

Effect of Sorafenib on Murine Liver Regeneration

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Hepatocellular carcinoma (HCC) is a common cause of cancer-related death. Sorafenib prolongs survival of patients with advanced disease and is approved for the systemic treatment of unresectable HCC. It possesses antiangiogenic and antiproliferative properties by way of inhibition of the receptor tyrosine kinases vascular endothelial growth factor receptor 2 (VEGFR-2) and platelet-derived growth factor receptor-beta 1/2 (PDGFR- β) and the kinase RAF. Sorafenib represents a candidate compound for adjuvant therapy in HCC patients. The aim of our study was to investigate whether sorafenib affects liver regeneration. C57BL6 mice received sorafenib orally at 30 mg/kg/day or its vehicle either for 14 days until the day before hepatectomy or starting the day after surgery or both. Animals were sacrificed 24, 72, and 120 hours after hepatectomy. Liver regeneration was calculated as a percent of initial liver weight. Bromodeoxyuridine (BrdU) incorporation and phospho-extracellular signal-regulated kinase (pERK1/2) were determined by immunohistochemistry on liver sections. VEGF-A, PDGF-BB, and hepatocyte growth factor (HGF) levels were measured in liver tissue homogenates. Histological analysis of scar tissue was performed. Treatment stopped 1 day before surgery had no impact on liver regeneration. Continuous sorafenib treatment and treatment started 1 day after surgery had statistically significant effects on liver regeneration at 120 hours compared to vehicle-treated control animals (72% \pm 12 versus control 88% \pm 15 and 70% \pm 13 versus control 86% \pm 5 at 120 hours, both $P \leq 0.02$). BrdU incorporation showed decreased numbers of positive nuclei in both groups receiving sorafenib after surgery. Phospho-ERK levels were reduced in sorafenib-treated animals. An increase of VEGF-A levels was observed in mice receiving sorafenib. Wound-healing complications were observed in animals receiving sorafenib after surgery and confirmed on histological sections. **Conclusion:** This preclinical study shows that sorafenib did not impact on liver regeneration when ceased before surgery; however, administration after hepatectomy affected late liver regeneration. (HEPATOLOGY 2011;53:577-586)

Hepatocellular carcinoma (HCC) belongs to the six most commonly diagnosed cancers worldwide and represents the third most common

Abbreviations: BrdU, bromodeoxyuridine; EGF, epidermal growth factor; ERK1/2, extracellular signal-regulated kinase; HCC, hepatocellular carcinoma; HGF, hepatocyte growth factor; JNK, c-jun N-terminal kinase; MAPK, mitogen-activated protein kinase; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; PDGFR- β , platelet-derived growth factor receptor-beta; 1/2; PI3-kinase, phosphatidylinositol 3-kinase; TGF α , transforming growth factor alpha; IL-6, interleukin-6; TNF, tumor necrosis factor; VEGFR-2, vascular endothelial growth factor receptor 2.

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cause of cancer-related death¹; moreover, its incidence is rising in the Western world.²⁻⁴ Conventional chemotherapy yields only marginal benefits and patients with unresectable or metastatic HCC have a poor prognosis.^{5,6} Curative strategies such as liver resection or local ablation are only amenable to patients with small tumors and preserved liver function. These approaches are associated with a reduction of the hepatic functional mass and are followed by compensatory liver regeneration. Sorafenib is a multikinase inhibitor with antiangiogenic properties; it has been shown to significantly improve the survival of patients with advanced HCC and preserved liver functions.^{7,8} Given these beneficial results, the indication for sorafenib could become extended to other HCC patients, i.e., as a neoadjuvant or adjuvant therapy given before or after local ablation/resection, respectively. This may increase the number of patients eligible for curative treatment and/or prolong survival of patients with more advanced disease.

Sorafenib acts by blocking the receptor tyrosine kinases VEGFR (vascular endothelial growth factor receptor) 1, 2, and 3, PDGFR- β (platelet derived growth

factor receptor-beta), Flt-3, c-Kit, fibroblast growth factor receptor-1, and the serine/threonine kinase RAF,^{9,10} thereby repressing tumor cell proliferation and angiogenesis. These same enzymes, however, also belong to pathways involved in liver regeneration, orchestrating the complex interplay of growth factor and cytokine signaling leading to restoration of liver mass.^{11,12}

The aim of our study was therefore to investigate the effects of sorafenib on liver regeneration. We performed our experiments using a murine model of partial hepatectomy. Our results show a mild effect on liver regeneration in animals that received sorafenib after liver resection.

Materials and Methods

Animals and Surgery. Experiments were performed in male C57/BL6 mice (Charles Rivers Laboratories, Germany) weighing 20–25 g. Animals were housed on a 12-hour light/dark cycle and were provided with mice chow and water ad libitum. Experimental animal protocols and animal procedures complied with the *Guide for the Care and Use of Laboratory Animals* (National Academy of Sciences, NIH Publication 86-23, revised 1996) and were approved by local regulatory authorities.

BAY 54-9085 (sorafenib tosylate) (Bayer HealthCare Pharmaceuticals, Montville, NJ) 30 mg/kg/day or its vehicle (Cremophor/ ethanol/ distilled water) was administered by gavage. The dosing volume used was 0.1 mL/10 g body weight. The proportions of Cremophor/ ethanol/ distilled water were 12.5% Cremophor, 12.5% ethanol, and 75% distilled water. For the animals receiving sorafenib, the drug was first dissolved in a 50% Cremophor / 50% ethanol mixture and water was then added to reach the final volume. Animals treated with the vehicle only received the analog fluid mixture without the drug. Cremophor EL was purchased from Sigma (Sigma Cat. No. C-5135). Animals were divided into three groups and their controls. For the first group, treatment was started 14 days before 2/3 hepatectomy and stopped 1 day before surgery; the second group received continuous sorafenib treatment beginning 14 days prior to surgery until the time of harvest; and the third group started treatment the day after 2/3 hepatectomy. Two-thirds hepatectomy was performed according to the method described by Higgins and Anderson.¹³ Under isoflurane anesthesia the left lateral and median lobes were ligated and resected. The abdominal muscular and skin walls were sutured separately with nonabsorbable material until harvesting. Animals were euthanized with Nembutal (50 mg/

kg intraperitoneal) 24, 72, and 120 hours after partial hepatectomy for the first two groups and at 72 and 120 hours for the third group starting sorafenib after surgery; liver, scar tissue, and blood samples were taken at endpoints (n = 7–14 animals/group). Liver regeneration was determined as the ratio of liver weight (g) at harvesting time/liver weight (g) at the time of partial hepatectomy. Liver weight at the time of hepatectomy was calculated using five animals sacrificed for this purpose.

