Sorafenib-Induced Hepatocellular Carcinoma Cell Death Depends on Reactive Oxygen Species Production In Vitro and In Vivo

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Abstract
Sorafenib is presently the only effective therapy in advanced hepatocellular carcinoma (HCC). Because most anticancer drugs act, at least in part, through the generation of reactive oxygen species, we investigated whether sorafenib can induce an oxidative stress. The effects of sorafenib on intracellular ROS production and cell death were assessed in vitro in human (HepG2) and murine (Hepa 1.6) HCC cell lines and human endothelial cells (HUVEC) as controls. In addition, 26 sera from HCC patients treated by sorafenib were analyzed for serum levels of advanced oxidation protein products (AOPP). Sorafenib significantly and dose-dependently enhanced in vitro ROS production by HCC cells. The SOD mimic MnTBAP decreased sorafenib-induced lysis of HepG2 cells by 20% and of Hepa 1.6 cells by 75% compared with HCC cells treated with 5 mg/L sorafenib alone. MnTBAP significantly enhanced by 25% tumor growth in mice treated by sorafenib. On the other hand, serum levels of AOPP were higher in HCC patients treated by sorafenib than in sera collected before treatment (P < 0.001). An increase in serum AOPP concentration ≥ 0.2 μmol/L chloramine T equivalent after 15 days of treatment is a predictive factor for sorafenib response with higher progression free survival (P < 0.05) and overall survival rates (P < 0.05). As a conclusion, sorafenib dose-dependently induces the generation of ROS in tumor cells in vitro and in vivo. The sera of Sorafenib-treated HCC patients contain increased AOPP levels that are correlated with the clinical effectiveness of sorafenib and can be used as a marker of effectiveness of the drug. Mol Cancer Ther; 11(10); 2284–93. ©2012 AACR.

Introduction
Hepatocellular carcinoma (HCC) is one of the most common lethal malignancies, accounting for 626,000 new cases per year worldwide (1). The incidence of HCC is increasing in the United States and Europe and it is the third highest cause of cancer-related death globally (1). HCC is generally diagnosed at an advanced stage or with progression after locoregional therapy and has a dismal prognosis, owing to the underlying liver disease and the lack of effective therapeutic options (2, 3). Sorafenib (BAY-43-9006 Nexavar, Bayer Pharmaceuticals Corp. and Onyx Pharmaceuticals Inc.), an oral kinase inhibitor of the VEGF, PDGF receptors, and Raf, decreases tumor growth and inhibits angiogenesis in advanced HCC (4, 5). Sorafenib exerts a direct effect on tumor-cell proliferation and survival but mainly acts on endothelial cells to inhibit tumor angiogenesis (6, 7). Finally, it is the only nonsurgical and nonradiological treatment that improves median overall survival and progression-free survival in patients with advanced HCC (5).

Sorafenib inhibits tumor cell proliferation and induces cell death through the inhibition of the RAF/MEK/ERK pathways (6). Human HCC-lesions display both high expression and enhanced activity of MAP kinase (MAPK) compared with adjacent non-neoplastic liver (8). Treating HCC cells with a MEK inhibitor reduces cell proliferation and induces apoptosis (9). Several studies have linked the oncogenic potential of ERK to the activation of anti-apoptotic proteins, such as Bcl-2, Bcl-XL, Mcl-1, IAP, and the repression of pro-apoptotic proteins, such as Bad and Bim (10). Moreover, the overactivation of the MEK/ERK pathway in liver tumor cells confers resistance to TGFB-β-induced cell death through impairing NOX4 up-regulation and subsequent production of reactive oxygen species (ROS), which is required for an efficient mitochondrial-dependent apoptosis (11, 12). The role of ROS in tumor biology has been extensively studied (13, 14). ROS are chemically reactive molecules that fulfill essential functions in living organisms. A moderate increase in...
ROS can promote cell proliferation and differentiation (15, 16), whereas excessive amounts of ROS can cause oxidative damages to lipids, proteins and DNA and lead to cell death (13, 14). Several chemotherapeutic drugs exert their cytotoxic effects through the generation of ROS (17). As sorafenib inhibits the MEK/ERK pathway that controls ROS production in HCC, one of the mechanisms through which sorafenib could exert its cytotoxic effects is the restoration of the susceptibility of HCC cells to ROS-induced lysis.

