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Received September 3, 2002; accepted October 25, 2002

ABSTRACT
Many drugs vary in potency and/or toxicity according to the time of day when they are administered. In this study, we investigated whether antitumor efficacy of angiogenesis inhibitor, TNP-470 [O-(chloroacetyl-carbamoyl) fumagillo], could be improved by optimizing the dosing schedule. Tumor-bearing mice were housed under standardized light/dark cycle conditions (lights on at 7:00 AM, off at 7:00 PM) with food and water ad libitum. The antitumor effect of TNP-470 (30 mg/kg s.c.) was more potent in mice injected with the drug at the early light phase than it was when administered at the early dark phase. The diurnal change in the antitumor effect of TNP-470 was parallel to that in its antiangiogenic activity. The variation in the effects of TNP-470 was closely related to the diurnal variations in its inhibitory action on methionine aminopeptidase activity in tumor masses. There was a significant dosing time-dependent change in the concentration of TNP-470 in plasma. The higher concentration of TNP-470 in plasma was observed when its antitumor and antiangiogenic activities were increased. These results suggest that therapeutic efficacy of TNP-470 can be enhanced by choosing the most appropriate time of day to administer the drug.

Angiogenesis, the formation of new vessels from pre-existing vessels, has been shown to be necessary for the progression and metastasis of malignant tumors (Folkman, 1971). Tumors require a supply of nutrients, oxygen, and various growth factors. In addition, they utilize the newly formed blood vessels as conduits to disseminate invasive tumor cells. Because the growth and metastasis of malignant tumors are dependent on angiogenesis, a novel anticancer treatment has been developed in which tumors are regressed by prolonged inhibition of angiogenesis. In comparison with conventional chemotherapy, antiangiogenic therapy has a number of clinical advantages, including low toxicity, lack of drug resistance, and easy access of the drugs to the targeted endothelial cells (Boehm et al., 1997). A variety of antiangiogenic agents are currently undergoing clinical trails for dormancy therapy of tumors.

TNP-470 (AGM-1470), a synthetic analog of fumagillin, has been shown to prevent angiogenesis by arresting the endothelial cell cycle (Hori et al., 1994), and it potently inhibits tumor growth and metastasis in a wide range of in vivo tumor models (Ingber et al., 1990; O’Reilly et al., 1995). TNP-470 arrests the endothelial cell cycle by inhibiting the activities of type II methionine aminopeptidase (MetAP-2), which appears to play an important role in cell cycle initiation (Griffith et al., 1997; Sin et al., 1997). Although TNP-470 has significant clinical advantages as a therapeutic agent for cancer treatment, its short serum half-life and dose-limiting side effects diminish the potency of this drug (Logothetis et al., 2001).

One approach to increase the efficacy of pharmacotherapy is to administer drugs at a time of day when they are most effective and/or best tolerated. Daily rhythmic variations in biological functions such as secretions of hormones and synthesis of proteins are thought to be important factors affecting the efficacy of drugs. Indeed, chronopharmacological

ABBREVIATIONS: TNP-470, O-(chloroacetyl-carbamoyl) fumagillo; MetAP-2, methionine aminopeptidase; Met-MCA, methionine 4-methyl coumaryl-7-amide; AMC, 7-amino-4-methylcoumarin; NONMEM, nonlinear mixed effect model; AUC, area under the plasma concentration-time curve; AGM-1883, fumagillo carbamate.
strategy can improve the effects of drugs and/or attenuate their toxicity (Koyanagi et al., 1997; Takane et al., 2000; Ohdo et al., 2001). Therefore, therapeutic efficacy of TNP-470 could be improved by optimizing the dosing schedule.

The purpose of this study was to investigate the influence of dosing time on antitumor and antiangiogenic activities of TNP-470 in mice. The mechanisms underlying the dosing time-dependent changes in the pharmacological action of TNP-470 was examined in terms of sensitivity of living organisms to the drug and its pharmacokinetics.

Materials and Methods

Materials. TNP-470 was provided as lyophilized powder by Takeda Chemical Industrial, Ltd. (Osaka, Japan). For treatment, the compound was suspended in a sterilized saline containing 3% ethanol and 5% arabic gum. The following materials were commercially obtained: methionine 4-methyl coumary-7-amide (Met-MCA) and 7-amino-4-methylcoumarin (AMC) from the Peptide Institute, Inc. (Osaka, Japan); and Dulbecco’s modified Eagle’s medium and fetal bovine serum from Invitrogen (Carlsbad, CA).

