AZD6244 (ARRY-142886) enhances the therapeutic efficacy of sorafenib in mouse models of gastric cancer

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Abstract

Gastric cancer is a deadly disease for which current therapeutic options are extremely limited. Vascular endothelial growth factor receptors and platelet-derived growth factor receptors regulate gastric cancer cell proliferation, invasion, and tumor angiogenesis. In the present study, we report that sorafenib therapy effectively inhibited tumor growth and angiogenesis in tumor xenografts. These were associated with reduction in the phosphorylation of vascular endothelial growth factor receptor-2 Tyr951, c-Kit Tyr568/570, platelet-derived growth factor receptor-β Tyr1021, and Akt Ser473 and Thr308, down-regulation of positive cell cycle regulators, increased apoptosis, and up-regulation of p27. Sorafenib treatment also caused up-regulation of p-c-Raf Ser338 and p-extracellular signal-regulated kinase (ERK) Thr202/Tyr204 in gastric cancer xenografts. The combination of sorafenib and MAP/ERK kinase inhibitor AZD6244 enhances the effectiveness of each compound alone. Potential effect of sorafenib/ AZD6244 included increase in proapoptotic Bim. Our data show that MAP/ERK kinase inhibition enhances the anti-tumor activity of sorafenib in vivo, supporting a rationale for multitargeted suppression of the angiogenesis and ERK signaling network in gastric cancer therapy. [Mol Cancer Ther 2009;8(9):2537–45]

Introduction

Gastric cancer is the fourth most common cancer and the second most common cause of cancer-related deaths worldwide (1); in 2002, there was an estimation of 934,000 new cases and 700,000 deaths annually (1). Although the global gastric cancer incidence has declined, the incidence remains high in Asian countries (2, 3). Although early diagnosis and treatment of gastric cancer significantly improves prognosis (4, 5), the 5-year survival rate is only 10% to 15% for those with advanced disease (6). At present, the combination of 5-fluorouracil and platinum analog is the most widely accepted standard regimen worldwide (7). However, the prognosis remains poor, particularly for late-stage gastric cancer, despite numerous efforts of various randomized studies on advanced gastric cancer (7, 8). Hence, there is clearly a need to identify other agents that may offer superior efficacy for patients with advanced gastric cancer.

Vascular endothelial growth factor (VEGF) receptors and platelet-derived growth factor (PDGF) receptors are important in endothelial cell proliferation, invasion, and angiogenesis (9). The Raf/MAP/extracellular signal-regulated kinase (ERK) kinase (MEK)/ERK pathway can be activated by many factors (10, 11), and its activation is associated with aggressive tumor behavior (12) and angiogenesis (13, 14). It has been shown that c-Raf up-regulates VEGF expression through HIF-1α (15). Previous studies have established an association between VEGF expression, increased microvessel density, and decreased survival in gastric cancer (16). Expression of VEGF is strongly correlated with tumor progression and poor prognosis in gastrointestinal malignancies, including gastric cancer (17). Preclinical studies on agents that target VEGF and its receptors in gastric cancer have shown significant antitumor effects (18, 19). Data from preclinical study suggested that simultaneous inhibition of VEGF receptor-2 and PDGF receptor-β might produce greater antitumor effects than inhibition of either receptor tyrosine kinase alone (20). Because VEGF receptors, PDGF receptors, and Raf/MEK/ERK cascade play critical roles in the development and progression of gastric cancer, the use of a multi-kinase inhibitor to block these signaling cascades could thus have therapeutic efficacy.

Sorafenib (BAY 43-9006; Nexavar, Bayer and Onyx Pharmaceuticals) is a multikinase inhibitor that has been shown to have efficacy against a wide variety of tumors in preclinical models (21, 22). Previous studies showed that sorafenib blocked tumor cell proliferation and angiogenesis by inhibiting c-Raf, V600E mutant B-Raf, and wild-type B-Raf, as...
well as VEGF receptor-2, VEGF receptor-3, PDGF receptor, FLT3, Ret, and c-Kit (21, 23). More recently, sorafenib has been shown to provide a significant improvement on overall survival in patients with hepatocellular carcinoma (24) and progression-free survival in renal cell carcinoma (25). It has also been reported that scattering of AGS gastric cancer cells induced by Helicobacter pylori infection was inhibited by sorafenib (26), further suggesting that this agent may be effective in treating gastric cancer.