Cell Proliferation. At harvesting time, liver sections were fixed in 10% buffered formalin and processed for staining with hematoxylin and eosin or for immunohistochemistry. The remaining liver was snap-frozen in liquid nitrogen and kept at -80°C until further use. For determination of hepatocyte proliferation, 1 mg bromodeoxyuridine (BrdU) was injected intraperitoneally 2 hours before sacrifice and BrdU incorporation was measured using the BrdU In-Situ Detection kit obtained from BD Pharmingen (BD Biosciences, San Jose, CA). BrdU incorporation was expressed as number of BrdU-positive nuclei/mm².

Immunohistology. Immunohistochemistry for phospho-extracellular signal-regulated kinase (pERK) was performed after deparaffinization, rehydration, and antigen retrieval in citrate buffer by incubation in blocking solution (Tris-Hcl buffer saline Tween-20 [TBST]/5% goat serum) for 1 hour, incubation with primary antibody (phospho-p44/p42 MAPK [Thr202-Tyr204] rabbit monoclonal antibody [mAb], 1:150, Cell Signaling) overnight at 4°C , incubation with secondary antibody (polyclonal goat antirabbit immunoglobulin/biotinylated; Dako) and signal detection by the avidin-biotin system. Imaging was performed with an Olympus microscope and analysis with the MetaMorph imaging software (Molecular Devices) in at least 3 fields per slide. Nuclear phospho-ERK expression was expressed as pERK-positive nuclei/total nuclei/mm².

Liver Homogenate for Enzyme-Linked Immunosorbent Assay (ELISA). Thirty mg of frozen liver tissues was weighed and lysed in 300 μL HEPES buffer (20 mM HEPES, pH 7.4; 1.5 mM EDTA; 0.5 mM PMSF; 1 \times protease inhibitor mix [complete mini tablets, Roche]; 1 \times phosphatase inhibitor [PhosStop, Roche]). Homogenate was collected after homogenization and centrifugation at 14,000 rpm for 10 minutes at 4°C . Protein concentration was measured according to Lowry et al.¹⁴ The amount of VEGF-A, PDGF-BB, and hepatocyte growth factor (HGF) present in whole liver protein extracts were measured using ELISA assays (VEGF-A, PDGF-BB measured with Quantikine immunoassay, R&D; HGF measured with RayBio ELISA

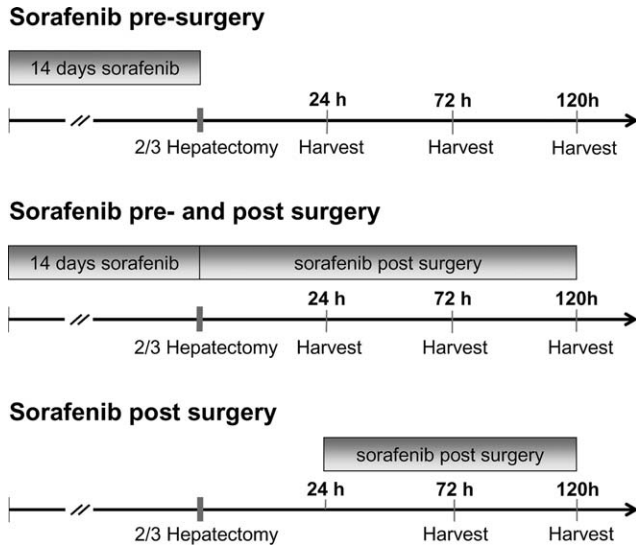


Fig. 1. Treatment scheme. In the “sorafenib pre-surgery” group sorafenib treatment was started 14 days prior to hepatectomy and stopped 1 day before the intervention. In the “sorafenib pre- and post-surgery” group sorafenib was started 14 days before surgery and continued until harvest. For those two groups animals were harvested at 24 hours, 72 hours, or 120 hours after hepatectomy. In the “sorafenib post-surgery” group sorafenib treatment was started 24 hours after hepatectomy and continued until the time of harvest at 72 or 120 hours.

Kit, RayBiotech) following the manufacturer’s instructions. Protein concentration of each liver homogenate was used to normalize the hepatic VEGF-A, PDGF-BB, and HGF levels.

Scar Tissue Analysis. Scar tissue of the peritoneal and muscular abdominal wall were collected at harvest and embedded in paraffin. Tissue was stained with the chromotrope-aniline blue method (CAB trichromic assay).¹⁵ Microscopic evaluation was performed with an Olympus microscope by a blinded investigator. In order to evaluate wound healing in the different treatment groups, the scar margins of the abdominal wall were assessed for bridging reactions. Both the 72-hour and the 120-hour timepoints were studied. Bridging reactions were defined as loci where inflammatory cells transverse the thin layer of collagen formed on the cut edge, participating in the granulation tissue that fills the wound cleft, and eventually linking up opposite scar margins.

Statistical Analysis. Data were analyzed with GraphPad Prism 4.0 software. Kruskal-Wallis and the Mann-Whitney test assessed the statistical significance of differences between mean values; P less than 0.05 was considered significant.

