Sorafenib treatment improves hepatopulmonary syndrome in rats with biliary cirrhosis

Ching-Chih CHANG*†, Chiao-Lin CHUANG*†, Fa-Yauh LEE†‡, Sun-Sang WANG†‡§, Han-Chieh LIN†‡, Hui-Chun HUANG†‡, Tzu-Hua TENG†, Shao-Jung HSU‡, Hsian-Guey HSIEH∥ and Shou-Dong LEE†¶

*Division of General Medicine, Department of Medicine, Taipei Veterans General Hospital, Taipei, Taiwan
†National Yang-Ming University School of Medicine, Taipei, Taiwan
‡Division of Gastroenterology, Department of Medicine, Taipei Veterans General Hospital, Taipei, Taiwan
§Department of Medical Affair and Planning, Taipei Veterans General Hospital, Taipei, Taiwan
∥Department of Medical Research and Education, Taipei Veterans General Hospital, Taipei, Taiwan
¶Cheng-Hsin General Hospital, Taipei, Taiwan

Abstract
HPS (hepatopulmonary syndrome) is characterized by oxygen desaturation in patients with chronic liver disease. The initiation of HPS comes from abnormal pulmonary vasodilatation and/or angiogenesis. In the present study, we evaluated anti-angiogenesis therapy using sorafenib in experimental HPS animals. HPS was induced by CBDL (common bile duct ligation) in rats. A 2-week 10 mg·(kg of body weight)$^{-1}$·day$^{-1}$ treatment regimen of sorafenib or distilled water (control) was initiated 2 weeks after the surgical procedure. Haemodynamics, liver biochemistry, plasma VEGF (vascular endothelial growth factor) measurements and blood gas analysis of the CBDL rats were performed. The livers of the CBDL rats were dissected for histopathology examination, and the lungs were examined by immunohistochemical staining, real-time PCR and Western blot analysis. In another two parallel groups, intrapulmonary shunts were determined. The AaP$_{O_2}$ (alveolar–arterial O$_2$ gradient) and plasma VEGF levels were reduced after sorafenib treatment [AaP$_{O_2}$, 7.2\(\pm\)3.4 mmHg in sorafenib-treated rats compared with 15.3\(\pm\)4.2 mmHg in controls ($P=0.004$); VEGF, 45.3\(\pm\)7.7 pg/ml in sorafenib-treated rats compared with 54.4\(\pm\)7.7 pg/ml in controls ($P=0.021$)]. Sorafenib attenuated pulmonary VEGF mRNA and VEGF, VEGFR-2 (VEGF receptor 2), phospho-VEGFR-2 and Akt protein expression. In addition, sorafenib significantly attenuated intrapulmonary angiogenesis and decreased the degree of intrapulmonary shunting by 33.7% (11.2\(\pm\)5.7% in sorafenib-treated rats compared with 16.9\(\pm\)5.9% in controls; $P=0.003$). Our findings suggest that sorafenib attenuates intrapulmonary shunting and decreases the AaP$_{O_2}$ in CBDL rats, implicating the improvement of HPS in this experimental animal model. The beneficial effect may be attributed to the reduction in intrapulmonary angiogenesis through inhibition of the VEGF/VEGFR-2/Akt pathway.

Key words: angiogenesis, hepatopulmonary syndrome, intrapulmonary shunt, liver cirrhosis, sorafenib

INTRODUCTION
HPS (hepatopulmonary syndrome) is characterized by a desaturation of oxygen in the arterial blood of patients with chronic liver disease [1]. Three important components of HPS are hypoxia with increased AaP$_{O_2}$ (alveolar–arterial O$_2$ gradient), increased intrapulmonary shunts and chronic liver disease (most in the patients with portal hypertension and liver cirrhosis). Increased intrapulmonary shunts can cause abnormal gas exchange and induce hypoxia in HPS patients [2,3]. Shunt formation can be derived from dilatation of the pre-existing vessels and/or angiogenesis, so the initiation of HPS may come from abnormal vasodilatation- and/or angiogenesis-induced overt shunts in the lung [4]. Results from liver transplantation centres indicate that the prevalence of HPS in cirrhotic patients range from 5 to 32% [5]. In the past, the efficacy of treatment was unsatisfactory except

Abbreviations: AaP$_{O_2}$, alveolar–arterial O$_2$ gradient; ALT, alanine aminotransferase; AST, aspartate aminotransferase; CBDL, common bile duct ligation; ET, endothelin; ERK, extracellular-signal-regulated kinase; H&E, haematoxylin and eosin; HPS, hepatopulmonary syndrome; HR, heart rate; MAP, mean arterial pressure; NOS, NO synthase; eNOS, endothelial NOS; iNOS, inducible NOS; MAPK, mitogen-activated protein kinase; P$_{O_2}$, partial pressure of O$_2$; PDGF, platelet-derived growth factor; PDGFR-κ, PDGF receptor-κ; P$_{CO_2}$, partial pressure of CO$_2$; PPVL, partial portal vein ligation; RT–PCR, reverse transcription–PCR; TBS-T, Tris-buffered saline plus tween 20; VEGF, vascular endothelial growth factor; VEGFR, VEGF receptor; vWF, von Willebrand factor.

