

Metronomic S-1 Chemotherapy and Vandetanib: An Efficacious and Nontoxic Treatment for Hepatocellular Carcinoma¹

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Abstract

BACKGROUND: Metronomic chemotherapy involves frequent, regular administration of cytotoxic drugs at non-toxic doses, usually without prolonged breaks. We investigated the therapeutic efficacies of metronomic S-1, an oral 5-fluorouracil prodrug, and vandetanib, an epidermal growth factor receptor and vascular endothelial growth factor (VEGF) receptor tyrosine kinase inhibitor, in models of hepatocellular carcinoma (HCC). **METHODS:** We compared anti-HCC effects and toxicity in the six treatment groups: control (untreated), maximum tolerated dose (MTD) S-1, metronomic S-1, vandetanib, MTD S-1 with vandetanib, and metronomic S-1 with vandetanib. Tumor microvessel density (MVD) and tumor apoptosis were evaluated by immunohistochemistry. The expression of VEGF and thrombospondin-1, an endogenous inhibitor of angiogenesis, was analyzed by Western blot. **RESULTS:** Metronomic S-1 significantly inhibited tumor growth, which was enhanced by combination with vandetanib. With respect to toxicities, MTD S-1 caused severe body weight loss and myelosuppression, whereas metronomic S-1 did not cause any overt toxicities. Moreover, metronomic S-1 or metronomic S-1 with vandetanib prolonged survival, the latter treatment providing the greatest benefit. Metronomic S-1 and metronomic S-1 with vandetanib decreased MVDs and increased apoptosis in tumor tissues. The expression of VEGF in tumor tissues was upregulated by vandetanib and metronomic S-1 with vandetanib, whereas the expression of thrombospondin-1 was upregulated by metronomic S-1 and metronomic S-1 with vandetanib. **CONCLUSION:** Metronomic S-1 with an antiangiogenic agent seems to be an effective and safe therapeutic strategy for HCC.

Neoplasia (2011) 13, 187–197

Introduction

Hepatocellular carcinoma (HCC) is the fifth most common solid tumor and the third leading cause of cancer-related deaths globally [1]. Although prognosis of early and intermediate stage HCC has improved owing to advances in treatments, there are few proven effective systemic therapies for advanced HCC [2]. In particular, conventional chemotherapy using cytotoxic drugs for advanced HCC has not been shown to improve survival. Almost all cases of HCC occur in patients with chronic liver disorders, such as liver cirrhosis. Patients with liver cirrhosis have liver dysfunction and also pancytopenia. These pathologies

Abbreviations: HCC, hepatocellular carcinoma; MTD, maximum tolerated dose; MVD, microvessel density; VEGF, vascular endothelial growth factor; EGF, epidermal growth factor; TSP-1, thrombospondin-1; HUVEC, human umbilical vascular endothelial cell. Address all correspondence to: Hideki Iwamoto, MD, Division of Gastroenterology, Department of Medicine, Kurume University School of Medicine, 67 Asahi-Machi, Kurumeshi, Fukuoka-ken, 830-0011, Japan. E-mail: iwamoto_hideki@med.kurume-u.ac.jp

¹All authors agreed to the submission of this article, and there is no conflict to disclose. Received 18 August 2010; Revised 6 December 2010; Accepted 8 December 2010

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DOI 10.1593/neo.101186

limit the use of conventional chemotherapy as a treatment strategy for HCC.

Conventional chemotherapy often involves pulsatile administration schedules using maximum tolerated doses (MTDs) of cytotoxic drugs. The long break periods between therapies not only allow recovery from various toxicities, especially myelosuppression, but also provide an opportunity, unfortunately, for the drug-treated tumors to recover as well [3]. In contrast, metronomic chemotherapy is given at frequent intervals using minimally or nontoxic doses without prolonged breaks. In several pre-clinical studies, such metronomic protocols have shown surprisingly effective antitumor effects, despite the reduced toxicity [4–6].

