Tyrosine Kinase Inhibitor SU6668 Inhibits Peritoneal Dissemination of Gastric Cancer Via Suppression of Tumor Angiogenesis

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Abstract. Background: Peritoneal dissemination of cancer involves several steps, including tumor cell attachment, invasion and growth in the peritoneum. Tumor angiogenesis is a prerequisite for the growth of disseminated tumor. Vascular endothelial growth factor (VEGF) and its receptor are major regulators of angiogenesis. Purpose: We examined the cytotoxic effects of SU6668, an inhibitor of VEGF tyrosine kinase receptors, on in vitro gastric cancer cell lines and human umbilical vascular endothelial cells (HUVEC); we also examined the antitumor effects of SU6668 on human gastric cancer cells administered intraperitoneally into nude mice. Materials and Methods: Direct cytotoxicity to gastric cancer cells (TMK-1, MKN-45 and MKN-74) and normal cells (HUVEC) was determined by the MTT assay and the bromodeoxyuridine (BrdU) incorporation assay, with and without VEGF-evoked growth stimulation in vitro. TMK-1 cells were transplanted intraperitoneally into nude mice, followed by twice daily oral administration of SU6668 (200 mg/kg/day) for two weeks starting on the first day after transplantation. Both the number and the wet weight of disseminated peritoneal tumor nodules were assessed. Results: In the MTT assay, SU6668 demonstrated low-grade cytotoxicity to the cell growth of three gastric cancer cells, with a 50% inhibitory concentration (IC_{50}) of 22.6 µg/ml for TMK-1, 31.8 µg/ml for MKN-45 and 26.7 µg/ml for MKN-74; HUVEC was sensitive to SU6668 with an IC_{50} of 8.9 µg/ml. In the BrdU assay, VEGF stimulated DNA synthesis in HUVEC, while the incorporation of BrdU was not affected by VEGF in gastric cancer cell lines. SU6668 inhibited VEGF-induced DNA synthesis in HUVEC, while BrdU incorporation of gastric cancer cell lines was inhibited by SU6668 without correlation to VEGF stimulation. Peritoneal dissemination of cancer in nude mice was significantly suppressed by SU6668 compared with a control group at the p<0.05 level. Conclusion: The mechanism of the antitumor activity of SU6668 may not involve direct toxicity to cancer cells, but may rather be an inhibitory effect on tumor angiogenesis, resulting in the inhibition of tumor dissemination in the peritoneum.

While declining in incidence, gastric cancer remains a major cause of cancer-related death in the Japanese population, particularly when it reaches the stage of peritoneal dissemination (1).

Peritoneal dissemination of gastric cancer requires several steps that include tumor cell attachment, invasion and growth in the peritoneum. Angiogenesis is critical for tumor development, growth and metastasis. Vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), platelet-derived growth factor (PDGF) and their tyrosine kinase receptors are major regulators of angiogenesis. SU6668 is a tyrosine kinase receptor inhibitor, developed by SUGEN, San Francisco, CA, USA, that inhibits these three distinct growth factor receptor targets, i.e. VEGF, FGF and PDGF (2). In vitro studies have confirmed that SU6668 inhibits growth factor-stimulated tyrosine phosphorylation. SU6668 also has significant antitumor activity against many types of tumor xenografts transplanted into nude mice. SU6668 inhibits angiogenesis through several mechanisms, including the induction of apoptosis in vascular endothelial cells and tumor cells.

In the present study, we examined the in vitro cytotoxicity of SU6668 to gastric cancer cell lines and to human umbilical vascular endothelial cells (HUVEC) using the MTT assay and the BrdU incorporation assay. We also studied the
inhibitory effects of SU6668 on peritoneal cancer dissemination in nude mice to assess its potential clinical utility as a novel agent against peritoneal dissemination of gastric cancer.