1 These authors contributed equally to this paper.

Correspondence: Professor Fa-Yauh Lee (email fylee@vghtpe.gov.tw) or Dr Sun-Sang Wang (email sswang@vghtpe.gov.tw).
for liver transplantation. Studies have reported a median survival of 24 months and a 5-year survival rate of 23% in HPS patients without liver transplantation, in contrast with a 5-year survival rate of 76% after liver transplantation [6]. However, emerging evidence has shown promising results of medical treatment based on recent advances in the understanding of the pathogenesis of HPS [4,7].

There are three postulated mechanisms for the pathogenesis of HPS: (i) ET (endothelin)-1-mediated overexpression of ET_{1b} receptors in the pulmonary vasculature; (ii) intrapulmonary macrophage or monocyte recruitment; and (iii) pulmonary angiogenesis [4]. Among these mechanisms, pulmonary angiogenesis plays a crucial role. Zhang et al. [4] demonstrated that HPS is associated with pulmonary angiogenesis, activation of Akt and eNOS (endothelial NOS [NO synthase]) in the pulmonary endothelium and increased VEGF (vascular endothelial growth factor) production in intrapulmonary monocytes. Angiogenesis is also an important factor in portal hypertension and liver cirrhosis. Fernandez et al. [8] showed that combined VEGF and PDGF (platelet-derived growth factor) blockade can reverse portal hypertension and improve hyperdynamic splanchnic circulation in portal hypertensive rats. Sorafenib, a VEGF and PDGF inhibitor, has been shown to reduce intrahepatic resistance, decrease portal pressure and the degree of collateral shunting, and improve liver fibrosis in rats with PPVL (partial portal vein ligation) and in CBDL (common bile duct ligation)-induced cirrhotic rats [9].

Sorafenib is a potent multiple-kinase inhibitor that blocks VEGFR (VEGF receptor)-2 and PDGFR-β (PDGF receptor-β), as well as the Raf serine/threonine kinases along the Raf/MAPK (mitogen-activated protein kinase)/ERK (extracellular-signal-regulated kinase) pathway [10]. This multiple-kinase inhibitor has been documented to have potent anti-angiogenesis effects and it has been approved clinically for the treatment renal cell carcinoma and hepatocellular carcinoma [11,12]. Mejias et al. [9] demonstrated that sorafenib treatment can result in a 25% reduction in portal pressure, as well as a remarkable improvement in hepatic damage and liver fibrosis in cirrhotic rats. In addition, in their study, sorafenib administration induced an approximately 80% decrease in splanchnic neovascularization and an 18% decrease in the extent of portal systemic collateral vessels in PPVL rats. Furthermore, treatment of CBDL rats with sorafenib has been shown to decrease portal pressure, in parallel with a reduction in hepatic Rho kinase expression and Rho kinase-mediated intrahepatic vascular constriction [13]. Sorafenib has also been shown to decrease the mRNA levels of TNFα (tumour necrosis factor α), VEGFR-1, VEGFR-2, TGFβ (transforming growth factor β), cyclo-oxygenase 1 and various genes that are involved in cellular proliferation, fibrogenesis, tissue remodelling, inflammation and angiogenesis pathways [14].

An ideal experimental animal model that simulates HPS clinically should include hypoxia with an increased Aa{\text{\textsubscript{P}}}O_{2} and elevated degrees of intrapulmonary shunting. Many animal models have been studied for HPS, including PPVL, thioacetamide-induced fulminant hepatic failure and CBDL [4,15,16]. Among these models, only the CBDL rat model mimics the pathophysiological findings in human HPS. A unique finding in CBDL rats is that cholangiocytes, a major source of ET-1, proliferate after CBDL and then increase circulating ET-1 levels [17]. ET-1 has been documented to be an important factor in the induction of HPS [18]. In addition, Gaudio et al. [19] reported that cholangiocytes secrete VEGF and express VEGFR-2 and VEGFR-3, both of which are amplified in the cholangiocytes of CBDL rats. Therefore overproduction of VEGF and ET-1 due to the proliferation of cholangiocytes in CBDL rats makes CBDL the preferred experimental model for the study of HPS.

Regarding the major role of angiogenesis in the development of HPS, we were interested in the influence of anti-angiogenesis therapy by sorafenib treatment in experimental cirrhotic rats with HPS. Using the CBDL-induced HPS animal model, we conducted the present study to evaluate the potentially therapeutic benefit of sorafenib in HPS.

