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Basis for dosing time-dependent change in the anti-tumor effect of imatinib in mice☆

Hiroo Nakagawa [a], *Takako Takiguchi* [a], *Mariko Nakamura* [b], *Atsuko Furuyama* [b], *Satoru Koyanagi* [a], *Hironori Aramaki* [c], *Shun Higuchi* [b], *Shigehiro Ohdo* [a,*]

[a] *Pharmaceutics, Division of Clinical Pharmacy, Department of Medico-Pharmaceutical Sciences, Faculty of Pharmaceutical Sciences, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, Japan*

[b] *Clinical Pharmacokinetics, Division of Clinical Pharmacy, Department of Medico-Pharmaceutical Sciences, Faculty of Pharmaceutical Sciences, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, Japan*

[c] *Department of Molecular Biology, Daiichi College of Pharmaceutical Sciences, 22-1 Tamagawa-cho, Minami-ku, Fukuoka 815-8511, Japan*

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PDGF, platelet-derived growth factor
ERK, extracellular signal-regulated kinases
MAP, mitogen-activated protein kinase
PI3K, phosphoinositol-3-kinase

ABSTRACT

Because a variety of receptor tyrosine kinases are involved in the mechanism of tumor progression, the development of a clinically useful tyrosine kinase inhibitor is expected as a therapeutic agent for the treatment of malignant cancers. Imatinib mesylate, known as Gleevec or STI-571, is a molecule that inhibits the function of various receptors with tyrosine kinase activity, such as Abl, the bcr–abl chimeric product, KIT, and platelet-derived growth factor (PDGF) receptors. In this study, we investigated the influence of dosing time on the ability of imatinib to inhibit tumor growth in mice. Tumor-bearing mice were housed under standardized light/dark cycle conditions (lights on at 07:00 h, off at 19:00 h) with food and water ad libitum. The growth of tumor cells implanted in mice was more severely inhibited by the administration of imatinib (50 mg/kg, i.p.) in the early light phase than when it was administered in the early dark phase. The dosing time-dependency of anti-tumor effects was parallel to that of imatinib-induced anti-angiogenic effect. The inhibitory effect of imatinib on tyrosine kinase activity of PDGF receptors, but not of KIT and Abl, varied according to its administration time. The dosing time-dependency of imatinib-induced inhibition of PDGF receptor activity was closely related to that of its anti-tumor effects. Our results suggest that the anti-tumor efficacy of imatinib is enhanced by administering the drug when PDGF receptor activity was increased. The potent therapeutic efficacy of the drug could be expected by optimizing the dosing schedule.

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* *Corresponding author*. Tel.: +81 92 642 6610; fax: +81 92 642 6614.
E-mail address: ohdo@phar.kyushu-u.ac.jp (S. Ohdo).

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1. Introduction

The identification of molecules with a function to help DNA replication contributes to the development of molecular target therapeutic agents for treatment of cancers. Imatinib mesylate, known as Gleevec or STI-571, is a tyrosine kinase inhibitor of KIT, Abl, and platelet-derived growth factor (PDGF) receptor kinases. The inhibition of kinase activities of bcr–abl and KIT by imatinib accounts for its clinical activity in chronic myeloid leukemia and gastrointestinal stromal tumors, respectively [1–5]. The constitutive activation of PDGF receptor tyrosine kinases has been observed in various types of malignant tumor cells [3,6]. Consequently, imatinib also exerts anti-tumor activity against cancer cells expressing both PDGF and their cognate receptors [7,8].

Daily rhythmic variations in biological functions are thought to affect the efficacy and/or toxicity of drugs: a large number of drugs cannot be expected to have the same potency at different administration times [9–14]. It has been suggested that drug administration at appropriate times of day can improve the outcome of pharmacotherapy by maximizing potency and minimizing the toxicity of the drug, whereas drug administration at inappropriate times of day can induce severe side effects [15,16]. A chronopharmacological strategy can also improve tumor response, overall survival, and dose-limiting toxicity in cancer patients that are subjects of ongoing prospective randomized clinical trials: however, many anti-cancer drugs are still administered without regard to the time of day. Identification of mechanism underlying the dosing time-dependency of anti-tumor effects of chemotherapeutic agents will help us to achieve better chronopharmacotherapy for cancer treatment.

This study was designed to examine the influence of dosing time on the ability of imatinib to inhibit tumor growth in mice. The mechanism underlying the dosing time dependent changes in the anti-tumor activity of imatinib was investigated from the viewpoint of sensitivity of tumor cells to the drug.

