Everolimus Augments the Effects of Sorafenib in a Syngeneic Orthotopic Model of Hepatocellular Carcinoma

Anne-Christine Piguet¹, Bettina Saar², Ruslan Hlushchuk⁴, Marie V. St-Pierre¹, Paul M.J. McSheehy⁵, Vesna Radojevic¹, Maresa Atthinos¹, Luigi Terracciano⁶, Valentin Djonov⁴, and Jean-François Dufour¹,³

Abstract

Sorafenib targets the Raf/mitogen-activated protein kinase, VEGF, and platelet-derived growth factor pathways and prolongs survival patients in advanced hepatocellular carcinoma (HCC). Everolimus inhibits the mammalian target of rapamycin, a kinase overactive in HCC. To investigate whether the antitumor effects of these agents are additive, we compared a combined and sequential treatment regimen of everolimus and sorafenib with monotherapy. After hepatic implantation of Morris Hepatoma (MH) cells, rats were randomly allocated to everolimus (5 mg/kg, 2×/week), sorafenib (7.5 mg/kg/d), combined everolimus and sorafenib, sequential sorafenib (2 weeks) then everolimus (3 weeks), or control groups. MRI quantified tumor volumes. Erk1/2, 4E-BP1, and their phosphorylated forms were quantified by immunoblotting. Angiogenesis was assessed in vitro by aortic ring and tube formation assays, and in vivo with Vegf-a mRNA and vascular casts. After 35 days, tumor volumes were reduced by 60%, 85%, and 55%, relative to controls, in everolimus, the combination, and sequential groups, respectively (P < 0.01). Survival was longest in the combination group (P < 0.001). Phosphorylation of 4E-BP1 and Erk1/2 decreased after everolimus and sorafenib, respectively. Angiogenesis decreased after all treatments (P < 0.05), although sorafenib increased Vegf-a mRNA in liver tumors. Vessel sprouting was abundant in control tumors, lower after sorafenib, and absent after the combination. Intussusceptive angiogenic transluminal pillars failed to coalesce after the combination. Combined treatment with everolimus and sorafenib exerts a stronger antitumoral effect on MH tumors than monotherapy. Everolimus retains antitumoral properties when administered sequentially after sorafenib. This supports the clinical use of everolimus in HCC, both in combination with sorafenib or after sorafenib.

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Introduction

Sorafenib is the only drug for which randomized control trials have shown an improved survival in advanced hepatocellular carcinoma (HCC; refs. 1, 2), and is the only systemic targeted therapy approved for clinical use in many countries. Sorafenib inhibits the kinase activity of Raf, an enzyme operative within the mitogen-activated protein kinase (MAPK) signaling pathway and inhibits the VEGF receptors (VEGFR) and platelet-derived growth factor receptor-β (PDGF-β). In many cases of HCC, Ras kinase is overexpressed or mutated and the Raf/MAPK pathway is activated (3). As a result of the inhibition of these target molecules, sorafenib decreases tumor microvessel density and exerts an antiproliferative effect on tumor cells (4). Despite these actions, sorafenib only extends the life expectancy of patients with HCC by a few months, suggesting that other signaling pathways remain active.

Additional pathways implicated in tumorigenesis include those signaling through phosphoinositide 3-kinase (PI3K)/Akt/mTOR, WNT/β-catenin, insulin-like growth factor, hepatocyte growth factor/c-met proto-oncogene and growth factor–regulated angiogenic signaling (VEGF, PDGF, epidermal growth factor; ref. 5). In this study we focused on the PI3K/Akt/mTOR signaling cascade. The mTOR, which is the downstream target of the serine/threonine kinase Akt, increases protein synthesis and cell proliferation in response to growth factors. Pharmacologic inhibition of mTOR by rapamycin and its analogues arrests the cell cycle by abrogating the PI3K/Akt-mediated proliferative signals. Moreover, mTOR inhibitors reduce the expression of VEGF, which is associated with tumor angiogenesis (6). We reported previously that
inhibition of mTOR significantly slows tumor growth, impairs the tumor angiogenesis that occurs by sprouting, and improves survival in an experimental HCC model (6). Everolimus, a rapamycin analogue, is the only mTOR inhibitor currently under investigation in clinical HCC trials, either as monotherapy or combined with other therapeutic options, such as bevacizumab, sorafenib, and transarterial chemoembolization with doxorubicin. As the activities of mTOR inhibitors and sorafenib occur at separate stages along 2 signaling pathways, their combination could be complementary and provide more effective suppression of HCC. Although the combination of sorafenib and rapamycin has shown synergistic inhibition of HCC xenografts (7), important information is lacking with respect to the mechanisms of this synergism and the specific effects of the drug combination on angiogenic processes. Additional uncertainties relate to the most effective means of administering the drug combination and whether patients who have been unresponsive or intolerant to sorafenib could subsequently benefit from an mTOR inhibitor.