Animals and Cells. Male ICR mice (5 weeks old) were purchased from Charles River Japan, Inc. (Kanagawa, Japan). They were housed in a light-controlled room (light on from 7:00 AM to 7:00 PM) at a room temperature of 24 ± 1°C and a humidity of 60 ± 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 of the mice. Two murine tumor cell lines (sarcoma 180 and B16 melanoma) were purchased from Dai Nippon Seiyaku (Osaka, Japan). Lewis lung carcinoma cells were supplied by the Cell Resource Center for Bio-medical Research, Tohoku University (Sendai, Japan). These tumor cells were maintained in Dulbecco’s modified Eagle’s medium and fetal bovine serum from Invitrogen (Carlsbad, CA).

Influence of TNP-470 dosing time on tumor growth. Three types of mouse tumor cells (sarcoma 180, Lewis lung carcinoma, and B16 melanoma) were inoculated into ICR male mice. TNP-470 (30 mg/kg) (closed squares, 7:00 AM; closed triangles, 7:00 PM) or vehicle (open circles, 7:00 AM) were administered subcutaneously every 2 days before drug treatment.

Measuring the Effect of Dosing Time on Antitumor Action of TNP-470. Tumor-bearing mice were injected with a single daily dose of TNP-470 (30 mg/kg s.c.) or vehicle once every other day at 7:00 AM or 7:00 PM. This dosage of TNP-470 has been reported to exert antitumor activity in mice (Ingber et al., 1990). In all mice, tumor volumes were measured throughout the duration of the experiment. The tumor volume was estimated according to the following formula: tumor volume (mm³) = 4/3πxyz, where 2x, 2y, and 2z are the three perpendicular diameters of the tumor. The mice were also weighed throughout the experiment.

Measuring the Effect of Dosing Time on Antiangiogenic Activity of TNP-470. The tumor-induced neovascularization was assessed by the dorsal air sac method (Asano et al., 1995). Briefly, cultured sarcoma 180 cells (1 × 10⁵) were packed into a membrane chamber, and the chamber was implanted into the dorsal air sac of mice (day 0). The mice were injected with a single dose of TNP-470 (30 mg/kg s.c.) or vehicle once every other day at 7:00 AM or 7:00 PM. 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.

Determination of Methionine Aminopeptidase activity. To determine variations in methionine aminopeptidase activity, tumor (sarcoma 180) masses were removed from six tumor-bearing control mice at 9:00 AM, 1:00 PM, 5:00 PM, 9:00 PM, 1:00 AM, and 5:00 AM. The tumor masses were homogenized with 500 μl of 10 mM HEPES-KOH (pH 7.5)/50 mM KCl/3 mM Mg(Gae)C/0.3 mM EDTA/10% glycerol/0.01% NaN₃/0.5% Triton X-100/100 μM phenylmethylsulfonyl fluoride/7 mM 2-mercaptoethanol. After removal of insoluble materials by centrifugation at 12,000g for 10 min, the resulting supernatants were subjected to an assay for methionine aminopeptidase activity. The assay mixture contained the following components in a total volume of 1 ml: 10 mM Met-MCA, 10 μl of the supernatant, and 10 mM HEPES-KOH buffer (pH 7.5). Incubation followed at 37°C for 30 min, and enzyme reaction was terminated by adding 10 μl of 10 mM EDTA solution. The released AMC was measured fluorometrically on a Hitachi 204 Fluorophotometer with excitation at 380 nm and emission at 460 nm. One unit was defined as the enzyme activity releasing 1 nmol of AMC per minute at 37°C. To study the influence of dosing time on TNP-470’s effect on methionine aminopeptidase activity, tumor-bearing mice were injected with TNP-470 (30 mg/kg s.c.) or vehicle at 7:00 AM or 7:00 PM. Their tumor masses were
removed at 2 h after TNP-470 or vehicle injection, and methionine aminopeptidase activity was measured as described above.

**Determination of TNP-470 Concentration in Plasma.** Tumor (sarcoma 180)-bearing mice were injected with TNP-470 (30 mg/kg s.c.) at 7:00 AM or 7:00 PM. Blood samples were drawn by cardiac puncture at 10, 20, 30, 45, 60, and 90 min after TNP-470 injection. Plasma was separated by centrifugation (1200g) and stored at −70°C with 2% sulfuric acid to ensure the stability of TNP-470. The acidified plasma samples were deproteinized with 2 volumes of acetonitrile by vortexing for 30 s, followed by centrifugation at 5600g for 15 min at 4°C. The supernatant was placed into a screw cap tube and evaporated to dryness under a flux of nitrogen. The dried residue was dissolved in 100 µl of acetonitrile, and the solution was reacted for 15 min with 200 µl of 5 mM sodium 8-quinolinethiolate. The solution was filtered through a 0.45 µm membrane filter (Millipore Corporation, Boston, MA). The filtrate was placed into autosampler vials and maintained at 4°C until injection onto the high-performance liquid chromatography system described below.