Results

Mice which were treated with sorafenib for 14 days and stopped treatment 1 day before partial hepatec-

tomy showed no impairment in liver regeneration when compared to the control group that received the vehicle only (Figs. 1, 2A). In contrast, the animals receiving continuous sorafenib treatment presented significantly lower liver mass restoration at 120 hours in comparison to the animals treated with the vehicle ($72\% \pm 12\%$ versus vehicle $88\% \pm 15\%$, $P < 0.02$). No significant differences were observed at earlier timepoints (24 hours, $62\% \pm 8\%$ versus vehicle $61\% \pm 16\%$; 72 hours, $74\% \pm 22\%$ versus vehicle $72\% \pm 22\%$) (Fig. 2B). A similar effect on late regeneration was observed in the group starting sorafenib treatment 1 day after 2/3 hepatectomy, with significantly reduced liver regeneration at 120 hours ($70\% \pm 13\%$ versus vehicle $86\% \pm 5\%$, $P < 0.003$; 72 hours, $62\% \pm 9\%$ versus vehicle $70\% \pm 12\%$, not significant [n.s.]) (Fig. 2C).

Cell proliferation was assessed by BrdU incorporation. At 24 and 72 hours after surgery the number of positive nuclei was significantly decreased in the liver of animals continuously treated with sorafenib in comparison to their controls (24 hours, 6 ± 3 versus vehicle 17 ± 9 nuclei/mm², $P < 0.001$; 72 hours, 74 ± 25 versus vehicle 144 ± 67 nuclei/mm², $P < 0.02$) (Fig. 3B). BrdU incorporation also revealed reduced cell proliferation at 72 hours in the group of mice starting treatment after surgery compared to their controls (23 ± 8 versus vehicle 99 ± 40 nuclei/mm², $P < 0.001$) (Fig. 3C). Both groups showed no significant difference at 120 hours after hepatectomy. Further, no differences were observed when comparing animals stopping sorafenib 1 day before surgery and their controls at any timepoint (Fig. 3A).

Sorafenib inhibits the serine/threonine kinase RAF; therefore, the inhibitory effect on the mitogen-activated protein kinase (MAPK) pathway was assessed by immunohistochemistry for pERK. At the time of hepatectomy (0 hours) (Fig. 4), vehicle-treated animals and mice receiving sorafenib after surgery showed comparable numbers of pERK-positive nuclei ($7.3\% \pm 5$ and $7.5\% \pm 4.7$ positive nuclei / total nuclei, respectively). Both groups starting sorafenib treatment 2 weeks prior to surgery showed significantly lower pERK levels when compared to the control group (0 hours, sorafenib presurgery $3.4\% \pm 2.6$ versus vehicle $7.3\% \pm 5$, $P \leq 0.01$; 0 hours, sorafenib pre- and postsurgery $3.0\% \pm 2.1$ versus vehicle $7.3\% \pm 5$, $P \leq 0.01$) and to the group starting sorafenib 1 day postsurgery (0 hours, sorafenib presurgery $3.4\% \pm 2.6$ versus postsurgery $7.5\% \pm 4.7$, $P \leq 0.05$; 0 hours, sorafenib pre- and postsurgery $3.0\% \pm 2.1$ versus postsurgery $7.5\% \pm 4.7$, $P \leq 0.05$). Twenty-four

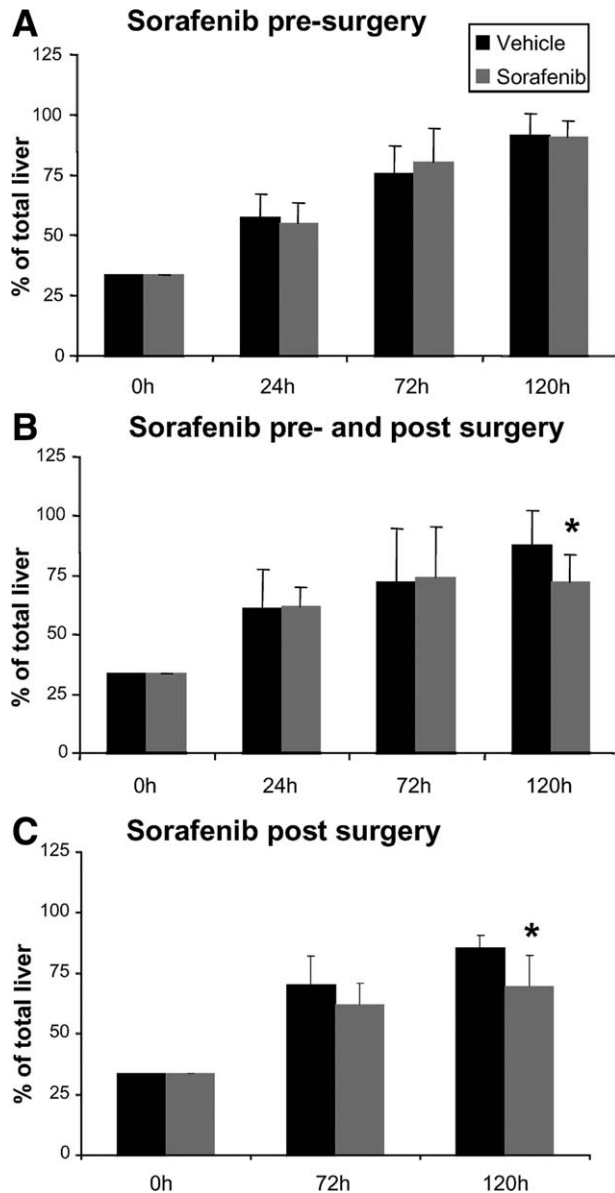


Fig. 2. Liver regeneration. Graphs representing liver regeneration at different timepoints; liver mass was calculated as percentage of total initial liver weight. (A) Animals treated with sorafenib or vehicle until 1 day before surgery. (B) Animals treated with sorafenib or vehicle continuously. (C) Animals treated with sorafenib or vehicle after surgery. Black bars: vehicle-treated mice; gray bars: sorafenib-treated mice. (A) No differences in liver regeneration were observed between vehicle- and sorafenib-treated animals. (B) Mice treated with sorafenib show impaired liver regeneration 120 hours after hepatectomy. (C) Sorafenib treatment compromises liver mass restoration 120 hours after surgery. Values are mean \pm SD. *Statistical significance ($P < 0.05$) versus vehicle-treated control mice.