In this study, we assessed the antitumor and angiogenesis effects of sorafenib alone and in combination with MEK inhibitor AZD6244 using patient-derived gastric carcinoma xenografts. Our results indicated that sorafenib inhibited cell proliferation and the growth of gastric cancer xenografts but up-regulated p-c-Raf Ser338 and p-ERK Thr202/Tyr204. The antitumor activity of sorafenib was significantly augmented through induction of apoptosis and inhibition of cell proliferation and angiogenesis when it was combined with AZD6244. Our results underscore the potential of a combined therapeutic approach with sorafenib and MEK inhibitors for the treatment of gastric cancer.

Materials and Methods

Reagents

Antibodies against cleaved caspase-3, Akt, p-Akt Ser473 and Thr308, cleaved poly(ADP-ribose) polymerase (PARP), p-c-Raf Ser259, p-MEK Ser217/221, and p-ERK Thr202/Tyr204 were from Cell Signaling Technology. Antibodies against Bim, Bax, Bcl-xL, Bad, Bel-2, c-Raf, ERK1/2, cyclin B1, cyclin A, cdk-2, cdk-4, cdk-6, cdc-2, p27, VEGF receptor-2, p-VEGF receptor-2 Tyr951, PDGF receptor-β, p-PDGFR receptor-β Tyr1021, c-KIT, p-c-KIT Tyr568/570, and α-tubulin were from Santa Cruz Biotechnology, Inc. Antibodies against Ki-67 and CD31 were from Lab Vision.

Effects of Sorafenib on the Growth of S.c. Gastric Cancer Xenografts

This study received ethics board approval at the National Cancer Centre of Singapore and Singapore General Hospital. All mice were maintained according to the Guide for the Care and Use of Laboratory Animals published by the NIH.

Patient-derived GC-28-1107 and GC-27-0208 gastric cancer xenografts were established into severe combined immunodeficient mice, as described (27). Briefly, primary gastric cancers were obtained from surgery, immediately placed in chilled RPMI 1640, and quickly transferred to the laboratory. Thin slices of tumor were diced into 2- to 3-mm pieces and washed thrice with RPMI 1640. These tumor pieces were minced into fine fragments that would pass through an 18-gauge needle and were then mixed 1:1 (v/v) with Matrigel (Collaborative Research) to give a total volume of 0.2 mL per injection. The tissue mixture was s.c. injected in both flanks of age 9 to 10 weeks severe combined immunodeficient mice (Animal Resources Center), as described (27).

Sorafenib (Nexavar, Bayer and Onyx Pharmaceuticals) and AZD6244 (AstraZeneca) were suspended in 30% Capsitol solution (vehicle) at an appropriate concentration. For monotherapy, mice bearing the GC-28-1107 or GC-27-0208 xenografts were given 10, 20, and 30 mg/kg sorafenib tosylate daily orally for 21 d. Each treatment group was comprised of 14 animals. Treatment started on day 15 after tumor implantation. By this time, the tumors reached the size of approximately 100 to 150 mm³. Animals were sacrificed 3 h after the last dose of sorafenib. Body and tumor weights were recorded, and the tumors were harvested for analysis.

To investigate the effects of AZD6244, sorafenib, and AZD6244 plus sorafenib on the growth of gastric cancer xenografts, mice bearing GC-28-1107 tumors and GC-27-0208 (14 per group) were orally administered with 200 μL of vehicle, 25 mg of AZD6244 per kg of body weight daily, 20 mg of sorafenib per kg body weight daily, or sorafenib plus AZD6244 for 21 d starting from day 15 after tumor implantation. By this time, the tumors reached the size of approximately 100 mm³. Tumor growth was monitored and tumor volume was calculated as described (28). At the end of the study, the mice were sacrificed with body and tumor weights being recorded, and the tumors were harvested for analysis.