The high-performance liquid chromatography system (Waters, Milford, MA) consisted of a model 600 solvent delivery system equipped with a model 474 autosampler compartment and a model 474 fluorescence detector. A 20-µl portion of the filtrate was injected into a Nova-pak C18 column (3.9 × 150-mm i.d.; Waters), maintained at 40°C. The derivatized TNP-470 was separated from by-products with 50% acetonitrile in water as the mobile phase. The flow rate was 1 ml/min, and the effluent was monitored fluorometrically at an excitation wavelength of 250 nm and emission wavelength of 428 nm.

**Nonlinear Mixed Effect Model analysis.** Nonlinear mixed effect model (NONMEM) is a computer program designed to analyze pharmacokinetics in study populations by pooling data (Beal and Sheiner, 1992). In this study, NONMEM was applied to the pharmacokinetic analysis of TNP-470 concentrations in plasma. The population pharmacokinetic parameters were calculated on an HT-9000 series 700 computer (Yokogawa-Hewlett Packard Ltd., Tokyo Japan) with the NONMEM program (version IV, level 1-1) following the two-compartment model with first-order absorption (the PREDPP program, subroutines ADVAN4 and TRANS4). Bayesian estimates of individual pharmacokinetic parameters were obtained by the NONMEM program's post hoc method. The statistical moment parameters such as area under the plasma concentration-time curve (AUC) were calculated by using the estimated individual pharmacokinetic parameters.

**Statistical Analysis.** The statistical significance of differences between groups was validated by the Bonferroni test for multiple comparisons and Student’s paired t test for comparison between two groups. The 5% level of probability was considered to be significant.

**Results**

**Influence of Dosing Time on Antitumor and Antiangiogenesis Activities of TNP-470.** Figure 1 shows the influence of dosing time on the ability of TNP-470 to inhibit tumor growth. Since no significant time-dependent difference was observed in the growth rate of tumor cells in mice treated with vehicle alone, the mean value of the tumor volume between 7:00 AM and 7:00 PM was shown as the control in Fig. 1. The growth of all three types of murine tumor cells was significantly suppressed by administration of TNP-470. However, the antitumor effect was more potent in mice injected with TNP-470 at 7:00 AM than in those that received the dose at 7:00 PM. Table 1 shows the influence of dosing time on the effect of TNP-470 on tumor growth rates. Twenty-one days after the start of the treatment, the growth rate of all three types of tumor cells (T/C) in mice injected at 7:00 AM was significantly smaller than that in mice injected at 19:00 (p < 0.01, respectively).

The photographs in Fig. 2 show the effect of dosing time on TNP-470’s ability 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, the neovascularization in mice given TNP-470 was clearly suppressed. The blood vessels in mice injected with the drug at 7:00 AM were virtually akin to those in the phosphate-buffered saline control mice.

**Influence of TNP-470 Dosing Time on Body Weight.** Changes in body weight over time during TNP-470 treatment are shown in Fig. 3. The body weight of mice injected with vehicle gradually increased during the experiment. Repeated administration of TNP-470 suppressed gains in body weight. However, the suppressive effect was more pronounced in mice injected with TNP-470 at 7:00 PM than at 7:00 AM.

**Influence of TNP-470 Dosing Time on Methionine Aminopeptidase Activity.** Under nondrugged states, methionine aminopeptidase activity in the tumor masses showed a significant diurnal variation, with higher levels from the late dark phase to the early light phase and lower levels from the late light phase to the early dark phase (Fig. 4A; p < 0.05). Methionine aminopeptidase activity at 2 h after TNP-470 injection (30 mg/kg s.c.) at 7:00 AM was significantly higher than it was after vehicle injection at 7:00 AM (Fig. 4B; p < 0.05). However, there was no significant difference between the enzyme’s activities in mice injected with the drug at 7:00 PM and vehicle injected at 7:00 PM.