hours after partial hepatectomy, pERK levels in the vehicle-treated control animals increased more than 4-fold; in contrast, pERK levels did not increase in the animals administered sorafenib before surgery only (24 hours, $4.3\% \pm 5.6$ versus vehicle $33.6\% \pm 10.6$, $P \leq 0.001$). Moreover, mice administered continuous sora-

fenib had even lower pERK levels (24 hours, $0.6\% \pm 0.8$ versus vehicle $33.6\% \pm 10.6$, $P \leq 0.001$). Note that the group starting sorafenib after surgery could not be assessed at 24 hours because this timepoint coincided with beginning of treatment. At 72 hours (Fig. 4; Supporting Information Fig. 1) the group that had stopped sorafenib 1 day before surgery showed comparable pERK levels as the vehicle-treated animals

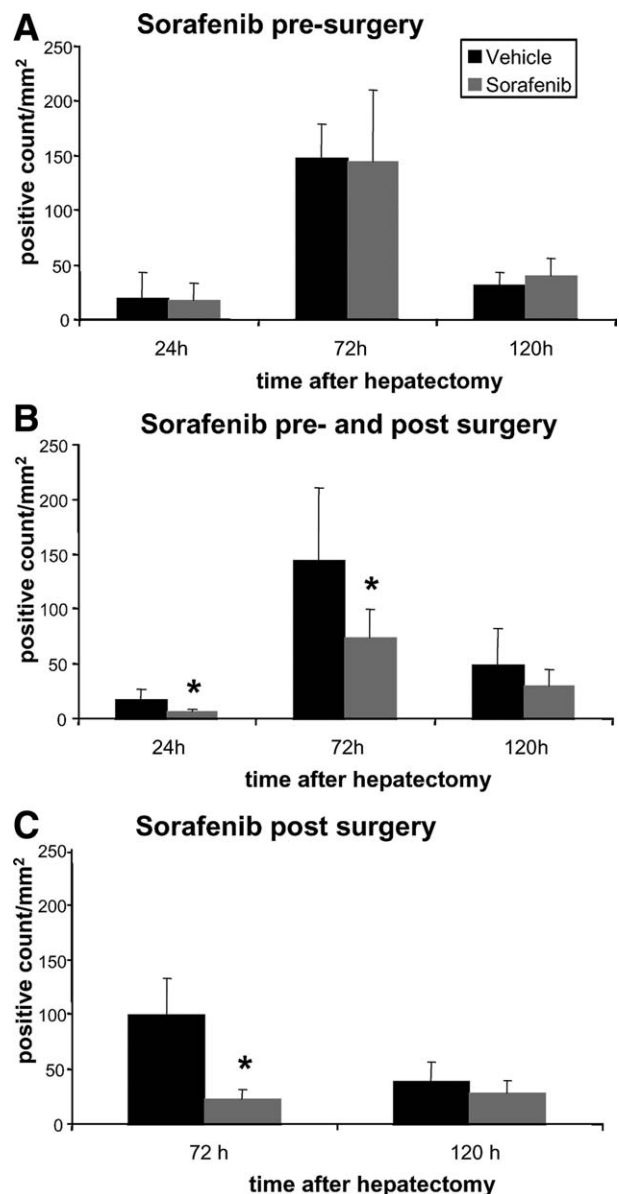


Fig. 3. Cell proliferation assessed by BrdU incorporation. (A) Mice treated before surgery. (B) Animals receiving continuous treatment. (C) Mice treated after surgery. No differences in cell proliferation were noted when treatment was stopped before hepatectomy (A). In the mice receiving continuous sorafenib, cell proliferation was reduced at 24 and 72 hours compared to controls (B). Similarly, BrdU incorporation was reduced at 72 hours in mice starting treatment 1 day after surgery (C). Black bars: vehicle-treated mice; gray bars: sorafenib-treated mice. Values are mean \pm SD. *Statistical significance ($P < 0.05$) versus vehicle-treated control mice.

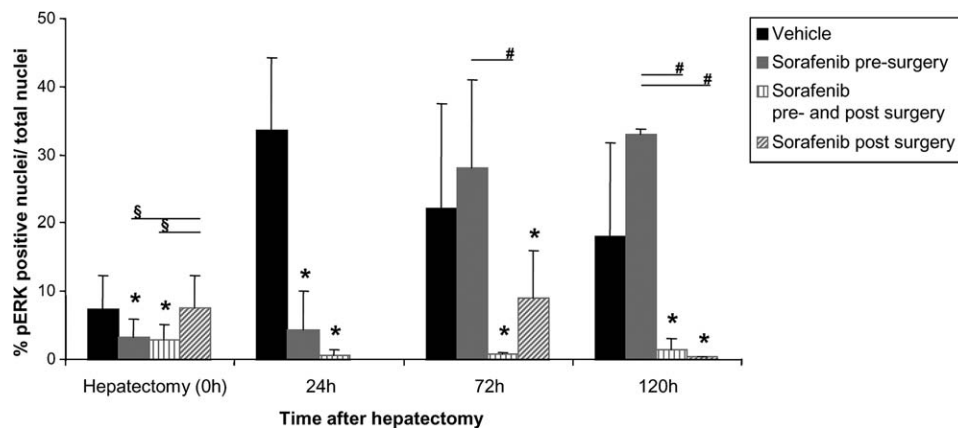


Fig. 4. Liver pERK levels. Quantification of immunohistochemical staining for pERK, percentage of pERK-positive nuclei per total nuclei. At the time of hepatectomy, both groups treated with sorafenib before surgery had a significant reduction of pERK when compared to controls and/or to animals starting treatment after surgery. At 24 hours, pERK levels failed to increase in the pre-surgery group and decreased in the continuous treatment group. Seventy-two hours after hepatectomy, ERK activity was comparable to baseline (0 hours) in the post-surgery animals. For the late timepoint, most phosphor-ERK was measured in the pre-surgery group. In contrast, both groups treated with sorafenib after surgery showed minimal expression. Values are mean \pm SD. *Statistical significance ($P < 0.05$) versus vehicle-treated control mice. §Statistical significance ($P < 0.05$) versus mice starting sorafenib after surgery.

