Combined Tyrosine and Serine/Threonine Kinase Inhibition by Sorafenib Prevents Progression of Experimental Pulmonary Hypertension and Myocardial Remodeling

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Background—Inhibition of tyrosine kinases, including platelet-derived growth factor receptor, can reduce pulmonary arterial pressure in experimental and clinical pulmonary hypertension. We hypothesized that inhibition of the serine/threonine kinases Raf-1 (also termed c-Raf) and b-Raf in addition to inhibition of tyrosine kinases effectively controls pulmonary vascular and right heart remodeling in pulmonary hypertension.

Methods and Results—We investigated the effects of the novel multikinase inhibitor sorafenib, which inhibits tyrosine kinases as well as serine/threonine kinases, in comparison to imatinib, a tyrosine kinase inhibitor, on hemodynamics, pulmonary and right ventricular (RV) remodeling, and downstream signaling in experimental pulmonary hypertension. Fourteen days after monocrotaline injection, male rats were treated orally for another 14 days with sorafenib (10 mg/kg per day), imatinib (50 mg/kg per day), or vehicle (n=12 to 16 per group). RV systolic pressure was decreased to 35.0±1.5 mm Hg by sorafenib and to 54.0±4.4 mm Hg by imatinib compared with placebo (82.9±6.0 mm Hg). In parallel, both sorafenib and imatinib reduced RV hypertrophy and pulmonary arterial muscularization. The effects of sorafenib on RV systolic pressure and RV mass were significantly greater than those of imatinib. Sorafenib prevented phosphorylation of Raf-1 and suppressed activation of the downstream ERK1/2 signaling pathway in RV myocardium and the lungs. In addition, sorafenib but not imatinib antagonized vasopressin-induced hypertrophy of the cardiomyoblast cell line H9c2.

Conclusions—The multikinase inhibitor sorafenib prevents pulmonary remodeling and improves cardiac and pulmonary function in experimental pulmonary hypertension. Sorafenib exerts direct myocardial anti hypertrophic effects, which appear to be mediated via inhibition of the Raf kinase pathway. The combined inhibition of tyrosine and serine/threonine kinases may provide an option to treat pulmonary arterial hypertension and associated right heart remodeling. (Circulation. 2008;118:2081-2090.)

Key Words: hypertension, pulmonary ■ monocrotaline ■ pulmonary heart disease ■ remodeling ■ sorafenib

Pulmonary arterial hypertension is a severe disease characterized by an elevation of pulmonary arterial pressure and progressive right heart failure. Both pulmonary arterial vasoconstriction and structural remodeling of the pulmonary vessels are integral features of the pathological processes contributing to an elevated pulmonary pressure in this disease. Although pulmonary vasodilation has been established as an effective therapy for pulmonary arterial hypertension, the vascular remodeling process remains a target for more causal and comprehensive treatment approaches. A variety of growth factors are involved in the abnormal cellular responses associated with pulmonary remodeling, including platelet-derived growth factor (PDGF), basic fibroblast growth factor, epidermal growth factor, and vascular endothelial growth factor (VEGF). Most growth factor receptors belong to transmembrane receptor tyrosine kinases, which activate the major signaling transduction pathways, including Ras mitogen-activated protein kinase, phosphatidylinositol 3-phosphate kinase, and phospholipase Cγ. On the basis of their role in malignant diseases, the identification of the molecular structure of receptor tyrosine kinases led to the development of several small-molecule inhibitors for the treatment of...
various types of cancer.\textsuperscript{10,11} In pulmonary hypertension, initial experimental studies demonstrated positive effects of epidermal growth factor and PDGF receptor inhibition on hemodynamics, remodeling, and survival.\textsuperscript{3,5} Individual clinical case reports from patients with pulmonary arterial hypertension indicate a beneficial effect of imatinib, which is an inhibitor of the tyrosine kinases PDGF receptor, BCR-ABL, and c-kit and is approved for treatment of chronic myeloid leukemia and gastrointestinal stromal tumor.\textsuperscript{12-14}

**Clinical Perspective p 2090**

In addition to receptor tyrosine kinases, serine/threonine kinases such as the Raf family and its downstream pathways represent an interesting target for intervention in pulmonary arterial hypertension on the basis of their role in vascular smooth muscle and myocardial hypertrophy.\textsuperscript{15,16} Unlike most tyrosine kinases, which are not expressed in the myocardium, serine/threonine kinases have been associated with myocardial hypertrophy. For example, the serine/threonine kinase Raf-1 is a critical determinant of myocardial hypertrophy after aortic banding in mice.\textsuperscript{16} Direct inhibition of these myocardial pathologies may therefore represent an additional tool to improve morbidity and mortality in patients with pulmonary arterial hypertension.