**Influence of Dosing Time on TNP-470 Pharmacokinetics.** As shown in Fig. 5, plasma TNP-470 concentrations at 10 min after TNP-470 injection (30 mg/kg s.c.) at 7:00 AM were significantly higher than those at 10 min after the drug’s injection at 7:00 PM (p < 0.05). Table 2 shows the pharmacokinetic parameters after TNP-470 injection. The analysis of pharmacokinetics was conducted using 72 plasma concentrations obtained from 72 mice. The final model derived from all data are as follows: CL (l/h) = 0.265 × 1.35<sup>PT</sup>, V<sub>c</sub> (liters) = 0.190, V<sub>p</sub> (liters) = 7.610, Q (l/h) = 0.589, and k<sub>a</sub> (1/h) = 8.781, where CL is total body clearance, V<sub>c</sub> and V<sub>p</sub> are the apparent volumes of distribution in the central and pe-

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**TABLE 1**

<table>
<thead>
<tr>
<th>Tumor</th>
<th>Time of Drug Injection (Clock Hours)</th>
<th>Statistical Significance</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>7:00 (7:00 AM)</td>
<td>19:00 (7:00 PM)</td>
</tr>
<tr>
<td>Sarcoma 180</td>
<td>0.232 ± 0.031</td>
<td>0.524 ± 0.079</td>
</tr>
<tr>
<td>Lewis lung carcinoma</td>
<td>0.193 ± 0.045</td>
<td>0.562 ± 0.098</td>
</tr>
<tr>
<td>B16 melanoma</td>
<td>0.263 ± 0.041</td>
<td>0.627 ± 0.080</td>
</tr>
</tbody>
</table>

Influence of dosing time on tumor growth rates (T/C) after TNP-470 (30 mg/kg s.c.) injection at 7:00 AM or 7:00 PM

Values are the mean ± S.E. of 8 to 10 mice. Statistical significance between the two groups was evaluated by Student’s t test. T/C = tumor volume of treated/mean tumor volume of control.
ripheral compartments, $Q$ is intercompartmental clearance, and $k_a$ is absorption rate constant. DT represents dosing time: DT $= 0$ if injection of the drug was at 7:00 AM; DT $= 1$ if injection of the drug was at 7:00 PM. Using the population parameters, individual pharmacokinetic parameters were calculated based on Bayesian estimates, and then the AUC was derived from them. CL was significantly larger in mice injected with TNP-470 at 7:00 PM than at 7:00 AM ($p < 0.01$). In contrast, AUC was significantly smaller in mice injected with TNP-470 at 7:00 PM than at 7:00 AM ($p < 0.01$).

**Discussion**

The role of angiogenesis in tumor progression and invasiveness is well recognized, and antiangiogenesis is becoming accepted as an effective therapeutic approach to the treatment of cancers. TNP-470 is one of the first antiangiogenic agents to be tested clinically, making it a valuable prototype for future trials of antiangiogenic agents. In this study, we show that the growth of all three types of tumor cells (sarco-
Values are the mean ± S.E. of six mice. *, p < 0.05 when the two dosing times were evaluated by using Student’s t test.

The daily rhythmic variations in biological functions such as receptor sensitivity and enzymatic activity are thought to be a crucial factor influencing drug susceptibility. TNP-470 binds covalently to MetAP-2, leading to the inhibition of its enzymatic activity. The inhibitory action is highly specific for MetAP-2, compared with that for its closely related isozyme MetAP-1 (Lowther et al., 1998). Down-regulation of MetAP-2 expression by an antisense oligonucleotide leads to inhibition of endothelial cell proliferation (Wang et al., 1998), suggesting that MetAP-2 plays an important role in the growth of endothelial cells. Therefore, the inhibition of MetAP-2 activity is considered one of the underlying molecular mechanisms by which TNP-470 blocks angiogenesis. In this study, we evaluated methionine aminopeptidase activity and its inhibition by TNP-470 in tumor masses because it was very difficult to measure the enzyme activity only in endothelial cells of tumor masses. Under nondrugged states, methionine aminopeptidase activity in the tumor masses followed a diurnal rhythm: activity was higher from the late dark phase to the early light phase than it was from the late light phase to the early dark phase. The expression of MetAP-2 protein in the tumor masses also varied diurnally (data not shown). Diurnal variations in methionine aminopeptidase activity may vary directly as the amount of enzyme present.

Since methionine aminopeptidase activity varies diurnally in normal states, it is important to determine whether this variation affects the pharmacological efficacy of TNP-470. After TNP-470 injection at 7:00 AM, the methionine aminopeptidase activity in tumor masses decreased significantly compared with that after vehicle injection at 7:00 PM. This result coincided with the dosing time-dependent change in the antitumor and antiangiogenic activities of TNP-470; namely, the pharmacological activities were more potent in mice injected with TNP-470 at the time when methionine aminopeptidase activity increased. Therefore, the changes in antitumor and antiangiogenic effects of TNP-470 may be closely related to the diurnal rhythm of methionine aminopeptidase activity in tumor masses.