Materials and Methods

Cell lines. Three human gastric carcinoma cell lines (TMK-1, MKN-45 and MKN-74) were used. TMK-1 was initially established as a serially transplanted human tumor xenograft in nude mice by Tokuda et al. (3) using a gastric cancer cell line isolated from a 21-year-old male patient with gastric cancer, established as a cancer cell line by Tahara et al. (4) and kindly supplied by Dr. S. Hirohashi, National Cancer Center, to our laboratory. The MKN-45, MKN-74 and human umbilical vascular endothelial cell (HUVEC) lines were purchased from Dainippon Seiyaku, Co. Ltd., Japan.

Agents. SU6668 was generously supplied by Taiho Pharmaceutical Co., Ltd., Tokyo, Japan.
Evaluation of cytotoxicity by MTT assay. We evaluated the in vitro chemosensitivity of the gastric cancer cell lines and HUVEC to SU6668 using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT, Sigma Chemical Co., St. Louis, MO, USA) assay as reported by Mosmann (5), with modifications (6-9). Single cells were suspended in RPMI-1640 medium (GIBCO, Gaithersburg, MA, USA) supplemented with 10% fetal bovine serum (FBS; CSL Limited, Australia) and then diluted to 1 x 10^5 cells/ml. One hundred ml aliquots containing 10^4 cells were plated into each of 96-well round-bottomed micro-titer plates (SUMILON, Sumitomo Bakelite Co., Tokyo, Japan). After pre-incubation with FBS for 24 hours, the cells were incubated for another 24 hours starved of FBS. SU6668 was dissolved in RPMI-1640 and 100 ml aliquots were added to each well, giving final concentrations of 1.9, 3.75, 7.5, 15, 30, 60 and 120 μg of SU6668 per ml. Control wells contained 100 μl cell suspension and 100 μl RPMI-1640, while 200 μl RPMI-1640 was used as a blank. Plates were incubated for 24 hours at 37°C in a humidified atmosphere containing 95% air and 5% CO2. A mixture of 0.4% MTT (Sigma) and 0.1 M sodium succinate (Wako Pure Chemical Ind., Ltd., Osaka, Japan), each dissolved in 10 μl phosphate-buffered saline and filtered through a 0.45-mm membrane filter (Millipore, Bedford, MA, USA), was then added and the plates were incubated for an additional 3 hours at 37°C. After the final incubation, 150 μl dimethyl sulfoxide (Nacalai Tesque, Kyoto, Japan) was added to each well to dissolve the MTT-formazan salt, and the plates were shaken mechanically for 10 minutes on a mixer (Model 250, Sonifier, Branson, MO, USA). The optical densities of each well were determined on a NJ-2300 microplate spectrophotometer at 540 nm and 630 nm (Immuno Reader, Nalgen Nunc International, Rochester, NY, USA). Inhibition rates (I.R.; %) were calculated using the following formula: (1 – A/B) x 100 (percentage), where A and B represent the mean absorbances of the treated and control wells, respectively.

Figure 2. Inhibitory effect of SU6668 on the cell proliferation of human gastric cancer cell lines and human umbilical vascular endothelial cells, with or without the presence of VEGF (100 μg/ml, 24 hours)

Figure 3. Ratio of BrdU uptake of human gastric cancer cell lines and human umbilical vascular endothelial cells in the presence of VEGF (100 μg/ml, 24 hours) to VEGF with SU6668. The concentrations of SU6668 used were 24 μg/ml for TMK-1 and MKN-45, and 8 μg/ml for HUVEC, which accounted for their IC50 detected by the MTT assay.
After I.R. was plotted against the concentration, the regression equation was conducted as I.R. = a x ln (concentration) – b, where a and b are variables. Fifty percent concentration (IC50) was calculated from the formula: IC50 = a^50 + b^a.

**Evaluation of cytotoxicity by BrdU incorporation assay.** The BrdU-incorporation inhibition assay was conducted according to the kit instructions (Roche Diagnostic Co. Ltd., Tokyo, Japan). In brief, TMK-1, MKN-45 and HUVEC were incubated for 48 hours in medium (RPMI-1640 and 10% FBS) with or without SU6668, medium with 100 μg of VEGF per ml.

