Sorafenib induces growth suppression in mouse models of gastrointestinal stromal tumor

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Abstract
Gastrointestinal stromal tumor (GIST) is the most common mesenchymal tumor of the gastrointestinal tract. Current therapeutic options include surgery and targeted molecular approaches such as imatinib and sunitinib. Our aim was to establish patient-derived GIST xenografts for the use of screening new drugs and improving current treatment regimens used in GIST. In this present study, we investigate the antitumor activity of sorafenib against patient-derived GIST xenografts. Murine xenograft models were given two oral doses of sorafenib daily for 30 days and growth of established tumor xenografts was monitored at least twice weekly by vernier caliper measurements. Western blotting was then used to determine changes in proteins in these xenografts before and after sorafenib therapy. Apoptotic and cell proliferation were analyzed by immunohistochemistry. Our data found that oral administration of sorafenib to mice, bearing patient-derived GIST xenografts, resulted in dose-dependent inhibition of tumor growth. Sorafenib-induced growth inhibition was associated with decreased cell proliferation, increased apoptosis, and reduction in tumor angiogenesis. Western blot analysis revealed that sorafenib inhibited C-Raf, phospho-extracellular signal-regulated kinase 1/2, and phospho-MEK1 (Thr286) slightly as well as phospho-c-Kit (Tyr668/Tyr570), phospho-platelet-derived growth factor receptor β (Tyr1021), and phospho-Flik1 (Tyr981), suggesting that sorafenib inhibited GIST growth by blocking the Raf/MEK/extracellular signal-regulated kinase pathway and angiogenesis. Sorafenib also induced cell cycle arrest, evident through increased levels of p15 and p27 and decreased levels of p21, cyclin A, cyclin B1, and cdc-2. Our study provides a strong rationale for the clinical investigation of sorafenib in patients with GIST as well as an established platform for further drug evaluation studies using GIST xenograft models. [Mol Cancer Ther 2009;8(1):152–9]

Introduction
Gastrointestinal stromal tumors (GIST) are the most common mesenchymal tumors arising from the gastrointestinal tract (1). The annual incidence of GIST ranges from 6.8 cases per million in the United States (2) to 14.5 cases per million in Sweden (3), whereby the lower estimate in some countries may be explained by the difference in case definitions (4). However, many small asymptomatic GISTs remain undetected; one study identified two GISTs per 1,000 autopsies done (5), suggesting that asymptomatic GISTs of low malignancy potential may not be such uncommon tumors in the elderly population.

Surgery remains the mainstay treatment for focal, non-metastatic GISTs (6). However, surgery is technically difficult as meticulous technique is necessary to avoid tumor rupture of the soft, fragile GIST lesions as to lower the risk of peritoneal recurrence (6, 7). Furthermore, complete resection is accomplished in only ~85% of patients with primary localized disease and the 5-year survival rate after removal of primary localized GIST is only ~50%, whereby tumor recurrences after surgery are also commonly observed (6–10). Surgery also has limited efficacy in patients with metastatic disease, majority of which will still experience tumor recurrence in 18 to 24 months despite undergoing complete resection of their primary tumor (3, 7–10).

The malignant behavior of GIST results from a gain-of-function mutation of the gene encoding for either c-Kit (11) or platelet-derived growth factor receptor (PDGFR)-α (12), whereby 90% of all GISTs have a mutation in the gene encoding for c-Kit (13) and 5% of all GISTs have a mutation in the gene encoding for PDGFRα (12). c-Kit and PDGFRα mutations appear to be mutually exclusive and <10% of all GIST patients do not have detectable mutations in the genes encoding for either c-Kit or PDGFRα (14, 15). The intracytoplasmic portion of both c-Kit and PDGFRα proteins belong to the family of type III receptor tyrosine kinases, whereby binding of the Kit or PDGF ligands to its corresponding receptor tyrosine kinase (c-Kit and

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Here, we report clinical benefits in GIST patients remain largely unknown. The mechanisms of action that lead to the sorafenib-mediated resistance GIST, whereby preliminary data suggest that sorafenib is active in these patients (28), the exact line therapy in patients with imatinib- and sunitinib-resistant GIST in a randomized phase II discon-

imatinib-resistant Kit mutants inhibit c-Kit kinase activity and cell growth of multiple tyrosine kinases (24, 25). Sorafenib has been shown to inhibit c-Kit kinase activity and cell growth of multiple imatinib-resistant Kit mutants in vitro (26) as well as induce partial responses lasting 11 months in 2 patients with imatinib-resistant GIST in a randomized phase II discontinued trial (27).