Sorafenib is an inhibitor of the serine/threonine kinases Raf-1 and b-Raf as well as the tyrosine kinases PDGF receptor, VEGF receptor, c-kit, and Flt-3, with IC\textsubscript{50} values between 6 and 70 nmol/L.\textsuperscript{10,17} It is approved for the treatment of renal cell carcinoma and hepatocellular carcinoma. The aim of the present study was to investigate the potential of sorafenib, a multikinase inhibitor, for treatment of experimental pulmonary hypertension. The effects of sorafenib on hemodynamics, vascular remodeling, and biomarker expression were compared with those of imatinib in vivo with the use of monocrotaline-induced pulmonary hypertension in rats and in vitro in the rat cardiomyoblast cell line H9c2.

**Methods**

All animal experiments were performed in accordance with the European Community guidelines for the use of experimental animals and with the German law for the protection of animals.

**Monocrotaline-Induced Pulmonary Arterial Hypertension**

Adult male Sprague-Dawley rats weighing 250 to 280 g were purchased from Charles River Laboratories (Sulzfeld, Germany). Rats were given a single subcutaneous injection of 60 mg/kg monocrotaline (Sigma-Aldrich Chemie GmbH, München, Germany) or vehicle under isoflurane anesthesia (2% vol/vol) as described previously.\textsuperscript{3} Fourteen days after the monocrotaline injection, the animals were randomized to receive sorafenib (10 mg/kg per day), imatinib (50 mg/kg per day),\textsuperscript{3} or vehicle by once daily gavage for 14 days until the day of the final hemodynamic and histological analysis on day 28. Sorafenib (Nexavar, BAY 43-9006) is a small-molecule kinase inhibitor that directly blocks the autophosphorylation of several receptor tyrosine kinases (VEGF receptors 2 and 3, PDGF receptor-\(\beta\), c-kit, and Flt-3) and inhibits downstream Raf serine/threonine kinase isoforms.\textsuperscript{17} Imatinib (Glivec, STI571) is an inhibitor of the tyrosine kinases BCR-ABL, PDGF receptor-\(\beta\), and c-kit.\textsuperscript{18} Pharmacokinetic profiling confirmed that at the doses chosen, oral administration of sorafenib and imatinib yielded free plasma concentrations of ~150 and 500 nmol/L, respectively, for >12 hours per day. These concentrations are well above the IC\textsubscript{50} values for all the aforementioned kinases.

**Hemodynamic Measurements and Tissue Preparation**

On days 14 and 28, respectively, after the injection of monocrotaline, the rats were anesthetized with pentobarbital (60 mg/kg IP). After tracheotomy, anesthesia was maintained by inhalation of isoflurane (1.8% vol/vol) under conditions of artificial ventilation (RUS-1321-RA, FMI Führ Medical Instruments GmbH, Seeheim/Ober-Beerbach, Germany). Flow was set at 0.5, and respiration volume was 10 mL/kg at 60 strokes per minute; inspiration to expiration ratio was 1:1, and the positive end-expiratory pressure was 1.0 cm H\textsubscript{2}O. A Millar microtip catheter (2F, SPR-320, FMI Führ Medical Instruments GmbH) was inserted into the right carotid artery to measure heart rate and systemic arterial pressure. A fluid-filled polyethylene catheter (PE-50) was inserted through the right jugular vein into the right ventricle (RV) for measurement of RV pressure. Cardiac output was measured by transpulmonary thermodilution technique. All hemodynamic measurements were performed with a PowerLab System with the use of Chart 5.0 software with the cardiac output module (AD Instruments GmbH, Spechbach, Germany). After exsanguination, the RV and lungs were snap-frozen on dry ice for RNA extraction and quantitative real-time polymerase chain reaction as well as Western blot analysis.