**MATERIALS AND METHODS**

**Animal model**

Male Sprague–Dawley rats weighing 200–250 g at the time of surgery were used. CBDL-induced liver cirrhosis was used as the HPS experimental model. Sham-operated rats were also used for parallel studies. The rats were housed in plastic cage and allowed free access to food and water. All rats were fasted for 12 h before the operation. The CBDL operation was performed as the previous report [20]. Briefly, under ketamine anaesthesia (100 mg/kg of body weight, intramuscular), the common bile duct was exposed and doubly ligated with 3-0 silk. The first ligature was then closed and the animals allowed to recover. To avoid coagulation defects, experimental rats received weekly vitamin K injections (50 μg/kg of body weight, intramuscular). A high yield of secondary biliary cirrhosis was created 4 weeks after the operation [20,21]. In all the experiments, the authors adhered to the American Physiological Society guiding principles for the care and use of laboratory animals (NIH publication no. 86-23, revised 1985). This study was approved by the Taipei Veteran General Hospital Animal Committee, Taiwan (IACUC 2011-090).

**Experimental design**

Sorafenib [10 mg (kg of body weight){\textsuperscript{-1}}day{\textsuperscript{-1}}; n = 6] or distilled water (control; n = 6) was administered orally by gavage to CBDL rats for 2 weeks. Treatment began 2 weeks after CBDL. At 4 weeks post-CBDL, haemodynamic measurements were made, and venous and arterial blood was collected from the sorafenib-treated and control rats. The liver biochemistry and VEGF levels were measured, and blood gas analysis was performed. The Aa{\text{\textsubscript{P}}}O_{2} was calculated as 150 − (P{\text{\textsubscript{c}}}O_{2}/0.8) − PO_{2} (where P{\text{\textsubscript{c}}}O_{2} and PO_{2} are partial pressure of CO_{2} and O_{2} respectively) [22]. Lungs were dissected for mRNA and protein studies, including iNOS (inducible NOS), eNOS, VEGF, VEGFR-1, VEGFR-2, phospho-VEGFR-2, PDGF, PDGFR-β, Akt, phospho-Akt, ERK-1/2 and phospho-ERK-1/2 in sorafenib-treated (n = 6) and
control (n = 6) CBDL rats. The liver and lung tissue were examined for histopathological changes using H&E (haematoxylin and eosin) staining; in addition, pulmonary angiogenesis was measured using immunohistochemical stains. In another two parallel CBDL rat groups, the degree of intrapulmonary shunting was determined using a colour microsphere technique in the sorafenib-treated (n = 20) and control (n = 24) groups. Besides, the sham-operated rats were also divided into two groups which received sorafenib (n = 11) or distilled water treatment (n = 10) respectively; then the haemodynamic, liver biochemistry measurement and blood gas analysis were performed. In another two parallel sham-operated rats (n = 8, sorafenib-treated; n = 7, control), the degrees of intrapulmonary shunting were also determined. The mortalities during experimental periods and adverse effects of sorafenib treatment were observed in these studied animals.

Systemic haemodynamic measurements
The right internal carotid artery of the rats was cannulated with a PE-50 catheter that was connected to a Spectramed DTX transducer. Continuous recordings of MAP (mean arterial pressure) and HR (heart rate) were performed on a multi-channel recorder (model RS 3400; Gould). The external zero reference was placed at the level of the mid-portion of the rat [23].

Determination of plasma VEGF levels
Plasma levels of VEGF were measured by a commercially available ELISA kit (R&D Systems), according to the manufacturer’s instructions. Briefly, standards and samples were pipetted into the wells containing a monoclonal antibody specific for VEGF. After washing, an enzyme-linked polyclonal antibody specific for VEGF was added to the wells. A substrate solution was then added to the wells. The colour development in proportion to the amount of VEGF bound was stopped with a stop solution. Standard curves were constructed using serial dilutions of recombinant VEGF. The intensity of the colour was measured at the absorbance of 450–600 nm with a Bio-kinetics Reader (Bio-Tek Instruments).

Intrapulmonary shunting analysis
The degrees of intrapulmonary shunting was determined using the technique described by Fallon et al. [22] and Zhang et al. [24], with minor modification. In brief, before microsphere injection, the animals underwent the placement of indwelling PE-50 femoral arterial and venous catheters. On the day of measurement, the animals underwent the placement of indwelling PE-50 with minor modification. In brief, before microsphere injection for a total of 90 s at a constant rate of 1.0 ml/min. The volume removed was replaced with an equal volume of sterile normal saline. Samples of the beads before venous injection and reference blood samples were coded. The numbers of coloured microspheres in the blood preparations were determined using a haemacytometer counting slide having a known volume. As the diameter of normal pulmonary microvasculature was less than 5 μm, most of the microsphere beads injected into the femoral vein would be trapped in the lung. However, microspheres would pass through the intrapulmonary shunting vessels in the HPS animal and were collected in the reference blood from the femoral artery. The total number of microspheres passing through the pulmonary microcirculation was calculated as the reference blood sample microspheres/mm × estimated blood volume. The estimated blood volume of each animal was derived from the following formula [25]:

\[
\text{Blood volume (ml)} = 0.06 \times \text{body weight (g)} + 0.77
\]

