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Hepatic arterial infusion of temsirolimus inhibits tumor growth of colorectal rat liver metastases even after a growth stimulating procedure like liver resection

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ABSTRACT

Background: Hepatic arterial infusion (HAI) of specific anti-tumor drugs can be more effective compared with systemic drug application. Herein, we studied whether HAI of temsirolimus is effective to inhibit tumor growth of colorectal liver metastases after liver resection.

Materials and methods: Twenty-four Wistar Albino Glaxo from Rijswijk (WAG/Rij) rats were randomized to four groups and underwent subcapsular implantation of CC531 colorectal cancer cells in the left liver lobe. In two groups, a 70% liver resection (Phx) was performed simultaneously. After 10 d, animals received either a HAI of temsirolimus (CCI-779) or saline solution (controls). Tumor growth was determined on d 10 and 13 using three-dimensional ultrasound. On d 13, tumor tissue was removed for histologic and immunohistochemical analysis.

Results: Sham controls revealed a tumor growth of ~40% from d 10 to d 13. HAI of temsirolimus completely inhibited this tumor growth. Controls with Phx showed a tumor growth of >60%. In contrast, HAI of temsirolimus in Phx animals did not only inhibit tumor growth but was even capable of decreasing the tumor size by ~8%. Immunohistochemical analysis of the tumors showed a decreased proliferation rate and an increased cleaved caspase-3 activity, which was associated with a significant reduction of platelet endothelial cell adhesion molecule (PECAM)-1-positive cells after HAI of temsirolimus.

Conclusions: HAI of temsirolimus inhibits tumor growth of CC531 colorectal liver metastases even if a growth-stimulating procedure like Phx is performed. Inhibition of tumor growth is provided by a decrease of tumor vascularization associated with an inhibition of tumor cell proliferation and an induction of tumor cell apoptosis.

1. Introduction

Fifty percent of colorectal carcinoma patients develop metastatic disease and the liver represents the most common site of metastasis [1]. Liver resection remains the only treatment that can achieve a significant long-term survival or even cure [1–3]. However, due to functional and morphological hepatic regeneration after liver resection, there is a considerable

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release of potent growth factors following such a procedure [4–6]. Thus, the recurrence of metastatic tumor growth might be stimulated [7]. In fact up to 70% of the patients suffer from recurrent hepatic metastatic disease after liver resection [8], and the tumor growth correlates with the amount of the resected hepatic tissue [7,9]. Although it is widely accepted that hepatic resection with curative intent is the standard of care for patients with resectable liver metastases, the treatment in such instances is not restricted to surgery alone and is yet multimodal [10]. Perioperative chemotherapy has led to a further improvement of the outcome of patients with colorectal liver metastasis [10]. However, there is still lack of evidence as to which regimen and application form of perioperative chemotherapy achieve the best results [10]. In this context, new cytotoxic drugs with antiproliferative and antiangiogenic potential have been investigated in different studies [11].

One of these novel agents is the rapamycin derivative temsirolimus (CCI-779), which provides a potent antitumor activity by inhibiting the protein kinase mammalian target of rapamycin (mTOR). This kinase is involved in the phosphatidylinositol 3-kinase/Akt signaling pathway and, therefore, plays a central role in the control of cell growth, cell survival, and angiogenisis *via* the mTOR complex 1 (mTORC1) and the mTOR complex 2 (mTORC2) [12,13]. The mTOR pathway is often aberrantly activated in cancers. The dysregulation of the mTOR pathway causes the antitumor effect of temsirolimus [14,15]. Preclinical data demonstrate antiangiogenic and antiproliferative properties of mTOR-inhibition *in vivo* and *in vitro* [5,16–18]. In addition, there are several clinical trials underway investigating the anticancer effect of rapamycin derivatives in different solid tumors including renal cancer, pancreatic neuroendocrine tumors, sarcomas, liver tumors, and colorectal cancer as well as in non-solid tumors such as non-Hodgkin lymphoma [13]. In all of these trials, the drug is always given systemically.