**Assessment of RV Hypertrophy**

After excision of the heart, its RV wall was separated from the left ventricular (LV) wall and the interventricular septum. The ratio of the weights of the RV to LV plus septum [RV/(LV + S)] was calculated as an index of RV hypertrophy.

**Histological Analysis**

Separate sets of animals were used for histological analysis of the lungs (n=6 per group). The lungs were perfusion-fixed with 10% buffered formalin, followed by immersion in a 3% paraformaldehyde solution. The degree of muscularization of small peripheral pulmonary arteries was assessed by double staining 3-\(\mu\)m sections with an anti-\(\alpha\)-smooth muscle actin antibody (dilution: 1:900, Sigma-Aldrich Chemie) and anti-human von Willebrand factor antibody (dilution: 1:900, Dako, Hamburg, Germany). Sections were counterstained with hematoxylin and examined by light microscopy with a computerized morphometric system (Qwin, Leica, Wetzlar, Germany). At \(\times40\) magnification, 60 to 80 intra-acinar vessels were analyzed per animal by an observer blinded to treatment. Arteries were categorized as muscular (with a complete medial coat of muscle), partially muscular (with only a crescent of muscle), or nonmuscular (no apparent muscle).\textsuperscript{3} The percentage of pulmonary vessels in each category was determined by dividing the number of vessels in that category by the total number counted in the same experimental group.

**Cell Culture**

H9c2 rat cardiomyoblasts (American Type Culture Collection) were cultured in Dulbecco’s modified Eagle’s medium with 10% fetal calf serum and subcultured before confluence. Cultures were utilized before fusion into myotubes occurred and were maintained through passage number 25. Arginine vasopressin is an endogenous peptide hormone and a stimulator of hypertrophy in cardiomyocytes\textsuperscript{19,20} as well as in H9c2 cells.\textsuperscript{21,22} Hypertrophy was induced by treatment of confluent H9c2 cells in 24 wells (2\(\times10^4\) cells) with arginine vasopressin in Dulbecco’s modified Eagle’s medium with 0.25% fetal calf serum. Medium was replaced daily. A dose-dependent hypertrophic effect was found, reaching 13-, 21-, and 19-fold induction of \(\beta\)-myosin heavy chain (MYHCB) expression at 1, 10, and 1000 nmol/L arginine vasopressin, respectively. For the subsequent kinase inhibition experiments, the concentration of arginine vasopressin was set to an excess of 1 \(\mu\)mol/L as described previously.\textsuperscript{21,22} An ATPlow cell viability assay (Promega) demonstrated cell viability rates of >85% at sorafenib or imatinib concentrations...
At concentrations >1 μmol/L, however, viability rates decreased to 4% and 0% (sorafenib) and were 114% and 56% (imatinib) at 3 and 10 μmol/L, respectively. Therefore, the concentrations of the kinase inhibitors were kept at <1 μmol/L in all experiments. We used real-time polymerase chain reaction–based quantification of the mRNA expression of MYHCB and transient receptor protein channel 6 (TRPC6) as markers of hypertrophy.23,24

RNA Extraction and Quantitative Real-Time Polymerase Chain Reaction

Samples of the RV were immediately snap-frozen on dry ice. Total RNA was extracted from RV samples and H9c2 cells with the use of the RNeasy fibrous tissue kit (Qiagen) and checked for integrity on a Bioanalyzer (Agilent). For reverse transcription, 1 μg of total RNA

Table. Primer and Probe Sequences for Real-Time Polymerase Chain Reaction Analysis of Rat ANP, Rat BNP, Rat COL1A2, Rat COL3A1, Rat TIMP1, Rat Osteopontin, Rat MYHCB, Rat TRPC6, and Rat β-2 Microglobulin