Although sorafenib is currently being studied as a third-line therapy in patients with imatinib- and sunitinib-resistant GIST, whereby preliminary data suggest that sorafenib is active in these patients (28), the exact mechanisms of action that lead to the sorafenib-mediated clinical benefits in GIST patients remain largely unknown. Here, we report in vivo antitumor activity and the possible mechanisms of action of sorafenib on patient-derived GIST xenografts.

Materials and Methods

Reagents

Sorafenib tosylate (Nexavar; 200 mg) was purchased from Bayer HealthCare Pharmaceutical. Research-grade Capsitol was purchased from CyDex. Antibodies against α-tubulin, cyclin A, cyclin B1, cdc-2, p21, p27, phospho-c-Kit (Tyr568/Tyr570), PDGFRβ, phospho-PDGFRβ (Tyr121), and phospho-Fli1 (Tyr493) were obtained from Santa Cruz Biotechnology. Antibodies against phospho-MEK (Thr296), phospho-ERK1/2 (Thr202/Tyr204), C-Raf, phospho-Raf (Ser259/Ser338), and cleaved poly(ADP-ribose) polymerase (PARP) were obtained from Cell Signaling Technology. Anti-B-Raf antibody was from Upstate. Antibodies against Ki-67 and CD31/platelet endothelial cell adhesion molecule-1 were obtained from NeoMarkers. Anti-phospho-MEK1 (Ser218/Ser222) antibody was from Epitomics. c-Kit antibody was from DAKO Japan. Conjugated secondary antibodies were supplied by Pierce. The chemiluminescent detection system was purchased from Amershams Pharmacia Biotech.

Establishment of GIST Xenografts

Prior written informed consent was obtained from patients. The study also received ethics board approval at the National Cancer Centre of Singapore as well as the Singapore General Hospital. All mice were maintained according to the “Guide for the Care and Use of Laboratory Animals” published by the NIH. They were fed until ad libitum with sterilized food and water and housed in negative pressure isolators with 12 h light/dark cycles. Patient-derived GIST xenografts were established into severe combined immunodeficient (SCID) mice as described (29). Briefly, primary GISTs were obtained from Operation Theater and immediately placed in chilled RPMI 1640. The tumors were kept in an ice bath 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/injection. The tissue mixture was s.c. injected in both flanks of SCID mice (Animal Resources Center) ages 9 to 10 weeks. For serial transplantation, tumor-bearing animals were anesthetized with diethyl ether and sacrificed by cervical dislocation. Tumors were minced under sterile conditions and injected in successive SCID mice as described above.

To examine the efficacy of sorafenib on GIST, sorafenib was dissolved in vehicle (30% Capsitol in water) at an appropriate concentration before treatment. For the dose-response experiment, mice bearing the GIST 18-1205 xenografts were given two oral doses of sorafenib daily (30 and 60 mg/kg sorafenib) for 30 days. To investigate the antitumor effects of sorafenib, mice bearing tumors were orally administered 30 mg/kg/d sorafenib for 30 days.
Control mice received only vehicle. Animals were sacrificed 6 h after the last dose. For each tumor, 6 to 8 mice were used. Growth of established tumor xenografts was monitored at least twice weekly by vernier caliper measurement of the length (a) and width (b) of tumor. Tumor volumes were calculated as \(\left[\frac{a \times b^2}{2}\right] \times \pi \times 6\).

At the end of each experiment, body weight and tumor weight were recorded. Tumors were harvested, frozen in liquid nitrogen, and fixed in buffer containing 10% formalin and embedded in paraffin for histologic study. Each experiment was repeated at least twice.