We asked whether the antiproliferative and antiangiogenic properties of everolimus and sorafenib in liver tumors are additive when administered in combination, whether their concomitant use improves survival, and whether administration of everolimus sequentially after sorafenib is beneficial. We chose an orthotopic syngeneic rat model of HCC and examined the effects of everolimus sequentially after sorafenib. We used an immunohistochemical marker to measure HIF-1α expression and its role in tumor angiogenesis. Our results provide a rationale for combining everolimus with sorafenib in HCC.

Materials and Methods

Animals and surgical procedures

Animal experiments were approved by the Local Animal Use Committee. The livers of male American Cancer Institute rats (Harlan), 10 to 12 weeks old, were surgically implanted with tumors derived from Morris hepatoma (MH)-3924A cells as previously described (6, 8).

Animal treatment protocol

On day 6 after tumor implantation, the rats were randomized to a group receiving either everolimus (5 mg/kg/2 × week; Novartis; structural formula in Supplementary Fig. S1A), sorafenib (7.5 mg/kg/d; Bayer HealthCare Pharmaceuticals; structural formula in Supplementary Fig. S1B), the combination of everolimus and sorafenib, or the successive treatment of sorafenib for 2 weeks followed by everolimus for 3 weeks, or to a control group. Drugs and vehicle were administered by gavage. Rats were euthanized on day 42 after tumor implantation. In a second series of experiments designed to measure survival, animals were treated until the appearance of signs of wasting or suffering that indicated distress (deterioration of the general state of the animals, loss of weight greater than 20%, severe piloeruption, hardier gland secretion, abnormal posture, and behavior), at which point they were euthanized. The investigators were blinded to treatment allocation.

MRI

Liver MRI with a commercial 3.0 Tesla system (TIM TRIO; Siemens) of tumors was first done on day 11 after tumor implantation and weekly thereafter. All animals received food and water ad libitum. Animals were anesthetized and placed prone and head first in an 8-channel-wrist coil. After visualization of the liver, a volume-adapted high-resolution T2-weighted Turbo Spin Echo sequence with fat suppression was acquired in the coronal plane and repeated in the axial acquisition direction (repetition time/echo time max/66; voxel size 0.3 × 0.3 × 2 mm³, matrix 384, turbo factor 14, acquisition time 4.48 minutes). Data were analyzed on a postprocessing workstation (Leonardo; Siemens). The largest diameter of the tumor was measured in 3 planes perpendicular to each other. The volume of tumor ellipsoids in mm³ was calculated as: \(4/3 \times \pi \times r_1 \times r_2 \times r_3\) (with \(r_1, r_2,\) and \(r_3\) representing perpendicular radii of the lesion).

Immunohistochemistry

Tumor necrosis was assessed by staining tumor sections with Giemsa and by quantifying the necrotic area by using the Metamorph software. Tumoral apoptosis was measured in paraffin-embedded sections by cleaved caspase 3 immunostaining (Cell Signaling). Tumoral hypoxia was measured in paraffin-embedded sections by hypoxia-inducible factor (HIF)-1α immunostaining (Abcam) with classification as follows: 0, no HIF-positive tumoral cells; 1, <10% HIF-positive tumoral cells; 2, 10%–20% HIF-positive tumoral cells; 3, 20%–50% HIF-positive tumoral cells; 4, >50% HIF-positive tumoral cells. Tumoral invasiveness was assessed in paraffin-embedded sections by immunohistochemical staining for E-cadherin (Abcam).