The diurnal changes in drug susceptibility may be caused not only by the diurnally regulated sensitivity of living organisms to drugs but also by drug pharmacokinetics (Koyanagi et al., 1997; Takane et al., 2000). TNP-470 rapidly disappeared from plasma of mice and was undetectable within 90 min after injection. However, peak concentrations were significantly higher after injection at 7:00 AM than they were after injection at 7:00 PM. Higher concentrations of TNP-470 coincided with increases in the drug’s antitumor and antiangiogenic activity. It is known that cytostatic inhibition of endothelial cell growth by TNP-470 can continue for several days, even after removal of the drug from the culture medium (Kusaka et al., 1994). Exposure to a higher concentration of TNP-470 is important for the duration of its inhibitory action on endothelial cell proliferation. Therefore, the dosing time-dependent difference in plasma TNP-470 concentration seems to contribute, at least in part, to that in antitumor and antiangiogenic effects of TNP-470. A significant dosing time-dependent difference was also demonstrated in the pharmacokinetic parameters of TNP-470, which showed higher CL for injection at 7:00 PM than at 7:00 AM. The variations in CL seem to be closely related to those in plasma TNP-470 concentrations. Assessment of the biotransformation of TNP-470 in human hepatocytes reveals that the drug is converted into the active metabolite, AGM-1883, and others (Cretton-Scott et al., 1996). All of the metabolites apparently have short elimination half-lives; they rapidly disappear from the plasma compartment (Placidi et al., 1995; Figg et al., 1997). The metabolism of TNP-470 is thought to be done by both microsomal epoxide hydrolase, which is a part of the mixed-function oxidase system, and esterase hydrolysis.

**TABLE 2**

Influence of dosing time on pharmacokinetic parameters after TNP-470 (30 mg/kg s.c.) injection at 7:00 AM or 7:00 PM

<table>
<thead>
<tr>
<th>Pharmacokinetic Parameter</th>
<th>Time of Drug Injection (Clock Hours)</th>
<th>Statistical Significance</th>
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<tbody>
<tr>
<td></td>
<td>7:00 (7:00 AM)</td>
<td>19:00 (7:00 PM)</td>
</tr>
<tr>
<td>CL (l/h/kg)</td>
<td>6.959 ± 0.069</td>
<td>9.407 ± 0.089</td>
</tr>
<tr>
<td>V. (l/kg)</td>
<td>4.794 ± 0.153</td>
<td>3.197 ± 0.031</td>
</tr>
<tr>
<td>AUC (µg/ml/h)</td>
<td>4.328 ± 0.042</td>
<td>3.197 ± 0.031</td>
</tr>
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N.S., not significant.
sis (Cretton-Scott et al., 1996). Furthermore, epoxides are subject to nonenzymatic rearrangement or direct excretion. There are significant diurnal rhythms in hepatic enzyme activity and renal function (Holeslaw et al., 1975; Halberg and Halberg, 1984; Cal et al., 1986). The diurnal variations in these physiological functions may be the mechanism underlying the diurnal variations in plasma TNP-470 concentration.

In clinical trials, TNP-470 sometimes causes adverse neurological effects such as anesthesia agitation and gait disturbance, and these adverse effects ultimately diminish the potency of this drug (Logothetis et al., 2001). Suppression of weight gain is often observed in experimental animals treated with effective doses of TNP-470 (Yanase et al., 1993; Wassberg et al., 1997; Katzenstein et al., 1999). It has been suggested that the suppressive effect may be in part secondary to adverse neurological effects which make it difficult for the animal to ingest food (Shusterman et al., 2001). In this study, the suppression of weight gain induced by TNP-470 was more serious in mice injected with the drug at 7:00 PM than in those that were injected at 7:00 AM. In general, nocturnal active rodents consume more than 80% of their food during the dark phase—if food is offered ad libitum (Damiola et al., 2000). Therefore, the administration of TNP-470 at the time when food is normally consumed may prevent the animal from eating normally.

The present study demonstrates that dosing time-dependent changes in antitumor and antiangiogenic effects of TNP-470 are closely related to the diurnal variations in methionine aminopeptidase activity and its pharmacokinetics. In addition, the adverse effect of TNP-470 may be attenuated by administering the drug at the time when it is best tolerated. These findings support the notion that choice of the most appropriate time of day for drug administration will be an aid to establish the rational chronotherapeutics of TNP-470 for dormancy therapy of tumors.

Acknowledgments
We are indebted to Takeda Chemical Industrial, Ltd. (Osaka, Japan) for providing TNP-470 used in this study.

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