However, locoregional chemotherapy, such as hepatic arterial infusion (HAI), is capable of increasing the anti-tumor effect of specific drugs when compared to systemic treatment [19,20]. Accordingly, we herein studied, in a rat model of colorectal liver metastases, whether HAI of temsirolimus is capable of inhibiting tumor growth both under normal conditions and after growth stimulation by liver resection.

2. Materials and methods

2.1. Animals

For the experiments, 24 male Wistar Albino Glaxo from Rijswijk (WAG/Rij) rats with a mean body weight of 344.4 g ± 6.2 g were used. The animals were kept in a temperature- and humidity-controlled 12-h light/dark cycle environment with free access to water and standard laboratory chow (Altromin; Lage, Germany). All experiments were approved by the local governmental ethics committee and were performed in accordance to the Guidelines for the Welfare of Animals in Experimental Neoplasia of the United Kingdom Coordinating Committee on Cancer Research (as described in 1998 in Br J Cancer 77: 1-10) and the Guide for Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, National Research Council; NIH Guide, vol. 25, no. 28, 1996).

2.2. Experimental protocol

Animals were randomized in four groups (Sham, Rapa, Phx Sham, and Phx Rapa; $n = 6$ each group). At first the tumor cell implantation was performed (d 0). Two groups of animals underwent liver resection (Phx) simultaneously (Phx Sham, Phx Rapa). Two groups of non-Phx animals served as controls (Sham, Rapa). Ten days after tumor cell implantation, the animals were relaparotomized and received a hepatic arterial infusion of either temsirolimus (Rapa and Phx Rapa) or a comparable amount of saline solution (Sham, Phx Sham). The HAI was followed by three-dimensional ultrasound imaging. Three days later (d 13) animals underwent relaparotomy and re-examination of the tumor volume by three-dimensional ultrasound imaging. Then animals were sacrificed and tumor tissue as well as hepatic tissue were sampled for histologic and immunohistochemical analysis. Blood samples were taken on d 10 and 13 before HAI and ultrasound examinations. Body weight was measured on d 0, 10, and 13.

2.3. Tumor cell implantation

For induction of the colorectal liver metastases, the animals were placed in supine position on an electronically regulated heating pad, which adjusted the body temperature to 37°C. Under isoflurane inhalation anesthesia a median laparotomy was performed and 5×10^5 tumor cells of the syngeneic CC531 colon carcinoma cell line were implanted under the capsule of the left liver lobe using a 27 G needle (Omnicon F; Braun, Melsungen, Germany) as described previously [20].

2.4. Liver resection

After tumor cell implantation, Phx animals underwent a ~70% hepatectomy by resection of the intermediate lobe, the right lobe, and the processus caudatus. The laparotomy was closed by a one-layer running suture.

2.5. Hepatic arterial infusion

HAI was performed on d 10. Therefore, the gastroduodenal side branch of the common hepatic artery was cannulated with a catheter (internal diameter 0.28 mm; Portex, Hythe, UK). The tip of the catheter was positioned at the branching with the common hepatic artery. During the HAI, the hepatic artery was not occluded and showed orthograde blood flow. Temsirolimus (Torisel; Pfizer, Berlin, Germany) was given in a dose of 0.3 mg/kg bodyweight. Controls received a comparable amount of saline solution. After HAI, the catheter was removed and the gastroduodenal artery was ligated. The laparotomy was closed by a one-layer running suture.

2.6. Three-dimensional ultrasound imaging

Using the 40 MHz ultrasound probe of the Vevo 770 high-resolution imaging system (VisualSonics, Inc., Toronto,

ON, Canada), the tumor volume was measured three-dimensionally on d 10 and 13. The ultrasound probe was attached to a stepping motor that moves the probe over the surface of the left liver lobe. Thus, parallel two-dimensional images were acquired at intervals of 50 μm. The data for the three-dimensional reconstruction of the tumor was achieved by off-line-outlining the tumor dimension on every 200 μm of the two-dimensional images. With these data, the integrated software of the Vevo 770 high-resolution imaging system calculated a polygonal three-dimensional image and, thus, the tumor volume.

