

Antiangiogenic agent SU6668 suppresses the tumor growth of xenografted A-431 cells

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Abstract. SU6668 is an antiangiogenic agent that acts as a tyrosine kinase inhibitor for the vascular endothelial growth factor (VEGF) receptor. We treated xenografted A-431, a human squamous cell carcinoma cell line, with SU6668 to investigate the efficacy of tumor dormant therapy using angiogenesis inhibitors for patients with squamous cell carcinoma. A-431 cells were transplanted into severe combined immunodeficient (SCID) mice. The treatment group was given SU6668 at a dose of 200 mg/kg orally twice a day for 3 weeks; the control group was given only the vehicle in the same manner and intervals. SU6668 suppressed tumor growth of A-431 cells in the xenograft model. Furthermore, tumor volume and the number of vessels were significantly reduced in the treatment group compared with those of the control. No significant differences in body weight were found between the treatment and control groups, and no toxic reactions occurred during the experiment. We concluded that this agent was safe, efficient and potentially useful for the treatment of patients with squamous cell carcinoma.

Introduction

The prognosis of patients with esophageal cancer is dismal (1). Although advances in esophageal cancer therapy, including surgical technique and chemoradiation therapy, have been remarkable, the 5-year survival rate for patients undergoing curative or palliative resection is 40.0-42.4% (2,3). The majority of patients with esophageal cancer in Japan have squamous cell carcinoma. New paradigms and therapeutic approaches, such as antiangiogenic therapy, need to be investigated to improve the treatment of this neoplasm.

Tumor angiogenesis is essential for the tumor growth and metastatic spread of several solid tumors (4), and is induced by several angiogenic growth factors. Among several dozen angiogenic growth factors identified thus far, vascular

endothelial growth factor (VEGF), fibroblast growth factor (FGF), and platelet-derived growth factor (PDGF) have been widely studied and reported. These angiogenic growth factors are related to poor prognosis in some solid tumors (5-10), and we have also reported that VEGF expression is associated with a poor outcome and lymph node metastasis in squamous cell carcinoma of the esophagus (11). The inhibition of angiogenesis is expected to play a key role in the treatment of squamous cell carcinoma. Numerous small molecules and biological agents capable of inhibiting tumor-induced vascularization, like marimastat, BMS275291, BAY12-9566, neovastat, SU5416, SU6668, and ZD6474, have been identified (12-18). We hypothesized that angiogenesis inhibitors targeting VEGF, might also be applicable as a tumor dormant therapy for patients with squamous cell carcinoma. SU6668, (z)-3-[2,4-dimethyl-5-(2-dihydro-3-ylidenemethyl)-1H-pyrrol-3-yl]-propionic acid, a small molecule, is a relatively broad-spectrum receptor tyrosine kinase inhibitor that inhibits the function of the VEGF, FGF and PDGF receptors (19). This agent affects tumor vascularization and the growth of some types of xenografts (13,19,20). A-431 is a squamous cell carcinoma cell line that can be implanted into severe combined immunodeficient (SCID) mice. Here, we examined the antitumor effects of SU6668 on squamous cell carcinoma xenografts of A-431 in SCID mouse to test our hypothesis.

Materials and methods

Cell line. A-431 cells were obtained from Riken (Tsukuba, Japan). The A-431 tumor cell line was successfully transplanted into SCID mice in our laboratory. Cells from an early generation were stored in liquid nitrogen, and new cultures were established every 2-3 months.

Mice. Severe combined immunodeficient (SCID) mice with a genetic background of BALB/c were obtained from the Saitama Laboratory Animal Center (Saitama, Japan). All mice were maintained under specific pathogen-free conditions using an isorack in our experimental animal center and fed sterile food and water *ad libitum*. The 4-week-old male mice weighing 20-22 g each (n=10) were used in the study. The mice were randomly placed in a control (n=5) or SU6668 treatment (n=5) group. All animal studies were conducted according to institutional guidelines approved by the Animal Care and Use Committee of our university.