S-1 is an orally novel cytotoxic 5-fluorouracil (5-FU) prodrug, which consists of tegafur and two biochemical modulators, 5-chloro-2,4-dihydropyridine and potassium oxonate [7]. 5-Chloro-2,4-dihydropyridine competitively inhibits dihydropyrimidine dehydrogenase approximately 180 times more effectively than uracil. Thus, S-1 gives rise to high concentrations of 5-FU in blood and tumor tissue for long-term periods since biochemical modulation [7,8]. A drug similar to S-1, namely, UFT, has been used successfully in metronomic preclinical studies [5]. Moreover, in the clinic it has been used successfully in randomized phase 3 trials in a metronomic fashion to treat in an adjuvant manner a variety of early stage cancers, after surgery, including non-small cell lung cancer [9] and breast cancer [10]. Because S-1 is thought to be more potent than UFT with respect to the effect of biochemical modulations, one might expect a stronger antitumor effect by using S-1 [7]. In this study, we describe a method of administering metronomic S-1 to treat HCC and compare it to conventional MTD S-1 chemotherapy, either alone or with an antiangiogenic drug.

Tyrosine kinase inhibitors such as sorafenib have proven activity in HCC patients and now represent one of the few effective systemic therapies for HCC [11]. Preclinical studies have also shown that the antitumor effect of metronomic chemotherapy can be significantly enhanced by combination with vascular endothelial growth factor (VEGF) pathway targeting agents [12,13]. In this study, we show here that metronomic S-1 might be a promising therapy to consider for concurrent daily combination with an oral antiangiogenic drug, in this case, vandetanib (ZD6474; AstraZeneca Pharmaceuticals, Macclesfield, UK). Vandetanib inhibits not only the catalytic function of VEGFR-2 but also EGF receptors (EGFRs), in contrast to sorafenib or sunitinib that do not affect EGFRs [14]. We evaluated the efficacies of vandetanib alone *in vivo* for HCC-bearing mice using various hepatoma cell lines that had different expressions of EGFR (submitted for publication). EGFR is known to contribute to 5-FU drug resistance, and 5-FU is the major metabolite of S-1 [15]. Therefore, there is a rationale for drug targeting of both EGF receptors and VEGF receptors along with metronomic chemotherapy, which was the purpose of this study. Thus, we investigated the efficacy of combining with each treatment schedule of S-1 and vandetanib using two HCC cell lines, which express low or high levels of EGFR, that is, KYN-2 and Huh-7, respectively. Overall, our results suggest that the combination treatment of metronomic S-1 plus vandetanib may be useful for the therapy of HCC.

Materials and Methods

Cell Lines and Culture

In human hepatoma cell lines, Huh-7 was originally purchased from CAMBREX Bio Science Walkersville, Inc (Walkersville, MD), and KYN-2 was provided by the Department of Pathology, Kurume Univer-

sity School of Medicine. Cells were maintained in Dulbecco modified Eagle medium (DMEM; Gibco Invitrogen Cell Culture Co, Auckland, New Zealand) supplemented with 10% fetal bovine serum (FBS).

Human umbilical vascular endothelial cells (HUVECs) were purchased from CAMBREX Bio Science Walkersville, Inc, and maintained with endothelial cell growth medium-2 (Clonetics, San Diego, CA) containing 5% FBS.

Animals and Drugs

Male 5-week-old nude mice (BALB/c *nu/nu*) were purchased from Kyudo KK (Fukuoka, Japan). All experiments were conducted in accordance with the National Institutes of Health guidelines for the Care and Use of Laboratory Animals.

5-FU was purchased from Kyowa Hakko Kogyo Co, Ltd (Tokyo, Japan). S-1 was provided by Taiho Pharmaceutical Co, Ltd (Tokyo, Japan). S-1 consists of a mixture of tegafur, gimeracil, and oteracil at molar ratio of 1:0.4:1 in 0.5% hydroxypropylmethylcellulose (HPMC) solutions. Vandetanib (ZD6474; Zactima) was provided by AstraZeneca Pharmaceuticals (Macclesfield, UK).

In Vitro Cell Proliferation Assay

As the tegafur component of S-1 is physiologically converted to 5-FU in the body, we evaluated the difference of antiproliferative effects *in vitro* of 5-FU using different schedules with both hepatoma cells and HUVECs. Approximately 1000 cells in 100 μ l of DMEM containing 10% FBS was added to each well of 96-well plate. After incubation for 24 hours, the medium was exchanged to the serum-containing medium with various concentrations of 5-FU (0, 1, 10, 100, 500, 1000, 10,000 ng/ml). Each cell line was exposed to 5-FU for 5 days. To evaluate the antiproliferative effect of “MTD” versus “metronomic” chemotherapy, exchange of the medium containing 5-FU was performed using different schedules. For the metronomic schedule, the medium containing 5-FU was exchanged daily as described previously [16]. For the MTD schedule, the medium containing 5-FU was not changed. After incubation, cell proliferation was evaluated by a tetrazolium-based assay (Cell Count Reagent SF; Nakalai Tesque, Inc, Kyoto, Japan).