The concentrations of SU6668 used were 24 μg/ml for TMK-1 and MKN-45, and 8 μg/ml for HUVEC, which accounted for their IC50 detected by the MTT assay. After cell washing twice, 10 μl of BrdU labelling solution was added and the cells were incubated for 12 hours. The cells were washed twice and fixed with 200 μl fixing solution at -20°C for 30 minutes. The cells were again washed twice, 100 μl nuclease was added to the solutions and the cells were incubated for 30 minutes. After another two washings, 100 μl anti-BrdU-POD immune body was added and the cells were incubated for another 30 minutes. After the buffer solution was used to wash the antibody three times, 100 μl substrate of the peroxidase was added, and the incubation was continued until the positive control indicated a green color for approximately 30 minutes. The absorbance was read by a microplate-reader at 405 nm with a reference wavelength of 490 nm.

**Mice.** Severe combined immuno-deficient (SCID) mice with a CB-17 genetic background were purchased from CLEA Japan Co. Ltd. (Tokyo, Japan). The mice were maintained under specific pathogen-free conditions using an Isorack, and fed on sterile food and water ad libitum in our experimental animal center. Six- to eight-week-old mice weighing 20-22 g were used for the experiments.

**Evaluation of antitumor activity.** The investigation of antitumor activity utilized an intra-peritoneal dissemination model that we have previously described (10-12). 1.0 x 10^5 TMK-1 cells were injected intraperitoneally on Day 0. 200 mg/kg/day of SU6668 per kg was administered orally starting on Day 1 for 2 weeks. The mice were sacrificed on Day 16, and the number and weight of disseminated peritoneal tumors were counted. The incidence of peritoneal dissemination showed a statistically significant decrease at a p<0.05 level, and the weight of tumor nodules was also reduced. However, among the two treated mice in whom peritoneal dissemination was observed, the number of nodules was not different than the average number of nodules in the control mice.

**Statistical analysis.** Statistical analysis was performed using the Chi-square test. p<0.05 was regarded as statistically significant.

**Results**

The MTT assay detected SU6668 concentration-dependent antitumor activity against the three gastric cancer cell lines and HUVEC (Figure 1). However, cytotoxicity to gastric cancer cell lines was observed only for high concentrations of SU6668, with an IC50 of 22.6 μg/ml for TMK-1, 31.8 μg/ml for MKN-45 and 26.7 μg/ml for MKN-74. On the other hand, SU6668 demonstrated a potent inhibitory effect on the cell growth of HUVEC, with an IC50 of 8.9 μg/ml.

VEGF increased BrdU incorporation into HUVEC 2.57-fold relative to non-VEGF-stimulated controls, but did not affect BrdU incorporation into the two gastric cancer cell lines (Figure 2). SU6668 inhibited BrdU incorporation into the gastric cancer cell lines; gastric cancer cell lines incorporated approximately half as much BrdU as the controls incorporated. This inhibitory effect of SU6668 was not affected by VEGF stimulation. The incorporation of BrdU into HUVEC was remarkably reduced by SU6668 with or without VEGF stimulation. Figure 3 shows the ratio of BrdU uptake inhibitory effect by dividing the value of (SU6668 + VEGF) by VEGF alone. The selective inhibitory effect of SU6668 on HUVEC is evident.