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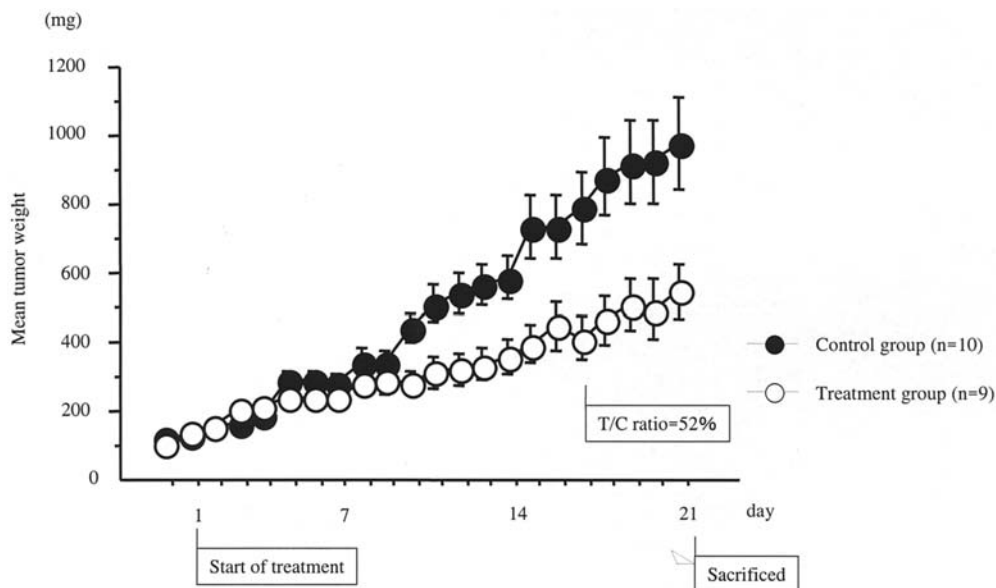


Figure 1. Antitumor activity of SU6668 against A-431 tumor cells transplanted into SCID mice. Tumor growth was suppressed in the treatment groups from 10 days after treatment until the end of the experiment. The T/C ratio was 52% at 17 and 18 days.

Reagents and antibodies. SU6668, (z)-3-[2,4-dimethyl-5-(2-dihydro-3-ylidenemethyl)-1H-pyrrol-3-yl]-propionic acid, was provided by Taiho Pharmaceutical Co., Ltd., Tokyo, Japan. SU6668 was formulated at a concentration of 50 mg/ml in an aqueous-base cremophor vehicle [16.92% 1 N sodium hydroxide solution, 24.6% cremophor EL (polyxyethylated castor oil), 1.56% benzyl alcohol, 35.14% PEG 400, and 16.79% deionized water]. To immunohistochemically quantify the tumor vessel counts, a monoclonal murine anti-human endothelial cell antibody, CD31 (clone JC/70A), was purchased from Dako (Denmark).

Tumor inoculation, measurement of tumor size and evaluation of agent activity. Initially, 1×10^7 A-431 cells were implanted into SCID mice in our laboratory. After tumor formation, the tumor was resected and a single fragment of tumor tissue, measuring approximately 3 x 3 x 3 mm, was inoculated into the subcutaneous tissue of the bilateral dorsum of ether-anesthetized mice using a trocar needle. The resultant tumors were measured (length and width) daily using sliding calipers by the same observer. Tumor weight was calculated according to the method of Geran *et al* using linear measurements and the formula: tumor weight (mg) = length (mm) x [width (mm)]² x 0.5 (21). On day 5 after tumor inoculation, when the tumors reached 100 to 300 mg, tumor-bearing mice were randomly allocated to test groups each consisting of 5 mice, and treatment was initiated.

The treatment group was given SU6668 at a dose of 200 mg/kg p.o. twice a day starting 5 days after transplantation for 21 days, while the control group was given only the vehicle. Animals were sacrificed 26 days after the initial treatment. The mice were sacrificed by cervical dislocation after adequate sedation with methoxyflurane had been confirmed using the toe pinch technique. The tumors were then excised and weighed. For CD31 staining, a section of the tumor tissue was fixed using the acetone-methylbenzoate-xylene (Amex) method and an immunohistochemical analysis was performed.

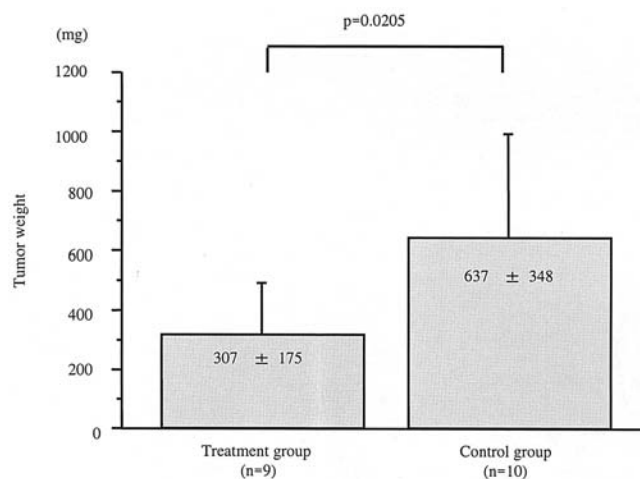


Figure 2. Actual tumor weight at the time of sacrifice. The mean tumor weight of the treatment groups (307 ± 175 mg) was significantly lower ($p=0.0205$) than that of the control groups (637 ± 348 mg).