**Gene Sequencing**

To examine the GIST kinase phenotype of our patient-derived GIST xenografts, PCR primer sequences designed to amplify exons 9, 11, 13, and 17 of \(c\)-Kit as well as exons 12, 14, and 18 of \(PDGFR\alpha\) were adapted from a previous study (30) and applied onto our xenograft models. PCR conditions were adapted and modified from another study (31). Briefly, PCR was carried out using 10\(\times\) PCR buffer (DyNAzyme Buffer), 10 mol/L of each deoxynucleotide triphosphate (Finnzymes), 10 pmol of each primer, and 2 units/\(\mu\)L DNA polymerase (Finnzymes Thermostable DyNAzyme II DNA Polymerase). The PCR conditions were 96\(^\circ\)C for 5 min and then 35 cycles of 30 s at 96\(^\circ\)C, 30 s at the annealing temperature of the primer, and 1 min at 72\(^\circ\)C followed by 1 cycle at 72\(^\circ\)C for 10 min. Purification of PCRs were carried out using Qiagen gel extraction kit and Exo-SAP purification from GE. Subsequent gene sequencing primers used were the same as those used for PCR. All PCRs were carried out in duplicate. Sequencing, using the dideoxy-chain termination method (ABI Big Dye 3.1v) and the ABI 3739xl sequencer, was done twice for each sample to rule out the possibility of PCR fidelity articles and was carried out in both directions.

**Western Blotting**

To study the dynamic changes in apoptosis, angiogenesis, Raf/MEK/ERK phosphorylation, and cell cycle progression by sorafenib, mice bearing 18-1205 tumors (~800 mm\(^3\)) were treated with vehicle or 30 mg/kg sorafenib for 3 or 5 days as described above. To determine changes in the indicated proteins, two to three independent tumors from 4 mice per group, vehicle- and sorafenib-treated mice were homogenized separately in lysate buffer. Total proteins (100 \(\mu\)g) were analyzed by Western blotting as described (29). All primary antibodies were used at a final concentration of 1 \(\mu\)g/mL. The blots were then visualized with a chemiluminescent detection system as described by the manufacturer.

**Immunohistochemistry**

Vehicle- and sorafenib-treated tumors on day 30 were collected, fixed, and paraffin-embedded as described (29). Sections (5 \(\mu\)m) were cut, dewaxed, and then rehydrated and subjected to antigen retrieval. After blocking endogenous peroxidase activity and reducing nonspecific background staining, the sections were incubated overnight at 4\(^\circ\)C with primary antibodies against cleaved PARP, Ki-67, and other markers as appropriate.
c-Kit, phospho-ERK1/2, and CD31. The slides were examined and pictures were taken using an Olympus BX60. The labeling index was obtained by counting the number of labeled cells among at least 500 cells per region and was then expressed as percentage values.

Results

To examine the GIST kinase phenotype of our patient-derived GIST xenografts, we sequenced exons 9, 11, 13, and 17 of c-Kit as well as exons 12, 14, and 18 of PDGFRA. Figure 1 shows that mutations of c-Kit exon 11 were found in both GIST 13-0308 and 18-1205 xenograft lines; the GIST 13-0308 has c-Kit exon 11 mutations 1671_1672insA, 1675_1683delATGTATGAA (Met522_Glu544del), and 1684delG, whereas GIST 18-1205 has c-Kit exon 11 mutation 1672_1685delCCCATGTATGAAGTA (Pro551_Val555del). No mutations were detected in exons 9, 13, and 17 of c-Kit as well as exons 12, 14, and 18 of PDGFRA in both GIST 18-1205 and 13-0308 lines (data not shown).

We then first investigated the dose-dependent tumor activity of sorafenib on GIST xenografts, whereby the tumor weights in mice, of the GIST 18-1205 xenograft line, treated with 30 and 60 mg/kg sorafenib were 58 ± 13% and 55 ± 11% of the controls, respectively (Fig. 2A). No overt toxicity of sorafenib was observed during the course of treatment as defined by weight loss, unkempt appearance or behavior, or mortality. Because the dosing schedule of 30 mg/kg/d gave the maximal dose-dependent growth inhibition, we selected this dose for our subsequent studies. Sorafenib, when given at a dose of 30 mg/kg/d, inhibited tumor growth of both xenograft lines (Table 1), whereby the mean tumor weights in sorafenib-treated GIST 18-1205 and 11-0308 xenograft lines were 58% and 55% of the vehicle-treated mice, respectively. Although there was no tumor regression observed, there was obvious reduction of tumor growth, evident through tumor volume measurements 15 and 30 days after treatment of the GIST 18-1205 xenograft line (Fig. 2B).