To determine whether ROS production is essential to sorafenib cytotoxicity, we studied the levels of ROS in HepG2 human and Hepa 1.6 murine HCC cells exposed to sorafenib in vitro. We then investigated the consequences of the selective inhibition of various ROS in vitro and in an in vivo mouse model of HCC. Finally, we showed ex vivo that the elevation of advanced oxidized protein products in patients’ sera 15 days following the initiation of sorafenib chemotherapy is correlated with the effectiveness of the drug.

Materials and Methods

Animals

C57/BL6 female mice between 6 and 8 weeks of age were used in all experiments (Harlan). Animals received human care in compliance with institutional guidelines (Accreditation number for animal research establishment: A-75-14-05, agreement number of Carole Nicco to conduct animal research: 75-1302).

Chemicals, cell lines, and culture

All chemicals were from Sigma (Saint Quentin Fallavier) except for sorafenib (BAY-43-906 Nexavar, Bayer Pharmaceuticals Corp. and Onyx Pharmaceuticals Inc.). Sorafenib (4-(4-(3-[4-Chloro-3-(trifluoromethyl)-phenyl]ureido)phenoxy)-N2-methylpyridine-2-carboxamide-4-methylbenzenesulphonate) has a molecular formula of C31H16ClF3N4O3C7H8O3S and a molecular weight of 637.0 (18). The chemical structure of sorafenib is presented in Fig. 1. Hepa 1.6 (mouse HCC cell line), HepG2 (human hepatocellular liver carcinoma cell line) were obtained from American Type Culture Collection. HUVEC (human umbilical venous endothelial cells) were obtained by digestion of umbilical cords with 0.1% collagenase as patients’ sera 15 days following the initiation of sorafenib chemotherapy is correlated with the effectiveness of the drug.

Figure 1. Molecular structure of sorafenib.

Cellular Production of O}_2^{−}, H}_2O}_2, and NO

Cells (2 × 10^4 cells per well) were seeded in 96-well plates (Costar, Corning, Inc.) and incubated for 48 hours with various concentrations of sorafenib alone or in association with either 400 μmol/L N-acetylcysteine (NAC), a precursor of reduced glutathione (GSH), or 100 μmol/L manganese (III) tetrakis (4-benzoic acid) porphyrin (MnTBAP), a mimic of superoxide dismutase (SOD), or 4 mmol/L diethyldithiocarbamate (DDC), a SOD inhibitor, or 40 μmol/L diphenyleneiodonium chloride (DPI), an inhibitor of flavonoid-containing enzymes like NADPH oxidase, or 40 mmol/L rotenone (inhibitor of mitochondrial complex I) or 40 mmol/L antimycin, an inhibitor of mitochondrial complex III, or with 40 mmol/L allopurinol, an inhibitor of xanthine oxidase, or with culture medium alone (19). Levels of intracellular superoxide anion O}_2^{−}, hydrogen peroxide H}_2O}_2 and nitric oxide NO were assessed spectrofluorometrically (Packard Bioscence) by oxidation of dihydroethidium (DHE, Molecular Probes) 2′,7′-dichlorodihydrofluorescein diacetate (H2DCFDA, Molecular Probes) 4,5-diaminofluorescein diacetate (DAF2-DA), respectively (20). The levels of O}_2^{−}, H}_2O}_2 and NO were calculated in each sample as follows: ROS rate (OD/min/10^6 cells) = (OD60 minutes after drug first contact – initial OD)/60 minutes/number of viable cells as measured by the crystal violet assay.