2.7. Immunohistochemistry

Proliferating cell nuclear antigen (PCNA) was used as an indicator of cell proliferation. Paraffin-embedded specimens, sectioned in 5 μm intervals, were incubated for 18 h at 4°C with a mouse monoclonal anti-PCNA antibody (1:50; Dako, Hamburg, Germany). For development of PCNA, a peroxidase-conjugated goat anti-mouse immunoglobulin G antibody (1:100; Dianova) was incubated for 30 min and 3.3′ diaminobenzidine was used as chromogen. Hemalaun was used for counterstaining. PCNA-positive cells were analyzed using the following score: 0 = <1%, 1: 1%–10%, 2 = 10%–30%, 3 = 30%–50%, 4 = >50% of PCNA-positive cells per high power field (HPF).

Cleaved caspase-3 (cysteine-aspartic proteases) was used as an indicator of apoptotic cell death. Sections of 5 μm of tumor-bearing specimens were incubated overnight at room temperature with a rabbit polyclonal anti-cleaved caspase-3 antibody (1:50; Cell Signaling Technology, Frankfurt, Germany). As secondary antibody a peroxidase-conjugated goat-anti-rabbit-immunoglobulin G antibody (1:100; Dianova, Hamburg, Germany) was used; 3.3′ diaminobenzidine served as chromogen. Counterstaining was performed with hemalaun. Twenty-five HPF per specimen were analyzed. Positively stained cells were counted and given as number per HPF.

Platelet endothelial cell adhesion molecule (PECAM)-1 was used as an indicator for tumor vascularization. The immunohistochemical detection of PECAM-1 expression was achieved using a primary mouse-anti-rat antibody (1:500, clone TLD-3 A12; Serotec, Puchheim, Germany) and a secondary peroxidase-conjugated goat-anti-mouse antibody (Dianova, Hamburg, Germany). PECAM-1-positive blood vessels were counted in 25 HPF per section, and are given as number per HPF.

2.8. Histology

At the end of the experiments (d 13), tissue specimens of the tumor-bearing livers were fixed in 4% phosphate-buffered formalin for 2–3 days and were then embedded in paraffin. Sections of 5 μm were cut and stained with hematoxylin-eosin for histologic analysis of hepatocellular injury. Hepatocellular injury was determined by analysis of hepatocellular cytoplasmic coarseness and vacuolization with a semiquantitative score (i.e., 0: none; 1: mild; 2: moderate; 3: severe) in 25 HPF per specimen.

2.9. Sampling and assays

Venous blood samples were taken on d 10 and 13 from the subhepatic vena cava before drug administration and ultrasound imaging. Using spectrophotometry aspartate aminotransferase and alanine aminotransferase serum activities were determined as indicators of hepatocellular injury.

2.10. Statistical analysis

All values are expressed as mean ± SEM. After analysis of normal distribution of data and homogeneity of variance, differences between the groups were assessed by one-way ANOVA followed by an appropriate post-hoc test including the correction of the α-error according to Bonferroni probabilities to compensate for multiple comparisons. Overall statistical significance was set at $P < 0.05$.

3. Results

3.1. Metastatic tumor engraftment and general health conditions

Ten days after tumor implantation, all animals showed a solitary tumor in the left liver lobe. There were no signs of extrahepatic disease. Animals were not affected by the tumor, showing normal feeding and cleaning habits. In addition, there were no signs of wound infection, although the abdominal wall closure seemed less stable at relaparotomy on d 13 in animals that underwent HAI of temsirolimus.