To gain better understanding of the potential mechanisms of sorafenib in GIST xenograft, we first investigated the phosphorylation statuses of well-established targets of sorafenib, such as phospho-eIF4E (Ser 209), C-Raf, and ERK1/2. Figure 2C shows reduced levels of phospho-eIF4E (Ser 209), C-Raf, and downstream targets of C-Raf in response to sorafenib treatment. Complete inhibition of ERK1/2 phosphorylation observed in sorafenib-treated xenografts illustrates that the reduction in phosphorylation of the MEK/ERK pathway parallels the decreased C-Raf activity in these tumors. A significant reduction in cdc-2 and, to a lesser extent, phospho-Akt (Ser473) levels was also noticed in sorafenib-treated xenografts.

We next examined the antiproliferative, antiangiogenic, and apoptotic effects of sorafenib in vivo. As shown in Table 1 and Fig. 3, the Ki-67 index in both sorafenib-treated xenograft lines, a cellular marker for proliferation, was significantly reduced (P < 0.01). The number of cleaved PARP-positive cells, a marker for apoptosis in tissue sections, was also found to be significantly elevated (P < 0.01) in both sorafenib-treated xenograft lines (Table 1; Fig. 3). Likewise, the microvessel density was remarkably reduced (P < 0.01), suggesting that sorafenib exhibited...
antiangiogenic properties in both GIST xenograft lines. As shown in Fig. 3, sorafenib also significantly reduced the number of cells expressing c-Kit, phospho-ERK1/2, as well as CD31+ endothelial cells but significantly increased cleaved PARP-positive cells on day 3 post-sorafenib exposure.

To study the dynamic changes in apoptosis and Raf/MEK/ERK phosphorylation by sorafenib, mice bearing 18-1205 tumors were treated with sorafenib for 3 or 5 days. As shown in Fig. 4A, C-Raf and phospho-Raf (Ser259/Ser338), but not B-Raf, were slightly reduced on day 3 and became barely detectable on day 5 following sorafenib treatment. The decrease in phosphorylation of C-Raf preceded the reduced phosphorylation of phospho-ERK1/2 in sorafenib-treated tumor models ($P < 0.01$; Fig. 4B). There also appears to be a slight decrease in levels of phospho-MEK1 (Thr286). In addition, sorafenib did reduce phosphorylation of phospho-c-Kit (Tyr568/Tyr570) and phospho-Flk1 (Tyr951) as well as phospho-PDGFRβ (Tyr587) to a lesser extent (Fig. 4A). Levels of cleaved PARP reached a maximum by day 3 and slightly declined on day 5, suggesting that apoptosis occurred as early as day 3 of treatment (Fig. 4B).

Because alterations in cell cycle proteins have been associated with cellular proliferation and clinical outcome (32), the status of cell cycle regulators in vehicle- and sorafenib-treated tumors after 3 and 5 days of treatment was also investigated. Figure 4B shows that significant increase in p27 and p15 as well as reductions in p21, cdc-2, cyclin B, and cyclin A in sorafenib-treated tumors were observed. There were no significant alterations in the levels of p16, cdk-4, and cdk-2 by sorafenib therapy (data not shown). This suggests that sorafenib may block the cell cycle progression in vivo.