Intracellular GSH measurement

Levels of intracellular GSH were measured spectrofluorometrically by monochlorobimane staining (21). Briefly, cells (2 × 10^4 cells per well) were seeded in 96-well plates (Costar, Corning, Inc.) and incubated for 48 hours with various concentrations of sorafenib alone or in association with either MnTBAP or NAC or culture medium alone. Cells were then washed once with PBS and incubated with 50 μmol/L monochlorobimane. Fluorescence intensity was measured after 15 minutes at 37°C. Excitation and emission wavelengths were 380 and 485 nm, respectively. Intracellular GSH level was expressed as arbitrary units of fluorescence intensity.

In vitro cell proliferation and viability assays

HepG2, Hepa 1,6, and HUVEC (2 × 10^4 cells/well) were seeded into 96-well plates and incubated for 48 hours in complete DMEM medium with increasing amounts of sorafenib alone, or in association with either 400 μmol/L NAC, or with 100 μmol/L MnTBAP or 200 μmol/L nitric oxide synthase inhibitor NG-nitro-L-arginine methyl ester (L-NAME). Cell proliferation was determined by pulsing the cells with [3H]thymidine (1 μCi/well) during the last

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16 hours of culture. Cell viability was evaluated by the crystal violet assay (20). Results were expressed as percentages of viable cells compared with untreated cells (that represent 100% viability).

**In vivo antitumor activity of sorafenib treatment**

Hepa 1.6 cells (2 × 10⁶) were injected subcutaneously into the back of the neck of C57/BL6 mice. When the tumor reached a mean size of 200 to 500 mm³, mice were randomized (around day 10) into each experimental and control groups according to tumor size, to start the treatment with a similar mean size in each group. Mice were treated with either sorafenib by oral route (50 mg/kg/day, n = 7), or intraperitoneal MnTBAP (15 mg/kg three times a week, n = 7), or PBS as control group, n = 7. Tumor size was measured with a calliper rule every 2 days. The tumor volume was calculated as follows: TV (mm³) = (L × W²)/2, where L is the longest and W the shortest radius of the tumor in millimeters. Results were expressed as means of tumor volumes ± SEM (n = 7 mice in each group).

**Patients and sera**

Twenty-six patients with advanced-stage HCC were enrolled in the study and treated with sorafenib at a validated dose of 400 mg twice daily until there was evidence of disease progression. Patients were classified as having advanced disease if they were not eligible for or had disease progression after surgical or locoregional therapies (5). All patients gave written informed consent. The study was in accordance with Declaration of Helsinki and was approved by the Comité de protection des personnes d’Île de France. All patients had a Child-Pugh class and a MELD score evaluation at baseline. The MELD score was 3.8 × log₁₀(bilirubin [mg/dL]) + 11.2 × log₁₀(INR) + 9.6 × log₁₀(creatinine [mg/dL]) + 6.4 × (etiology: 0 if cholestatic or alcoholic, 1 otherwise) as previously published (22). Sorafenib was administered at 50% of the planned dose if any severe adverse event related to the drug occurred, and in frail patients (5). Treatment interruptions and up to two dose reductions (first to 400 mg once daily, and then to 400 mg every 2 days) were decided in case of drug-related adverse effect. Treatment continued until the occurrence of either radiologic progression as defined by RECIST criteria (23), or symptomatic progression. A retrospective review of electronic medical records of patients with advanced HCC treated with sorafenib was carried out in our institution from October 2008 to December 2010. Further analysis of clinical toxicity and interpretation of body composition from computed tomography images were approved by the Institutional Review Board. Sera were collected from each patient 7 days before and 14 days after sorafenib start.

**Patients treatment and toxicity assessment**

Adult patients with advanced HCC received sorafenib at a starting dose of 200 mg twice a day or 400 mg twice a day according to their ECOG PS and comorbidities, at the discretion of the treating physician. Patients were treated in an outpatient setting, and toxicity was assessed at visits on days 14 and 28 after the initiation of sorafenib (or before if clinically indicated), then monthly. Sorafenib was reduced to 200 mg twice a day in case of severe toxicity (grade 3 or 4 toxicity according to the NCI-CTCAE v3.0), except for patients with grade 3 hypertension in whom anti-hypertensive drugs were introduced according to current guidelines (24). If toxicity was not thereby resolved, treatment was terminated and if so, patients were returned to the initially scheduled dose. A dose-limiting toxicity was defined as any toxicity leading to dose reduction or to termination of treatment. Only dose-limiting toxicities occurring during the first month of treatment were examined for the present analysis.