Real-time quantitative PCR

Total RNA was extracted from liver by an RNeasy Mini Kit (Qiagen) then was reverse transcribed by SuperscriptIII Reverse Transcriptase (Invitrogen) and a random hexamer mix. The probe and primers for Vegf-a were obtained from TaqMan Gene Expression Assays (Applied Biosystem) and quantitative PCR was carried out by using an ABI PRISM 7500 Sequence Detection System and the TaqMan universal PCR Master Mix, according to standard protocols. The \(C_t\) for each gene were standardized against ribosomal RNA (18s) to obtain the \(\Delta C_t\) values. The \(\Delta C_t\) values were calculated by subtracting the \(C_t\) values of animals treated with vehicle from \(C_t\) values of rats treated with the different drugs. Relative fold increases or decreases were calculated by the formula, \(2^{-\Delta C_t}\). All reactions were carried out in triplicate.

Vascular casting

As previously described (9), the liver vasculature was perfused with a freshly prepared solution of Mercox...
(Vilene Company) containing 0.1 mL accelerator per 5 mL resin. One hour after perfusion, the tumors were excised and macerated in 15% potassium hydroxide. After 3 to 4 weeks, the casts were washed and dehydrated in ethanol and desiccated under vacuum. Samples were layered with gold to a thickness of 10 nm and examined in a Philips XL 30 FEG scanning electron microscope.

**Cells and culture conditions**

MH-3924A cells were obtained from DKFZ and not further authenticated. Isolated rat aortic endothelial cells were cultured as described by Semela and colleagues (6) and characterized by immunofluorescence with antibodies to von Willebrand factor/factor VIII and CD31 (platelet/endothelial cell adhesion molecule). Human hepatic sinusoidal endothelial cells (HHSEC; ScienCell Research Laboratories) were characterized by the supplier by immunofluorescence with antibodies to von Willebrand factor/factor VIII and CD31 (platelet/endothelial cell adhesion molecule).

**3H-Thymidine incorporation assay**

MH and rat aortic endothelial cells were serum-starved overnight and then incubated with various concentrations of everolimus and sorafenib for 24 hours. 3H-Thymidine (0.2 μCi/mL; Amersham Biosciences) was added in the presence of drugs and the incubation continued for 24 hours. Cell proliferation was measured by counting the incorporation of 3H-thymidine. Experiments were repeated 3 times in triplicate.

**Rat aortic ring assay**

Aortic rings were prepared as previously described (6). Everolimus and/or sorafenib were added 24 hours after preparation and rings were incubated for 5 days. For sequential drug treatment, rings were incubated with sorafenib for 2 days, the medium was changed, and everolimus was added. At day 5, the rings were fixed and stained according to a Diff-Quick solution II protocol (Diff-Quick Stain Set; Baxter-Dade AG). Vascular outgrowth was quantified by counting the sprouts.

**Tube formation assay**

HHSEC (4 × 10⁵) were incubated in 24-well plates coated with Matrigel in the presence or absence of everolimus and/or sorafenib. After 72 hours, the area covered with vascular tubes was quantified by using the Metamorph software (Molecular Devices). Experiments were conducted 3 times in duplicate.

**Immunoblot analysis**

Liver tissue was homogenized in radioimmunoprecipitation assay buffer [150 mmol/L NaCl, 1% NP-40, 0.5% Na-deoxycholate, 0.1% SDS, and 50 mmol/L Tris-HCl (pH 7.4)] containing protease and phosphatase inhibitors (Roche). Protein concentration was assayed according to Lowry (10). Equal amount of proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes, blocked for 1 hour with 5% nonfat milk, then incubated overnight at 4°C with cleaved caspase 3, phospho-Erk1/2, phospho-4E-BP1, phospho-Elk1, and phospho-Akt (Ser473) antibodies (Cell Signaling). After washing, the membranes were incubated with peroxidase-conjugated secondary antibody (Pierce) and signals were revealed by using an enhanced chemiluminescence detection system (Perkin Elmer) and a Fujifilm LAS.100 CCD camera coupled to a computer by using the AIDA 2.1 software (Raytest). Membranes were stripped and reincubated with antibodies against total Erk1/2, 4E-BP1, Elk1, and Akt (Cell Signaling). Membranes were stripped again before incubation with anti-β-actin antibody (Sigma-Aldrich Chemie GmbH) and protein was normalized for actin expression.