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<th>Gene</th>
<th>Symbol</th>
<th>Forward Primer (5′-3′)</th>
<th>Reverse Primer (5′-3′)</th>
<th>Probe (5′-FAM-3′-TAMRA)</th>
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<td>Atrial natriuretic peptide</td>
<td>ANP</td>
<td>CCTCTTCCTGGCCTTTTTG</td>
<td>TGTGTTGGACCCCGACTGT</td>
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<td>Brain natriuretic peptide</td>
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<td>ATCACTTGGAGGTGTCACCA</td>
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<tr>
<td>Collagen, type I, α-2</td>
<td>COL1A2</td>
<td>CCCAGCCAAGAATGACATCAC</td>
<td>TCAAACTGCTGCCCACCAT</td>
<td>TGGCCAGGCAACACAGATGTTCT</td>
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<tr>
<td>Collagen, type III, α-1</td>
<td>COL3A1</td>
<td>TGGGCGGTTTTTCACACATATT</td>
<td>ACTCTCTATTGTCGTTAAACAGACCTTG</td>
<td>CCAATGCAATTTCAAGACACTGA</td>
</tr>
<tr>
<td>Tissue inhibitor of metalloproteinase 1</td>
<td>TIMP1</td>
<td>CCGAGCGAGGATGTTTCTTC</td>
<td>GGAGCTGATGTTGCAAAATTTC</td>
<td>TGGCCGGCGCTTTGAAAGGA</td>
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<tr>
<td>Osteopontin</td>
<td>OPN</td>
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<td>GATCTGCTAGATTCTACGGAG</td>
<td>AGACATGCGAGAGAGGACTTTG</td>
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<tr>
<td>β-Myosin heavy chain 7</td>
<td>MYHCB</td>
<td>TGGAGAAGCCAGAACACGCAG</td>
<td>CCTGTCGTTGAGTGACATTTA</td>
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<tr>
<td>Transient receptor potential cation channel 6</td>
<td>TRPC6</td>
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Figure 1. Effect of treatment with imatinib and sorafenib on monocrotaline-induced pulmonary hypertension. A, RV systolic pressure (RVPsys). B, RV end-diastolic pressure (RVEDP). C, Cardiac index (CI). D, Stroke volume (SV). E, Systemic arterial pressure (SAP). F, Arterial oxygenation (Pao2). Con indicates control; Plac, placebo; Ima, imatinib; Sora, sorafenib; and MCT, monocrotaline. Data are mean±SEM (n=12 per group). *P<0.05 vs monocrotaline/placebo day 28; †P<0.05 vs monocrotaline/placebo day 14; #P<0.05 vs monocrotaline/imatinib.
was first digested with RNase-free DNase I (Gibco) for 15 minutes at room temperature and than reverse-transcribed with the use of Promisccript (Promega) in a total reaction volume of 40 μL according to the standard protocol of the kit supplier. Real-time polymerase chain reaction was performed as described previously.25 Primer and probe sequences are given in the Table. Cycle threshold values were corrected for ribosomal protein L32, β-2 microglobulin, and β-actin mRNA levels to exclude different starting amounts of total RNA. We observed that the calculated expression level was independent of the housekeeping gene used (ribosomal protein L32, β-2 microglobulin, or β-actin) in RV samples or between treated and untreated H9c2 cells. We therefore decided to normalize all mRNA expression data to β-2 microglobulin. The resulting expression levels are given in arbitrary units.

In RV samples, atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP) were used as markers of hypertrophy, and osteopontin, tissue inhibitor of metalloproteinase 1 (TIMP1),26 collagen 3α1 (COL3A1), and collagen 1α2 (COL1A2) were used as markers of cardiac fibrosis.

Western Blotting
Whole-cell protein extracts were prepared from either RV or total lung biopsy samples in lysis buffer containing 50 mmol/L Tris-HCl, pH 7.4, 50 mmol/L NaCl, 5 mmol/L EDTA, 1% Triton X-100, 0.05% sodium dodecyl sulfate, 50 mmol/L NaF, 10 mmol/L β-glycerophosphate, sodium pyrophosphate, 100 μmol/L Na3VO4, and protease inhibitor cocktail (Roche Diagnostics Corp, Indianapolis, Ind). Lysates were normalized and separated on 7.5% or 10% polyacrylamide gels and transferred to polyvinylidene fluoride membranes. After they were blocked, the membranes were probed with primary anti-p44/42 mitogen-activated protein kinase antibody (Cell Signaling Technology, Beverly, Mass), anti-phospho-p44/42 mitogen-activated protein kinase (Tyr204) antibody (Santa Cruz Biotechnology, Inc, Santa Cruz, Calif), anti-c-Raf antibody (Cell Signaling), and anti-phospho-C-Raf (Ser 338) antibody (Cell Signaling) at 1:1000 dilution. Anti-GAPDH (Novus Biologicals, Littleton, Colo) antibody was used at 1:4000 dilution as control for equal protein loading. After they were washed 5 times in phosphate-buffered saline plus 0.1% Tween, blots were incubated with appropriate secondary antibodies conjugated to horseradish peroxidase. After additional washes, antigen-antibody complexes were visualized by the ECL chemiluminescence detection system (Amersham Biosciences, Uppsala, Sweden).