**Assay of advanced oxidation protein products in sera**

Advanced oxidation protein products (AOPP) were measured by spectrophotometry as previously described (25). The assay was calibrated using chloramine-T. The absorbance was read at 340 nm on a microplate reader (Fusion, PerkinElmer). AOPP concentrations were expressed as µmol/L of chloramine-T equivalents.

**Assay of nitrite and nitrate levels in sera**

Nitrite levels were measured by direct Griess reaction (26), which is the simplest and most commonly used assay. Nitrate levels were measured by subtraction of the nitrite levels from total NO measured by indirect Griess reaction. Nitrate serum levels reflect NO synthesis in patients treated by sorafenib.

**Statistical analysis**

The statistical significance of differences between experimental treated groups and untreated controls was analyzed by Student’s t test for comparison of means. A level of P < 0.05 was accepted as significant, *P < 0.05; **P < 0.01; ***P < 0.001 versus controls. Descriptive statistics were used to report patients and disease characteristics [median, ranges, 95% CI], and treatment-induced toxicities. PFS and OS were measured from the date of first administration of sorafenib to the date of disease progression or death for the former, and the date of death for the latter. Survival analyses were carried out using the Kaplan–Meier method with the log-rank test. Toxicity and activity data were collected until December 15, 2011. Calculations were done with NCSS 2007 software (NCSS).

**Results**

**ROS production by sorafenib-stimulated HCC cell lines is dose-dependent**

Sorafenib increased the production of O₂⁻⁻, NO and H₂O₂ in HepG2, Hepa 1.6 and HUVEC cell lines (Fig. 2). At the highest concentration of sorafenib tested, O₂⁻⁻ levels were increased by 33% (P < 0.05), 300% (P < 0.001) and 40% (P < 0.05) in HepG2, Hepa 1.6 and HUVEC cell lines, respectively. Adding 400 µmol/L NAC to sorafenib did
not modify $O_2^{\cdot -}$ production in HepG2, Hepa 1.6 and HUVEC cells (Fig. 2A). Adding 100 $\mu$mol/L MnTBAP to 5 mg/L sorafenib, inhibited $O_2^{\cdot -}$ production in the three cell lines tested ($P < 0.05$ for HepG2, Hepa 1.6 and HUVEC). Adding 40 $\mu$mol/L DPI to 5 mg/L sorafenib inhibited $O_2^{\cdot -}$ production in HepG2 cells, Hepa 1.6 and HUVEC cells ($P < 0.05$ for HepG2, Hepa 1.6 and HUVEC).

Superoxide anions can originate from the mitochondrial complex I or III of the respiratory chain, or from the cytosolic enzymes NAD(P)H oxidase or xanthine oxidase. Adding rotenone and antimycin that inhibit the mitochondrial respiratory chain complexes I and III, respectively, increased the $O_2^{\cdot -}$ production in HepG2 cells, Hepa 1.6 and HUVEC cells ($P < 0.05$) without modifying the
production of superoxide anion induced by sorafenib because the $\mathrm{O}_2^-$ increase in cells treated with sorafenib was constant whether rotenone and antimycin was added or not (Fig. 2B). Adding 40 mmol/L allopurinol, a xanthine oxidase inhibitor decreased the basal production of $\mathrm{O}_2^-$ in HepG2 cells, Hepa 1.6 and HUVEC cells ($P < 0.05$) without modifying the production of superoxide anion induced by sorafenib (Fig. 2B). Adding 40 mmol/L DDC, a SOD inhibitor, enhanced $\mathrm{O}_2^-$ production in HepG2 cells, Hepa 1.6 and HUVEC cells ($P < 0.01$) without modifying the production of superoxide anion induced by sorafenib.