**Statistical analysis**

Data points represent the mean values ± SD. Data were compared by applying the nonparametric Mann-Whitney U test. *P* < 0.05 was considered statistically significant.

**Results**

**Effect of everolimus–sorafenib on tumor progression**

MRI 35 days after tumor implantation showed smaller tumors in the treated livers than in the untreated livers (Fig. 1A). Tumor volumes were significantly smaller in the combined sorafenib–everolimus group than all other groups (*P* < 0.01 vs. control; *P* < 0.05 vs. sorafenib; *P* < 0.01 vs. everolimus and vs. sequential everolimus–sorafenib; Fig. 1B). The sequential sorafenib–everolimus treatment and everolimus monotherapy were equally effective. Monotherapy with sorafenib was the least effective. At harvest, tumor sizes were 166 ± 53 mm³ after combined sorafenib and everolimus, 421 ± 84 mm³ after everolimus, 511 ± 183 mm³ after sorafenib for 2 weeks then everolimus for 3 weeks, 805 ± 317 mm³ after sorafenib, and 1,139 ± 238 mm³ for the untreated group. The median survival was longest for the combined everolimus–sorafenib group (70 days, *P* < 0.001 vs. control and sorafenib groups), shorter for sorafenib monotherapy (63.5 days), and shortest for the controls (57 days; Fig. 1C). Rats tolerated the treatments for the duration of the study (42 days). The body weights ranged from 234 ± 12 g for the combination group to 273 ± 14 g for the control group. However, the longer treatment imposed by the design of the survival study was associated with tooth fractures in the rats treated with sorafenib. Tooth fractures were noted after 8 weeks, and could have caused a secondary weight loss. Although the extent of tumor necrosis was not significantly affected by any of the treatments, a higher trend of necrosis was noted in the combined everolimus–sorafenib group (Fig. 2A; Supplementary Fig. S2A). Treatment with everolimus or sorafenib was associated with apoptosis, more so after sorafenib, as
assessed by immunoblots and immunohistochemical detection of cleaved caspase 3 (Fig. 2B and C). The degree of hypoxia in tumors was estimated by HIF-1α immunostaining. The number of HIF-1α-positive tumor cells was higher in the tumors treated with everolimus, sorafenib, and the combination than in the control group (P < 0.01). The number of HIF-1α-positive cells was also significantly greater in the sequential tumors treated with sorafenib–everolimus than in controls but achieved a lower statistical score (P < 0.05; Fig. 2D; Supplementary Fig. S2B).

Antiproliferative and antiangiogenic effects of everolimus–sorafenib in vitro

Relative to control conditions, the proliferation of rat endothelial cells decreased by 40% in the presence of everolimus (20–200 nmol/L). The effect was not dose dependent and was not potentiated by the addition of sorafenib (Fig. 3A). Sorafenib alone from 100 nmol/L to 10 μmol/L had no effect. In contrast, the proliferation of hepatoma MH cells was insensitive to everolimus alone (20–2,000 nmol/L). Sorafenib alone was antiproliferative only at the highest concentration (10 μmol/L). The addition of everolimus decreased the minimal effective concentration of sorafenib to 5 μmol/L (Fig. 3B).

The effect of sorafenib and everolimus on angiogenesis in vitro was measured in 2 ways. In the aortic ring assay, everolimus 200 nmol/L and sorafenib 100 nmol/L alone significantly decreased sprouting from aortic rings by 60% (Fig. 4A). The combination of drugs, either in a concomitant or sequential regimen, decreased sprouting even further (Fig. 4A). In fact, vessel sprouting was most inhibited by the sequential sorafenib–everolimus treatment protocol. The tube formation assay measures the ability of endothelial cells to form a linear structure and in contrast to the ring aortic assay, operates without the confounding influences of pericytes and fibroblasts. Endothelial tube formation was significantly impaired by everolimus both in monotherapy and in combination (Fig. 4B). Sorafenib had no effect in this assay.