Intrapulmonary shunts were calculated as an intrapulmonary shunt fraction:

\[
\text{Intrapulmonary shunt fraction (%) = (total number of microspheres passing through the pulmonary microcirculation/total beads injected into the venous circulation)} \times 100.
\]

Real-time quantitative RT–PCR (reverse transcription–PCR)
A portion (1 μg) of total RNA was reverse-transcribed to cDNA with Superscript II RT and poly dT priming according to the manufacturer’s instructions (Life Technologies). Quantitative RT–PCR was carried out on a LightCycler (LightCycler 480; Roche Diagnostics) and a standard LightCycler amplification cycle protocol was established for each gene. The sequence of the specific primers are shown in Table 1.

According to the manufacturer’s instructions, a LightCycler master mix was prepared using 2 μl of LightCycler-FastStart DNA Master SYBR Green I® (Roche Diagnostics) with forward and reverse primers at a final concentration of 0.5 μM each and MgCl₂ at 3 mM. A total volume of 18 μl of LightCycler master mix was pipetted into LightCycler glass capillaries and 2 μl of the cDNA product (diluted 10-fold) was added as a PCR template. The capillaries were capped, placed in the LightCycler carousel and briefly centrifuged in a specific LightCycler centrifuge. Thermal cycling was carried out as follows. The first segment of the amplification cycle consisted of a denaturation programme of 95°C for 10 min. The second segment consisted of a four-step denaturation (15 s at 95°C), primer annealing (5 s at 58°C), elongation (72°C for 10s) and quantification program repeated for 40 cycles. The third segment consisted of a melting curve programme (95°C for 0 s, 57°C for 15 s and a linear temperature transition at 0.05°C/s from 57 to 95°C with continuous fluorescence acquisition). The final segment consisted of a cooling programme to 40°C. An internal housekeeping gene control, β-actin, was used to normalize the differences in RNA isolation, RNA degradation, and the efficiencies of the reverse transcription. The relative amounts of mRNA of the mediators measured were calculated as the ratio to β-actin expression. LightCycler analysis software (Roche Diagnostics) allowed quantitative analysis of the PCR.
Western blot analysis

The protein extracts were made by pulverization in a grinder with liquid nitrogen, then 1 ml of lysis buffer [PBS containing 1% Nonidet P40, 0.5% sodium deoxycholate, 0.1% SDS and 0.05% protease inhibitor cocktail solution; Roche Diagnostics] was added for each 100 mg of powdered sample. The protein concentration was determined for each sample using the Bradford method [26]. An aliquot of 20–40 μg of protein from each sample was dissolved in sample buffer (63 mmol/l Tris/HCl, pH 6.8, containing 2% SDS, 10% glycerol, 5% mercaptoethanol and 0.005% Bromophenol Blue) and 10 μg of positive control were separated by denaturing SDS/PAGE (10% gel) (Mini-PROTEAN® 3 Cell; Bio-Rad Laboratories). Pre-stained protein markers (SDS/PAGE Standards; Bio-Rad Laboratories) were used for molecular-mass determination. The proteins were then transferred on to a PVDF membrane (Immun-Blot® PVDF Membrane; Bio-Rad Laboratories) using a semi-dry electroblotting system (Trans-Blot® SD Semi-dry Electrophoretic Transfer Cell; Bio-Rad Laboratories) for 1.5 h at 4 °C. To block non-specific binding, membranes were blocked for 30 min with 3% non-fat dried skimmed milk powder in TBS-T (Tris-buffered saline plus Tween 20) and 1% Tween 20. Blots were incubated with the first antibody [anti-eNOS, anti-iNOS (Transduction Laboratories and Santa Cruz Biotechnology); anti-VEGF, anti-VEGFR-1, anti-phospho-VEGFR-2, anti-VEGFR-2, anti-PDG, anti-PDGFR-β, anti-Akt (Ser473), anti-phospho-Akt (Ser473) (Santa Cruz Biotechnology), anti-ERK-1/2 (Millipore), anti-phospho-ERK-1/2 (Cell Signaling Technology) diluted with 3% non-fat dried skimmed milk powder in TBS-T (Tris-buffered saline plus Tween 20; 25 mmol/l Tris/HCl, pH 7.4, 137 mmol/l NaCl, 2.7 mmol/l KCl and 1% Tween 20). Blots were incubated with the first antibody [anti-eNOS, anti-iNOS (Transduction Laboratories and Santa Cruz Biotechnology); anti-VEGF, anti-VEGFR-1, anti-phospho-VEGFR-2, anti-VEGFR-2, anti-PDG, anti-PDGFR-β, anti-Akt (Ser473), anti-phospho-Akt (Ser473) (Santa Cruz Biotechnology), anti-ERK-1/2 (Millipore), anti-phospho-ERK-1/2 (Cell Signaling Technology) diluted with 3% non-fat dried skimmed milk powder in TBS-T] for 90 min at room temperature (25 °C) and washed. The blots were then incubated for 90 min with the secondary antibody [HRP (horseradish peroxidase)-conjugated goat anti-(mouse IgG) antibody, diluted with 3% non-fat dried skimmed milk powder in TBS-T; Sigma] and washed. Subsequent detection of the specific protein was performed by enhanced chemiluminescence [BCIP (5-bromo-4-chloroindol-3-yl phosphate)/NBT (Nitro Blue Tetrazolium) solution; Amresco]. With a computer-assisted video densitometer and digitalized software (Kodak Digital Science™ ID Image Analysis Software), the blots were scanned, photographed and then the signal intensity (integral volume) of the appropriate bands was analysed.