2. Materials and methods

2.1. Materials

Imatinib (STI-571, Gleevec) was kindly provided by Novartis Pharma (Basel, Switzerland). The compounds were stored at room temperature and diluted in sterile saline before use. For the treatment of tumor-bearing mice, the drug was used at an intraperitoneal (i.p.) dose of 50 mg/kg. The dose of imatinib was decided based on a previous study of anti-tumor activity in mice [17].

2.2. Animals and cells

Male ICR mice (5 weeks old) were purchased from Charles River Japan Inc. (Kanagawa, Japan). They were housed in groups of 3–10 per cage in a light-controlled room (lights (100 lx) on from 07:00 to 19:00 h) at a room temperature of $24 \pm 1\,^{\circ}C$ and a humidity of $60 \pm 10\%$ with food and water ad libitum. The animals were adapted to the light/dark cycle for 2 weeks before the experiments. During the dark period, a dim red light was used to aid treatment. Three murine tumor cell lines (Sarcoma 180, Lewis lung carcinoma and B16 melanoma) were supplied by the Cell Resource Center for Biomedical Research, Tohoku University (Sendai, Japan). These tumor cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum at 37 °C in a humidified 5% CO_2 atmosphere. A 50 μl volume of 1×10^7 viable tumor cells was inoculated into the right hind footpad of each mouse.

2.3. Experimental design

To investigate the influence of dosing time on the ability of imatinib to inhibit tumor growth or tumor-induced angiogenesis, tumor-bearing mice were injected with a single daily dose of imatinib (50 mg/kg, i.p.) or saline at 09:00 or 21:00 h every day. To explore the temporal profiles of tyrosine kinase activity of KIT, Abl, and PDGF receptors in the implanted tumor cells, cell lysates from the tumor masses were prepared at 09:00, 13:00, 17:00, 21:00, 01:00 or 05:00 h. To explore the influence of dosing time on the ability of imatinib to inhibit the tyrosine kinase activity of KIT, Abl or PDGF receptor in the implanted tumor cells, tumor-bearing mice were injected with imatinib (50 mg/kg, i.p.) or saline at 09:00 or 21:00 h. At 1 h after drug injection, tyrosine kinase activity was assessed by immunoblotting. The influence of dosing time on the ability of imatinib to inhibit the phosphorylation of ERK and Akt in the implanted tumor cells was investigated by the same procedure.

2.4. Determination of the anti-tumor effect

Mice inoculated with sarcoma 180 cells were given a single daily injection of imatinib (50 mg/kg, i.p.) or saline for 19 days. In all mice, tumor volumes were measured throughout the experiment. The tumor volume was estimated according to the following formula: tumor volume (mm^3) = $4\pi xyz/3$, where $2x$, $2y$, and $2z$ are the three perpendicular diameters of the tumor. The mice were also weighed throughout the experiment.

2.5. Determination of anti-angiogenic effect

Tumor-induced neovascularization was assessed by the dorsal air-sac method [20]. Briefly, cultured sarcoma 180 cells (1×10^7) were packed into a membrane chamber, which was implanted into the dorsal air sac of mice (day 0). The mice were injected with a single daily dose of imatinib (50 mg/kg, i.p.) or saline for 6 days. On day 7, the mice were deeply anesthetized with ether and killed. The newly formed blood vessels in the subcutaneous regions adjacent to the implanted chamber were photographed under a dissecting microscope.

2.6. Immunoblot analysis

Tumor masses were removed at indicated time points as described above and homogenized with ice-cold lysis buffer

(50 mM Tris–HCl, pH 7.8, 150 mM NaCl, 5 mM EDTA, 0.5% Nonidet P-40, 2 mM PMSF, 1 mM Na_3VO_4). After the removal of insoluble materials by centrifugation at 12,000 × *g* for 15 min at 4 °C, the resulting supernatants were assayed to detect each protein. Protein concentration was determined using a BCA protein assay kit (Sigma). The supernatant proteins (20 μg) were separated on SDS-polyacrylamide gels, and transferred to a polyvinylidene difluoride membrane. The membranes were reacted with antibodies against phosphorylated or non-phosphorylated KIT, Abl, α-PDGF receptor, β-PDGF receptor, ERK or Akt (Santa Cruz Biotechnology Inc., Santa Cruz, CA). Specific antigen/antibody complexes were made visible using horseradish peroxidase-conjugated secondary antibodies and Super Signal Chemiluminescent Substrate (Pierce Biotechnology Inc., Rockford, IL). The image from the immunoreacted membrane was digitized. The band intensity of each protein was quantified using NIH Image software. The amount of phosphoprotein was normalized by the amount of total protein.