Assessment of Proliferation and Apoptosis
Proliferation was assessed by proliferating cell nuclear antigen (PCNA) immunohistochemical staining and apoptosis by terminal deoxynucleotidyltransferase–mediated dUTP nick-end labeling (TUNEL) assay as described previously.3 Briefly, for PCNA immunohistochemical staining, consecutive 3-μm lung tissue sections were deparaffinized. Tissues sections were blocked in 3% BSA/PBS for 30 minutes at 37°C and incubated with anti-PCNA rabbit polyclonal antibody (Santa Cruz Biotechnology, Inc) for overnight at 4°C in PBS. For indirect immunofluorescence, slides were incubated with Alexa Fluor 555–labeled goat anti-rabbit IgG (Molecular Probes, Eugene, Ore). After incubation, all sections were counterstained with nuclear DAPI staining and mounted with Dako fluorescent mounting media (DakoCytomation, Carpinteria, Calif). Apoptosis in lung sections were visualized by the TUNEL method (In Situ Cell Death Detection Kit Roche, Mannheim, Germany) as specified by the manufacturer. At the end of the procedure, the slides were analyzed by fluorescence microscopy (Leica Instruments, Nussloch, Germany) after DAPI staining (DakoCytomation).

Statistical Analysis
Data are expressed as mean±SEM. Differences between groups were evaluated by the use of 1-way ANOVA, followed by Student-Newman-Keuls post hoc analysis for multiple comparisons (GraphPad Prism 4.0 software GraphPad Software Inc, San Diego, Calif). A P value of <.05 was regarded as significant.

The authors had full access to and take full responsibility for the integrity of the data. All authors have read and agree to the manuscript as written.
Results

Hemodynamic Parameters and RV Hypertrophy

Fourteen days after injection of monocrotaline, RV systolic pressure had increased to 41.0 ± 1.0 versus 25.5 ± 0.5 mm Hg in vehicle controls, and RV/(LV+S) had increased to 0.34 ± 0.01 versus 0.26 ± 0.01 (both *P < 0.05; n = 12 per group from a separate series).

Twenty-eight days after injection of monocrotaline (placebo group), RV systolic pressure and RV hypertrophy were more pronounced compared with the values at day 14. In addition, compared with nonmonocrotaline controls, cardiac output, stroke volume, and blood oxygenation were reduced. RV systolic pressure was drastically increased to 82.9 ± 6.0 mm Hg compared with 27.0 ± 0.5 mm Hg in saline-injected control animals (Figure 1A). This was paralleled by extensive RV hypertrophy: the RV/(LV+S) ratio was 0.51 ± 0.02 compared with 0.24 ± 0.01 in saline-treated controls (Figure 2A). In parallel, RV end-diastolic pressure was increased (7.7 ± 1.3 versus 2.0 ± 0.2 mm Hg), and cardiac index (20.8 ± 2.1 versus 28.0 ± 1.2 mL/100 g) and stroke volume were decreased (0.18 ± 0.02 versus 0.32 ± 0.02 mL; Figure 1B through 1D). These hemodynamic changes were accompanied by a significant decrease in arterial P O2 (Figure 1F). No significant differences in systolic arterial pressure were found in the systemic circulation between the groups (Figure 1E).