Adding 400 μmol/L NAC to 5 mg/L sorafenib inhibited H$_2$O$_2$ production in the cell lines tested ($P < 0.05$ for HepG2, $P < 0.001$ for Hepa 1.6 and $P < 0.001$ for HUVEC, versus 5 mg/L sorafenib alone; Fig. 2C). Adding 100 μmol/L MnTBAP still augmented the generation of H$_2$O$_2$ in the cell lines (at 5 mg/L sorafenib: $P < 0.001$ for HepG2, $P < 0.05$ for Hepa 1.6 and $P < 0.05$ for HUVEC). NO production induced by sorafenib was dose-dependent (Fig. 2D) and increased with the concentration of sorafenib (at 5 mg/L sorafenib: $P < 0.05$ for HepG2, Hepa 1.6 and HUVEC).

Sorafenib exerts dose-dependent cytostatic and cytotoxic effects in vitro

Incubation of HepG2, Hepa 1.6 cells or HUVEC with increasing amounts of sorafenib dose-dependently decreased their proliferative rates and their viability. At 5 mg/L, sorafenib decreased the proliferative rates by 98%, 90% and 26%, respectively ($P < 0.001$ for HepG2 and Hepa 1.6, $P < 0.05$ for HUVEC; Fig. 4A), and their viability rates by 19%, 24% and 18%, respectively ($P < 0.05$ for HepG2, Hepa 1.6 and HUVEC). To determine the metabolic pathways through which sorafenib exerts its cytostatic and cytotoxic effects of sorafenib, the molecule was associated with MnTBAP, or NAC, or L-NAME.

Figure 3. Kinetic analysis of ROS production by HepG2, Hepa 1.6, and HUVEC cells. HepG2, Hepa 1.6, and HUVEC were incubated with 2.5 mg/L sorafenib alone or in association with 400 μmol/L N-acetylcysteine, 100 μmol/L MnTBAP, or 40 μmol/L DPI. Measurements of superoxide anion ($\mathrm{O}_2^-$; A) and hydrogen peroxide (B) were realized by spectrophotometry as described in Material and Methods. Results are expressed as mean OD ± SEM. Data from at least three independent experiments have been pooled. Statistical significance: *, $P < 0.05$ versus culture medium alone (basal level). Error bars represent SEM.
Adding 100 μmol/L MnTBAP to 5 mg/L sorafenib significantly inhibited the cytostatic effects (P < 0.001 for HepG2 and Hepa 1.6, P < 0.05 for HUVEC; Fig. 4A) and cytotoxic effects of sorafenib (P < 0.001 for HepG2, Hepa 1.6 and HUVEC; Fig. 4B). By contrast, neither 400 μmol/L NAC nor L-NAME altered the cytostatic or cytotoxic effects of sorafenib (Fig. 4A, B). In cells exposed to 5 mg/L sorafenib for 48 hours, the GSH pool was depleted by 40%, 53% and 44% in HepG2, Hepa 1.6 and HUVEC, respectively (P < 0.001 for HepG2, P < 0.001 for Hepa 1.6 and P < 0.001 for HUVEC; Fig. 4C). As expected, NAC partially reversed the depletion of GSH (at 5 mg/L sorafenib: P < 0.05 for HepG2, P < 0.05 for Hepa 1.6 and P < 0.01 for HUVEC), and MnTBAP worsened the depletion of GSH induced by 5 mg/L sorafenib (at 5 mg/L sorafenib: P < 0.001 for HepG2, P < 0.001 for Hepa 1.6 and P < 0.001 for HUVEC), (Fig. 4C); As MnTBAP but neither NAC nor L-NAME could abrogate the cytostatic and cytotoxic effects of sorafenib on the cell lines tested, O₂⁻/C⁰/C⁰ is the main ROS induced by sorafenib and responsible, at least in part, for its cytostatic and cytotoxic properties.