The efficacy of sorafenib in reducing tumor growth was determined by the treatment (T)/control (C) ratio, wherein T and C are the mean weights (milligrams) of sorafenib-treated and vehicle-treated tumors, respectively, on treatment day 21. T/C ratios ≤ 0.42 are considered an active response according to the Drug Evaluation Branch of the Division of Cancer Treatment, National Cancer Institute criteria.

Western Blot Analysis

To determine changes in indicated proteins, three to four independent tumors from vehicle- and drug-treated mice were homogenized separately in lysis buffer, as described (27). Eighty micrograms of proteins from a single tumor or cell lysate were subjected to Western blot analysis. The blots were then visualized with a chemiluminescent detection system (Amersham, Pharmacia Biotech).

Immunohistochemical Analysis

Tumor tissue samples were processed for paraffin embedding, and 5-μm sections were prepared. The sections were immunostained with CD31, Ki-67, and cleaved PARP antibodies to assess microvessel density, cell proliferation, and apoptosis, respectively, as described (29). The number of Ki-67-positive cells among at least 500 cells per region was counted and expressed as percentage values. For the quantification of mean microvessel density in sections stained for CD31, 10 random fields at a magnification of ×200 were captured for each tumor.

Statistical Analysis

For quantitation analysis, the sum of the density of bands corresponding to protein blotting with the antibody under study was calculated and normalized with the amount of α-tubulin. After normalization with α-tubulin, changes in the expression of the protein under study in treated samples were expressed relatively to the basal levels of this protein in vehicle-treated sample. Differences in the levels of protein under study, tumor weight at sacrifice, Ki-67 index, mean...
microvessel density, and cleaved PARP-positive cells were compared using Student's $t$ test. For all statistical analysis, significance was established at $P < 0.05$.

**Results**

We first evaluated the ability of sorafenib to suppress the growth of patient-derived GC-28-1107 and GC-27-0208 gastric cancer xenografts. As shown in Fig. 1, the tumor weights in mice treated with sorafenib for 21 days were significantly lower than those in vehicle-treated mice. Suppression of tumor growth was seen even at a low dose of 10 mg/kg/d. No overt toxicity, as defined by weight loss, unkempt appearance, mortality, and behavior, was observed in all sorafenib-treated groups during the course of treatment. With the 20 mg/kg/d dose regimen of sorafenib, the $T/C$ ratios for GC-28-1107 and GC-27-0208 xenografts were 0.39 and 0.32, respectively, whereby $T$ and $C$ represent the mean weights (milligrams) of sorafenib- and vehicle-treated tumors on day 21 during the treatment respectively. Because the ratios observed in two xenografts studied were <0.42, it was considered an active response (Drug Evaluation Branch of the Division of Cancer Treatment, National Cancer Institute criteria). This 20 mg/kg dose regimen was then used for all subsequent studies because it gave good growth inhibition and minimal toxicity.

We next examined the antiproliferative, apoptotic, and antiangiogenic effects of sorafenib in treated tumor xenografts. Representative CD31, Ki-67, and cleaved PARP stainings for vehicle- and sorafenib-treated GC-28-1107 tumor xenografts are shown in Fig. 2. The mean percentage of CD31-positive endothelial cells in GC-28-1107 xenografts treated with sorafenib was significantly decreased ($P < 0.05$). This observation is consistent with inhibition of the VEGF receptor and PDGF receptor signaling pathways in endothelial cells. In addition, a significant increase in apoptosis (percentage of cleaved PARP-positive cells) and a significant decrease in proliferation (percentage of Ki-67-positive cells) were observed ($P < 0.05$; Fig. 2D). Extended treatment with sorafenib also caused central tumor necrosis (Fig. 2C). Similar results were obtained when patient-derived GC-27-0208 xenograft was used.