3.2. Body weight

Analysis of the body weight from d 0–d 13 showed a significant decrease in Rapa, Phx Sham, and Phx Rapa compared with Sham controls (Table 1). However the body weight was not decreased below 10% of the initial value at d 0.

3.3. Metastatic tumor growth

Three-dimensional ultrasound analyses revealed an almost 40% increase of tumor volume from d 10–d 13 in Sham controls (Fig. 1). Of interest, HAI of temsirolimus (Rapa) completely inhibited tumor growth during this time period (Fig. 1). In Phx Sham controls, the tumor size even increased by more than 60%. HAI of temsirolimus after Phx (Phx Rapa)

Table 1 – Change of body weight.

Group	Body weight [%]
Sham	−2.3 ± 0.4
Rapa	−7.5 ± 0.4*,†
Phx Sham	−9.8 ± 0.8*,†
Phx Rapa	−7.6 ± 0.6*,†

Change of body weight [%] from d 0 to d 13. Animals underwent hepatic arterial infusion (HAI) either of saline (Sham, Phx Sham) or temsirolimus (Rapa, Phx Rapa) 10 d after tumor implantation with (Phx Sham, Phx Rapa) or without (Sham, Rapa) simultaneous liver resection. Data are given as mean ± SEM.
* $P < 0.05$ *versus* Sham.
† $P < 0.05$ *versus* Phx Sham.

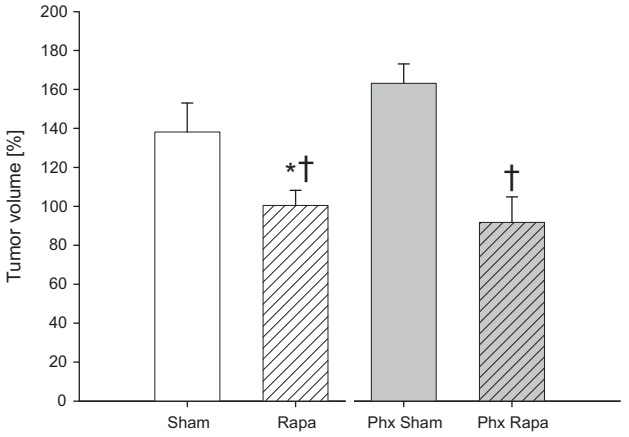

Fig. 1 – Tumor growth from d 10 to d 13 (given as tumor volume in percent) of animals undergoing hepatic arterial infusion either of saline (Sham, Phx Sham) or temsirolimus (Rapa, Phx Rapa) 10 d after tumor implantation with (Phx Sham, Phx Rapa) or without (Sham, Rapa) simultaneous liver resection. Data are given as mean ± SEM; *P < 0.05 *versus* Sham; †P < 0.05 *versus* Phx Sham.

did not only inhibit tumor growth but even reduced the tumor size by 8% during the 3-d time period (Fig. 1).

3.4. *Tumor cell proliferation*

As expected, the immunohistochemical analysis of PCNA-positive cells in sham controls (Sham) showed almost no proliferation in the normal liver tissue. In animals that underwent Phx (Phx Sham, Phx Rapa), the number of PCNA-positive cells in the normal liver was found significantly increased. Interestingly, the application of temsirolimus by HAI (Rapa, Phx Rapa) additionally increased the number of PCNA-positive cells in the liver tissue compared with the corresponding sham groups (Sham, Phx Sham) (Fig. 2).

In the tumor tissue, all groups exhibited a high proliferation rate. However, in temsirolimus-treated animals (Rapa, Phx Rapa) the number of PCNA-positive cells was significantly decreased compared with Sham even after Phx (Fig. 2).

3.5. *Apoptotic tumor cell death*

Analysis of cleaved caspase-3-positive cells showed a significant increase of apoptotic cell death in the liver tissue after HAI of temsirolimus compared to the corresponding sham controls. Within the tumor tissue this effect was even more pronounced (Fig. 3).