(72 hours, 28% \pm 12.9 versus vehicle 22.1% \pm 15.5, n.s.), whereas pERK levels remained barely detectable in the group receiving continuous sorafenib (72 hours, 0.8% \pm 0.3 versus vehicle 22.1% \pm 15.5, and versus sorafenib presurgery 28% \pm 12.9, $P \leq 0.05$). In the group of mice starting sorafenib treatment after surgery, pERK levels were not increased at 72 hours (72 hours, 8.9% \pm 7.1 versus vehicle 22.1% \pm 15.5, $P \leq 0.05$). Finally, the 120-hour timepoint revealed the highest number of pERK positive nuclei in animals treated before surgery only (120 hours, sorafenib pre-surgery 33% \pm 0.9 versus vehicle 18.1% \pm 13.6, n.s.). The group administered sorafenib pre- and post-surgery still showed very low pERK levels at 120 hours (120 hours, 1.4% \pm 1.7 versus vehicle 18.1% \pm 13.6, $P \leq 0.05$); moreover, in the group starting sorafenib 1 day after surgery, pERK-positive nuclei were barely detectable (120 hours, 0.3% \pm 0.2 versus vehicle 18.1% \pm 13.6, $P \leq 0.001$).

Next, hepatic VEGF-A levels were quantified from whole liver lysates by ELISA in the three treatment groups. After 2 weeks of sorafenib treatment, a significant increase in VEGF-A was observed at baseline (0 hours, measured at the time of hepatectomy). A 1.5-fold and 2-fold increase was measured in the mice receiving sorafenib prior to hepatectomy and in mice administered continuous sorafenib treatment compared to vehicle-treated animals (0 hours, sorafenib presurgery 38.2 \pm 6.7 pg/ μ g versus vehicle 25.4 \pm 3.0 pg/ μ g, $P < 0.0001$; and 0 hours, continuous sorafenib 42.6 \pm 6.6 pg/ μ g versus vehicle 20.5 \pm 5.0 pg/ μ g,

$P < 0.0001$, respectively) (Fig. 5A,B). In the group that stopped sorafenib before partial hepatectomy, the initial increase in VEGF levels was not maintained and no differences were seen at any of the timepoints post-surgery (Fig. 5A). The group receiving continuous sorafenib and the group starting treatment after surgery had significantly higher hepatic VEGF levels compared to vehicle control animals at 72 and 120 hours (continuous sorafenib group: 72 hours, 44.8 \pm 3.6 pg/ μ g versus vehicle 21.4 \pm 3.9 pg/ μ g, $P < 0.01$, and 120 hours, 60.0 \pm 12.0 pg/ μ g versus vehicle 20.7 \pm 3.8 pg/ μ g, $P < 0.05$; in the sorafenib postsurgery group: 72 hours, 43.8 \pm 11.1 pg/ μ g versus vehicle 23.3 \pm 6.4 pg/ μ g, $P < 0.001$, and 120 hours, 32.9 \pm 4.1 pg/ μ g versus vehicle 17.0 \pm 3.5 pg/ μ g, $P < 0.0001$) (Fig. 5B,C). Surprisingly, continuous sorafenib administration did not alter hepatic VEGF levels measured at 24 hours compared to controls (24 hours, 25.8 \pm 5.1 pg/ μ g versus vehicle 24.0 \pm 11.7 pg/ μ g) (Fig. 5B).

No differences were observed for PDGF-BB protein levels measured by ELISA in whole-liver lysates. The sorafenib-treated animals showed similar levels of PDGF as the vehicle-treated mice at all timepoints in all three treatment groups (data not shown).

HGF protein levels revealed a modest increase of liver HGF protein levels at 24 hours after hepatectomy in the control animals receiving vehicle treatment (Supporting Information Fig. 2). This increase was not observed in animals treated with sorafenib prior to 2/3 hepatectomy; HGF levels at 24 hours were even decreased in mice receiving continuous sorafenib treatment (24 hours,