Effect of combined everolimus–sorafenib on tumors in vivo

Compared with controls, the mRNA levels of Vegf-a were increased by 86% after sorafenib (P < 0.05) although this effect was blunted to 49% on addition of everolimus.
Everolimus alone did not produce a significant effect in Vegf-a mRNA. Everolimus alone decreased phosphorylation of 4E-BP1 in total tumor tissue, whereas the addition of sorafenib tended to blunt this effect (Fig. 5B; Supplementary Fig. S3A). Sorafenib alone decreased the phosphorylation of Erk1/2 in tumors but this effect was lost in combination with everolimus (Fig. 5B; Supplementary Fig. S3B). Sorafenib alone also decreased the phosphorylation of the Erk1/2 target, Elk1, in tumors (Supplementary Fig. S3C and D). The phosphorylation of Akt on Ser473, which is a site phosphorylated by the complex mTORC2, was increased in the tumors treated with sorafenib and everolimus (Fig. 5C; Supplementary Fig. S3E).

Vascular casts revealed abundant sprouting in tumors of the control group, whereas sprouting was reduced in sorafenib-treated tumors and absent in tumors treated with everolimus and sorafenib (Fig. 6A). Vessels were pierced by pillars in the sorafenib-treated tumors as a sign of nonsprouting intussusceptive angiogenesis. In the combination group, pillars were frequent but remained small and were positioned irregularly. Histologic analysis of the periphery of the tumor and application of a technique of digital quantification showed that in the combination group, an invasive front intercalated extensively into the surrounding tissue (Fig. 6B; Supplementary Fig. S4A). In contrast, the periphery of the tumors in the control group was regular and limited by a capsule. The tumor periphery was examined by immunohistochemical staining for expression of E-cadherin. In the tumors cells, E-cadherin was prominent and cytoplasmic regardless of the treatment group. In adjacent nontumoral hepatocytes at the interface, a peripheral membrane staining of E-cadherin was noted but only

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**Figure 2.** Effect of the combination of everolimus (Evero) and sorafenib (Sora) on tumor necrosis (A), tumor apoptosis (B and C), and tumor hypoxia (D). A, extent of necrosis, assessed by Giemsa staining, in tumors of rats implanted with MH cells. The percentage of necrotic area relative to the total area was quantified. The necrotic area was similar in all groups although necrosis was slightly but not significantly increased in the combination group (n = 6 for vehicle and sequential treatments, n = 5 for everolimus and sorafenib treatments, and n = 4 for combination treatment), B and C, apoptosis in tumoral tissue assessed by the presence of cleaved caspase 3. Immunoblots of cleaved caspase 3 (Cl-caspase 3; B) were compared in liver tumors from rats untreated (vehicle) and treated with everolimus and sorafenib, alone or in combination. From each treatment group, 2 to 3 representative immunoblots are shown. Actin was used as a loading control. Immunostaining for cleaved caspase 3 (C; magnification, ×10) in tumors representing untreated (vehicle) and treated groups. Compared with the untreated group, the amount of cleaved caspase 3 tended to increase in tumors of animals treated with everolimus, sorafenib, the combination of both drugs, or with the sequential treatment. D, hypoxia of liver tumors assessed by HIF-1α immunostaining (magnification, ×10). Compared with the untreated group, the number of HIF-1α-positive cells was significantly increased by everolimus, sorafenib, the combination of drugs, and the sequential treatment.
in the control group. This feature was not seen in the hepatocytes adjacent to the invading tumor cells in the everolimus–sorafenib group (Supplementary Fig. S4B). Despite the invasive characteristics of the limiting edges of the treated tumors, histologic analyses of the lungs of animals from all groups indicated no distant metastases.