Both imatinib and sorafenib substantially improved hemodynamic and morphological parameters in monocrotaline-treated rats. Compared with vehicle, treatment with either imatinib or sorafenib reduced RV systolic pressure to 54.0 ± 4.4 and 35.0 ± 1.5 mm Hg and RV end-diastolic pressure to 3.8 ± 0.6 and 2.6 ± 0.2 mm Hg, respectively (Figure 1A and 1B). Both sorafenib and imatinib reduced RV hypertrophy (Figure 2A), but in the sorafenib group, the hypertrophy index RV/(LV+S) was significantly lower (0.26 ± 0.01) than in the imatinib group (0.42 ± 0.02; Figure 2A). Systemic arterial pressure was not statistically different across groups (Figure 1E). Both kinase inhibitors normalized the reduced cardiac index and stroke volume and improved arterial oxygenation in animals with monocrotaline-induced pulmonary arterial hypertension (Figure 1C, 1D, and 1F).

Figure 3. Effects of imatinib and sorafenib on monocrotaline-induced mRNA expression of biomarkers of hypertrophy and remodeling in the right ventricle. A, ANP. B, BNP. C, Osteopontin. D, TIMP1. E, COL3A1. F, COL1A2. Con indicates control; Plac, placebo; Ima, imatinib; Sora, sorafenib; and MCT, monocrotaline. Data are mean ± SEM of n = 8 animals per group. *P < 0.05 vs monocrotaline/placebo day 28; †P < 0.05 vs monocrotaline/placebo day 14; ‡P < 0.05 vs monocrotaline/imatinib group.
In control animals without pulmonary arterial hypertension, 14-day treatment with sorafenib had no effect on RV systolic pressure (control/placebo, 28.2 ± 0.66 mm Hg versus control/sorafenib, 26.3 ± 0.56 mm Hg) and RV hypertrophy index (control/placebo, 0.28 ± 0.01 versus control/sorafenib, 0.26 ± 0.01; n=8 per group).

**Histological Analysis**

Injection of monocrotaline resulted in a stepwise media hypertrophy and a pronounced increase in muscularization of the distal pulmonary arteries (diameter 25 to 50 μm) after 14 and 28 days compared with pulmonary arteries from control rats (Figure 2). At day 28, the majority of distal vessels in control rats were nonmuscularized, whereas the fraction of fully muscularized vessels was drastically increased in monocrotaline-treated rats. Treatment with both kinase inhibitors significantly reduced the number of fully muscularized pulmonary arteries and resulted in an increase of nonmuscularized pulmonary arteries compared with the monocrotaline-placebo group.

**RV Gene Expression and Phosphorylation**

In the RV, expression of ANP and BNP was significantly elevated in monocrotaline rats compared with untreated controls (Figure 3A and 3B). Both imatinib and sorafenib strongly suppressed the expression of these hypertrophy marker genes. The effect on BNP was stronger with sorafenib than with imatinib (Figure 3B).

COL1A2 and COL3A1 mRNA expression levels in RV myocardium were moderately increased by monocrotaline and reduced to control levels by both kinase inhibitors (Figure 3C and 3D). Likewise, expression levels of TIMP1 and osteopontin were strongly increased by monocrotaline alone and were reduced to near normal levels by both imatinib and sorafenib (Figure 3E and 3F).

Treatment of pulmonary hypertensive rats with sorafenib suppressed phosphorylation of Raf-1 and ERK1/2 in the lungs (Figure 4) as well as in the RV (Figure 5). By contrast, imatinib did not affect the phosphorylation ERK1/2 and Raf-1 in the myocardium (Figure 5).

**Effects on Proliferation and Apoptosis of Vascular Smooth Muscle Cells**

In monocrotaline-induced pulmonary hypertension, medial hypertrophy of distal pulmonary vessels was associated with an increased proliferation of vascular cells demonstrated by prominent PCNA-positive cells. Treatment with sorafenib considerably reduced PCNA-positive cells (Figure 6A). Apoptosis was virtually absent in pulmonary resistance vessels of control animals and very low in pulmonary arteries of monocrotaline-challenged animals. The number of TUNEL-positive apoptotic cells was substantially increased in pulmonary arterial vessel walls of rats treated with sorafenib (Figure 6B).

**Effects on H9c2 Cells**

Sorafenib dose-dependently suppressed the arginine vasopressin–induced expression of the hypertrophy marker genes MYHCβ and TRPC6 (IC₅₀ ≈ 300 nmol/L each), whereas cotreatment with imatinib had no suppressive effect (Figure 7).