2.7. *Statistical analysis*

The statistical significance of differences between groups was validated by the Bonferroni test for multiple comparisons and Student's *t*-test for comparison between two groups. A 5% level of probability was considered significant.

3. Results

3.1. *Influence of dosing time on the anti-tumor effect of imatinib*

In the first set of experiments, we investigated whether the anti-tumor efficacy of imatinib varied according to its administration time. In our preliminary experiments, the expressions of KIT, Abl, and PDGF receptors were observed in cultured sarcoma 180, Lewis lung carcinoma, and B16 melanoma cells (Supplemental data 1); therefore, we used those cells in this study. Since there was no significant time-dependent difference in the growth of these implanted tumor cells, the mean value of the tumor volume between 09:00 and 21:00 h was set as the control. Although the growth of all three different types of tumor cells was suppressed by the administration of imatinib, the anti-tumor effect was more potent in mice injected with the drug at 09:00 than at 21:00 h (Fig. 1). Nineteen days after the start of treatment, the volume of all three types of tumor cells in mice injected at 09:00 h was significantly smaller than in mice injected at 21:00 h ($p < 0.01$, respectively). The anti-tumor efficacy of imatinib varied according to its administration time.

As new blood vessel formation is essential for tumor growth, we also investigated the influence of imatinib dosing time on tumor-induced angiogenesis. Sarcoma 180 tumor cells showed potent angiogenic activity in mouse dorsal air sac model [18]; therefore, the tumor cell lines were used in this study. The photographs in Fig. 2 show the influence of dosing time on ability of imatinib to inhibit tumor-induced angiogenesis. Seven days after sarcoma 180 tumor cells were implanted, neovascularization from surrounding blood vessels was observed in the control mice in the region adjacent to the implanted chamber; however, neovascularization in mice given imatinib was clearly suppressed. The blood vessels in mice injected with the drug at 09:00 h were not obviously different from those in the phosphate-buffered saline control mice. The dosing time-dependency of anti-angiogenic activity of imatinib was parallel to that of its anti-tumor efficacy.

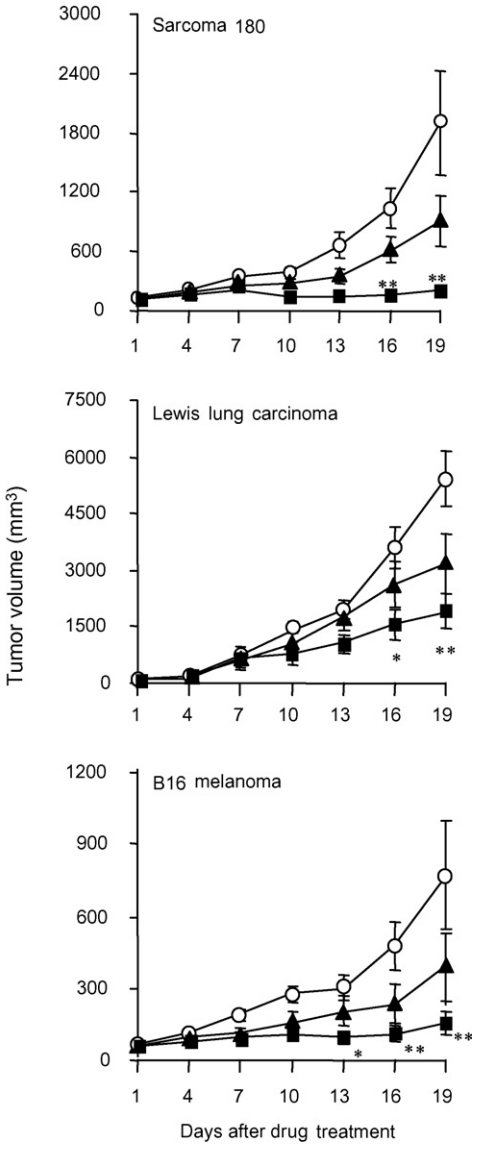

Fig. 1 – Influence of dosing time on the ability of imatinib to inhibit tumor growth. Three types of tumor cells (Sarcoma 180, Lewis lung carcinoma, and B16 melanoma) were inoculated into mice. Imatinib (50 mg/kg) ((■) 09:00 h; (▲) 21:00 h) or saline (○) was injected intraperitoneally every day for 19 days. Each point is the mean ± S.E. of 8–10 mice. $^{**}p < 0.01$, $^{*}p < 0.05$ when compared with the corresponding saline-treated group using the Bonferroni test.