Determination of the Optimal Dose for S-1 Using Metronomic Chemotherapy

We determined the optimal metronomic dose of S-1 according to a previous report, which involved evaluating different doses of a chemotherapy drug both for antitumor effects and toxicity, with the aim of determining a dose that has minimal toxicity but retains good efficacy [17]. A total of 5×10^6 Huh-7 cells were injected into the flank regions of nude mice. Therapy with different doses of S-1 was initiated when the estimated tumor volume ($0.52 \times \text{length} \times \text{width}^2$) reached 150 to 200 mm^3 . Mice received S-1 orally administered by gavage with the following agents on a daily basis for 14 days: 1) HPMC as the control group; 2) S-1, 7.5 mg/kg per day; 3) S-1, 5.0 mg/kg per day; 4) S-1, 2.5 mg/kg per day; or 5) S-1, 1.0 mg/kg per day. Tumor-bearing mice were randomly divided into groups of 10 mice. The mice were killed at day 15 after start of treatment. The inhibition rate of tumor growth (IR %) was calculated as follows: $\text{IR \%} = (1 - \text{mean RTV of treatment group} / \text{mean RTV of control group}) \times 100$, where RTV indicates the relative tumor volume: tumor volume on killing / tumor volume on initial treatment. For comparison of the toxicity in each group, mouse body weights were measured every 3 days. Peripheral leukocyte count and hemoglobin (Hb) concentrations of these mice were also measured at day 15.

Tumor Growth and Toxicity Assessment in the Subcutaneous Tumor Transplant Model

We selected as the optimal metronomic dosage for S-1, 5.0 mg/kg per day based on our aforementioned study. We selected the MTD for S-1 15 mg/kg per day for 7 days, followed by a 7-day break period, based on previous published findings [6]. To compare the antitumor effect and toxicity caused by MTD or metronomic S-1, long-term experiments were performed using the Huh-7 subcutaneous transplant model. Mice were randomly divided to six groups: 1) HPMC as the control group; 2) MTD S-1, 15 mg/kg per day p.o. for 1 week, followed by a 1-week break period for a cumulative dose of 95 mg/kg; 3) metronomic S-1, 5 mg/kg per day p.o. for 2 weeks without any break period for a cumulative dose of 70 mg/kg; 4) vandetanib 25 mg/kg per day p.o. for 2 weeks; 5) MTD S-1 with vandetanib; or 6) metronomic S-1 with vandetanib. Each group consisted of 10 mice. It is important to note that the cumulative metronomic doses were distinctly less than the cumulative MTD. In other words, whereas the schedule used was “dose-dense,” it was not “dose intense.” The aforementioned schedules were performed in two cycles, 4 weeks in total. Estimated tumor volumes were measured every 3 days, and all mice were killed after 4 weeks of treatment. For comparison of the toxicity in each group, mouse body weights were measured every 3 days. Peripheral leukocyte count and hemoglobin (Hb) concentrations in these mice were also measured at sacrifice.

Tumor Growth and Survival Assessment in the Orthotopic Transplant Model

We also examined tumor growth using an orthotopic liver transplant model. The mice were implanted with 2×10^6 KYN-2 cells into the left lobe liver. Mice were randomly divided into six groups, as outlined above, and therapy was initiated 7 days after implantation of tumor cells. Each group consisted of 10 mice. The mice were killed at day 29 of initial treatment, and tumor volumes were evaluated.

In addition, a survival study was also performed using the KYN-2 orthotopic transplant model for the six groups as mentioned above. Each group consisted of 10 mice. In the group for survival observation, animals were killed according to (pre)clinical signs of weakness, for example, anorexia, or greater than 20% weight loss, and days of life were recorded from initial treatment.

Immunohistochemical Staining of CD31, PCNA, and TUNEL

The sections of all tumor tissues obtained from KYN-2 orthotopic transplant model were boiled for 30 minutes by high pH target retrieval solution (DAKO Japan, Kyoto, Japan) for antigen retrieval. The sections were incubated with rabbit anti-human CD31 antibody (diluted 1:300; Abcam, Inc, Tokyo, Japan) and rabbit anti-human PCNA antibody (diluted 1:250; Abcam, Inc) at 4°C overnight. Then the avidin-biotin procedures were subsequently performed using a Vectastain ABC Kit (Vector Laboratories, Inc, Burlingame, CA). The sections were reacted with 0.005% H_2O_2 -3,3'-diaminobenzidine at room temperature for 1 minute. For quantification of microvessel density (MVD), CD31-positive vessels were counted in randomly selected 30 areas per five tumors in each treatment group at 200-fold magnification.

The terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) method was performed for the evaluation of apoptosis in each of the treated tumor tissues. TUNEL labeling was performed using the ApopTag Kit (Chemicon, Temecula, CA) according to the manufacturer's instructions. The stained sections of tumors of each group were reviewed, and the apoptosis index,

determined by TUNEL staining, was determined by counting at least 1000 cells in five randomly selected high-power fields (magnification, $\times 200$).

Expression of Thrombospondin-1 and VEGF in Tumor Tissues

We examined the expression of VEGF and thrombospondin-1 (TSP-1) in treated tumor tissues using Western blot analysis. TSP-1 is a known endogenous antiangiogenic protein [18]. Five samples of each treatment group and control group were loaded in equal conditions, respectively. Thirty micrograms of protein was loaded onto a NuPAGE 4% to 12% Bis-Tris gel (Invitrogen, CA). Membranes were incubated with rabbit anti-TSP-1 antibody (1:350 dilution; Abcam, Inc) or rabbit anti-VEGF antibody (1:500 dilution; Abcam, Inc) at 4°C overnight. Equal protein loading was assessed by mouse anti- β -actin antibody (1:1000 dilution; Sigma, St Louis, MO). After incubation with HRP-conjugated anti-rabbit immunoglobulin G (1:10,000 dilution; GE Healthcare UK Ltd, Buckinghamshire, UK) or HRP-conjugated anti-mouse immunoglobulin G antibody (1:5000 dilution; GE Healthcare UK Ltd) for 1 hour, immunoreactive bands were stained by an enhanced chemiluminescence Western blot analysis system using LAS 4000 mini (Fujifilm, Tokyo, Japan) and were calculated with the amount of luminescence in each sample using multigauge software (Fujifilm). The relative amount of luminescence in each treatment group for the control group was expressed as [(treatment group VEGF or TSP-1 / treatment group β -actin) / (control group VEGF or TSP-1 / control group β -actin)] and compared with each group.

Statistical Analysis

All experimental data were expressed as mean \pm SD. Differences between groups were examined for statistical significance using the Mann-Whitney *U* test, the Kruskal-Wallis test, and nonparametric analysis of variance. If the one-way analysis of variance was significant, differences between individual groups were estimated using the Fisher least significant difference test. Overall survival was estimated according to the Kaplan-Meier method and compared using the log-rank test. $P < .05$ was considered to be statistically significant.

Results

Comparison of Antiproliferative Effects of Metronomic versus MTD Type Chemotherapy In Vitro

The 50% inhibitory concentration (IC_{50}) levels of metronomic and MTD schedules of 5-FU, the major metabolite of S-1, for each cell line are shown in Table 1. The antiproliferative effects of 5-FU for each cell line were found to be dose-dependent (Figure 1, A–C). The IC_{50} levels for the MTD and metronomic schedule for Huh-7 cells were 3.84 and 0.77 μ M, respectively (Figure 1A). The IC_{50}

Table 1. IC_{50} Levels of MTD and Metronomic Schedule in Hepatoma Cell Lines and Endothelial Cell.

	5-FU IC_{50} (μ M)	
	MTD	Metronomic
Hepatoma cell lines		
Huh-7	3.84	0.77
KYN-2	7.69	3.84
Endothelial cell		
HUVECs	7.7	0.76