Because sorafenib has been shown to target cell proliferation through the Raf/MEK/ERK signaling pathway (21, 22), we investigated whether sorafenib-induced growth suppression in patient-derived xenografts was associated with the inactivation of this pathway. As shown in Fig. 3B, the levels of c-Raf and p-c-Raf Ser259 in GC-28-1107 tumors treated with 20 mg/kg of sorafenib were ~73% and 61% of those observed in the controls, respectively. Surprisingly, treatment of GC-28-1107 xenograft with sorafenib also resulted in up-regulation of p-c-Raf Ser338, p-MEK Ser217/221, and p-ERK Thr202/Tyr204, suggesting that sorafenib treatment may lead to activation of the Raf/MEK/ERK signaling pathway. In addition, phosphorylation of Akt Ser473 and Thr308 was also decreased by sorafenib. Although total Akt was decreased by sorafenib, the magnitude of reduction was less than phosphorylated Akt, suggesting that sorafenib may inhibit Akt pathway by reducing Akt and its phosphorylation.

Similar data were obtained when the sorafenib-treated GC-27-0208 tumors were analyzed. To gain further into mechanistic action of sorafenib, we conducted additional experiments wherein tumors were collected on days 1 to 4 of dosing. Western blot analysis revealed that phosphorylation of PDGF receptor-$\beta$ and VEGF receptor-2, and Akt was significantly reduced on day 3 of dosing. Reduction in

**Figure 1.** Effects of sorafenib on growth of patient-derived GC-28-1107 and GC-27-0208 gastric cancer xenografts. GC-28-1107 and GC-27-0208 tumors were s.c. implanted in severe combined immunodeficient mice, as described in Materials and Methods. Mice bearing tumor xenografts were treated with vehicle or indicated doses of sorafenib for 21 d, as described in Materials and Methods. Each group consisted of 14 mice. Representative vehicle- and sorafenib-treated tumors and means of tumor weights for GC-28-1107 (A) and GC-27-0208 (B). The data were expressed as the mean weight of 14 tumors ± SE. *, $P < 0.05$ as determined by Student’s $t$ test. Experiments were repeated twice with similar results.
c-Raf, cyclin D1, p-Akt, and cyclin B1 was observed on day 2 of therapy. p-c-Raf Ser338 was increased by day 2 and reached maximal level by day 3 of treatment. Significant increase in phospho-ERK1/2 and apoptosis was noticed on day 3 of sorafenib treatment (Supplementary Fig. S2).

Because Bcl-2 family of proteins regulates apoptosis in mammalian cells (30, 31), and caspase-3 has a central role in PARP cleavage and the proteolytic cleavage of PARP is a biochemical event during apoptosis (32), we determined whether these apoptotic pathways were activated by sorafenib treatment. Figure 3B shows that the 89-kDa cleaved PARP and 17- to 19-kDa cleaved caspase-3 fragments were readily detected in GC-28-1107 tumors treated with 10 mg/kg sorafenib. Figure 3C shows that, whereas Bad expression was increased by ~4 folds, Bcl-2 levels were decreased by sorafenib in a dose-dependent fashion. Bcl-xL and Bax levels in sorafenib-treated tumors were not significantly affected by sorafenib treatment. The data suggest that there is a shift in the dynamic balance between the outputs of proapoptotic and antiapoptotic proteins. Similar results were obtained when patient-derived GC-27-0208 xenograft was used.

Because cell cycle regulators play an important role in the development and progression of gastric cancer, we analyzed the expression of cell cycle regulatory proteins in sorafenib-treated tumor xenografts. Figure 3C shows that the levels of cyclin D1, cyclin A, Cdk-2, Cdk-4, Cdk-6, cdc-2, and cyclin B1 in sorafenib-treated GC-28-1107 tumors were significantly reduced (P<0.01). The results suggest that sorafenib may also inhibit cell cycle progression in vivo by reducing the expression of positive cell cycle regulators.