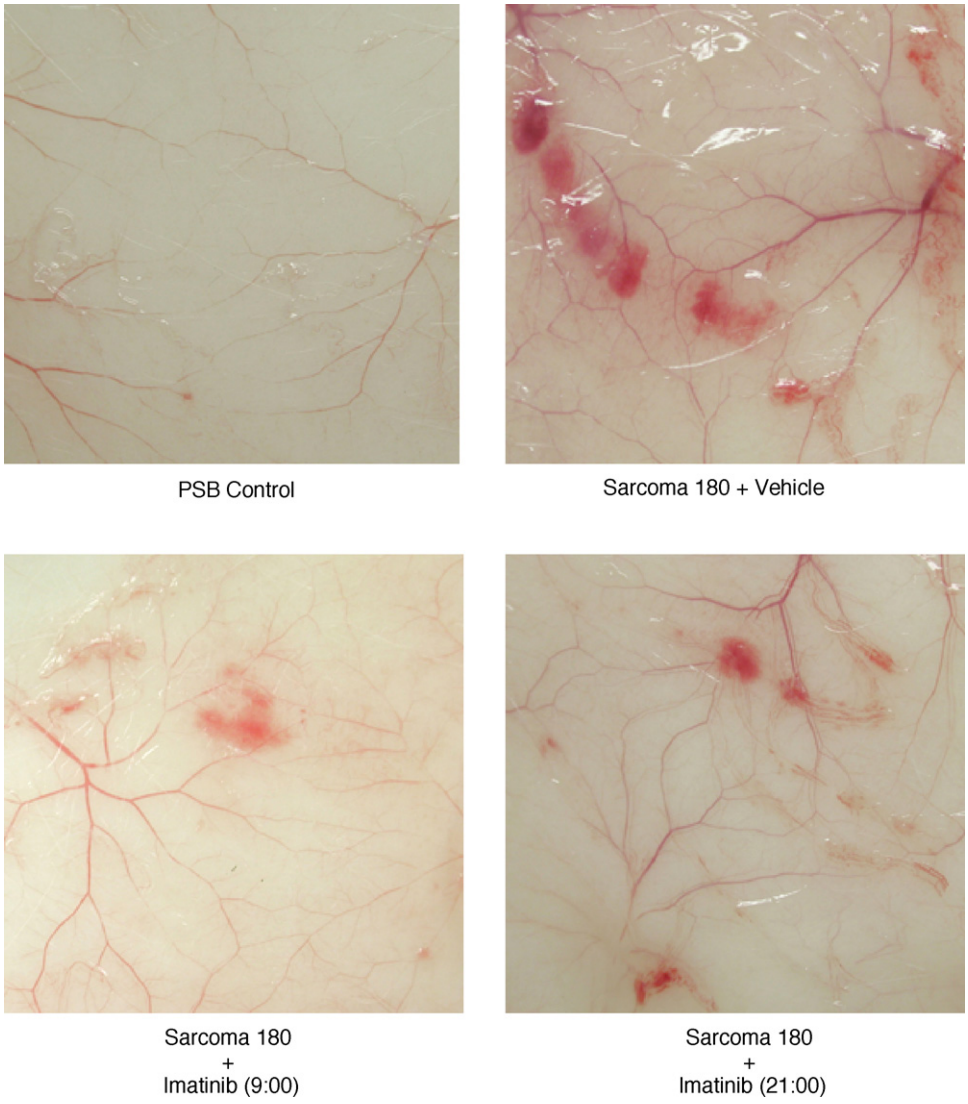

Fig. 2 – Influence of dosing time on the ability of imatinib to inhibit tumor-induced angiogenesis. Representative photographs of sarcoma 180-induced angiogenesis in mice injected intraperitoneally with imatinib (50 mg/kg) (09:00 or 21:00 h) or saline every day for 7 days. The control chamber contained phosphate-buffered saline instead of sarcoma 180 cells. Data shown were confirmed in three independent experiments.

3.2. *Influence of dosing time on the ability of imatinib to inhibit the tyrosine kinase activity of KIT, Abl, and PDGF receptors*

Imatinib exerts its anti-tumor activity by inhibiting the function of various receptors with tyrosine kinase activity, such as KIT, Abl, and PDGF receptors. Although there were no significant 24-h variations in the tyrosine phosphorylation of KIT and Abl in the sarcoma 180 tumor cells (Fig. 3A and B), the tyrosine phosphorylation of α- and β-PDGF receptors showed significant 24-h oscillations ($p < 0.05$, respectively; Fig. 3C). An enhanced phosphorylation state of PDGF receptors was observed from the late dark phase to the early light phase. Similar time-dependent variations in the phosphorylation of PDGF receptors were also found in the implanted Lewis lung carcinoma and B16 melanoma tumor cells (data not shown).