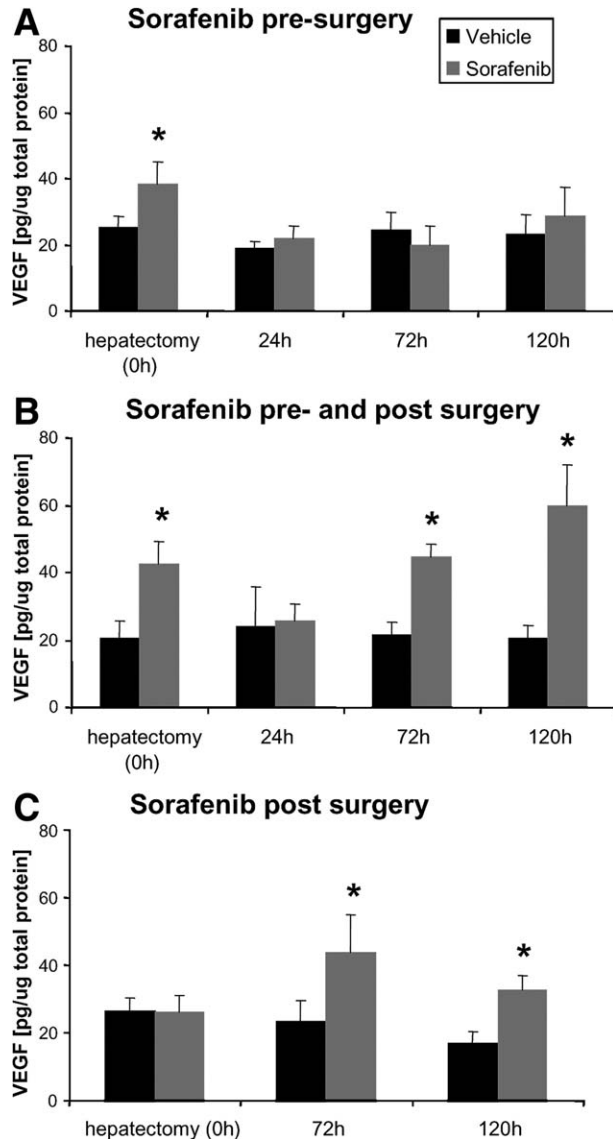


Fig. 5. VEGF hepatic levels. VEGF protein content measured by ELISA from whole liver lysate, showing baseline (hepatectomy) and protein levels at sacrifice. (A) Animals treated with sorafenib or vehicle until 1 day before surgery. (B) Animals treated with sorafenib or vehicle continuously. (C) Animals treated with sorafenib or vehicle after surgery. Black bars: vehicle-treated mice; gray bars: sorafenib-treated mice. Baseline levels increased after 2 weeks of sorafenib treatment but returned to control levels 24 hours after hepatectomy in the group stopping the drug before surgery (A). VEGF levels are higher in the sorafenib-treated animals at baseline (0 hours). No difference was observed after 24 hours, but at 72 and 120 hours, higher VEGF levels were measured in the mice receiving sorafenib, with a 3-fold increase at 120 hours compared to controls (B). Equal amounts of hepatic VEGF were seen at baseline; VEGF levels were increased at 72 and 120 hours in mice starting sorafenib after surgery compared to control animals (C). Values are mean \pm SD. *Statistical significance ($P < 0.05$) versus vehicle-treated control mice.

continuous sorafenib 16.5 ± 4.0 ng/mg versus vehicle 30.7 ± 4.8 ng/mg, $P < 0.01$). Further, we noted a consistent increase of HGF levels at 120 hours after hepatectomy in the mice receiving continuous sorafenib treat-

ment and the mice starting sorafenib after surgery, although this was not significant.

At the time of sacrifice, the abdominal scar was excised and the suture removed carefully. Although the scar margins of the control animals remained sealed, mice receiving sorafenib until harvest had more fragile scars, i.e., the margins were not sealed or separated in a zip-like fashion upon minimal traction. Histological analysis of scar tissue revealed differences in the scar of vehicle- and sorafenib-treated animals. The scars of vehicle control animals presented tissue remodeling of the muscular wall with dense granulation tissue filling the wound cleft (Fig. 6, top panel). Quantification of bridging reactions revealed no significant differences 72 hours after surgery. However, at 120 hours we observed significantly less bridges in animals that had received sorafenib treatment after surgery compared to vehicle controls (120 hours, continuous sorafenib 1.8 ± 1.1 versus vehicle 4.2 ± 1.8 , $P < 0.05$; sorafenib postsurgery 1.8 ± 1.4 versus vehicle 4.2 ± 1.8 , $P < 0.01$) (Fig. 7). Moreover, the scars of animals that were treated with sorafenib after surgery showed less intense tissue remodeling and granulation tissue was less dense or barely present (Fig. 6, lower panels).

Discussion

Our preclinical results show that sorafenib administration that is stopped 1 day before hepatic resection had no effect on liver regeneration in this study, whereas liver regeneration was impaired at the late timepoint examined (120 hours) when sorafenib was administered postoperatively.

Liver regeneration is a complex process that depends on the activation of several growth signal pathways. Sorafenib inhibits the serine/threonine kinase activity of RAF in the RAF/MEK/ERK signaling pathway and the receptor tyrosine kinase activity of the VEGF receptor-2.⁹ Liver regeneration studies have shown that a variety of growth factors and cytokines, acting by way of their respective receptors, activate complementary signaling pathways that elicit cellular proliferation and liver mass restoration. Among these intracellular mediator is the RAS/RAF/MEK pathway, resulting in the activation of ERK1/2.¹² Growth factors such as EGF, HGF, and TGF α and different cytokines (interleukin-6 [IL-6], TNF [tumor necrosis factor]) trigger ERK1/2 activation.¹⁶⁻¹⁸ This mitogenic cascade is inhibited by sorafenib at the level of RAF. Our analysis of phosphorylated ERK by immunohistochemistry showed decreased levels in the sorafenib-treated animals, with an important inhibition of ERK activation after