**Discussion**

In this study, we demonstrate that the multikinase inhibitor sorafenib reverses pulmonary hypertension and myocardial hypertrophy in monocrotaline-treated rats. On the basis of its inhibitory effect on both tyrosine and serine/threonine kinases, the beneficial effects of sorafenib appear to be due to a dual pulmonary and myocardial mode of action.
The pulmonary vascular alterations in pulmonary arterial hypertension are characterized by (1) proliferation of smooth muscle cells;3,27 (2) migration of smooth muscle cells and myofibroblasts;28 and (3) resistance of smooth muscle cells to apoptosis due to upregulation of bcl-229 and survivin.27 Interestingly, these characteristics resemble important features of tumor cells. Consequently, as postulated 10 years ago,30 the tyrosine kinase inhibitor imatinib improved hemodynamics, pulmonary vascular remodeling, and survival in monocrotaline-treated rats3 and showed encouraging results in preliminary clinical studies in patients with pulmonary arterial hypertension.13,14 Good evidence exists that the effects of imatinib are mediated via inhibition of the PDGF receptor, which has been found to be upregulated in lung explants from patients with pulmonary arterial hypertension.2,3 However, besides the PDGF receptor, imatinib is a potent inhibitor of several other tyrosine kinases (such as the receptor for stem cell factor, c-kit, or BCR-ABL kinase18).

Figure 5. Effects of imatinib and sorafenib on phosphorylation of Raf-1 and ERK1/2 in the RV. Western blots are representative of 2 individual RVs from each group. Quantification of Raf-1 and ERK1/2 phosphorylation (normalized to Raf-1 and total ERK1/2) is shown in the bar graphs. Con indicates control; Plac, placebo; Ima, imatinib; Sora, sorafenib; MCT, monocrotaline; and ND, not detectable. *P<0.05 vs monocrotaline/placebo day 28; †P<0.05 vs monocrotaline/placebo day 14; #P<0.05 vs monocrotaline/imatinib group.

Figure 6. Effects of sorafenib on proliferation and apoptosis of pulmonary artery smooth muscle cells. Proliferation (A) was assessed via staining for PCNA (red nuclei are PCNA-positive cells). Apoptosis (B) was assessed by in situ TUNEL assay (green cells are TUNEL-positive cells). MCT indicates monocrotaline. Bar=20 μm.
whose roles in pulmonary hypertension appear less clear. For example, c-kit–positive dendritic cells are known to accumulate in the walls of pulmonary vessels of patients with pulmonary hypertension. In addition, the involvement of BCR-ABL kinase has recently been discussed. Kerkelä et al2 reported cardiotoxic effects of imatinib on neonatal mouse cardiomyocytes, which may be mediated by BCR-ABL kinase, but cardiotoxicity seems to be rare in patients.33

The multikinase inhibitor sorafenib inhibits the tyrosine kinases PDGF receptor, VEGF receptors 2 and 3, c-kit, and Flt-3 (but not BCR-ABL) in the nanomolar range.17 On the basis of its unique kinase inhibition profile targeting tyrosine as well as serine/threonine kinases,17 we hypothesized that sorafenib might prove useful for pulmonary hypertension. Our data demonstrate a substantial effect on all major indices of cardiac and pulmonary function, including RV systolic pressure, cardiac output, and arterial oxygen pressure, in monocrotaline-treated rats. In agreement with the functional benefit, morphometric indices of RV hypertrophy and pulmonary arterial muscularization were significantly reduced. In addition, our results indicate that sorafenib favorably affects key features of pulmonary remodeling, such as proliferation and reduced apoptosis of pulmonary vascular smooth muscle cells. Besides its inhibitory effects on tyrosine kinases, sorafenib exerts its action via inhibition of serine/threonine kinases, as evidenced by the reduction of the phosphorylation of ERK1/2 and Raf-1 in the lungs of pulmonary hypertensive rats. Of note, RV hypertrophy and arterial oxygenation were significantly improved at day 28 (after 14 days of sorafenib treatment) compared with the 2-week time point (and no active treatment). This indicates that sorafenib was able to restore some key pathological features of pulmonary hypertension, beyond mere prevention of further progression of the disease. In addition to its pulmonary benefit, sorafenib exerts direct antiremodeling effects in the myocardium. In the cardiomyoblast cell line H9c2, sorafenib produced a substantial decrease in all markers of hypertrophy, whereas imatinib had no effect. As demonstrated by the reduced phosphorylation of Raf-1 and ERK1/2 in RV myocardium, this benefit is likely to be mediated via inhibition of the Raf-MEK-ERK pathway that is downstream of Ras and one of the main hypertrophy signaling pathways in the heart.16,34