3.6. *Tumor vascularization*

In non-hepatectomized animals that underwent HAI of temsirolimus, the number of PECAM-1-positive cells in the metastatic tumors was found to be significantly reduced compared with non-hepatectomized sham controls. In parallel, after Phx HAI of temsirolimus also significantly reduced the number of PECAM-1-positive cells (Fig. 4).

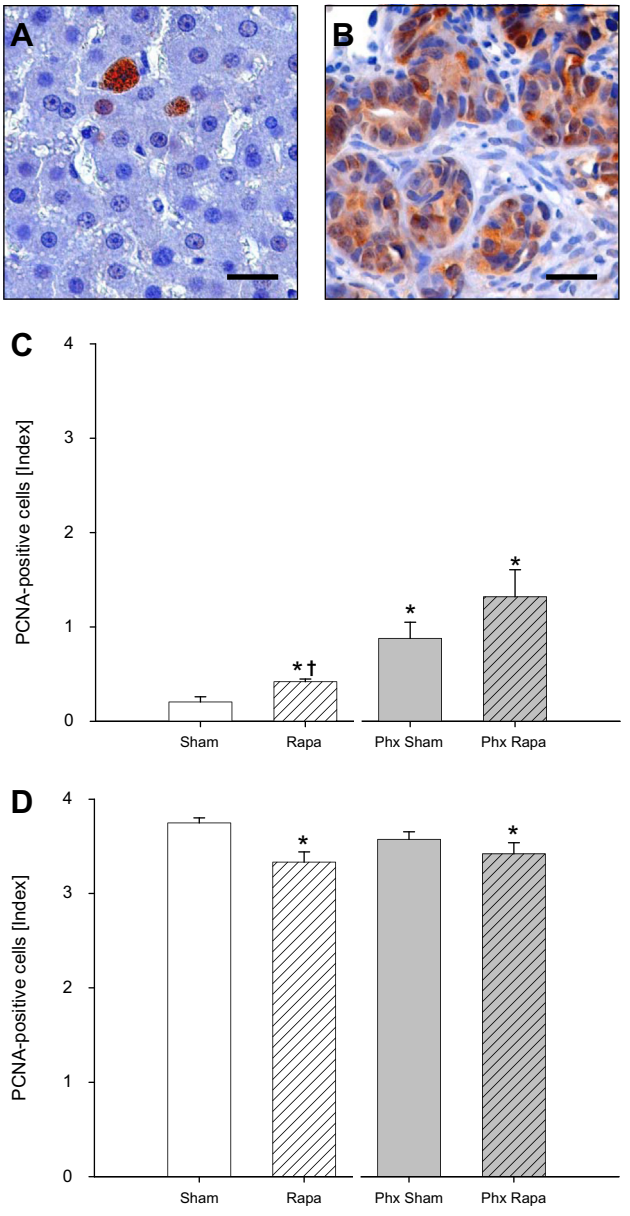

Fig. 2 – Immunohistochemical sections of proliferating nuclear cell antigen (PCNA) expression in the liver (A) and the tumor tissue (B); (A) displays a section of the liver and (B) displays a section of a tumor from an animal of the HAI-Rapa group. Panels (C) and (D) show the PCNA data (given as index: 0: <1%, 1: 1%–10%, 2: 10%–30%, 3: 30%–50%, 4: >50% of PCNA-positive cells) in the liver (C) and in the tumor tissue (D) of animals undergoing hepatic arterial infusion either of saline (Sham, Phx Sham) or temsirolimus (Rapa, Phx Rapa) 10 d after tumor implantation with (Phx Sham, Phx Rapa) or without (Sham, Rapa) simultaneous liver resection. Data are given as mean ± SEM; *P < 0.05 *versus* Sham; †P < 0.05 *versus* Phx Sham. Bars represent 100 μm. (Color version of figure is available online.)