Because Western blot analysis showed that up-regulation of p-c-Raf Ser338 and p-ERK Thr202/Tyr204 was detected in sorafenib-treated tumors (Fig. 3B), we wished to determine whether inhibition of ERK signaling pathway by AZD6244 would enhance the antitumor activity of sorafenib in gastric cancer. As shown in Supplementary Fig. S2, AZD6244, when given at the dose of 25 mg/kg, was sufficient to block phosphorylation of ERK1/2. To investigate the effects of sorafenib and AZD6244 on tumor growth in severe combined immunodeficient mice, cohort groups were composed of mice bearing GC-28-1107 and GC-27-0208 xenografts randomly assigned to receive vehicle alone, single agents (20 mg/kg sorafenib or 25 mg/kg AZD6244), or the combination therapy (sorafenib plus AZD6244). Agents were provided for a period of 21 days using a dosage schedule of once daily. Therapy initiated after tumor establishment (100–150 mm³), thereby more closely mimicking a clinical therapeutic trial rather than a less relevant prophylactic regimen. Treatment regimen was highly tolerated; no significant weight loss was observed.

Figure 4A shows that treatment with AZD6244 alone moderately affected GC-28-1107 xenograft growth rate. Average tumor volume was lower than that of control mice at each follow-up time point. Sorafenib alone induced significant tumor growth inhibition compared with control or AZD6244 alone treatment (P<0.05). Combined AZD6244 and sorafenib was markedly inhibitory compared with control, AZD6244 alone, or sorafenib alone (P<0.01). Moreover, in GC-28-1107 and GC-27-0208 lines, treatment with sorafenib significantly reduced tumor weight compared with control (P<0.05), whereas AZD6244 alone had modest effect on tumor weight (Table 1). Average tumor weight in AZD6244-treated group was slightly lower than that of control mice. However, combination therapy showed significant effect on tumor weight when compared with sorafenib alone (Table 1). Although the combination at full dose (30 mg/kg sorafenib/25 mg/kg AZD6244) exerted a potent antitumor activity, it did not cause tumor regression (Supplementary Fig. S2).
We investigated the phosphorylation status of the VEGF receptor-2, PDGF receptor-β, Akt, and ERK, as well as apoptotic markers in treated tumors. Sorafenib induced significant reductions in the levels p-PDGF receptor-β Tyr1021 (P < 0.05). Although sorafenib alone decreased the levels of p-VEGF receptor-2 Tyr951, reduction in this marker was most marked when the drugs were used in combination, suggesting the VEGF receptor and PDGF receptor pathways are inactivated in sorafenib- and sorafenib/AZD6244-treated tumors (Fig. 4B). As expected, sorafenib alone and sorafenib/AZD6244 induced down-regulation of p-c-Raf Ser259, c-Raf, cyclin B1, cyclin D1, cdk-2, cdk-6, Mcl-1, and Bcl-2 but up-regulation of p-c-Raf Ser338, p-MEK Ser217/221, and Bad. Sorafenib alone markedly reduced phospho-Akt Ser473, whereas this inhibitory effect was lost in sorafenib/AZD6244. In addition to abolishment of sorafenib-induced up-regulation of p-ERK, sorafenib/AZD6244 also caused a marked up-regulation of Bim, cleaved caspase-3, and cleaved PARP when compared with sorafenib and AZD6244 alone (Fig. 4B). Similar data were obtained when samples from GC-27-0208 line were analyzed (Supplementary Fig. S1).

Next, we evaluated representative tumor sections from four treatment arms using immunohistochemistry. Staining results for CD31, Ki-67, and cleaved PARP are similar to those for gastric tumors above. Sorafenib alone and in combination with AZD6244 induced a decrease in large blood vessels and percentage of Ki-67–positive cells in GC-28-1107 xenograft line compared with control (P < 0.05; Fig. 5 and Table 1). In addition, apoptosis (cleaved PARP–positive cells) was observed in AZD6244- and sorafenib-treated tumors. Combination therapy showed significant increase in cleaved PARP–positive cells when compared with sorafenib and AZD6244 alone (P < 0.05; Fig. 5 and Table 1). The results suggest that AZD6244 produced a predominantly apoptotic effect with a minimal effect on proliferation, whereas 20 mg/kg-dose sorafenib was antiangiogenic and apoptosis-inductive. Interestingly, combination therapy resulted in decreased proliferation and elevated levels of apoptosis (Table 1).