Histopathological and immunohistochemical staining studies

The liver and lung tissues of rats were dissected free and fixed in 10% formalin solution. They were dehydrated in graded ethanol, cleaned in xylene and embedded in paraffin. Sections were cut into 4-μm slices, deparaffinized in xylene and rehydrated through a series of ethanol solutions. The sections were stained with H&E and examined by light microscopy. vWF (von Willebrand factor), produced uniquely by endothelial cells and megakaryocytes, has been routinely used to identify vessels in tissue sections [27]. The immunohistochemical staining technique, using anti-vWF antibody, was performed to determine angiogenesis in the lung tissue. The sections were heated in a microwave oven for antigen retrieval. Endogenous peroxidase was inhibited using 3% H2O2 in methanol. Immunostaining was performed using the primary antibodies against vWF [1:100 dilution of mouse monoclonal anti-(human vWF); MCA127T; AbD Serotec], followed by the biotinylated anti-mouse IgG (H + L) (Vector Laboratories) as the secondary antibody. Detection of biotinylated antibody was performed using the VECTASTAIN®-Elite ABC kit from Vector Laboratories. For the chromogen, DAB (diaminobenzidine) was used, which resulted in a brown colour at the antigen site. Finally, the sections were counterstained with Mayer’s haematoxylin and they were covered with mounting medium. Normal mouse serum was used on negative control sections for immunoreactivity.

Drugs

Sorafenib (Nexavar®) was purchased from Bayer Schering Pharmaceuticals. All of the solutions were freshly prepared on the day of experiment.

Statistical analysis

The results are expressed as means ± S.D. Statistical analyses were performed using an unpaired Student’s t test. Results were considered statistically significant at a two-tailed P value of less than 0.05.

RESULTS

Mortality and adverse effects in the sorafenib-treated and control CBDL and sham-operated rats

None of the sorafenib-treated or control CBDL and sham-operated rats died during the experimental period. In addition, no obviously adverse effects were observed in the sorafenib-treated CBDL and sham-operated rats.

Haemodynamic effects, liver biochemistry and arterial blood gas analysis in sham-operated rats

Table 2 shows the body weight, baseline haemodynamic parameters, liver biochemistry and arterial blood gas data of the sorafenib-treated and control CBDL and sham-operated rats.

Table 1 Primer pairs for the selected genes

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Sense</th>
<th>Antisense</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-Actin</td>
<td>5′-CGCCCTAGGCAACCAGGGTG-3′</td>
<td>5′-GCTGGGTTGTGAGGCTCTCAA-3′</td>
</tr>
<tr>
<td>iNOS</td>
<td>5′-AGGCCACCTGATGATCCTGC-3′</td>
<td>5′-GCTGGCTCTGAGGCTCTTG-3′</td>
</tr>
<tr>
<td>eNOS</td>
<td>5′-GGAATGAGCAATGCACTGA-3′</td>
<td>5′-GCCAGTCTAGACCAGCATACA-3′</td>
</tr>
<tr>
<td>VEGF</td>
<td>5′-AGCAAGGCGCAAGAATCCC-3′</td>
<td>5′-TAACTCAACTGCTCCGCCC-3′</td>
</tr>
<tr>
<td>VEGFR-1</td>
<td>5′-AGGAGAGACCTGGAACCTGCTCT-3′</td>
<td>5′-ATCTCGGCTGTCAGCGCATAG-3′</td>
</tr>
<tr>
<td>VEGFR-2</td>
<td>5′-TAGCCACAGCAAGACTGTGAGG-3′</td>
<td>5′-TGAGGTGAGAGATGGGTAGG-3′</td>
</tr>
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</table>
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Table 2  Body weight, haemodynamic parameters, liver biochemistry and arterial gas analysis in sham-operated rats