Discussion

We previously reported that inhibition of the mTOR pathway, which is activated in many cases of HCC (11–13), decreases VEGF levels, impairs tumor angiogenesis, and results in smaller tumors and longer survival in a rat MH-3924A model of HCC (6). We now show the benefits of combining an mTOR inhibitor with sorafenib, an inhibitor of B-Raf and Raf-1 kinases, as well as VEGF and PDGF receptor. Our findings show that combined everolimus and sorafenib is a more potent antitumor regimen than either agent alone, exerts a stronger anti-angiogenic effect than either agent alone, and improves survival in this HCC model. Everolimus also retains its antitumoral potency in vivo when administered sequentially after sorafenib, a finding that carries important clinical implications. Our positive results contrast

Figure 3. Effect of the combination of everolimus and sorafenib on rat aortic endothelial and MH cell proliferation. A, cell proliferation was measured by \(^{3}H\)-thymidine incorporation in rat aortic endothelial cells. Sorafenib did not affect proliferation of aortic endothelial cells. Everolimus 20 nmol/L significantly decreased proliferation by 40%. There was no additive effect when sorafenib and everolimus were combined. *, \(P < 0.05\) vs. complete medium. B, in MH cells, sorafenib 10 \(\mu\)mol/L inhibited the proliferation, in contrast to everolimus. The minimum effective concentration of sorafenib was decreased to 5 \(\mu\)mol/L on the addition of everolimus. Experiments were carried out 3 times in triplicate. *, \(P < 0.05\) vs. complete medium; #, \(P < 0.05\) vs. sorafenib 5 \(\mu\)mol/L; †, \(P < 0.05\) vs. sorafenib 10 \(\mu\)mol/L; and $, \(P < 0.05\) vs. everolimus 20 nmol/L + sorafenib 10 \(\mu\)mol/L. DMSO, dimethylsulfoxide.
with those of Newell and colleagues, who found no difference in tumor growth with this combination (14). This disparity is perhaps explained by our propitious choice of an orthotopic syngeneic model, which is a closer representation of HCC than the xenograft model chosen by previous investigators (7, 14, 15).

HCC is a hypervascular tumor, relying on angiogenesis for growth (16). Focal hypoxia is a potent angiogenic stimulus and both everolimus and sorafenib treatment regimens exerted their antitumoral effects within a local environment that was subject to such stimuli, as shown by the increased expression of HIF-1α in all treated tumors (Fig. 2D). The upregulation of Vegf-a mRNA in the tumor by sorafenib, an effect also reported with other receptor tyrosine kinase inhibitors such as vatalanib (17) and sunitinib (18), can be attributed to a feedback response to the suppressed VEGF receptor signaling (19). Despite this, hypoxia-driven neovascularization was not seen in the treated liver tumors. Rather, everolimus and sorafenib impaired angiogenesis and altered the structure of the tumor vascular architecture (Fig. 6A). In keeping with our previous findings, where inhibition of mTOR with sirolimus effected a switch in tumor angiogenesis from sprouting to intussusception (6), the combination of everolimus–sorafenib promoted an increase in the number of vascular pillars but these pillars remained small, which suggests an inability to fuse, and consequently an impairment of the process of intussusception as a means of tumor vascularization. We ascribe the superior ability of the everolimus and sorafenib
combination to slow tumor growth to this impaired tumor angiogenesis and vascularization.

The innate resistance of the MH-3924A cells to the antiproliferative actions of mTOR inhibition and the low sensitivity to Raf/Erk (extracellular signal regulated kinase) blockade in vitro (Fig. 3B) did not preclude a response to everolimus and sorafenib when these cells were implanted as solid tumors in vivo. Other investigators have reported similar antitumoral responses when insensitive cell lines were seeded in vivo (20, 21). The everolimus mediated decrease in proliferation and migration of endothelial cells in vitro and impairment of vessel sprouting point to antiangiogenesis as the means by which the resistant tumors became sensitized to mTOR inhibition in vivo. Lane and colleagues postulated that the antiangiogenic effects of everolimus were due to the combination of a reduced VEGF production in tumor cells and direct action on mTOR signaling in nontumor pericytes and endothelial cells (20). We have previously reported that another mTOR inhibitor, sirolimus, decreased VEGF-a in MH-3924A–derived tumors under different experimental circumstances (6). A similar reduction was not detected with everolimus, although the sorafenib-induced increase in VEGF-a mRNA tended to be less acute in the presence of everolimus (Fig. 5A). Our findings appear more consistent with inhibition of mTOR signaling in endothelial cells (Figs. 3A, 4). The basis for the resistance of MH-3924A cells to everolimus has not been investigated. The presence of an oncogenic mutation in PI3K catalytic a-subunit and PTEN loss of function has been linked to mTOR inhibitor sensitivity (22). Conversely, selected K-Ras mutations have been linked to mTOR inhibitor insensitivity, as has overexpression of the myc oncogene (23). The MH-3924A cells likely overexpress K-Ras because gene amplification was detected in several MH cell lines (24) but a more extensive genotyping is required to fully explain the response to mTOR signaling inhibition. Genotyping may also explain the basis for the low sensitivity of MH-3924A cells to sorafenib (Fig. 3B). Tumor cell lines containing an activating receptor tyrosine kinase mutation are more sensitive to sorafenib whereas cell lines in which multiple signaling pathways drive growth are less sensitive (25).