Discussion

It has been shown that the Raf/MEK/ERK pathway participates in signal transduction pathways that regulate growth factor response, angiogenesis, tumor cell proliferation, and apoptosis (reviewed in ref. 33). Although implicated in gastrointestinal cancer pathogenesis (15, 16, 18, 19, 34), the therapeutic value of targeting angiogenesis and the Raf/MEK/ERK pathway in gastric cancer has been limited. This study was done to assess the antitumor activity of sorafenib either alone or in combination with AZD6244 on gastric cancer xenografts and to investigate the underlying antitumor mechanisms.
We have shown that patient-derived cancer xenograft lines responded favorably to sorafenib and sorafenib/AZD6244 therapies whereby sorafenib and sorafenib/AZD6244 suppressed the growth of patient-derived gastric cancer xenografts. Sorafenib-induced growth suppression is associated with inhibition of cell proliferation, as determined by decrease in the number of Ki-67-positive cells and levels of positive cell cycle regulatory proteins such as cyclin D1, Cdk-2, Cdk-4, Cdk-6, cdc-2, cyclin B1, and cyclin A. In addition, sorafenib and sorafenib/AZD6244 have an effect on gastric cancer cell apoptosis through the caspase mediated mitochondrial pathway. In vivo, sorafenib and sorafenib/AZD6244 also exerts antiangiogenic effects evidenced by reduction in the levels of p-VEGF receptor-2 and p-PDGFR receptor-β and lowered microvessel density of tumors harvested from sorafenib- and sorafenib/AZD6244-treated tumors. Up-regulation of p-c-Raf Ser338, p-MEK, and p-ERK Thr202/Tyr204 was also observed in sorafenib-treated tumors. Pharmacologic inhibition of the ERK pathway by AZD6244 enhances the antitumor effect of sorafenib in patient-derived gastric cancer. We further show that such inhibition leads to increase in Bim protein, inhibition of tumor cell proliferation, and increased apoptosis.

In the present study, we test the efficacy of sorafenib on two patient-derived gastric cancer xenograft lines. Although both lines responded to sorafenib in a similar fashion, more patient-derived gastric cancer xenografts are needed to...
validate the above observations. Although the use of patient-derived xenografts is an improvement over the traditional cell lines–derived xenografts, both ectopic xenograft models still have limitations as ideal models for testing new drugs because of the use of immunocompromised mice and lack of interaction between cancer cells and their surrounding tumor microenvironment made up of stromal cells and immune cells. One solution to the tumor microenvironment is surgical orthotopic implantation, in which the intact fragments of gastric tumor taken directly from a patient are transplanted into the stomach of immunodeficient mice. Although establishment of surgical orthotopic