We next investigated whether the inhibitory effect of imatinib on the tyrosine kinases varied according to its administration time. Because imatinib concentration in sarcoma 180 tumor masses peaked about 1 h after drug injection (Supplemental data 2), tyrosine kinase activities in the implanted tumor cells were assessed at 1 h after imatinib injection. The tyrosine phosphorylation of KIT and Abl in the implanted tumor cells was significantly suppressed by imatinib administration at both 09:00 and 21:00 h ($p < 0.01$, respectively; Fig. 4A and B); the inhibitory effects were not significantly different between the two dosing times.

The tyrosine phosphorylation of α- and β-PDGF receptors on the implanted sarcoma 180 tumor cells after imatinib injection (50 mg/kg. i.p.) at 09:00 h was significantly lower than after saline injection at the corresponding dosing time ($p < 0.05$ for α- PDGF receptors, $p < 0.01$ for β-PDGF receptors; Fig. 4C and D). However, there was no significant difference in

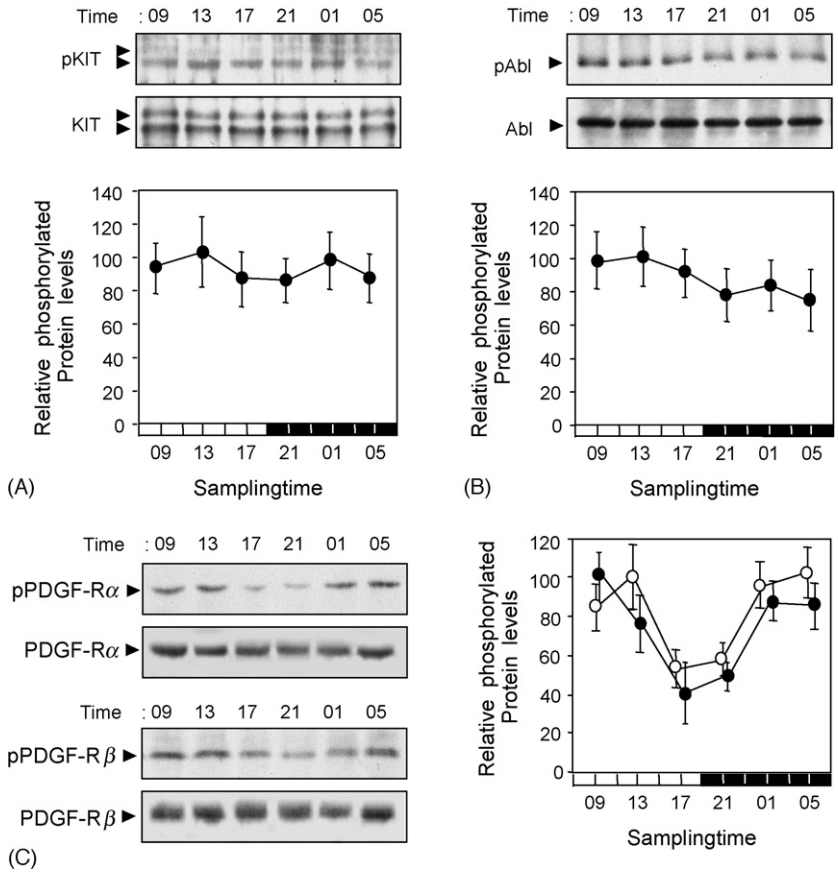

Fig. 3 – Temporal profiles of tyrosine kinase activity of KIT, Abl, and PDGF receptors in sarcoma 180 tumor masses. (A and B) Temporal profiles of phosphorylation of KIT (A) and Abl (B) proteins in tumor masses. Values are normalized by the total amount of each receptor protein. For plots of intensity, the mean peak value of each receptor is set at 100. Each value represents the mean ± S.E. (n = 3–4). The horizontal bar at the bottom indicates light and dark cycles. The upper panels show representative immunoblotting for 24-h variations in the phosphorylation of KIT and Abl (pKIT and pAbl). (C) Temporal profiles of phosphorylation α- and β-PDGF receptors (pPDGF-Rα and pPDGF-Rβ) in tumor masses. Values are normalized by the total amount of each receptor protein. For plots of intensity, the mean peak value of each receptor is set at 100. Each value represents the mean ± S.E. (n = 3–4). The horizontal bar at the bottom indicates light and dark cycles. The protein abundances for both pPDGF-Rα and pPDGF-Rβ exhibit significant 24-h variations ($p < 0.05$, ANOVA). The left panels show representative immunoblotting for the phosphorylation of each PDGF receptor protein.

the phosphorylation state of PDGF receptors between the saline group and the group given imatinib at 21:00 h. These results suggest that the inhibitory effect of imatinib on the tyrosine phosphorylation of PDGF receptors varies according to its administration time.

