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The Therapeutic Efficacy of STI-571, a c-kit Receptor Tyrosine Kinase Inhibitor, Used in Combination with TNP-470, an Angiogenesis Inhibitor, in the Treatment of Glioma

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ABSTRACT

The c-kit tyrosine kinase inhibitor STI-571 (imatinib mesylate) exhibits therapeutic activity against chronic myeloid leukemia and gastrointestinal stromal tumors. The median inhibitory concentration of STI-571 against proliferation of SRB10A mouse glioma cells was 8 μM. We also tested the growth-inhibitory effects of an angiogenesis inhibitor, TNP-470, given in combination with STI-571. Combination therapy with STI-571 and TNP-470 inhibited the growth of subcutaneous and intracranial gliomas more strongly than did either agent alone. The present study suggests that gliomas can be treated with a combination of molecularly targeted tumor cell agents and angiogenesis inhibitors. (Jikeikai Med J 2008 ; 55 : 1-6)

Key words : glioma, c-kit, STI-571, platelet-derived growth factor, TNP-470, molecularly targeted therapy

INTRODUCTION

Fundamental insights into signal transduction and cell-cycle control have been obtained in the past decade and are now being translated into a new generation of signal transduction/cell-cycle inhibitors. One such new drug is STI-571 (imatinib mesylate), a c-kit and bcr-abl tyrosine kinase inhibitor that has been shown to have significant activity in patients with chronic myelogenous leukemia bearing the bcr-abl tyrosine kinase produced by the Philadelphia chromosome[1,2] and in patients with gastrointestinal stromal tumors bearing c-kit tyrosine kinase[3]. STI-571 is now being tested in clinical trials for the treatment of lung cancer and gliomas[4,5].

On the other hand, tumor angiogenesis is an important process for the survival and growth of solid tumors, including gliomas. Several angiogenesis inhibitors are being tested in clinical trials[6]. The fumagillin analogue TNP-470 is an angiogenesis inhibitor that blocks endothelial proliferation[7]. In animal models, TNP-470 is effective for the treatment of a wide variety of tumors and metastases[8-10]. The combination of TNP-470 and other anticancer drugs can produce significant synergistic effects that can affect both tumor cells and endothelial cells[11].

The present study was designed to assess the therapeutic potential of a novel molecular targeting agent, STI-571, and an angiogenesis inhibitor, TNP-470, for use in patients with intracranial malignant gliomas.

MATERIALS AND METHODS

Cell lines and animals : The SRB10A mouse glioblastoma cells (kindly provided by Dr. K. Sakamoto,

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Gumma University, Japan), rat RT2 glioblastoma cells (kindly provided by Dr. G. Yancey Gillespie, University of Alabama, Birmingham, Birmingham, AL, USA), rat 9L glioblastoma cells, and U87MG and T98G human glioblastoma cells (purchased from American Type Culture Collection, Rockville, MD, USA) were maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (FBS). Philadelphia-chromosome-positive KU812 human chronic myeloid leukemia cells (Riken, Tokyo, Japan) were grown in RPMI 1640 medium supplemented with 10% FBS. Six-week-old female B10A/SgSn Slc mice were purchased from Japan SLC Inc. (Hamamatsu, Japan). Animal studies were approved by the Animal Care and Use Committee of The Jikei University School of Medicine and performed in accordance with its guidelines.

Western blot analysis: Confluent cells were lysed at 4°C in a lysis buffer containing 50 mM Tris-HCl (pH 7.5), 50 mM NaCl, 0.1 mg/ml phenylmethylsulfonyl fluoride, 100 μM benzamidine, and 100 mM Na_3VO_4. Cell lysates, each containing 100 μg of protein, were subjected to sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE; 7.5% polyacrylamide gel). The blots were then incubated with a rabbit polyclonal antibody against the carboxy-terminal domain of the human p145 c-kit tyrosine kinase (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and detected with the ECL Western blotting analysis system (Amersham, Arlington Heights, IL, USA).

In vitro cytotoxic assay: Cells of the KU812, SRB10A, RT2, 9L, T98G, and U87MG lines (5×10^3 cells/100 μl each) were plated into individual wells of a 96-well microtiter plate coated with collagen (Iwaki, Chiba, Japan). The cells were also treated for 72 hours with increasing concentrations of STI-571 (0-1,000 μM). Cells were then treated with Cell Counting Kit-8 solution (Dojindo, Tokyo, Japan). Absorbance was measured with a microplate reader (Ultramark Microplate Reader, Bio-Rad Laboratories, Hercules, CA, USA) at a wavelength of 450 nm.