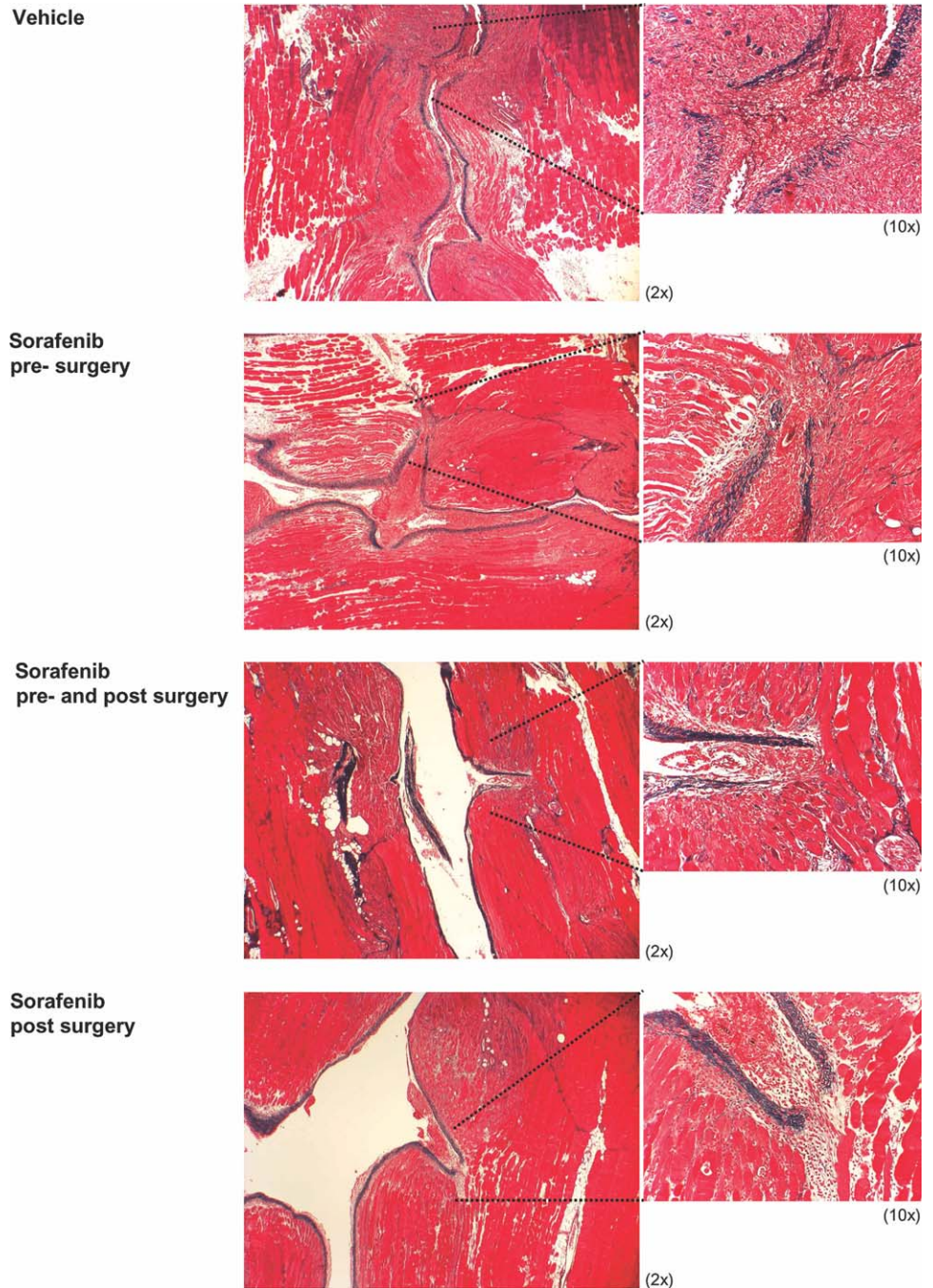


Fig. 6. Wound healing: histology. Wound healing was assessed on histological sections of the abdominal scar. Examples of scarring tissue for each group at 120 hours after surgery are shown. The top panel shows wound healing in a control animal; dense granulation tissue links up the wound edges, appearing as a bridge (inset). The granulation tissue is looser or scarce in the animals receiving sorafenib after surgical intervention (bottom panels); there are fewer bridges and the muscular tissue appears less reactive.

hepatectomy but also diminished baseline phospho-ERK contents at the time of hepatectomy in the animals that had received sorafenib treatment prior to surgery. It is interesting that liver regeneration still occurred reasonably effectively in spite of almost complete pERK ablation in the mice treated with sorafenib after resection. The importance of phospho-ERK1/2 activity in hepatocyte proliferation is not univocal. Although some reports support a key role of the MAPK pathway in regulating hepatocyte proliferation,¹⁹⁻²¹ others observed a discrepancy between

ERK1/2 activity and cellular proliferation.²² Further and similar to our findings, Borowiak et al.²³ showed only mild effects on liver regeneration for conditional *Met* mutant mice 5-7 days after hepatectomy, despite low phospho-ERK1/2 levels and reduced cell proliferation. These data and our findings, together with reports assessing the roles of other signal transducers such as JNK1/2, p38, and the PI3-kinase,^{22,24,25} indicate a redundancy of the system rather than exclusive roles of selected pathways. HGF is an important player in the liver regeneration process; its receptor *Met* is

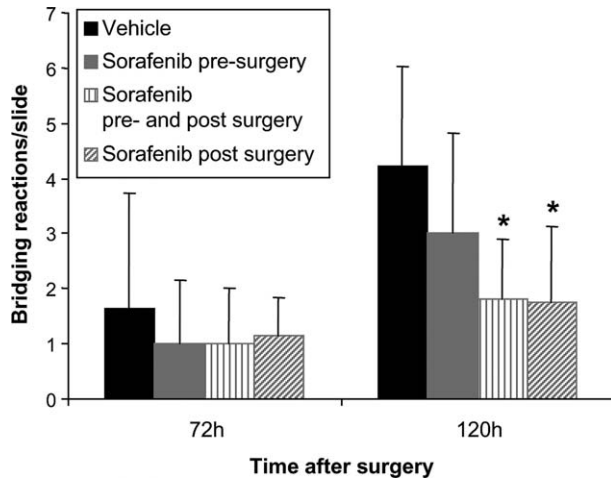


Fig. 7. Wound healing: quantification. The number of bridging reactions per tissue slide was counted. Quantification revealed most bridging reactions in the control animals as compared to the three treatment groups, with significantly less sealing observed at 120 hours in the two groups receiving sorafenib after surgery. Values are mean \pm SD. *Statistical significance ($P < 0.05$) versus vehicle-treated control mice.

rapidly activated after partial hepatectomy.¹¹ Downstream signaling is mediated in part by PI3K/Akt, RAS/RAF/MEK/ERK, and the transcription factor STAT3, resulting in cell survival and cell proliferation.^{26,27} Overall, our analyses of liver HGF protein levels showed minimal effects of sorafenib on HGF levels. However, our experimental setup did not focus on the very early events of liver regeneration (before 24 hours). Apart from a reduction of HGF protein content at 24 hours in the mice continuously treated with sorafenib, HGF levels did not appear to be reduced by sorafenib treatment. These data suggest that the regenerative process could still occur by way of other signaling cascades than RAS/RAF/MEK, providing in part an explanation as to why regeneration occurred despite minimal phospho-ERK induction.