We observed a greater reduction in RV systolic pressure and RV hypertrophy in the sorafenib group than in the imatinib group. However, this functional benefit did not completely translate into a lower degree of pulmonary artery muscularization, which was similar after treatment with both drugs. Differential effects on the capacitance of the pulmonary circulation are unlikely because cardiac index and stroke volume were almost identical in the sorafenib and imatinib groups. A potential explanation is the prevention of cardiac hypertrophy and hence hypercontractility in the RV by sorafenib but not imatinib. However, in the RV homogenates, the upregulated remodeling markers ANP, BNP, osteopontin, and TIMP1 and the collagens 3α1 and 1α2 were significantly decreased in response to both agents, indicating that the cardiac antiremodeling occurs at least partly as a secondary phenomenon due to RV unloading.

VEGF and its receptors are targets for several antitumor drugs, including the approved compounds sorafenib (Nexavar), Bevacizumab (Avastin), and Sunitinib (Sutent). In the lungs, VEGF may play a role in maintenance of the lung structure.35 Lung-specific VEGF knockout mice develop an emphysema phenotype,36 and chronic VEGF inhibition can induce emphysema37 or exacerbate hypoxia-induced pulmonary arterial remodeling.38 In a separate set of experiments, therefore, we analyzed morphological features of sorafenib in monocrotaline-treated rats, such as mean linear intercept, septal wall thickness, and airspace content, and observed no significant changes (data not shown). In addition, treatment of healthy rats with sorafenib for 2 weeks did not reveal any adverse effects on RV pressure or hypertrophy. Taken together, these findings lead us to believe that the overall kinase inhibition profile of sorafenib may offer a unique opportunity to treat the functional and morphological consequences of pulmonary arterial hypertension through a dual pulmonary and cardiac mode of action.

In summary, we describe the successful therapeutic use of the multikinase inhibitor sorafenib in an animal model of pulmonary arterial hypertension. The inhibition of the Ras/Raf pathway appears to contribute to the effect of sorafenib in right heart hypertrophy and pulmonary vascular remodeling. The combined inhibition of tyrosine and serine/threonine kinases may provide an option to treat pulmonary arterial hypertension and associated right heart remodeling.

Figure 7. Effects of imatinib and sorafenib on arginine vasopressin–induced mRNA expression of MYHCB (A) and TRPC6 (B) in H9c2 cells after 72-hour incubation. Data are mean±SEM of 1 representative experiment performed in triplicate. *P<0.05 vs monocrotaline/placebo; #P<0.05 vs monocrotaline/imatinib.
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Disclosures
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References
Pulmonary arterial hypertension is a life-threatening disease that is characterized by a variety of remodeling processes in the pulmonary vasculature and the right ventricle, including migration of pulmonary endothelial and vascular smooth muscle cells as well as hypertrophy, fibrosis, and dysregulation of extracellular matrix in the right ventricular myocardium. Current vasodilatory therapy does not causally address the underlying deviations in intracellular signal transduction leading to pulmonary vascular and myocardial remodeling. Interestingly, dysregulation of kinase signaling pathways is a key characteristic of many cancer tissues as well as in pulmonary hypertension. Overactivation of receptor tyrosine kinases has been linked to endothelial and pulmonary vascular smooth muscle cell proliferation, whereas serine/threonine kinases are connected to smooth muscle and myocardial hypertrophy and remodeling. In the present study, we demonstrate that the combined tyrosine and serine/threonine kinase inhibitor sorafenib can prevent pulmonary and myocardial remodeling in a rat model of pulmonary arterial hypertension. As a result of its dual pulmonary and myocardial action, combined kinase inhibition may represent a promising and more causal approach to treat pulmonary arterial hypertension.
Combined Tyrosine and Serine/Threonine Kinase Inhibition by Sorafenib Prevents Progression of Experimental Pulmonary Hypertension and Myocardial Remodeling
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