The antitumor effects of treatments were assessed by evaluating the inhibition rate and calculated using the formula: $100 - \text{lowest T/C value (\%)} \text{ during the experiment}$, where T is the relative mean tumor weight of the treated group and C is the relative mean tumor weight of the control group at any given time. The presence of antitumor activity was defined as an inhibition rate $\geq 58\%$; this cutoff value was determined using the value of $1 - (0.75)^3$, which corresponds to a 25% reduction in tumor diameter (22).

Immunohistochemical analysis. To quantify the tumor vessel counts, immunohistochemical staining was performed using the AmeX method (23). Tissue specimens were fixed in acetone at -20°C overnight. The specimens were dehydrated in acetone at 4°C for 15 min, again at room temperature for 15 min, and rinsed twice in methyl benzoate and xylene at room temperature for 15 min. Tissues were permeated with paraffin at 60°C for 3 h, then embedded in paraffin and cut

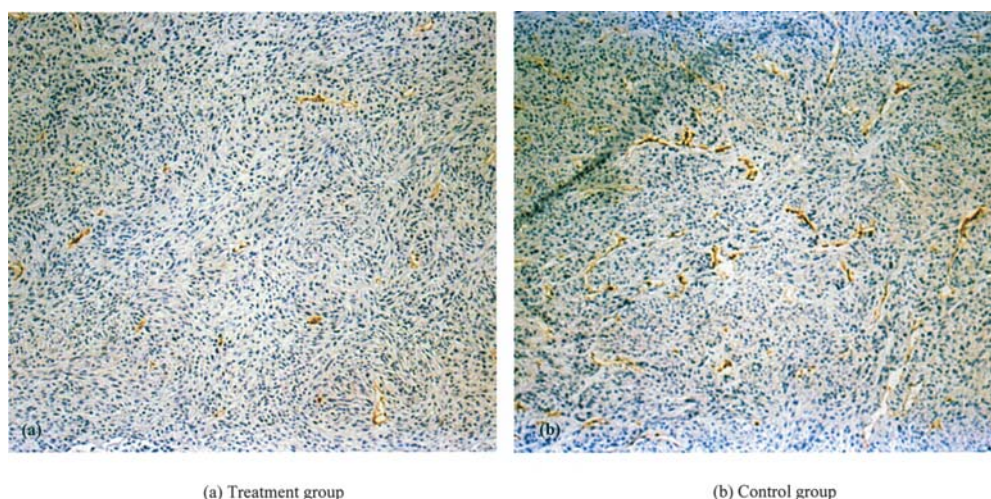


Figure 3. Immunohistochemical staining for CD31. Endothelial cells were positively stained. (a) Treatment group and (b) control group.

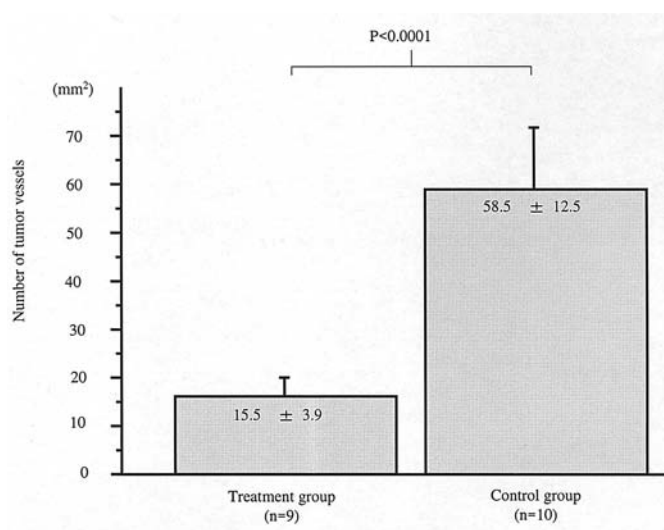


Figure 4. Number of tumor vessels. The mean density of tumor vessels in the treatment group ($15.5 \pm 3.9/\text{mm}^2$) was significantly lower than that in the control group ($58.5 \pm 12.5/\text{mm}^2$) ($p < 0.0001$).

into 4- μm sections for immunohistochemistry with primary antibodies to CD31 (endothelial cells). Five random 0.159- mm^2 fields at a $\times 100$ magnification were captured for each tumor using light microscopy.