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**Table I. Inhibition of BrdU incorporation by SU6668 with or without VEGF-stimulation.**

<table>
<thead>
<tr>
<th>Condition</th>
<th>TMK-1</th>
<th>MKN-45</th>
<th>HUVEC</th>
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<tbody>
<tr>
<td>medium + SU6668</td>
<td>0.41</td>
<td>0.32</td>
<td>0.10</td>
</tr>
<tr>
<td>VEGF + SU6668</td>
<td>0.50</td>
<td>0.31</td>
<td>0.11</td>
</tr>
<tr>
<td>medium + VEGF</td>
<td>0.91</td>
<td>0.83</td>
<td>2.57</td>
</tr>
</tbody>
</table>

**Table II. Preventive effect of SU6668 on peritoneal dissemination of TMK-1 gastric cancer cell line in SCID mouse.**

<table>
<thead>
<tr>
<th>Peritoneal dissemination</th>
<th>Control</th>
<th>Treated</th>
</tr>
</thead>
<tbody>
<tr>
<td>(+)</td>
<td>7</td>
<td>2</td>
</tr>
<tr>
<td>(-)</td>
<td>0</td>
<td>6</td>
</tr>
<tr>
<td>Total</td>
<td>7</td>
<td>8</td>
</tr>
</tbody>
</table>

1.0 X 10^5 TMK-1 cells were injected intraperitoneally on Day 0. 200 mg/kg/day of SU6668 per kg was administered orally starting on Day 1 for 2 weeks. The mice were sacrificed on Day 16, and the number and weight of disseminated peritoneal tumors were counted. The incidence of peritoneal dissemination showed a statistically significant decrease at a p<0.05 level, and the weight of tumor nodules was also reduced. However, among the two treated mice in whom peritoneal dissemination was observed, the number of nodules was not different than the average number of nodules in the control mice.
Intra-peritoneal injection of TMK-1 into nude mice resulted in peritoneal dissemination of gastric cancer in 7 out of 7 control mice, while only 2 out of 8 SU6668-treated mice developed peritoneal dissemination (Table I). This difference in the incidence of peritoneal dissemination between the control and treated groups was statistically significant at the \( p<0.05 \) level. Of the 2 mice in the SU6668-treated group who developed peritoneal dissemination, 1 had three nodules and the other had five nodules; the average weight of these eight nodules was 15 mg. Although the mean number of disseminated nodules among the control mice was 4.4±2.2, these nodules had a mean weight of 150±190 mg (Table II). Thus, while SU6668 did not reduce the number of tumor nodules in the 2 mice with peritoneal dissemination, the weight of their tumor nodules was reduced compared with tumor nodules in the control mice.

**Discussion**

In the present study, we investigated the direct cytotoxicity of SU6668 to three gastric cancer cell lines and HUVEC in vitro, and the preventive effect of SU6668 on a peritoneal dissemination model of TMK-1 in SCID mice. Because conventional chemotherapy is not specific for cancer cells, resulting in toxic and sometimes fatal side-effects, a number of promising new anticancer drugs are being developed which target intracellular pathways or extracellular cell molecules. In particular, various tyrosine kinase receptors have been identified as regulators of tumor or tumor blood vessel growth (13). The tyrosine kinase receptor inhibitors include the following agents: imatinib mesylate (Gleevec), inhibiting the BCR-ABL tyrosine kinase and mutated c-kit; gefitinib (Iressa), inhibiting the EGF-receptor tyrosine kinase; and trastuzumab (Herceptin), inhibiting the Her2/neu tyrosine kinase. SU6668 is an example of a multitarget tyrosine kinase inhibitor.

Laird et al. reported that oral or intraperitoneal administration of SU6668 in nude mice resulted in significant growth inhibition of human tumor xenografts of gliomas, melanomas and tumors of lung, colon, ovarian and epidermoid origin (14). Furthermore, SU6668 treatment suppressed tumor angiogenesis of C6 glioma xenografts in the dorsal skin fold chamber model using intravital multifluorescence video microscopy. Shaheen et al. reported that both SU6668 and SU5416, a tyrosine kinase inhibitor for Flk-1/KDR, inhibited hepatic metastasis by murine colon cancer, CT-26 (15). In their experiments, BALB/c mice underwent splenic injection with CT-26 to induce hepatic metastases. Daily intraperitoneal injections of SU5416 or SU6668 inhibited hepatic metastases by 48.1% and 55.3%, respectively. In the treated groups, SU5416 and SU6668 inhibited microvessel formation and cell proliferation and increased apoptosis in tumor and endothelial cells. The authors also observed the preventive effect of SU6668 in other experiments using the splenic injection model of CT-26 colon cancer cells into BALB/c mice to generate liver metastases (16). SU6668 increased median survival by 58% \( (p<0.001) \) and caused a progressive increase in tumor cell and endothelial cell apoptosis. Pericyte vessel coverage and tumor vascularity were also significantly decreased in the SU6668-treated group. Based upon these data, the authors suggested that SU6668 may affect tumor endothelial cell survival directly through inhibition of VEGF stimulation and indirectly via decreased pericyte coverage.