*P < 0.001 and †P = 0.037 compared with the sorafenib-treated group.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Sorafenib (n = 11)</th>
<th>Control (n = 10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>354 ± 16</td>
<td>352 ± 43</td>
</tr>
<tr>
<td>MAP (mmHg)</td>
<td>114 ± 14</td>
<td>114 ± 15</td>
</tr>
<tr>
<td>HR (beats/min)</td>
<td>365 ± 50</td>
<td>382 ± 45</td>
</tr>
<tr>
<td>ALT (units/l)</td>
<td>102 ± 21</td>
<td>58 ± 10*</td>
</tr>
<tr>
<td>AST (units/l)</td>
<td>223 ± 89</td>
<td>153 ± 41*</td>
</tr>
<tr>
<td>Total bilirubin (mg/dl)</td>
<td>0.1 ± 0.01</td>
<td>0.09 ± 0.01</td>
</tr>
<tr>
<td>P02 (mmHg)</td>
<td>91.1 ± 6.3</td>
<td>95.6 ± 5.5</td>
</tr>
<tr>
<td>PCO2 (mmHg)</td>
<td>40.1 ± 3.1</td>
<td>37.4 ± 3.9</td>
</tr>
<tr>
<td>AaPO2 (mmHg)</td>
<td>8.8 ± 2.9</td>
<td>8.4 ± 0.9</td>
</tr>
</tbody>
</table>

sorafenib-treated and control sham-operated rats. The haemodynamic, plasma levels of total bilirubin and arterial blood gas data were similar between the sorafenib-treated and control groups. The plasma levels of AST (aspartate aminotransferase; P = 0.037) and ALT (alanine aminotransferase; P < 0.001) were elevated after sorafenib treatment in the sham-operated rats (Table 2).

Haemodynamic effects, liver biochemistry, plasma VEGF levels and arterial blood gas analysis in CBDL rats

Table 3 shows the body weight, baseline haemodynamic parameters, liver biochemistry, AaPO2 and plasma VEGF levels of the sorafenib-treated and control CBDL rats. The MAP, HR and plasma levels of AST, ALT and total bilirubin were similar between the two groups. The plasma VEGF levels were significantly lower in the sorafenib-treated group (P = 0.021; Table 3). AaPO2 was also significantly reduced after sorafenib treatment (P = 0.004; Table 3).

Degree of intrapulmonary shunting in sorafenib-treated CBDL and sham-operated rats

Sorafenib treatment significantly decreased the intrapulmonary shunting of the CBDL-induced cirrhotic rats compared with the control group (11.2 ± 5.7% in sorafenib-treated rats compared with 16.9 ± 5.9% in the controls; P = 0.003) (Figure 1A). A 33.7% reduction in the degree of intrapulmonary shunting was recorded. In contrast, sorafenib treatment did not influence the intrapulmonary shunting degrees in the sham-operated rats (6.2 ± 2.8% in sorafenib-treated rats compared with 5.3 ± 1.9% in controls; P > 0.05) (Figure 1B).

mRNA and protein expression of eNOS, iNOS, VEGF, VEGFR-1, VEGFR-2, phospho-VEGFR-2, PDGF and PDGFR-β in the pulmonary tissue of CBDL rats

In the pulmonary tissue from the sorafenib-treated CBDL rats, the expression of VEGF mRNA was significantly lower than that of the control group (0.061 ± 0.017 in sorafenib-treated rats compared with 0.082 ± 0.008 in controls; P = 0.02) (Figure 2).
Expression of phospho-Akt, ERK-1/2 and phospho-ERK protein levels in the pulmonary tissue of CBDL rats

The protein levels of ERK-1 and ERK-2 were not changed after sorafenib treatment (all \( P > 0.05 \)) (Figure 4A). The phosphorylation of ERK-1/2 were similar between sorafenib-treated and control groups (\( P > 0.05 \); Figure 4B). The protein levels of phosphorylated Akt were significantly decreased after sorafenib treatment (\( P < 0.05 \); Figure 4C).

Effects of sorafenib treatments on the histopathological change of liver and lung tissues and influences of pulmonary angiogenesis of CBDL rats

The liver tissues of CBDL rats had marked proliferation of bile ducts and extensive fibrosis as identified by H&E staining. Sorafenib treatment did not influence microscopic histopathological appearance of the liver in the H&E staining (Figures 5A and 5B). Similarly, sorafenib treatment did not influence histopathological appearance of lung in CBDL rats (results not shown). However, sorafenib treatment significantly attenuated the vWF-contained cellular densities in the lung tissues, which meant abolishment of pulmonary angiogenesis of CBDL rats. The representative lung tissues were shown using immunohistochemical stains of vWF (Figures 5C and 5D).