Liver regeneration depends not only on hepatocyte proliferation but also on endothelial cell proliferation and angiogenesis.²⁸ VEGF is a key mediator of angiogenesis and also participates in the induction of growth factors in the regenerating liver.^{29,30} It indirectly promotes hepatocyte proliferation by stimulating HGF production in sinusoidal endothelial cells (via VEGF receptor 1),³¹⁻³³ the transient inhibition of HGF observed at 24 hours in the continuously treated animals may reflect the inhibition of endothelial VEGFR-1 by sorafenib. Endothelial cell proliferation, migration, and survival is mediated by VEGFR-2.^{34,35} Mice heterozygous for VEGFR-2 were reported to maintain normal proliferative capacity of the parenchyma and the sinusoidal endothelial cells following

partial hepatectomy.³⁶ We observed a pharmacodynamic effect of elevated VEGF levels in the liver of animals treated with sorafenib. Interestingly, sorafenib treatment alone, prior to surgical intervention, had already induced an increase in VEGF levels at baseline (0 hours). Hepatocytes are the source of VEGF in the regenerating liver, but VEGF can be produced by most cells in mammals.²⁹ The increase in VEGF levels observed at 3 and 5 days after hepatectomy in the mice receiving sorafenib treatment are likely produced by the replicating hepatocyte population; the source of elevated VEGF prior to liver resection may be diverse, accounting for a systemic adaptation of impeded VEGF signaling elicited by sorafenib. Similarly, sunitinib, a multitarget receptor tyrosine kinase inhibitor, was reported to induce increases in VEGF levels and other proangiogenic factors in mice.³⁷

Sorafenib treatment did have an effect on liver mass restoration in the animals receiving the drug postoperatively, independent of drug administration prior to surgery or starting the day after the operation. Liver regeneration was impaired in these mice, albeit mildly and only at a late timepoint. Similar observations were noted in a study looking at the effects of anti-VEGF therapy after partial hepatectomy.³⁸ For the earlier timepoints studied, the difference in liver mass recuperation was not significant, suggesting that inhibition of the RAS/MAPK/ERK pathway and the VEGFR kinase is not critical to initiate liver regeneration, but plays a role in sustaining the process. A possible explanation for the late appearance of the antiangiogenic effect is that, chronologically, replication of endothelial cells follows replication of hepatocytes.³⁹ On the other hand, an earlier effect concerning hepatocyte proliferation was observed, as assessed by BrdU incorporation. The proliferation assay showed significantly reduced DNA synthesis at early timepoints (24 and 72 hours), pointing to an inhibitory effect on parenchyma restitution. Considering clinical settings, these findings may be of importance for patients receiving sorafenib while being treated with a local ablative therapy such as transarterial chemoembolization (TACE) or radiofrequency ablation (RFA). It also may be of relevance for patients who are subjected to portal vein ligation to induce a compensatory hypertrophy in view of a hemihepatectomy.

An important finding of our work is that sorafenib stopped the day before surgery had no impact on liver regeneration in this preclinical study. It did not impair hepatocyte proliferation nor ERK phosphorylation; only the hepatic VEGF levels were increased at baseline, returning to control values as early as 1 day

postoperatively. The compound sorafenib is a competitive inhibitor, implying reversibility of its actions, and has a half-life of 25-48 hours^{40,41} in man. These findings suggest that patients receiving sorafenib while waiting for liver transplantation may receive a small-for-size liver, without having a negative effect of prior sorafenib treatment on liver size adaptation. Further, considering an indication for sorafenib as a neoadjuvant treatment, our data suggest that drug administration may be continued until the day preceding surgery without compromising liver mass recuperation.

Wound healing was affected in mice treated with sorafenib after surgical intervention. Scar tissue of the muscular abdominal wall revealed histological differences that we were able to quantify. Wound healing was also markedly affected at the skin level. The inhibitory effect of sorafenib on vasculogenesis is a plausible explanation for these observations; another mechanism may be the inhibition of ERK phosphorylation. Mice carrying a conditional c-Met mutant show impaired wound healing of the skin.⁴² The c-Met mutated keratinocytes are unable to form a hyperproliferative epithelium, essential for the reepithelialization of the wound. These keratinocytes show altered signal transduction, including markedly reduced ERK1/2 phosphorylation, upon growth factor stimulation. In order to determine the impact of sorafenib on wound healing in a clinical setting, prospective studies are needed; however, it is recommended that sorafenib be discontinued prior to surgery. The findings of this preclinical study suggest there is a potential for an increased rate of complications if treatment is not discontinued prior to, or started rapidly after, surgery. The complications we observed consisted mainly of scar dehiscence presenting as incomplete sealing of the abdominal wall. This study could not evaluate parameters relevant for transplantation such as vessel suture remodeling of the hepatic branches and the biliary system.

We showed that sorafenib does have an effect on cytokine levels after partial hepatectomy. Interestingly, the levels of VEGF increased under sorafenib treatment. This is an important finding because plasma VEGF levels have been reported to correlate with tumor VEGF expression and to have a prognostic significance in HCC patients treated by resection or TACE.⁴³⁻⁴⁶ The interpretation of VEGF as a parameter to predict recurrence and patient outcome should take into account a concomitant therapy with sorafenib.

Our study has limitations. It was performed in an animal model and it is unclear how it will translate in the clinic. It was performed in noncirrhotic animals, when most of the patients affected by HCC have

underlying cirrhosis. Cirrhosis impairs liver regeneration; whether this is further affected by sorafenib is unknown and is not addressed by our experimental model. According to guidelines, HCC resection is possible only in highly selected cirrhosis patients with normal bilirubin and no portal hypertension. Our data do not allow us to predict the effect of sorafenib in such a setting. Also, our model does not take into account other patient characteristics such as concomitant metabolic disorders, i.e., fatty liver disease or diabetes, susceptible to interfere with normal liver regeneration or wound healing.

In conclusion, this is the first preclinical study analyzing sorafenib and liver regeneration. To date there have been no clinical reports on liver regeneration under sorafenib treatment. We show that sorafenib affects late liver mass restoration when it is administered after surgery, although this effect is mild in our murine model. Liver regeneration is not impaired by sorafenib when treatment is discontinued before intervention.

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