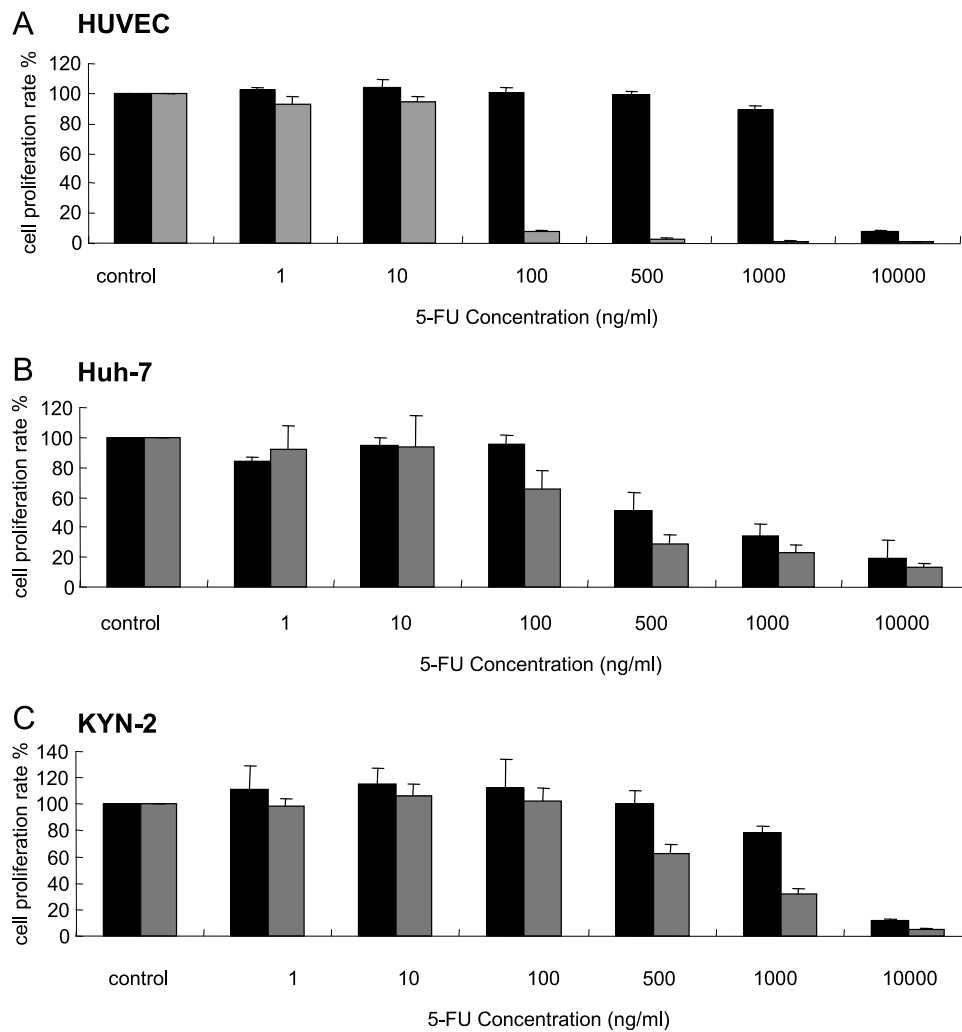


Figure 1. Inhibitory effect of metronomic chemotherapy for each cell line tested in a cell proliferation assay. To evaluate the antiproliferative effect of “MTD” and “metronomic” chemotherapy *in vitro*, exchange of the medium containing 5-FU was performed in different schedules. For the metronomic schedule, the medium containing 5-FU (0, 1, 10, 100, 500, 1000, and 10,000 ng/ml) was exchanged once a day. For the MTD schedule, the medium containing 5-FU was not changed. Data are shown as a ratio of the control and expressed as mean \pm SD of 10 samples. * $P < .001$ compared with each schedule. Dark gray-shaded columns show MTD schedule, and light gray-shaded columns showed metronomic schedule. (A) HUVEC. HUVEC was cultured with 100 μ l of endothelial cell growth medium-2 with 5% FBS containing 5-FU. (B) Huh-7. (C) KYN-2. Hepatoma cells were cultured with 100 μ l of DMEM with 10% FBS containing 5-FU.

levels for KYN-2 were 7.69 and 3.84 μ M, respectively. For the hepatoma cell lines, the metronomic schedule inhibited cell proliferation at approximately 1/2 to 1/4 concentrations of 5-FU compared with MTD schedule (Table 1). The metronomic schedule for HUVECs inhibited cell proliferation at apparently lower levels (IC₅₀ levels, 0.76 μ M) approximately 1/10 the concentration of 5-FU compared with MTD schedule (IC₅₀ levels, 7.7 μ M; Table 1).