Statistical analysis. Tumor volume, body weight, tumor weight and tumor vessel density were compared between the two groups using an unpaired Student's t-test. Statistical significance was defined as $p < 0.05$.

Results

Toxicity. Treatment toxicity was monitored by observing the loss of animal body weight. No significant differences in body weight were found between the treatment and control groups, and no toxic reactions occurred. No deaths or body weight loss $>4.3\%$ were seen in either the control or treated mice during the experiment.

Effect on tumor growth. Fig. 1 shows the suppression of A-431 tumor growth by SU6668 in SCID mice. Relative to the control mice, the mean tumor volumes were smaller in the treatment groups from 10 days after treatment until the end of the experiment, with a T/C ratio of 52% at 17 and 18 days after treatment. However, SU6668 significantly reduced the actual tumor weight (637 mg in the control group and 308 mg in the SU6668 group; $p = 0.02$) (Fig. 2).

Effect on tumor angiogenesis. We assessed tumor angiogenesis using immunohistochemical staining for CD31 to detect vessels in the tumors (Fig. 3). The density of tumor vessels in the treatment group ($15.5/\text{mm}^2$) was significantly lower ($p < 0.0001$) than that in the control group ($58.5/\text{mm}^2$) (Fig. 4).

Discussion

In this study, we found that the daily oral administration of SU6668 significantly suppressed the growth of A-431 tumors in SCID mouse. While the mean relative tumor ratio was $>48\%$, the actual tumor weight of the treatment group was statistically lower than that of the control group.

The suppression of tumor growth caused by daily treatment with SU6668 was apparently related to a marked decline in blood perfusion in the tumor. In this study, the number of tumor vessels in the treatment group was significantly fewer than that in the control group. Previous studies have shown that SU6668 inhibits tumor growth in mice by inhibiting the proliferation of vascular endothelial cells and inducing apoptosis (19,20). The SU6668-induced inhibition of angiogenic receptor tyrosine kinase activity is associated with rapid vessel killing in tumors, leading to broad and potent antitumor effects (24). Our results confirmed those of previous reports, providing further evidence that the inhibition of angiogenesis-related receptor tyrosine kinase activity suppresses tumor growth, and SU6668 suppresses tumor growth through its inhibitory effects on tumor angiogenesis.

No significant differences in body weight were found between the treatment and control groups, and no toxic reactions occurred during the experiment. As none of the mice died during the experiment, the adverse effects of this

agent appear to be minimal. Angiogenesis inhibitors target angiogenic endothelial cells and are not likely to produce or exacerbate either local or systemic toxicity caused by radiotherapy and chemotherapy (25). The appearance of antiangiogenic drugs has caused a paradigm shift in approaches to cancer therapy, and cancer can now be considered a 'chronic disease' because antiangiogenic therapy may keep patients alive with a minimal tumor load (26). Antiangiogenic drugs are also expected to change current strategies for cancer therapy.

Another strategy to increase the efficacy of antiangiogenic therapy is to combine antiangiogenic agents with conventional cytotoxic therapies, such as radiation therapy and chemotherapy (27). SU6668, a potent therapeutic agent, is potentially useful for suppressing tumor growth and enhancing the response of tumors to radiotherapy (26,28). An increase in response by tumors to radiation after exposure to SU6668 may improve the efficacy of chemoradiation therapy in patients with squamous cell carcinoma. Furthermore, combined antiangiogenic and immune therapy may represent a new strategy for cancer treatment (29). Xiong *et al* reported that it was feasible to conduct a phase I trial of SU6668 using functional computed tomography scans and dynamic contrast enhanced magnetic resonance imaging (DCE-MRI) (30). DCE-MRI is a promising approach to non-invasively monitor the sequence of vascular events that occur during tumor growth, making it useful for characterizing the activity of antiangiogenic and vascular targeting agents (31). Some studies have also suggested that SU6668 inhibits peritoneal dissemination and prolongs survival in mouse models (32,33). We conclude that SU6668 is safe, efficient and potentially useful for the treatment of squamous cell carcinoma, and may in the near future change treatment strategies for patients with squamous cell carcinoma.

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