DISCUSSION

In the present study, we show for the first time that sorafenib can attenuate pulmonary angiogenesis to reduce the degree of intrapulmonary shunting and decrease \( \Delta aP_{O_2} \) in CBDL-induced HPS rats, implicating that 2 weeks of sorafenib treatment can improve HPS in an experimental animal model. The decreased densities of vWF-contained endothelial cells in the pulmonary tissues, as determined by immunohistochemical staining, suggest that sorafenib treatment attenuates the pulmonary angiogenesis of CBDL rats. In addition, sorafenib treatment significantly decreased the plasma levels of VEGF, abolished the expression of pulmonary VEGF mRNA and decreased VEGF, VEGFR-2 and phospho-VEGFR-2 protein levels. Therefore the beneficial effects of sorafenib on HPS rats may be related, at least partially, to the inhibition of the VEGF/VEGFR-2 related angiogenesis pathway.

VEGF is secreted by endothelium and modulates cellular function via autocrine and paracrine mechanisms [28]. It can induce an angiogenic signal mainly through VEGFR-2, which is expressed in endothelial cells that directly or indirectly contribute to the formation of vascular wall [29]. The VEGF/VEGFR-2 pathway enrolls endothelial cell in a sprouting angiogenesis programme [30]. To the best of our knowledge, the present study is the first to demonstrate that inhibition of VEGF/VEGFR-2 by sorafenib can attenuate intrapulmonary shunting and improve HPS. In agreement with our findings, using recombinant adenovirus encoding human endostatin plus angiostatin to inhibit VEGF, Zhang et al. [4] demonstrated that down-regulation
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Figure 4 Protein levels of pulmonary ERK-1/2, phospho-ERK and phospho-Akt in sorafenib-treated and control CBDL rats

(A and B) Sorafenib treatment did not change the expression of ERK-1/2 and phosphorylated ERK protein levels. However, the expression of phospho-Akt protein levels was significantly abolished after sorafenib treatment (C).

of the VEGF-mediated angiogenic pathway could improve HPS in CBDL rats as manifested by decreased intrapulmonary vascular density, decreased AaPo2 and lung VEGF, VEGFR-2 levels. Interestingly, in their report, VEGF, phospho-VEGFR-2 and eNOS were all attenuated by VEGF inhibition. However, we found that only VEGF, VEGFR-2 and phospho-VEGFR-2, but not NOS, were abolished after sorafenib treatment in the CBDL rats. The difference in results may be derived from the different strategies of VEGF inhibition. Besides, our findings may suggest that NO takes a minor role in the pathogenesis of HPS. A previous study to evaluate the genetic risk factors of HPS in patients with advanced liver disease has also shown that polymorphisms in genes of angiogenesis increased the risk of HPS; however, the vasoregulatory genes, such as NOS, were not associated with the risk of HPS [31]. The report is consistent with our findings that angiogenesis plays a major role in the mechanism of HPS.

VEGFR-2-mediated VEGF activation of endothelial cells is critical for angiogenesis. Two major pathways that regulate this process are the PI3K (phosphoinositide 3-kinase)/Akt and MAPK/ERK pathways [32,33]. In agreement with a previous report [4], the pulmonary protein levels of phospho-Akt were significantly decreased after inhibition of angiogenesis in the CBDL rats. Regarding the ERK pathway, sorafenib treatment has been shown previously to significantly abolish the protein levels of ERK in the liver, aorta and mesentery vasculatures of portal hypertensive and cirrhotic rats [9,13]. However, in the present study, sorafenib treatment did not significantly diminish the pulmonary ERK protein levels in the CBDL rats. The possible explanations include different regimens of sorafenib treatment and different target organ presentations (liver and vessels against lung). Nevertheless, it is possible that the downstream signalling pathway of sorafenib-induced pulmonary VEGFR-2 attenuation may be
through an Akt-dependent, but not ERK-dependent, pathway. As the process of angiogenesis is very complicated and sorafenib is a multiple-kinase inhibitor, the precise mechanism of sorafenib treatment in HPS needs further investigations.

The pulmonary complications of cirrhosis include HPS and portopulmonary hypertension. The portopulmonary hypertension is defined by elevated pulmonary arterial pressures in cirrhotic patients. The patients with portopulmonary hypertension also present with hypoxia, but have no change in Aa\n\n\nO_2. Besides, the pathogenesis of HPS results from overt intrapulmonary vasodilation, but portopulmonary hypertension is related to the significant vasoconstriction. It is interesting to note that sorafenib treatment also attenuated pulmonary hypertension in the experimental animal model [34]. Using a hypoxia and VEGFR-1, VEGFR-2 blockade-induced pulmonary hypertensive rodent model, Moreno-Vinasco et al. [34] demonstrated that sorafenib treatment can improve haemodynamics and attenuate the pulmonary hypertension. In agreement with our findings, they also found that sorafenib administration inhibited the VEGF/VEGFR-2 pathway and attenuated the vWF-containing pulmonary endothelial cells. In the present study, we did not measure the pulmonary arterial pressure in CBDL rats, thus we could not confirm how many CBDL rats developed portopulmonary hypertension. However, the mechanism of portopulmonary hypertension is completely different from HPS. The CBDL rat model is a well-documented HPS animal model that is featured by increased Aa\n\n\nO_2 and increased intrapulmonary shunts [22]. The results of the present study suggest that sorafenib administration improves the experimental HPS, but not portopulmonary hypertension.