**Effect of sorafenib on tumor growth in vivo**

Mice bearing Hepa 1.6 tumors were treated with either sorafenib alone or in association with MnTBAP (Fig. 5). Mice treated with sorafenib alone presented significantly smaller tumors than untreated mice (-79%, P < 0.001 at
Sorafenib has a well-documented effectiveness in HCC and cytostatic effects of sorafenib are mediated via O$_2^-$/H$_2$O$_2$ and generation of ROS responsible for protein oxidation. No difference in the adverse effects of sorafenib was observed between the two groups and no difference in limiting toxicity (P < 0.05; Supplementary Table 1). Best response at 3 months was significantly higher in ≥0.2 AOPP group than in <0.2 AOPP group considering mRECIST criteria or Choi criteria (mRECIST criteria: 30% versus 13%; P < 0.05; Choi criteria: 60% versus 13%; P < 0.05) (Fig. 6B). Median progression-free survival (Fig. 6D) was significantly higher in the ≥0.2 AOPP group (174 days [range: 34 to 482]) than in the ≤0.2 AOPP group (50 days [range: 9 to 136], P < 0.05). Median overall free survival (Fig. 6D) was significantly higher in the ≥0.2 AOPP group (315 days [range: 138 to 1075]) than in the ≤0.2 AOPP group (191 days [range: 43 to 705], P < 0.05).

### Table 1. Baseline characteristics of hepatocellular carcinoma patients treated with sorafenib

<table>
<thead>
<tr>
<th>Patient Characteristics</th>
<th>AOPPd &lt;0.2 AOPP Group</th>
<th>AOPPd ≥0.2 AOPP Group</th>
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<tr>
<td>Age, years, Average (SD)</td>
<td>63.1 (15.9)</td>
<td>66.6 (11.0)</td>
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<td>Sex, Male/Female (%)</td>
<td>80/20</td>
<td>88/12</td>
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<td>ECOG 0/1/2, (%)</td>
<td>30/50/20</td>
<td>44/50/6</td>
</tr>
<tr>
<td>Specific metastatic sites, n</td>
<td>1.6 (2.0)</td>
<td>1.9 (2.9)</td>
</tr>
<tr>
<td>Lung</td>
<td>4</td>
<td>5</td>
</tr>
<tr>
<td>Liver</td>
<td>10</td>
<td>15</td>
</tr>
<tr>
<td>Other</td>
<td>4</td>
<td>7</td>
</tr>
<tr>
<td>Duration of disease, months, Average (SD)</td>
<td>11.2 (9.3)</td>
<td>13.4 (11.9)</td>
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<tr>
<td>Cause of Cirrhosis, n</td>
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<td>5</td>
</tr>
<tr>
<td>Hepatitis C only</td>
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<td>7</td>
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<tr>
<td>Hepatitis B only</td>
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<td>1</td>
</tr>
<tr>
<td>Alcohol only</td>
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<td>3</td>
</tr>
<tr>
<td>Child Pugh classification</td>
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<td>11/5/0</td>
</tr>
<tr>
<td>A/B/C, n</td>
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<td>5</td>
</tr>
<tr>
<td>MELD score*</td>
<td>10 (4–16)</td>
<td>12 (6–19)</td>
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<tr>
<td>Weight (kg), Average (SD)</td>
<td>66.8 (13.7)</td>
<td>75.6 (9.8)</td>
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<td>BMI, Average (SD)</td>
<td>23.9 (5.8)</td>
<td>25.5 (4.0)</td>
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<td>Biologic Results</td>
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<td>Albuminemia (g/dL)</td>
<td>31.8 (22–36)</td>
<td>37.7 (31–44)</td>
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<td>Pre-albuminemia</td>
<td>0.10 (0.05–0.18)</td>
<td>0.18 (0.09–0.28)</td>
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<tr>
<td>Bilirubinemia</td>
<td>31.0 (4–81)</td>
<td>12.7 (4–22)</td>
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<td>ALT</td>
<td>80.9 (31–143)</td>
<td>48 (15–127)</td>
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<tr>
<td>AST</td>
<td>125.8 (24–418)</td>
<td>77 (21–168)</td>
</tr>
</tbody>
</table>

**Abbreviations:** SD, standard deviation; ECOG, Eastern Cooperative Oncology Group Criteria Performance Status; BMI, body mass index (weight/height$^2$).