<table>
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<tr>
<th>Lines of xenografts</th>
<th>Treatments</th>
<th>Body weight at sacrifice (g)</th>
<th>Tumor weight (mg)</th>
<th>% T/C</th>
<th>Microvessel* density</th>
<th>Ki-67 index (%)</th>
<th>Cleaved PARP (%)</th>
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</thead>
<tbody>
<tr>
<td>GC-28-1107</td>
<td>Vehicle</td>
<td>21.4 ± 0.65</td>
<td>1160 ± 102^a</td>
<td>100</td>
<td>57 ± 4.5^a</td>
<td>1.5 ± 0.3^a</td>
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<td></td>
<td>Sorafenib</td>
<td>20.1 ± 0.55</td>
<td>454 ± 49^b</td>
<td>39</td>
<td>4 ± 1^b</td>
<td>18 ± 2.3^b</td>
<td>12.2 ± 2^b</td>
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<td></td>
<td>AZD6244</td>
<td>20.5 ± 0.4</td>
<td>880 ± 83^a</td>
<td>75.8</td>
<td>11 ± 2.5^a</td>
<td>47 ± 4^b</td>
<td>7 ± 1.5^b</td>
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<tr>
<td></td>
<td>Sorafenib + AZD6244</td>
<td>20.1 ± 0.7</td>
<td>207 ± 22.5^c</td>
<td>17.8</td>
<td>2 ± 0.5^c</td>
<td>8 ± 1.5^c</td>
<td>28 ± 3^c</td>
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<tr>
<td>GC-27-0208</td>
<td>Vehicle</td>
<td>21.8 ± 0.45</td>
<td>1185 ± 73.5^a</td>
<td>100</td>
<td>11.8 ± 2.5^a</td>
<td>60.8 ± 4.5^a</td>
<td>1.3 ± 0.4^a</td>
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<tr>
<td></td>
<td>Sorafenib</td>
<td>20.3 ± 0.5</td>
<td>385 ± 29^b</td>
<td>32.4</td>
<td>5.3 ± 1.5^b</td>
<td>21.9 ± 3^b</td>
<td>11.9 ± 1.5^b</td>
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<tr>
<td></td>
<td>AZD6244</td>
<td>20.2 ± 0.4</td>
<td>980 ± 77^a</td>
<td>82.7</td>
<td>10.1 ± 2^a</td>
<td>45 ± 4^a</td>
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<td>19.9 ± 0.45</td>
<td>142 ± 26.5^c</td>
<td>12</td>
<td>3.2 ± 1^b</td>
<td>8 ± 2^c</td>
<td>27 ± 2.5^c</td>
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</tbody>
</table>

NOTE: Differences in body weight and tumor weight at sacrifice, Ki-67 index, and percentage of cleaved PARP–positive cells (mean ± SE) among treatment groups were analyzed by Student’s t test. Treatments with different letters are significantly different from one another (P < 0.05). Efficacy of sorafenib, AZD6244, and sorafenib/AZD6244 was determined by % T/C, wherein T and C are the mean weights (mg) of drug-treated and vehicle-treated tumors on day 21 during the treatment, respectively. Experiments were repeated at least twice with similar results.

*Mean microvessel density of 10 random 0.159 mm² fields. Original magnification, ×200.

Figure 5. Effects of sorafenib, AZD6244, and sorafenib/AZD6244 therapies on angiogenesis, cell proliferation, and apoptosis of GC-28-1107 xenograft. Representative pictures of blood vessels stained with anti-CD31, proliferative cells stained with anti–Ki-67, and apoptotic cells stained with anti–cleaved PARP antibodies in vehicle-, sorafenib-, AZD6244-, and sorafenib/AZD6244-treated tumors. Original magnification, ×200. Treatment with sorafenib/AZD6244 resulted in decreased Ki-67–positive cells and increased cleaved PARP–positive cells. Experiments were repeated twice with similar results.
gastric cancer xenografts is technically difficult, we still believe that such tumor models reflect more closely to the disease in human for preclinical testing of targeted therapies in gastric cancer.

At present, the molecular mechanisms responsible for the induction of apoptosis and inhibition of angiogenesis by sorafenib in gastric cancer xenografts are not fully understood. Our in vivo data suggest that sorafenib causes a shift in the dynamic balance between the outputs of proapoptotic and antiapoptotic pathways by decreasing Bcl-2 and increasing Bad. These may be responsible, in part, for the apoptotic activity observed in sorafenib-treated tumors. As shown in Fig. 1, the tumor size attained at the end of the experiment is ~39% of the control tumors for GC-28-1107 and 32% for GC-28-0207. At the molecular levels, sorafenib inhibits tumor growth despite normal activation of the Raf/MEK/ERK pathway, suggesting that sorafenib exerts the antitumor effects mainly through its antiangiogenic activity rather than inhibition of the Raf/MEK/ERK pathway. Our hypothesis of sorafenib inhibiting tumor angiogenesis is further supported by our data showing that extended treatment with sorafenib caused central tumor necrosis (Fig. 2C). Because recruitment of pericytes into tumors involves VEGF receptor/PDGF receptor system (35, 36) and activation of the Akt pathway can recapitulate the effects of VEGF on blood vessels (37), inhibition of the VEGF receptor-2, PDGF receptor-β, and Akt signaling pathways by sorafenib would effectively inhibit tumor angiogenesis and subsequent increase in hypoxia and tumor apoptosis/necrosis.