A number of tyrosine kinase inhibitors, such as imatinib, sunitinib and sorafenib, have been shown to regulate splanchnic neovascularization and improve portal hypertension [35,36]. However, only sorafenib has been approved to treat hepatocellular carcinoma and extensively used in cirrhotic patients with hepatocellular carcinoma [12]. Coriat et al. [37] demonstrated that sorafenib decreases portal venous inflow without modifying aortic blood flow and ayzygos venous blood flow in cirrhotic patients with advanced hepatocellular carcinoma, and that it provides clinical benefits in cirrhotic patients. In animal studies, sorafenib has been shown to reduce portal pressure and ameliorate the degree of portal–systemic shunting in PPVL and CBDL rats [9]. These human and animal studies point out the beneficial effect of
sorafenib in liver cirrhosis and portal hypertension. Regarding HPS as a late and serious complication of liver cirrhosis, our findings support that sorafenib may be beneficial for the treatment of HPS in cirrhotic patients in the future.

Although we found that sorafenib treatment can improve experimental HPS, extrapolating findings of an animal study to clinical trials should be done with caution. Some adverse effects of anti-angiogenesis therapy in cirrhotic patients have been reported, including interstitial pneumonitis and variceal bleeding [38,39]. In addition, Henneberg et al. demonstrated that 7 days of sorafenib treatment [60 mg·(kg of body weight)\(^{-1}\)·day\(^{-1}\)] caused impaired liver biochemistry and damaged hepatic histology in CBDL rats [40]. They found that sorafenib-induced hepatic injury in both CBDL and sham-operated rats, but worse in CBDL rats, which implicated that high-dose sorafenib treatment may have direct hepatic toxicity in cirrhotic rats [40]. It is noteworthy that the regimen of 14 days of sorafenib treatment [10 mg·(kg of body weight)\(^{-1}\)·day\(^{-1}\) in the present study] elevates the plasma levels of AST and ALT in sham-operated rats. However, the liver biochemistry is similar between the sorafenib-treated and control CBDL rats. The direct hepatic toxicity in normal and cirrhotic condition awaits further investigations.

In conclusion, sorafenib treatment can improve experimental HPS in CBDL-induced cirrhotic rats. The effects of sorafenib-induced attenuation of intrapulmonary shunts are most likely due to inhibition of pulmonary angiogenesis through blockade of the VEGF/VEGFR-2/Akt pathway. However, the extrapolation of anti-angiogenesis therapy to clinical use must be done cautiously because of the possible adverse effects of sorafenib.

**CLINICAL PERSPECTIVES**

- HPS is characterized by oxygen desaturation in patients with chronic liver disease. The initiation of HPS arises from abnormal pulmonary vasodilatation and/or angiogenesis.

- In the present study, we evaluated anti-angiogenesis therapy using sorafenib in experimental HPS animals. A 2-week 10 mg·(kg of body weight)\(^{-1}\)·day\(^{-1}\) sorafenib treatment significantly decreased the Aa\(\Delta\)Po\(_2\) and plasma VEGF levels and attenuated the pulmonary VEGF, VEGFR-2, phospho-VEGFR-2 and Akt protein expression. In addition, sorafenib significantly attenuated intrapulmonary angiogenesis and decreased the degree of intrapulmonary shunting by 33.7%.

- Our findings suggest that sorafenib treatment can improve HPS in this experimental animal model. The beneficial effect may be attributed to the reduction of intrapulmonary angiogenesis through inhibition of VEGF/VEGFR-2/Akt pathway. However, the extrapolation of anti-angiogenesis therapy to clinical use must be done cautiously because of the possible adverse effects of sorafenib.

**REFERENCES**


**AUTHOR CONTRIBUTION**

Ching-Chih Chang designed the study and wrote the paper. Chiao-Lin Chuang assisted with data analysis. Sun-Sang Wang and Fa-Yauh Lee conceived the project and revised the paper. Han-Chieh Lin, Hui-Chun Huang and Shou-Dong Lee conceived the project. Tzu-Hua Teng, Shao-Jung Hsu and Hsian-Guey Hsieh provided technical assistance. All of the authors contributed to the final version of the paper.

**ACKNOWLEDGEMENTS**

We thank Chieh-Hsin Liu and Yi-Chou Chen for their excellent technical assistance.

**FUNDING**

This work was partly supported by the Taipei Veterans General Hospital, Taipei, Taiwan [grant number V100C-173].


Received 30 January 2012/1 October 2012; accepted 9 October 2012
Published as Immediate Publication 9 October 2012, doi: 10.1042/CS20120052