Our results were consistent with these previous reports. In the present study, SU6668 showed a potent direct cytotoxicity to HUVEC with an IC\(_{50}\) of 8.9 \( \mu \)g/ml. SU6668 cytotoxicity to the three gastric cancer cell lines was less evident, as reflected in the high 50% inhibitory concentrations that were observed. VEGF stimulation of HUVEC cell proliferation but did not affect cell proliferation in the gastric cancer cell lines, as detected by the BrdU incorporation assay. This VEGF stimulation of HUVEC cell proliferation was significantly suppressed by SU6668, while the effect of SU6668 on the gastric cancer cell lines was not affected by the presence of VEGF. These results suggest that SU6668 has a specific cytotoxicity to vascular endothelial cells represented by HUVEC and a limited cytotoxicity to gastric cancer cell lines, which may contribute to the clinical application.

The present study also found that SU6668 successfully prevented the peritoneal dissemination of TMK-1 cells in a SCID mice model. We developed this model in 1999, demonstrating that the intraperitoneal injection of TMK-1 cells into SCID mice resulted in peritoneal dissemination of gastric cancer mimicking clinical carcinomatous peritonitis (10). In that experiment, we found that R-94138, a matrix metalloproteinase inhibitor, inhibited the peritoneal dissemination of TMK-1 in SCID mice, and we also found that the combination of mitomycin C and R-94138 increased the amount of inhibition. We subsequently tested the flavonoid nobiletin found in *Citrus depressa Rutaceae*, a popular citrus fruit in Okinawa, Japan (11), and marimastat, a broad-spectrum matrix metalloproteinase (MMP) inhibitor (12). Nobiletin inhibited the formation of disseminated peritoneal TMK-1 nodules by reducing the enzymatic activity of MMP-9. Marimastat also inhibited peritoneal dissemination through the down-regulation of gelatinase, as evidenced by a reduction in the total weight, number and microvascular density of the disseminated tumor nodules. The present study demonstrated that SU6668 inhibited the peritoneal dissemination of TMK-1 tumor cells, probably through activity against vascular endothelial cells.
SU6668 is in phase I trials in the UK and US, and phase I/II trials involving hematological and solid tumors are expected to commence shortly (17). The current phase I dose-escalation study has enrolled 74 patients to determine the toxicities of SU6668 in fed and fasting patients. Having completed once- or twice-daily oral administration of SU6668 at doses of 100 to 2,400 mg/m² to patients diagnosed as having advanced malignancies, accrual in this phase I study is continuing in order to define the toxicities of doses >200 mg/m² twice-daily. Some toxicities, including nausea, vomiting, fatigue and tumor pain, have been observed. SUGEN entered into a collaboration with the Cancer Research Campaign (CRC) to conduct a phase I trial of SU6668 using an i.v. formulation. However, after the first six patients had been treated, the trial was halted due to problems with the i.v. formulation.

In conclusion, SU6668 inhibits peritoneal dissemination of human gastric cancer cells in SCID mice. The mechanism of the antitumor activity of SU6668 is probably not direct toxicity to cancer cells, but rather an inhibitory effect on tumor angiogenesis, resulting in the inhibition of tumor dissemination in the peritoneum. Further clinical investigation of SU6668 is warranted.

References

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