3.3. *Influence of dosing time on the ability of imatinib to inhibit the phosphorylation of ERK and Akt*

The autophosphorylation of PDGF receptors can induce the proliferation of cells through activation of ERK/MAP and PI3K/Akt signaling pathways [20,21]. In final set of experiment, we investigated the influence of imatinib dosing time on the activity of ERK and PI3K/Akt signaling pathways. Under non-drugged state, ERK phosphorylation in the implanted sarcoma 180 tumor cells showed obvious 24-h oscillation (Fig. 5A upper panel). An enhanced phosphorylation state of ERK was observed from the late dark phase to the early light phase, corresponding with the 24-h variations in tyrosine phosphorylation of PDGF receptors (Fig. 3C). The phosphorylation of ERK in the implanted tumor cells at 1 h after imatinib injection (50 mg/kg. i.p.) at 09:00 h was significantly lower than after saline injection at the corresponding dosing time ($p < 0.01$; Fig. 5A lower panel); however, there was no significant difference in the amount of phosphorylated ERK between the saline group and the group given imatinib at 21:00 h. Under non-drugged state, Akt phosphorylation in the implanted sarcoma 180 tumor cells also showed obvious 24-h oscillation (Fig. 5B upper panel). A dosing time-dependent difference was observed in the inhibitory effect of imatinib on Akt phosphorylation (Fig. 5B lower panel), although the phosphorylation of Akt in the implanted tumor cells was significantly inhibited by imatinib administration at both time points ($p < 0.01$, respectively). These results suggest that the inhibitory effect of imatinib on PDGF receptor signaling varies according to its administration time.