*Values reported are median (25th percentile to 75th percentile).

**Assay of nitrate and AOPP levels in sera from patients with HCC treated by sorafenib**

Twenty-six patients treated by sorafenib for HCC were included in the present study. Patients' characteristics are presented in Table 1. No difference was observed in nitrate serum levels between the beginning of the treatment (day 0) and day 15 (mean: 22.8 versus 22.6; Figs. 5A and 6A). As nitrite and/or nitrate serum reflect NO synthesis, these results confirm that NO does not mediate sorafenib anti-tumor effectiveness. Serum AOPP levels in patients treated by sorafenib were higher after 15 days' treatment than before treatment (P < 0.05; Fig. 6A). The patients were distributed into two groups: one group with an increase in AOPP ≤0.2 μmol/L chloramine T equivalent (<0.2 AOPP, n = 10), and one group with ≥0.2 μmol/L chloramine T equivalent (≥0.2 AOPP, n = 16). The latter group comprised patients in whom sorafenib effectively generated ROS responsible for protein oxidation. No difference in the adverse effects of sorafenib was observed between the two groups and no difference in limiting toxicity (P = NS), diarrhea (P = NS), hypertension (P = NS), hand foot syndrome (P = NS) nor asthenia (P = NS; Supplementary Table 1). Best response at 3 months was significantly higher in ≥0.2 AOPP group than in <0.2 AOPP group considering mRECIST criteria or Choi criteria (mRECIST criteria: 30% versus 13%; P < 0.05; Choi criteria: 60% versus 13%; P < 0.05) (Fig. 6B). Median progression-free survival (Fig. 6D) was significantly higher in the ≥0.2 AOPP group (174 days [range: 34 to 482]) than in the ≤0.2 AOPP group (50 days [range: 9 to 136], P < 0.05). Median overall free survival (Fig. 6D) was significantly higher in the ≥0.2 AOPP group (315 days [range: 138 to 1075]) than in the ≤0.2 AOPP group (191 days [range: 43 to 705], P < 0.05).

**Discussion**

This study reports for the first time that the cytotoxic and cytostatic effects of sorafenib are mediated via O$_2^-$/H$_2$O$_2$. Sorafenib has a well-documented effectiveness in HCC.
cell lines and is presently the only validated treatment for HCC (6, 27). As previously published, we confirm that sorafenib exerts dose-dependent cytostatic and cytotoxic effects in vitro (6) and report that HCC cell lines produce various ROS following exposure to sorafenib in vitro. Furthermore, the concentration of AOPP in the sera from patients treated by sorafenib is a predictive marker of both progression-free survival and overall survival.