Figure 5. Effect of everolimus (Evero) and sorafenib (Sora) treatment on gene expression of Vegf-a and on the phosphorylation of target proteins in tumoral tissue. A, tumoral mRNA expression of Vegf-a. mRNA was extracted from liver tumors and the level of Vegf-a was measured by quantitative real-time PCR. Compared with the vehicle (control) group, Vegf-a mRNA increased after sorafenib. *, P < 0.05 vs. vehicle. Tumors treated with the combination of everolimus and sorafenib expressed higher mRNA levels of Vegf-a than everolimus alone. ††, P < 0.05 vs. everolimus; n = 6 for sequential treatment, n = 5 for vehicle and everolimus treatments, n = 4 for sorafenib and combination treatment. B, effect of treatment on the expression and phosphorylation of 4E-BP1 and Erk1/2 in tumors. Immunoblots of phosphorylated 4E-BP1 and Erk1/2, which are target proteins of everolimus and sorafenib, respectively, were quantified. The membranes were then stripped and reprobed with antibodies against 4E-BP1 and Erk1/2 to measure total levels of each protein. Actin was used as a loading control. The immunoblots shown are representative of each group. C, effect of treatment on the phosphorylation of Akt (Ser473) in tumoral tissue. The phosphorylation of Akt (Ser473) was assessed by immunoblotting. The membrane was then stripped and reprobed with antibodies against Akt to measure the total level of the protein. Actin was used as a loading control. The immunoblots shown are representative of results from each group.
multiple signaling pathways are likely important for proliferation of MH-3924A cells. Sorafenib can also exert antitumor actions independent from the MEK/ERK pathway. Sorafenib is apoptotic in tumor tissue (Fig. 2B and C), an effect likely explained by the reduced phosphorylation of the initiation factor eIF4E and downregulation of the antiapoptotic protein Mcl-1 (25). Sorafenib blocks the VEGFR-2 receptor, an effect which could be linked to antiangiogenesis through decreased endothelial cell survival. Endothelial cell survival is assured through antiapoptotic signaling, which normally occurs through Akt/protein kinase B via the PI3K-dependent pathway and upregulation of antiapoptotic protein signaling (26). In support of this, sorafenib decreased the phosphorylation of Akt in endothelial cells in vitro (data not shown).