Sorafenib inhibits multiple receptor tyrosine kinases in addition to Raf, such as VEGF receptors, c-Kit, and PDGF receptors (21). Previous studies (21, 22) employed the in vitro kinase assays with recombinant proteins to determine the effects of sorafenib on c-Raf, B-Raf, MEK-1, and ERK activity. These studies have emphasized the importance of the Raf/MEK/ERK signaling pathway in sorafenib-induced apoptosis in human cancer cell lines (21, 23). They showed that p-ERK was inhibited by sorafenib but did not reveal any information whether sorafenib inhibited c-Raf expression. In the present study, we observe that treatment of gastric cancer xenografts with sorafenib triggers phosphorylation of ERK1/2. Further investigation revealed that sorafenib therapy is also accompanied by a down-regulation of c-Raf and p-c-Raf Ser259 but up-regulation of p-c-Raf Ser338 and p-MEK1/2 Ser217/221. It has been reported that phosphorylation of c-Raf at Ser259 prevents c-Raf activation and dephosphorylation of c-Raf at Ser259 is an essential part of the c-Raf activation process (38). Based on the above information, it is possible that inhibition of p-c-Raf Ser259 by sorafenib facilitates the phosphorylation of c-Raf at Ser338, which in turn phosphorylates MEK and ERK. Activation of Raf is not unique to sorafenib because other c-Raf inhibitors, such as SB 203580 (39) and ZM 336372 (40), also trigger a remarkable activation of c-Raf in vivo, suggesting that c-Raf can suppress its own activation by a direct feedback phosphorylation.

It remains to be determined whether elevation of p-ERK is a unique feature of the GC-28-1107 and GC-28-0207 xenografts. Up-regulation of p-ERK by sorafenib as observed in this study raises the concern whether this dual feedback loop will reduce the efficacy of sorafenib in long-term gastric cancer therapy, as shown for rapamycin-mediated Akt activation (41). Indeed, cotreatment of mice bearing patient-derived gastric cancer xenografts with AZD6244 and sorafenib is more effective in inhibiting tumor growth, inducing apoptosis, and up-regulating proapoptotic Bim expression than either single agent alone. The mechanism(s) responsible for AZD6244 to enhance apoptotic activity of sorafenib is not known. It is possible that up-regulation of Bim by AZD6244/sorafenib would allow more Bim to bind to and antagonize antiapoptotic effect of Bcl-2 and Bcl-xL, leading to Bax-dependent apoptosis release, caspase activation, and cell death. The results indicate that up-regulation of p-ERK partly counteracts the apoptotic activity of sorafenib and suggest that AZD6244 can be combined with sorafenib for the treatment and/or prevention of gastric cancer.

In summary, we have shown that sorafenib suppressed cell proliferation and induced apoptosis in gastric cancer xenografts. Sorafenib treatment also up-regulated p-c-Raf Ser338 and p-ERK Thr202/Tyr204 in gastric cancer xenografts, which represents a Raf/ERK feedback loop. Pharmacologic inhibition of the MEK/ERK pathway by AZD6244 enhanced the antitumor effect of sorafenib in ectopic models of gastric cancer. We further showed that such inhibition leads to inhibition of tumor cell proliferation and increased apoptosis. Our findings as well as recent work by others (42, 43) highlight the value of pathway-targeted combination therapy to achieve maximal blockade of signaling pathways for cancer treatment and underscore the potential of a combined therapeutic approach with sorafenib and MEK inhibitors for the treatment of gastric cancer.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

References


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