**Discussion**

This study is the first report, to the best of our knowledge, wherein GIST is found to have grown significantly in SCID mice such as to allow for serial transplantation. Two of the 7 primary tumors could be grown as xenografts, whereas the GIST 18-1205 and 11-0308 xenograft lines were first detected 58 and 62 weeks post-transplantation, respectively, as such to sufficiently allow a second-passage and histologic documentation of GIST 2 years later. Both of the xenograft lines have mutations of c-Kit exon 11 (Fig. 1). The GIST 13-0308 has c-Kit exon 11 mutations 1671_1672insA, 1675_1683delATGTATGAA (Met522_Glu544del), and 1684delG, whereas GIST 18-1205 has c-Kit exon 11 mutation 1672_1685delCCCATGTATGAAGTA (Pro551_Val555del). These xenografts are still currently propagating in our laboratory. The xenografting of primary GIST appears to be the only currently available means to permit the propagation of a significant proportion of these tumors. Although the establishment of these models was technically difficult and the tumors were found to be relatively slow-growing, we still believe that such tumor models, whereby the implanted tumor tissue retains most of its normal architecture and function, more accurately reflect the in vivo situation compared with in vitro studies, particularly in studies for drug evaluation. However, such patient-derived xenografts also have limitations as GIST models; due to the use of immunocompromised mice, we are thus unable to investigate the role of the immune system in GIST, particularly the role of the immune system in mediating responses to therapy of signaling inhibitors such as sorafenib. Nonetheless, the development of our GIST xenograft model should be an important development in the GIST field, notably when there are <10 reported cell culture models for GIST.

Given the above caveats, we have shown that two patient-derived GIST xenograft lines responded to sorafenib therapy. Sorafenib-induced tumor growth suppression was associated with inhibition of cell proliferation as determined by decrease in Ki-67 index (Table 1; Fig. 3) and increase apoptosis as determined by increase in levels of cleaved PARP (Table 1; [Figs. 2C, 3], and 4B), whereby these antitumor properties of sorafenib were seen as early as 3 days post-treatment. Sorafenib also exerted antiangiogenic effects evident by lowered microvessel density and fewer CD31+ cells (Table 1; Fig. 3). In addition, we also found reduced levels of phospho-PDGFRβ (Tyr587) and phospho-Flk-1 (Tyr951; Fig. 4A) in tumors harvested from

**Table 1. Effect of sorafenib on body weight and tumor weight at sacrifice, cell proliferation (Ki-67), microvascular density (CD-31), as well as apoptosis (cleaved PARP) in mice treated with either vehicle or sorafenib (30 mg/kg) in GIST 18-1205 and 11-0308 xenograft lines**

<table>
<thead>
<tr>
<th>Lines of xenografts</th>
<th>Treatments</th>
<th>Body weight (g)</th>
<th>Tumor weight (mg)</th>
<th>Ki-67 index (%)</th>
<th>Cleaved PARP (%)</th>
<th>Microvessel density*</th>
</tr>
</thead>
<tbody>
<tr>
<td>18-1205</td>
<td>Vehicle</td>
<td>25.8 ± 0.9</td>
<td>1,475 ± 312</td>
<td>14 ± 5</td>
<td>&lt;0.01</td>
<td>11 ± 4</td>
</tr>
<tr>
<td></td>
<td>Sorafenib</td>
<td>23.7 ± 1.1</td>
<td>869 ± 180</td>
<td>5 ± 3</td>
<td>13 ± 6</td>
<td>6 ± 3</td>
</tr>
<tr>
<td>11-0308</td>
<td>Vehicle</td>
<td>24.2 ± 0.8</td>
<td>1,365 ± 287</td>
<td>11.6 ± 4</td>
<td>&lt;0.01</td>
<td>10 ± 3</td>
</tr>
<tr>
<td></td>
<td>Sorafenib</td>
<td>23.5 ± 1.1</td>
<td>746 ± 114</td>
<td>5 ± 3</td>
<td>11.4 ± 5</td>
<td>4 ± 3</td>
</tr>
</tbody>
</table>

GIST 18-1205 and 11-0308 xenografts were s.c. on the right flank of male SCID mice as described in Materials and Methods. Mice were treated with either 200 μl vehicle or 30 mg/kg sorafenib for 30 d starting from 60 d after tumor implantation as described in Materials and Methods. Mean microvessel density, Ki-67 index, and apoptosis were determined by immunohistochemical staining with antibodies against CD31, Ki-67, and cleaved PARP, respectively. Data are mean ± SE. Differences in body weight at harvest, microvessel density, Ki-67 index, and cleaved PARP-positive cells between vehicle and sorafenib-treated tumors were analyzed by ANOVA.

*Mean microvessel density of 10 random 0.159 mm² fields at ×100 magnification.