Cell proliferation assay: SRB10A cells (5×10^4 cells/5 ml medium) were plated onto 60-mm collagen-coated dishes and after 24 hours were treated with STI-571 (kindly provided by Novartis Pharmaceuticals) at a concentration of 10 μM. All cells were then trypsinized and counted with a cell counter (Coulter Counter, Northwell, UK).

Animal Experiments

Subcutaneous tumor model: A total of 1×10^6/100 μl of SRB10A cells were injected subcutaneously into the flanks of B10A mice. Five days after tumor implantation, STI-571 treatment was started at a dose of 100 mg/kg/day via intraperitoneal injection, and TNP-470 (kindly provided by Takeda Pharmaceuticals, Osaka, Japan) was administered at dose of 30 mg/kg every other day via intraperitoneal injection. Saline was administered to control animals. Tumor volume was estimated with the following formula: tumor volume (mm^3) = length $\times$ width$^2\times1/2$.

Intracranial tumor model: Five thousand SRB10A cells were injected into the right caudate nucleus of B10A mice using a small-animal stereotactic frame (Kopf) as previously described[9]. The animals were treated with STI-571 (100 mg/kg daily) and TNP-470 (30 mg/kg every other day) via intraperitoneal injection.

Statistical analysis: Data are the means $\pm$ SD of 3 independent experiments. The significance of differences between experimental values was assessed with Student's t-test, and $P<0.05$ was considered to indicate statistical significance. Survival among the different groups was assessed using log-rank analysis of Kaplan-Meier survival curves.

RESULTS

Glioma cell lines express the c-kit receptor: We examined the expression of the c-kit receptor in several glioma cell lines. Western blot analysis of crude protein lysates prepared from glioma cells and KU812 was performed with a rabbit polyclonal antibody against the c-kit carboxy terminal domain. The U87MG, RT2, and SRB10A cells lines exhibited

immunoreactive bands on SDS-PAGE. As a positive control, cellular extracts were prepared from KU812 cells, which showed a broad and intense signal (Fig. 1).

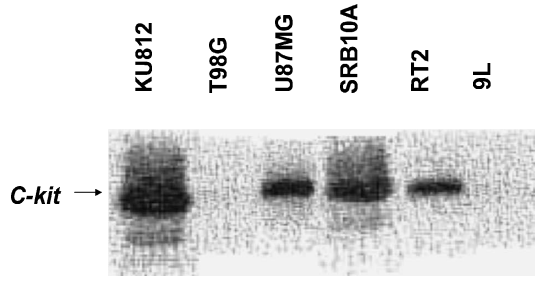
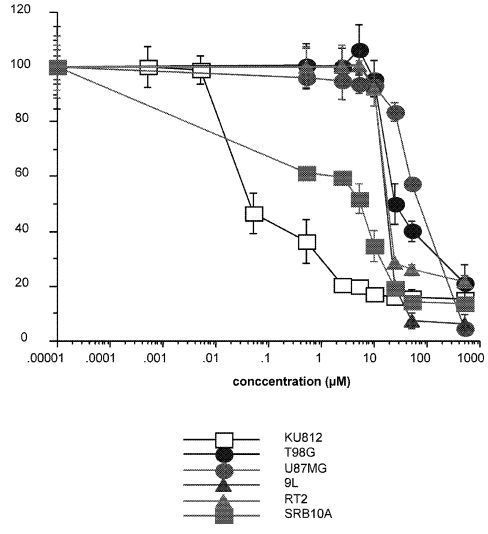


Fig. 1. Western blot analysis for c-kit expression in glioma cells. Cell lysates were prepared with lysis buffer, and proteins were separated by means of SDS-PAGE (7.5% polyacrylamide gel) and immunoblotted with a rabbit polyclonal c-kit antibody. The C-kit receptor was expressed by Philadelphia-chromosome-positive KU812 human chronic myeloid leukemia, U87MG human glioma, RT2 rat glioma, and SRB10A mouse glioma. Both T98G human glioma and 9L rat glioma were negative for c-kit.

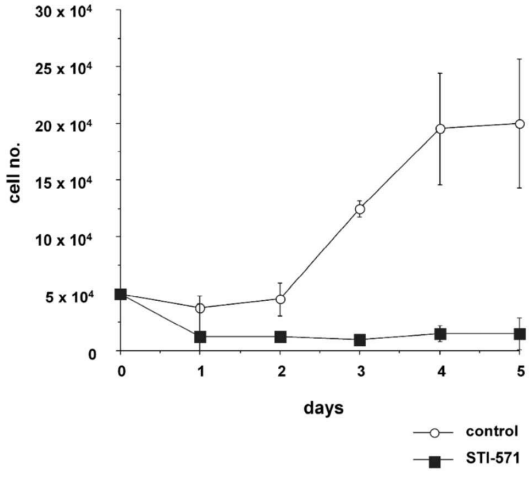
Inhibition of cell proliferation by STI-571: The antiproliferative effects of STI-571 were assayed in a series of glioma cell lines, including SRB10A. Cultured cells were incubated in media containing 10% FBS and increasing concentrations of STI-571 (0-1,000 μM). Then, 72 hours after treatment, cytotoxicity was assessed. As shown in Fig. 2(a), the median inhibitory concentration of STI-571 against SRB10A cell proliferation was 8 μM.