3.7. Histomorphologic analysis

The determination of the cytoplasmic coarseness showed a more pronounced injury after HAI of temsirolimus compared with sham controls (Table 2). Also, vacuolization of the hepatocytes was found to be slightly more pronounced in animals undergoing HAI of temsirolimus (Table 2).

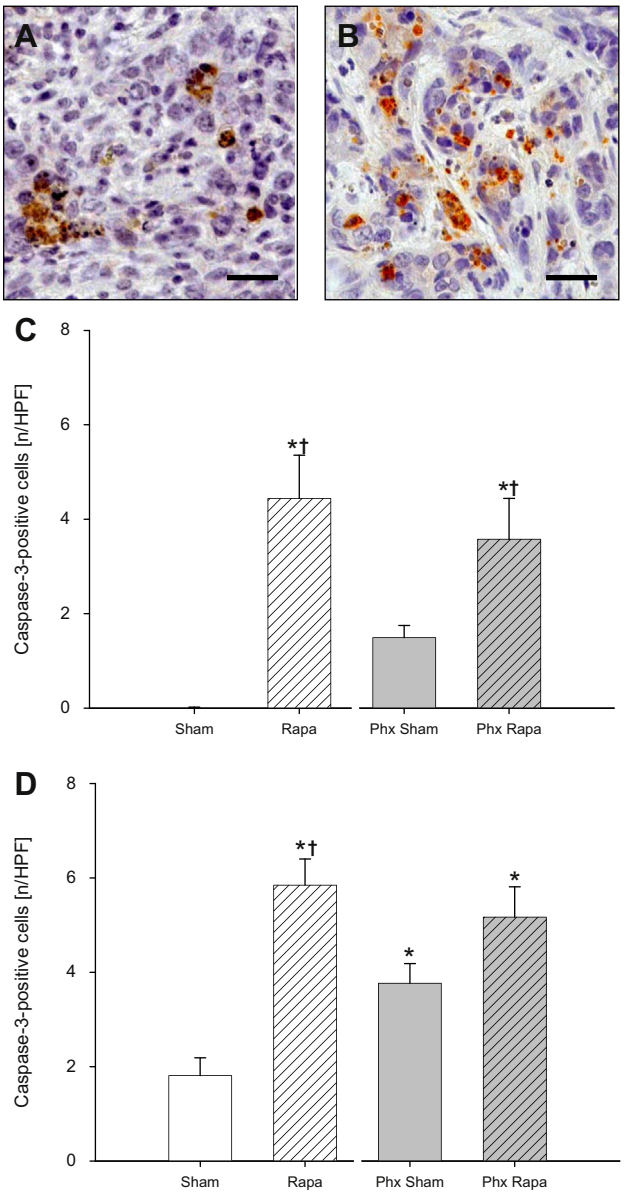

Fig. 3 – Immunohistochemical analysis of cleaved caspase-3 as an indicator of apoptotic cell death. Panel (A) shows the tumor tissue of an animal from the Phx Sham group. Panel (B) displays the tumor tissue of an animal from the Phx Rapa group. Panels (C) and (D) demonstrate the quantitative analysis of cleaved caspase-3-positive cells (given as number per HPF) in the liver (C) and in the tumor tissue (D) of animals undergoing hepatic arterial infusion either of saline (Sham, Phx Sham) or temsirolimus (Rapa, Phx Rapa) 10 d after tumor implantation with (Phx Sham, Phx Rapa) or without (Sham, Rapa) simultaneous liver resection. Data are given as mean ± SEM; *P < 0.05 *versus* Sham; †P < 0.05 *versus* Phx Sham. Bars represent 100 μm. (Color version of figure is available online.)

