-signaling pathway, including
bRASV600. With this ability, Regorafenib may also demonstrate a
direct inhibition of tumor growth rather than interfering with the
interaction between cancer cells and endothelial cells. The application
of Regorafenib is, however, limited due to adverse drug effects.

In summary, we have shown that E7080 (Lenvatinib), a multi-
tyrosine kinase inhibitor, suppresses in vivo angiogenesis at a dosage
of 5 mg/kg and delays the growth of xenografts with KRAS mutation.
These findings suggest a therapeutic potential of E7080 for treatment

Figure 5. The E7080-mediated influence of microvascularization induces tumor hypoxia. (A) Representative immunohistochemical
staining of untreated and E7080 treated tumors (LS174T). Quantitative analysis of LS174T derived xenografts (B) and HCT116 derived
xenografts (C), both KRAS mutated. Tumor sections were stained towards Ki67, CD31, CD34, and CAIX (carbonic anhydrase 9) using
standard immunohistochemical procedures as described in Material and Methods. Results are shown as Tukey boxplot with first, second
(the median) and third quartiles of four to seven animals (Figure 4) per group. The lower whisker represents the 1.5 interquartile
range (IQR) of the lower quartile, and the top whisker represents the 1.5 IQR of the upper quartile. The point indicates an outlier. *P < 0.05,
**P < 0.01, ***P < 0.001 to control tumors. For the results of CRC xenografts derived from patient resection specimens with KRAS
mutation see Supplementary Figure 3.
of patients with KRAS mutated CRC that have no benefit from a first line treatment with Cetuximab [43]. In addition, our findings implicate the need to combine E7080 with chemotherapeutics such as FOLFOX in future studies to further enhance its efficacy.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.neo.2014.09.008.

Acknowledgments
The authors are grateful to Dr. Irina Chodnevskaja, Sabine Gahn, Veronika Heimbach, Michaela Kapp, Monika Koospal, Bettina Mühlung, and Manuela Schneider for their skilful assistance with the experiments; and to Dr. S. Leo, KEYENCE INTERNATIONAL (Belgium). The work was supported by the Deutsche Forschungsgemeinschaft (DFG), Grant 1516/2-1 (TH), and by funds from the Interdisciplinary Centre for Clinical Research (IZKF) of the University of Würzburg (B-186 to AW, B-121 to AT, and D-150 to CO). The authors assume full responsibility for the contents of the research. This publication was funded by the German Research Foundation (DFG), and the University of Würzburg is in the funding program “Open Access Publishing”.

References
[1] Siegel R, Desantis C, and Jemal A (2014). Colorectal cancer statistics, 2014. CA Cancer J Clin 64(2), 104–117.
[2] Lorenz M, Staib-Sebler E, Hochmuth K, Heinrich S, Gog C, Vetter G, Encke A, Muller HH (2000). Surgical Resection of Liver Metastases of Colorectal Cancer J Clin Oncol 18(3), 722–737.
[3] Valantzas K, Kodama K, Takase K, Sugih NH, Yamamoto Y, Iwata M, and Tsuoura A (2013). Antitumor activities of the targeted multi-tyrosine kinase inhibitor lenvatinib (E7080) against RET gene fusion-driven tumor models. Cancer Lett 340(1), 97–103.
[4] Cunningham D, Humblet Y, Siena S, Khayat D, Bleiberg H, Santoro A, Bets D, Bouché O, Mineur L, and Barone C, et al (2013). Regorafenib monotherapy in metastatic colorectal cancer: a randomized, double-blind, placebo-controlled, phase III study. J Clin Oncol 31(16), 200–209.
[5] Goel HL and Mercurio AM (2013). VEGF targets the tumour cell. Nat Rev Cancer 13(13), 871–882.
[6] Kletou M, Eichler P, Liehr T, Stöcker W, and Schütz F (2008). VEGF-A-induced neovascularization is critically dependent on VEGF-A-induced angiogenesis. J Cell Sci 121(1), 27–31.
[7] Boedeker D and Gockel W (2009). Vascular tumour growth is highly aggressive, orthotopic colon cancer model. Mol Cancer Ther 8(5), 1195–1206.
[8] Muecker K, Zorn D, and Weigmann B (2010). VEGF-A-induced angiogenesis is essential for the development of large metastatic xenografts in nude mice. Mol Cancer Ther 9(2), 407–418.
[9] Hanahan D and Folkman J (1996). Patterns and emerging mechanisms of the vascular endothelial growth factor in angiogenesis. Cell 88(3), 313–320.
[10] Mulder K, Scarfe A, Chua N, and Spratlin J (2011). The role of bevacizumab in metastatic colorectal cancer. J Exp Med 208(3), 485–496.
Olive PL, Aquino-Parsons C, MacPhail SH, Liao SY, Raleigh JA, Lerman MI, and Stanbridge EJ (2001). Carbonic anhydrase 9 as an endogenous marker for hypoxic cells in cervical cancer. *Cancer Res* **61**(24), 8924–8929.

Lock FE, McDonald PC, Lou Y, Serrano I, Chafe SC, Ostlund C, Aparicio S, Winum JY, Supuran CT, and Dedhar S (2013). Targeting carbonic anhydrase IX depletes breast cancer stem cells within the hypoxic niche. *Oncogene* **32**(44), 5210–5219.

Gatenby RA and Gillies RJ (2004). Why do cancers have high aerobic glycolysis? *Nat Rev Cancer* **4**(11), 891–899.

Cairns RA, Harris IS, and Mak TW (2011). Regulation of cancer cell metabolism. *Nat Rev Cancer* **11**(2), 85–95.

Hanahan D and Weinberg RA (2011). Hallmarks of cancer: the next generation. *Cell* **144**(5), 646–674.

Winder T and Lenz HJ (2010). Vascular endothelial growth factor and epidermal growth factor signaling pathways as therapeutic targets for colorectal cancer. *Gastroenterology* **138**, 2163–2176.

Amado RG, Wolf M, Peeters M, Van Cutsem E, Siena S, Freeman DJ, Juan T, Sikorski R, Suggs S, and Radinsky R, et al (2008). Wild-type KRAS is required for panitumumab efficacy in patients with metastatic colorectal cancer. *J Clin Oncol* **26**(10), 1626–1634.

Yamada K, Yamamoto N, Yamada Y, Nokiizaka H, Fujisawa Y, Hirata T, Koizumi F, Nishio K, Koyama N, and Tamura T (2011). Phase I dose-escalation study and biomarker analysis of E7080 in patients with advanced solid tumors. *Clin Cancer Res* **17**, 2528–2537.

Boss DS, Glen H, Beijnen JH, Keesen M, Morrison R, Tait B, Copalu W, Mazur A, Wanders J, and O’Brien JP, et al (2012). A phase I study of E7080, a multitargeted tyrosine kinase inhibitor, in patients with advanced solid tumours. *Br J Cancer* **106**, 1598–1604.

Molina AM, Hutson TE, Larkin J, Gold AM, Wood K, Carter D, Motzer R, and Michaelson MD (2014). A phase 1b clinical trial of the multi-targeted tyrosine kinase inhibitor lenvatinib (E7080) in combination with everolimus for treatment of metastatic renal cell carcinoma (RCC). *Cancer Chemother Pharmacol* **73**, 181–189.