The *in vitro* growth of SRB10A tumor cells, which were the glioma cells most sensitive to STI-571, was completely inhibited by treatment with STI-571 at a concentration of 10 μM (Fig. 2(b)).

In vivo tumor growth and combination therapy with STI-571 and TNP-470: The *in vivo* growth of SRB10A cells that had been subcutaneously injected into mice was assessed. The growth of the SRB10A tumor syngeneic grafts was monitored following the daily intraperitoneal injection of 100 mg/kg of STI-571 for 16 consecutive days. The growth of STI-571-treated SRB10A grafts was inhibited by 70% relative

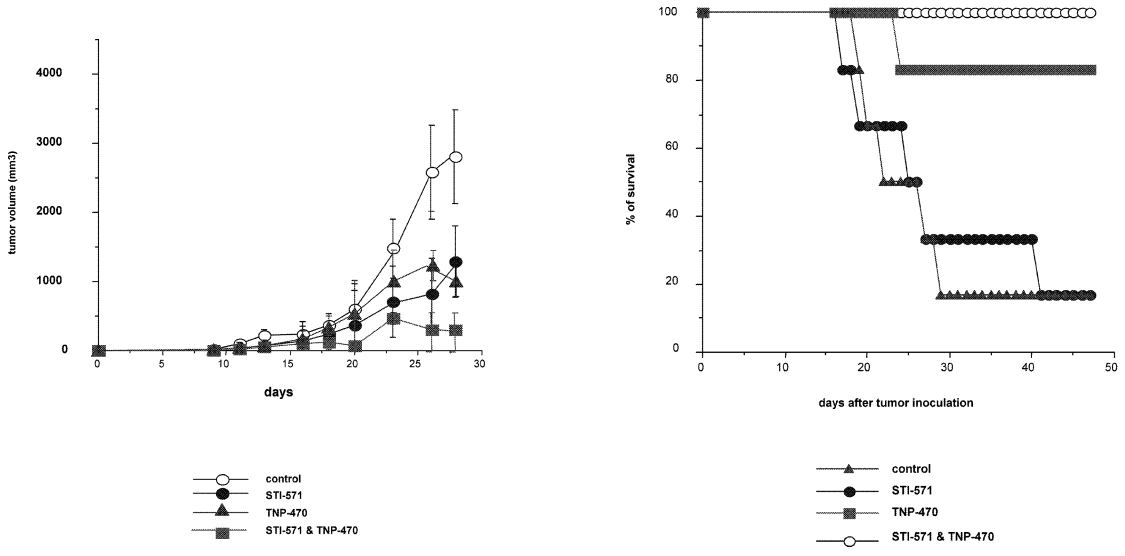


(a)



(b)

Fig. 2. (a) *Sensitivity of glioma cell lines to STI-571 in vitro.* The sensitivity of cultured glioma cell lines to STI-571 was examined. Cells were plated on 96-well plates (5×10^3 cells/well) and exposed to various concentrations of STI-571 for 72 hours. Cytotoxicity was determined with a Cell Counting Kit-8. Absorbance at 450 nm was measured with a microplate reader. The mean and SD were determined from 3 independent measurements.
(b) *In vitro growth rate of SRB10A glioma cells treated with STI-571.* The growth rate of SRB10A mouse glioma cells exposed to STI-571 (10 μM) was determined. Over a 5-day period, cell numbers were counted in triplicate with a hemocytometer (mean: bars: SD).



(a) (b)

Fig. 3. (a) The growth rate of subcutaneous SRB10A tumors treated with STI-571 and TNP-470. SIT-571 (100 mg/kg) and TNP-470 (30 mg/kg) showed additive inhibitory effects on the growth of SRB10A tumors ($n=6$/each group). bars: SD.
(b) Survival of intracranial SRB10A tumors treated with STI-571 and TNP-470 ($n=6$/each group). Tumor-bearing mice treated with a combination of STI-571 and TNP-470 survived longer than did mice treated with either STI-571 or TNP-470 alone. When compared with the results of the subcutaneous tumor model, intracranial SRB10A tumors were completely inhibited by the combination of STI-571 and TNP-470 ($n=6$/each group).

to control tumors (Fig. 3(a)). On the whole, mice receiving STI-571 appeared healthy throughout the course of the experiment.