The molecular mechanism by which everolimus and sorafenib combine to exert effective antitumor activity in MH-3924A-derived tumors has been partly elucidated in our studies. The combination therapy did increase the pAkt Ser473/Akt ratio in our tumor model (Fig. 5C; Supplementary Fig. S3), which was likely because of the mTOR complex 2–dependent phosphorylation of Akt in tumoral cells. However, a higher AKT phosphorylation is not necessarily incompatible with a reduction in tumor growth (27, 28). In fact, induction of p-AKT secondary to mTOR inhibition was shown to be independent of the antiproliferation cellular response to everolimus and modulation of AKT phosphorylation alone does not predict effects on downstream signaling (28). Therefore, the antitumoral benefit of the combined treatment in our tumor model depends on pathways unrelated to the tumoral Akt signaling. The combination of everolimus and sorafenib also annulled the hypophosphorylation of 4E-BP1 elicited by everolimus alone in tumor tissue (Supplementary Fig. S3). However, the phosphorylation of the ribosomal protein S6 was completely inhibited in tumors treated with both everolimus and the combination (data not shown), which confirms the pharmacologic inhibition of the mTOR complex 1. As the MH-3924A cell line is insensitive to everolimus, and inhibition of S6 kinase and hypophosphorylated 4E-BP1 has been shown in cell lines that were both sensitive and resistant to everolimus (28), we conclude that the ratio p4E-BP1/4E-BP1 in total tumoral tissue is not an adequate pharmacodynamic marker for the antitumoral effects of combined everolimus and sorafenib. The addition of everolimus to sorafenib annulled the effect of sorafenib on the phosphorylation of Erk1/2 and downstream pElk1 (Supplementary Fig. S3). One explanation is that Erk1/2 may be phosphorylated by a kinase other than Raf. A second explanation is that the phosphorylation of Erk1/2 could be regulated by cross-talk between PI3K/Akt and MAPK signaling due to a feedback loop affecting the S6K-PI3K-Ras pathway (29). The increase in Erk...
phosphorylation observed after the combined everolimus and sorafenib is not incompatible with better antitumoral properties because of the likelihood that MEK/ERK-independent mechanisms are responsible for the reduced growth of MH-3924A tumors. We argue that everolimus and sorafenib together reduced tumor growth in vivo more effectively than monotherapies primarily because of the combined effects of inhibition of mTOR signaling in endothelial cells and perhaps in nontumor pericytes (20), and of sorafenib-induced tumor cell apoptosis and reduced antiapoptotic signaling in endothelial cells and perhaps in other supportive cells of the vasculature.

A comparison of the peripheral tumor regions revealed distinct histologic differences between treatment and control groups. The tumor front, which was linear and often encapsulated in the control group, appeared irregular and invasive after treatment, and prominently intermingled with the surrounding tissue to produce isolated islands of tumor tissue. These features were particularly evident in the combination group. Although this suggested a more invasive tumor phenotype (30), no distant metastases were detected in any of the animals at the time of harvesting. We speculate that the treated tumors differed from the untreated tumors because the treated tumors have evolved in an antiangiogenic environment and must rely on the blood supply at the peripheral edges to support growth. Whether the differences in staining pattern of E-cadherin at the tumor interface influence the pattern of invasiveness observed is not known at this point (31).

Despite a superior reduction in tumor growth, the effect of the everolimus–sorafenib combination on the median survival remained modest in comparison with the other treatment options. Our survival study was designed with conditions wherein rats were euthanized when explicit endpoint criteria linked to distress had been reached. Because the endpoint criteria would have precipitated termination of the study for the distressed animals, the effect of the combination everolimus–sorafenib on the median survival was modest. We would expect a larger clinical effect on the median natural survival of patients.

The sequential administration of everolimus after sorafenib may be clinically useful in certain circumstances. In clinical trials, 40% of the HCC patients treated with sorafenib develop side effects severe enough to warrant discontinuation of the treatment. Such patients deprived of targeted therapy are then exposed to a rebound effect, which has been shown experimentally, although no mechanism was postulated (32). This rebound effect could be provoked partly by an increased concentration of growth factors such as VEGF, as we report here, which may fuel tumor growth if left unopposed. Our results offer reassurance that patients can still benefit from an alternative systemic targeted therapy after sorafenib and that everolimus can still exert its antiangiogenic effects. However, the extent of the clinical improvement that can be offered to patients remains to be verified. One must also carefully consider whether the combination of an inhibitor of mTOR and sorafenib will be tolerated by patients with liver cirrhosis.

In conclusion, our results present mechanistic insights into the treatment of HCC with everolimus either in combination with sorafenib or in subsequent treatment and provide the experimental basis for testing this combination in clinical trials.

Disclosure of Potential Conflicts of Interest

P.M.J. McSheehy is an employee at Novartis. J.-F. Dufour is a consultant with Bayer, Novartis, Roche, and Sanofi and received commercial research grants from Bayer, Novartis, and Roche.

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Anne-Christine Piguet, Bettina Saar, Ruslan Hlushchuk, et al.

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