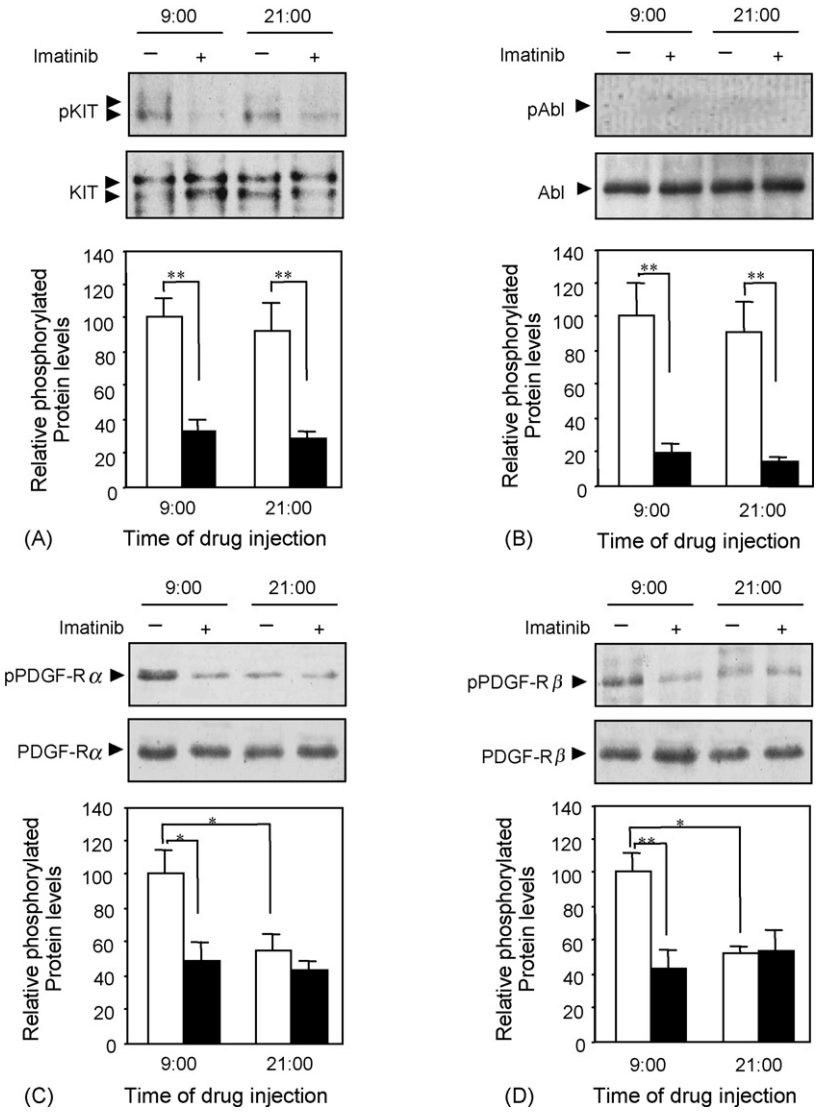

Fig. 4 – Influence of dosing time on the ability of imatinib to inhibit the tyrosine kinase activity of KIT (A), Abl (B), and PDGF receptors (C and D) in tumor masses. Mice inoculated with sarcoma 180 cells were given a single injection of imatinib ((■) 50 mg/kg, i.p.) or saline (□) at 09:00 or 21:00 h. At 1 h after the drug injection, tyrosine kinase activity was assessed by immunoblotting. Values are normalized by the total amount of each receptor protein. For plots of intensity, the mean value of the saline-treated group at 09:00 is set at 100. Each value represents the mean ± S.E. ($n = 3–4$). $^{**}p < 0.01$; $^{*}p < 0.05$ compared between the two groups using the Bonferroni test. Upper panels show representative immunoblotting for the phosphorylation of each receptor protein. + and − indicate injections with imatinib and saline, respectively.

4. Discussion

This study demonstrated that the growth of all three types of tumor cells (sarcoma 180, Lewis lung carcinoma and B16 melanoma) implanted in mice was inhibited by treatment with imatinib and that the effectiveness of the drug varied with the time of day when it was administered. The drug's anti-tumor effect was pronounced when given at the peak time of the phosphorylation of PDGF receptors. The time-dependent change in anti-tumor effect of imatinib was parallel change in its anti-angiogenic activity. Similar dosing time-dependent differences in the anti-tumor and anti-angiogenic effects are also observed in other type of drugs [18,19].

Daily rhythmic variations in biological functions such as receptor expression and signal transduction are thought to affect the efficacy and/or toxicity of drugs. Imatinib exerts its anti-tumor activity by inhibiting the function of various tyrosine kinase receptors such as KIT, Abl, and PDGF receptors. Although potent activation of all these tyrosine kinases receptors was observed in our tested tumor cell lines, the activity of PDGR receptors, but not of KIT and Abl, in the implanted tumor cells showed significant 24-h oscillation. Several ligands, which bind to PDGF receptors, can induce their dimerization and autophosphorylation, leading to the activation of cell growth and survival signaling pathways [21]. One possible mechanism accounting for the 24-h variation in the tyrosine kinase activity of PDGF receptors is that there is time-dependent change in the binding amounts of ligands to