To determine whether glioma growth can be inhibited by a molecularly targeted agent given in combination with another anticancer drug, we examined the growth-inhibitory effects of an angiogenesis inhibitor, TNP-470, co-administered with STI-571. Initially, subcutaneous SRB10A tumor syngeneic grafts were treated with intraperitoneal injection of 100 mg/kg of STI-571 daily and 30 mg/kg of TNP-470 given every other day. As shown in Fig. 3(a), growth-inhibitory effects were observed both with TNP-470 alone and with STI-571 alone. However, combination therapy with STI-571 and TNP-470 inhibited subcutaneous tumor growth more strongly than did either STI-571 or TNP-470 alone ($p<0.05$; Student's t-test).

For a more practical assessment of the potential of STI-571 as a therapeutic agent for human brain tumors, we inoculated SRB10A mouse glioma cells into the caudate nucleus of B10A mice and administered the drugs via intraperitoneal injection. As shown in Fig. 3(b), systemic treatment with STI-571 alone did not prolong the survival of mice bearing intracranial tumors. However, the co-administration of STI-571 and TNP-470 significantly prolonged the survival of tumor-bearing mice compared with treatment with either STI-571 alone ($p<0.001$; log-rank test) or TNP-470 alone ($p<0.05$; log-rank test). There were no undesirable effects of combination treatment with STI-571 and TNP-470 on animal behavior under ordinary conditions.

DISCUSSION

In the present report, we examined the sensitivity of various glioma cell lines to STI-571. The c-kit proto-oncogene was clearly identified in some of the glioma cell lines. Of the glioma cell lines examined, SRB10A expressed c-kit at the highest level. A previous report has shown that the c-kit receptor plays a significant role in the generation and maintenance of human colonic tumor xenografts[12]. How-

ever, one study has found that the percentage of patients with small cell lung cancer who had evidence of c-kit receptor expression was lower than would be expected on the basis of a review of the literature[5]. Another study has shown that only 40% of small cell lung cancer specimens had detectable c-kit receptors on immunohistochemical examination[13]. Therefore, these studies suggest that a test for c-kit expression should be required for enrollment in any clinical trial of STI-571.

We have shown that STI-571 plays a role in the proliferative potential of mouse glioma cells cultured in the presence of growth factors. A previous report has found that the cell growth of xenografts of the c-kit-expressing human colon cancer cell line HT29 in nude mice is significantly inhibited by STI-571[12]. The growth-suppressive effect of STI-571 is directly related to its ability to induce programmed cell death in cultured HT29 cells. The cytotoxic effect is caused by the involvement of the c-kit receptor in cell survival.

With respect to the sensitivity of glioma cell to STI-571, Kilic et al. have demonstrated that STI-571 inhibits the growth of U343MG and U87MG human glioblastoma xenografts in the brains of nude mice[14]. They have proposed that the main mechanism of STI-571-induced growth inhibition is cell cycle arrest rather than apoptosis. A recent study has demonstrated that specific c-KIT gene mutations in exon 9 and exon 11 occur in gastrointestinal stromal tumors and chronic myelogenous leukemia after treatment with STI-571[15]. It remains to be determined whether gliomas possess such KIT mutations. In the present study, we demonstrated that some glioma cell lines, including SRB10A, express c-kit.

The progressive growth of gliomas is dependent on the presence of an adequate blood supply and on vascular development ; in fact, angiogenesis is a rate-limiting factor in this process. TNP-470 is an angiogenesis inhibitor and a potent inhibitor of the growth of vascular endothelial cells[6]. TNP-470 inhibits the growth of vascular endothelial cells *in vitro* and inhibits angiogenesis and tumor growth in various animal models *in vivo*[8-10].

In the present study, combination therapy with STI-571 and TNP-470 inhibited the growth of subcutaneous and intracranial gliomas. Among mice bearing intracranial tumors, those treated with the combination of STI-571 and TNP-470 survived significantly longer than did those treated with either STI-571 or TNP-470 alone. *In vivo* treatment of gliomas with STI-571 was more effective when combined with TNP-470.

Our results suggest that gliomas can be treated with combined modalities that target both tumor cells and host microenvironmental factors, including the tumor vasculature. Combination therapies with agents that target endothelial cells to block angiogenesis and c-kit tyrosine kinase to prevent tumor growth represent an effective strategy for treating brain tumors.

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