Sorafenib exerts cytostatic and cytotoxic effects on HCC cell lines and on endothelial cells. Concomitantly, sorafenib can enhance the production of superoxide anion, hydrogen peroxide, and nitric oxide in those cells. The use of specific ROS modulators shows that dismutation of superoxide anions induced by a SOD mimic (MnTBAP; ref. 28) prevents sorafenib cytotoxicity whereas neither the H$_2$O$_2$ scavenger NAC (29) nor the NO synthase inhibitor L-NAME (30) have any effect on sorafenib cytotoxicity. Those data highlight the prominent role of superoxide anion in sorafenib antiproliferative and cytotoxic effects. Superoxide anion is the first radical produced within the cells. It can interact with various substrates such as proteins, DNA or lipids and cause irreversible cellular damages (31). Direct antiproliferative and cytotoxic effects of superoxide anion have already been observed in various types of tumor cells such as, for example, B16 mouse melanoma (32). Superoxide anion can originate from the cytosolic enzymes NAD(P)H oxidase, the xanthine oxidase or the complexes I and III of the mitochondrial respiratory chain. The xanthine oxidase inhibitor, allopurinol, and the complexes I and III of the mitochondrial respiratory chain inhibitors, antimycin and rotenone, had no significant impact on sorafenib-induced superoxide anion production. The use of DPI that inhibits the NAD(P)H oxidase clearly shows that in sorafenib-treated cells O$_2^-$/H$_2$O$_2$ is produced by NAD(P)H oxidase. These data are in line with finding that the treatment of HCC cells with NAD(P)H oxidase inhibitors, including DPI, significantly blunts both the generation of ROS and the induction of apoptosis (33). This suggests that NAD(P)H oxidase is the major source of ROS in human HCC cells (34). The activation of NAD(P)H oxidase can be directly caused by the molecule sorafenib. It can also be strengthened in vivo by the VEGF-mediated effects of sorafenib. Indeed, while inhibiting VEGF signalling and reducing neoangiogenesis (35), sorafenib leads to tumor hypoxia and triggers the production of ROS (35).

Our work emphasizes the involvement of the oxidative stress in the treatment of HCC patients by sorafenib and provides a new serum marker to evaluate the effectiveness of sorafenib. As sorafenib induces ROS that can modulate tumor cell death and proliferation (36, 37), we quantified the concentration of oxidized proteins in the sera of HCC patients. Proteins or lipids or DNA can be the targets of the oxidative burst (38). It has been shown that the concentration of advanced oxidation protein product can be used to monitor the in vivo oxidative stress in humans (39) as it reflects the intensity of the oxidative insult in various pathologic conditions. In our study, the

Figure 6. A, measurement of advanced oxidation protein products (AOPP) levels in 26 sera from patients either before sorafenib or after 15 days of treatment. AOPP were measured by spectrophotometry as previously described (25). B, best response rates at 3 months considering mRECIST criteria or Choi criteria. Best response rate correspond to either complete response, partial response, or stable disease. Progression-free survival (C) and overall free survival (D) in HCC patients receiving sorafenib therapy.
initial level of AOPP before any treatment is not predictive for sorafenib effectiveness (data not shown). However, the elevation of serum AOPP after 15 days’ treatment is significantly correlated with sorafenib clinical effectiveness. Indeed, a serum level of AOPP $\geq 0.2$ μmol/L chloramine T equivalent ($\geq 0.2$ AOPP) correlates with both progression-free survival and overall survival suggesting an efficient dose exposure. Accordingly, given the inter-individual variability of the values of the sorafenib area under the plasma concentration–time curve over 12 hours (AUC), the lack of effect observed in patients with $<0.2$ AOPP may be linked to an underexposure (40). To date, the bulk of data on dose exposure to sorafenib is growing and suggests a potential benefit for sorafenib dose-escalation strategies in renal cell carcinoma (41). Considering those results and the sorafenib pharmacokinetic analysis, the assay of serum AOPP might become a useful and early indicator of sorafenib effectiveness.

In conclusion, sorafenib effectiveness is mediated by ROS and particularly by $O_2$ production. The effect of this production on protein oxidation is an early predictive marker of sorafenib effectiveness. In response to sorafenib, HCC cancer cells produce massive amounts of ROS that in return induce tumor cell death (6). No or weak ROS production predicts a lack of effectiveness. Thus, we hypothesize that the effectiveness of sorafenib in HCC patients depends on the balance between the ability of tumor cells to produce ROS and their ability to tolerate an exposure to sorafenib. Therefore, monitoring ROS production induced by sorafenib in the absence of limiting toxicity could allow planning a dose escalation of the molecule. Clinical studies should be designed to best this hypothesis.

Disclosure of Potential Conflicts of Interest

F. Goldwasser has acted as a paid consultant for Bayer. No potential conflicts of interest were disclosed by the other authors.

Our research article involved patients and written informed consent was obtained from all patients. The study was in accordance with the Declaration of Helsinki and was approved by the Comité de protection des personnes d’Île de France.

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