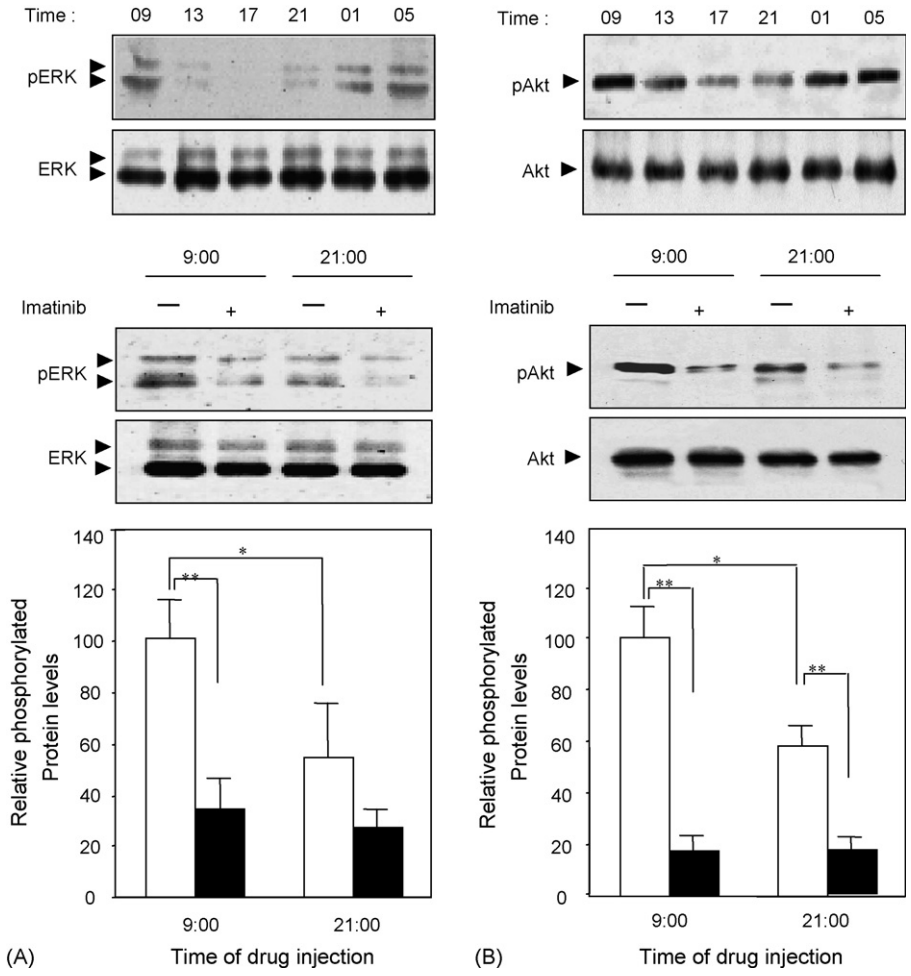

Fig. 5 – Influence of dosing time on the ability of imatinib to inhibit the phosphorylation of ERK (A) and Akt (B) in tumor masses. Mice inoculated with sarcoma 180 cells were given a single injection of imatinib ((■) 50 mg/kg, i.p.) or saline (□) at 09:00 or 21:00 h. At 1 h after drug injection, tyrosine kinase activity was assessed by immunoblotting. Values are normalized by the total amount of each protein. For plots of intensity, the mean value of the saline-treated group is set at 100. Each value represents the mean ± S.E. ($n = 3–4$). $^{**}p < 0.01$; $^{*}p < 0.05$ compared between the two groups using the Bonferroni test. Upper panels show representative immunoblotting for 24-h variation in the phosphorylation of ERK (A) and Akt (B) in tumor masses.

PDGF receptors. Our previous study has demonstrated that the expression of vascular endothelial growth factor (VEGF), a member of PDGF family of mitogens, in sarcoma 180 tumor cells implanted in mice shows a significant 24-h oscillation. The oscillation in the expression of VEGF gene is governed by the core components of the circadian clock [19]. It is therefore possible that the molecular components of the circadian clock affect the expression of PDGF genes, thereby causing the oscillation in its protein production. However, to our knowledge, no previous study has reported the daily oscillation in PDGF production from the tumor cells. Further study will be required to clarify the mechanism of 24-h variation in PDGF receptor activity in tumor masses.

The tyrosine kinase activity of both α- and β-PDGF receptors was significantly suppressed by imatinib injection at 09:00 h, whereas receptor activity was not significantly inhibited by drug injection at 21:00 h. The dosing time-dependent change in the inhibitory action of imatinib seemed to be dependent on the rhythmic variation in the tyrosine kinase activity of PDGF receptors. As PDGF receptors are involved in multiple tumor-associated processes including the autocrine growth stimulation of tumor cells, induction of tumor angiogenesis, and recruitment of tumor fibroblasts [22], the doing time-dependency of imatinib-induced inhibition of PDGF receptor activity may contribute to that of its anti-tumor effect. This notion is also supported by our findings that inhibitory effect of imatinib on ERK/MAPK and PI3K/Akt signaling varied according to its administration time. The PDGF signaling cascade involves both ERK/MAP kinase and PI3K/Akt pathways [20,23]. The inhibition of ERK and Akt phosphorylation by their specific inhibitors (PD9805913 and LY2940021, respectively) induces the low proliferation of fibrosarcoma and endothelial cells [24–27]. In fact, treatment of cultured sarcoma 180 cells with imatinib for 48 h resulted in a significant dose-dependent decrease in their proliferation, accompanied by the inhibition of phosphorylation of PDGF

receptors as well as that of ERK and Akt (Supplemental data 3). Taken together, the dosing time-dependency of the imatinib-induced inhibition of ERK/MAPK and PI3K/Akt signaling seems to be involved in the mechanism of that of its anti-tumor efficacy.

Although dosing time-dependent changes in drug susceptibility are caused not only by the circadian-regulated sensitivity of living organisms to drugs but also by drug pharmacokinetics [28–30], there was no significant difference between imatinib concentration in tumor masses after drug injection at 09:00 and 21:00 h (Supplemental data 2); therefore, imatinib pharmacokinetics is unlikely to contribute to dosing time-dependent changes in the pharmacological action of the drug.

The findings in this animal model suggest a mechanism underlying the dosing time-dependent change in the anti-tumor effect of imatinib. Rhythmic change in the tyrosine kinase activity of PDGF receptors on tumor cells seems to influence anti-tumor effect of imatinib. Potent anti-tumor activity of imatinib could be expected by administering the drug at when the PDGF receptor function increased. Our results may provide a clue to select the most appropriate time of day for administration of PDGF receptor tyrosine kinase inhibitor.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bcp.2006.08.002.

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