$P < 0.01$ versus vehicle in same xenograft line.
sorafenib-treated xenografts compared with vehicle-treated tumors. As a result of the slow growth of GIST xenografts, the T/C ratio, whereby T and C represent the mean tumor weight of sorafenib- and vehicle-treated xenografts, respectively, harvested at the end of 30 days, was calculated to be only 0.55 to 0.58. However, we believe that this ratio would continue to decrease if we had continued treatment beyond 30 days.

Figure 3. Immunohistochemical analysis of GIST xenografts. The GIST 18-1205 xenograft line was s.c. implanted in SCID mice and treated with sorafenib as described in Materials and Methods. Sections from vehicle- and sorafenib-treated tumors on day 30 were stained with antibodies against c-Kit, Ki-67, phospho-ERK1/2, cleaved PARP, and CD-31 as described in Materials and Methods. Representative samples. Experiments were repeated twice with similar results. Magnification, ×800 for c-Kit and ×400 for Ki-67, phospho-ERK1/2, cleaved PARP, and CD31.
A previous study has shown that 77% of GISTs stain positive for Ras p21 (33), underscoring members of the Raf/MEK/ERK signaling pathway as potential future targets of molecular therapy against GIST. In this present study, C-Raf, ERK1/2, phospho-ERK1/2, phospho-MEK1 (Figs. 2B and 4A and B), c-Kit, and phospho-c-Kit (Tyr568/Tyr570, Figs. 3 and 4A) in GIST xenografts were significantly reduced following sorafenib therapy, suggesting that sorafenib blocks the Raf/MEK/ERK pathway as well as c-Kit activation. Our observations are consistent with previous studies, which found sorafenib to inhibit tumor cell proliferation through selectivity for the Raf/MEK/ERK signaling pathway (24, 25) and c-Kit tyrosine kinase activity in vitro (24–26).

In this study, we also showed that sorafenib-induced growth inhibition is associated with increases in p27 and p15 as well as reductions in p21 and cyclin B1, suggesting that sorafenib induces cell cycle arrest in late G1-S phase. In addition, sorafenib also decreases cyclin A and cdc-2, indicating that sorafenib also blocks the cell cycle in the G2-M phase. Hence, based on all of the above data, we hypothesize that sorafenib-mediated inhibition of c-Kit, PDGFRβ, Flk1, and the Raf/ERK/MEK pathway results in the enhanced translation of p27, p15 and suppression of p21, cyclin B1, cyclin A, and cdc-2 expression. These result in inhibition of both angiogenesis and cell cycle progression.

Cell cycle proteins such as cyclin A, cyclin B1, and cdc-2 have been identified as markers of aggressive disease in GIST (32). There have also been correlations with low expression of p27 and the recurrence-free survival of GIST patients (32). These observations raise the possibility that sorafenib can be used to downgrade GIST. Both associations highlight the potential use of sorafenib, either as monotherapy or in combination with other molecular therapies, as an effective adjuvant therapy postsurgery.

There is already an ongoing phase II National Cancer Institute-sponsored multicenter investigation of sorafenib in imatinib- and sunitinib-resistant GIST (28). However, here we show that sorafenib inhibits tumor growth when given alone, suggesting that at least some patients may be amenable to single-agent therapy. However, the synergistic combination of sorafenib with other molecular targeted agents or cytotoxic agents are also worthy of investigation. Building on our experiences with GIST xenografts, we also propose subsequent studies such as comparing the effects of imatinib, sunitinib, and sorafenib on GIST xenografts. From our data, the obvious static effect of sorafenib on GIST xenograft tumor growth also highlights its potential as an effective adjuvant therapy for presurgical or postsurgical GIST patients.

In summary, we have shown that the tyrosine kinase receptor c-Kit, proangiogenic growth factor receptors Flk1 and PDGFRβ, as well as the Raf/ERK/MEK signaling pathway play an important role in cell proliferation, cell survival, and angiogenesis. Our study also shows that oral delivery of sorafenib inhibits these pathways and causes growth inhibition of patient-derived GIST xenografts, indicating that sorafenib may be a useful drug for treatment of GIST.
Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

References

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