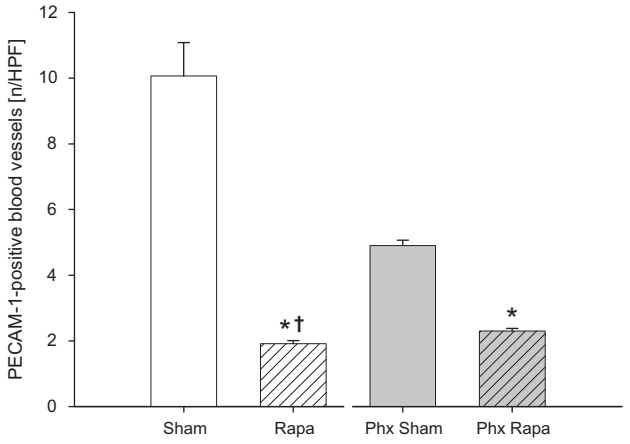

Fig. 4 – Immunohistochemical analysis of PECAM-1 positive blood vessels (given as number per HPF) in the tumor tissue of animals undergoing hepatic arterial infusion either of saline (Sham, Phx Sham) or temsirolimus (Rapa, Phx Rapa) 10 d after tumor implantation with (Phx Sham, Phx Rapa) or without (Sham, Rapa) simultaneous liver resection. Data are given as mean ± SEM; *P < 0.05 vs. Sham; †P < 0.05 *versus* Phx Sham.

3.8. Analysis of liver enzymes

Analysis of the liver enzyme aspartate aminotransferase and alanine aminotransferase as indicators of hepatocellular injury did not show significant differences comparing animals of the treatment groups to sham controls (data not shown).

4. Discussion

The major finding of the present study is that a single application of temsirolimus given *via* HAI is effective to inhibit tumor growth of rat colorectal liver metastasis by decreasing of tumor vascularization, inhibiting tumor cell proliferation,

Table 2 – Hepatocellular injury.

Group	Cytoplasmic coarseness	Vacuolization
Sham	1.03 ± 0.16	0.89 ± 0.24
Rapa	1.97 ± 0.11*,†	1.73 ± 0.19
Phx Sham	1.39 ± 0.14	1.04 ± 0.09
Phx Rapa	2.54 ± 0.20*,†	1.52 ± 0.16

Hepatocellular injury given as a semiquantitative score (i.e., 0: none; 1: mild; 2: moderate; 3: severe). Animals underwent hepatic arterial infusion (HAI) either of saline (Sham, Phx Sham) or temsirolimus (Rapa, Phx Rapa) 10 d after tumor implantation with (Phx Sham, Phx Rapa) or without (Sham, Rapa) simultaneous liver resection. Data are given as mean ± SEM.

* $P < 0.05$ *versus* Sham.

† $P < 0.05$ *versus* Phx Sham.

and inducing tumor cell apoptosis. This is observed also after the growth-stimulating procedure of liver resection.

Liver resection remains the treatment of choice of colorectal liver metastases [10]. However, liver regeneration after major hepatic resection is associated with a considerable release of potent growth factors, including hepatocyte growth factor (HGF), epidermal growth factor (EGF), and vascular endothelial growth factor (VEGF) [4,6,21]. Several studies have shown that hepatectomy accelerates local tumor growth and may, thus, lead to tumor recurrence [25–27]. Of interest, in a recent animal study, Rupertus *et al*. indicate that targeting mTOR represents an interesting strategy to prevent tumor recurrence after hepatectomy for colorectal metastasis [5]. Rapamycin and its derivatives target the kinase mTOR. This kinase is involved in the phosphatidylinositol 3-kinase /Akt signaling pathway and plays a central role in the control of angiogenesis, cell growth, and cell survival *via* the mTORC1 and the mTORC2 [12,13]. Several observations support the importance of the mTOR pathway in cancer pathogenesis. Oncogenic activation of mTOR signaling induces processes required for cancer cell growth, survival, and proliferation [14]. Preclinical data of *in vitro* and *in vivo* studies showed that aberrant mTOR pathway activation through oncogene stimulation contributes to tumor growth, angiogenesis, and metastasis [22]. The proto-oncogenes and mTOR downstream effectors 4EBP1 and eIF4 E are implicated in cellular transformation and their overexpression in colonic tumors is associated with poor prognosis [15]. Thus, dysregulation of multiple elements of the mTOR pathway has been reported and, therefore, the importance of considering mTOR-targeting in cancer therapy is reinforced [15].

In the present study, we investigated the anti-tumor activity of the rapamycin-derivative temsirolimus (CCI-779) in a rat model of colorectal liver metastasis. Temsirolimus has more favorable pharmacokinetic and solubility properties than rapamycin [15]. Like rapamycin, it forms a complex with the intracellular receptor FKBP12. This complex binds to mTOR and inhibits mTORC1 downstream signaling. It is known that the mTORC1 pathway plays an important role in colorectal cancer [24]. The inhibition of mTORC2, which induces antiproliferative effects, depends on the type of rapamycin-derivative and the cancer cell that is targeted [15]. However, temsirolimus is a cell type-specific mTORC2 inhibitor [23]. This is in line with our results, indicating a significant reduction of PCNA-positive cells in the tumor tissue of CC531 colorectal rat liver metastases after application of temsirolimus. In addition, temsirolimus provides its anti-tumor effects by down-regulation of VEGF [15]. Accordingly, we detected a significant reduction of PECAM-1-positive cells in the tumor tissue, indicating a significant reduction of tumor vascularization.

In addition, we found a significant induction of apoptosis when temsirolimus was applied. This might be due to direct cytotoxic effects as well as to the hypoxic effect provided by the antiangiogenic activity of the drug [12,28]. However, in most tumor types, mTOR inhibitors have predominantly led to disease stabilization rather than tumor regression [15]. So far, rapamycin analogues were always given systemically in clinical studies [29–37]. In contrast, in the present study, a single application of 0.3 mg/kg body weight temsirolimus, which corresponds to doses given clinically, applied *via* hepatic arterial infusion, was effective not only to inhibit tumor growth but to induce tumor regression. This effect could be detected when the growth-stimulating hepatic resection was performed.

HAI is a locoregional mode of chemotherapy, which bears the advantage of increasing the local concentration of a specific anti-tumor agent and may, thus, increase its anti-tumor activity within the affected organ [38,39]. In colorectal liver metastases, this advantageous effect is provided by the special blood supply. Liver metastases receive the nutritive blood flow from the hepatic arterial system [40]. Part of the arterial blood first drains into the peribiliary plexus before entering the liver sinusoids [41]. Liver metastases are supplied by these arterial vessels [42], which leads to a prolonged exposure time of arterially applied drugs to the metastases. This probably caused the increased anti-tumor activity after HAI [43].

Of interest, the application of temsirolimus resulted in a slight but significant increase of PCNA-positive cells in normal liver tissue. The mechanism behind this finding is not clear. It is well known that VEGF induces vasodilation [44] and that rapamycin inhibits VEGF [17] and vasodilation [45]. Rapamycin is thought to even induce vasoconstriction [46]. Thus, it may be speculated that temsirolimus induces slightly hypoxic conditions in normal liver tissue, which are known to stimulate cell proliferation as observed in the present study. In contrast, in tumors, rapamycin has been shown to induce microthrombosis [18]. This may result in complete anoxia with the consequence of more pronounced apoptotic cell death and reduced cell proliferation, as also observed in the present study.

Taken together, we demonstrate that HAI of temsirolimus is effective in inhibiting tumor growth of CC531 colorectal liver metastases independent of whether a growth-stimulating procedure like Phx was performed prior to the application of the drug. Thus, HAI of temsirolimus might be considered as an interesting treatment strategy of colorectal liver metastasis, especially following liver resection. Because temsirolimus is already approved for use in the treatment of solid tumors, our results may encourage the initiation of a phase I clinical trial in patients with colorectal liver metastases.

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The authors declare that they have no conflict of interest.

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