Determination of the Optimal Dose of S-1 for Metronomic Chemotherapy In Vivo: Maximum Tumor Growth Inhibition with Minimal Toxicity

In the 7.5- and 5.0-mg/kg-per-day S-1 treatment groups, there were significant differences in suppression of tumor growth compared with the control group ($P < .05$; Figure 2A), and dosages lower than 2.5 mg/kg per day S-1 were not statistically effective compared

with the control group. In addition, we evaluated body weight loss and myelosuppression toxicities associated with administration of S-1 (Figure 2, B–D). With respect to body weight loss, there was no significant difference between each group (Figure 2B). But only the 7.5-mg/kg-per-day group showed severe toxicity as determined by reductions in Hb concentration and leukocyte count ($P < .001$, compared with the control group; Figure 2, C and D). Therefore, we selected 5.0 mg/kg per day as the optimal metronomic dosage of S-1, which was used in all subsequent experiments.

Evaluation of the Antitumor Effect and Toxicity for Metronomic S-1 Chemotherapy in the Subcutaneous Transplant Tumor Model

In the assay for tumor growth, statistical differences were observed between the control group and all treatment groups (Figure 3A).

Metronomic S-1 potently inhibited tumor growth compared with MTD S-1 ($P < .01$). The mean tumor volumes were $4810.5 \pm 1440.9 \text{ cm}^3$ in the control group, $3212.6 \pm 1364.7 \text{ cm}^3$ in the MTD S-1 group, $1927.1 \pm 652.9 \text{ cm}^3$ in the metronomic S-1 group, and $2331.4 \pm 662.1 \text{ cm}^3$ in the vandetanib group, respectively. The mean tumor volumes in the MTD S-1 plus vandetanib group and metronomic S-1 plus vandetanib group were 2026.7 ± 1106.7 and $1383.7 \pm 697.5 \text{ cm}^3$, respectively. The greatest inhibition of tumor growth was induced by the metronomic S-1 in combination with vandetanib (Figure 3A). In addition, we evaluated toxicity in each of Huh-7 subcutaneous tumor treatment groups (Figure 3, B–D). In leukocyte count, there were no significant differences in the groups (Figure 3B). In Hb concentration, the control group was $12.84 \pm 1.82 \text{ g/dl}$, the MTD S-1 group was $9.77 \pm 3.63 \text{ g/dl}$, the metronomic S-1 group was $11.73 \pm 3.27 \text{ g/dl}$, and the vandetanib group was $12.34 \pm 2.77 \text{ g/dl}$. For the combination treatments, the MTD S-1 plus vandetanib group was $8.24 \pm 1.64 \text{ g/dl}$, and for the metronomic S-1 plus vandetanib group, it was $11.74 \pm 1.55 \text{ g/dl}$ (Figure 3C). With respect to rate of body weight loss, in the MTD S-1 monotherapy and MTD S-1 with vandetanib groups, the values observed were $10.48\% \pm 6.85\%$ and $8.59\% \pm 5.02\%$ reduction compared with the control group, respectively. Vandetanib, metronomic S-1, and the combination therapy resulted in $5.64\% \pm 4.23\%$, $3.04\% \pm 2.23\%$, and $-0.51\% \pm 5.56\%$ reduction compared with the control group,

respectively (Figure 3D). Both the MTD S-1 and MTD S-1 plus vandetanib treatment groups experienced severe body weight loss and reduced Hb concentrations compared with the control group (Figure 3, C and D). In marked contrast, the metronomic S-1 monotherapy and metronomic S-1 with vandetanib groups did not manifest any overt toxicity (Figure 3, B–D).

Evaluation of Antitumor Efficacy Using Metronomic S-1 Chemotherapy in an Orthotopic Liver Transplant Model

For tumor volume assessments, all treatments except MTD S-1 monotherapy were effective compared with the control group (Figure 4A). Tumor volumes at sacrifice were $4186.0 \pm 1128.0 \text{ cm}^3$ in the control group, $3259.0 \pm 788.7 \text{ cm}^3$ in the MTD S-1 group, $1501.3 \pm 1002.2 \text{ cm}^3$ in the metronomic S-1 group, and $1582.0 \pm 354.9 \text{ cm}^3$ in the vandetanib group. There was a significant difference between metronomic S-1 and MTD S-1 in tumor growth inhibition ($P < .05$; Figure 4A). For the combination treatment groups, tumor volumes were $931.1 \pm 331.7 \text{ cm}^3$ in the MTD S-1 plus vandetanib group and $875.0 \pm 369.4 \text{ cm}^3$ in the metronomic S-1 plus vandetanib group. There was no significant difference between the metronomic S-1 plus vandetanib group and the MTD S-1 plus vandetanib group. However, the greatest inhibition of tumor growth was detected in the metronomic S-1 plus vandetanib treatment group ($P < .001$; Figure 4A).

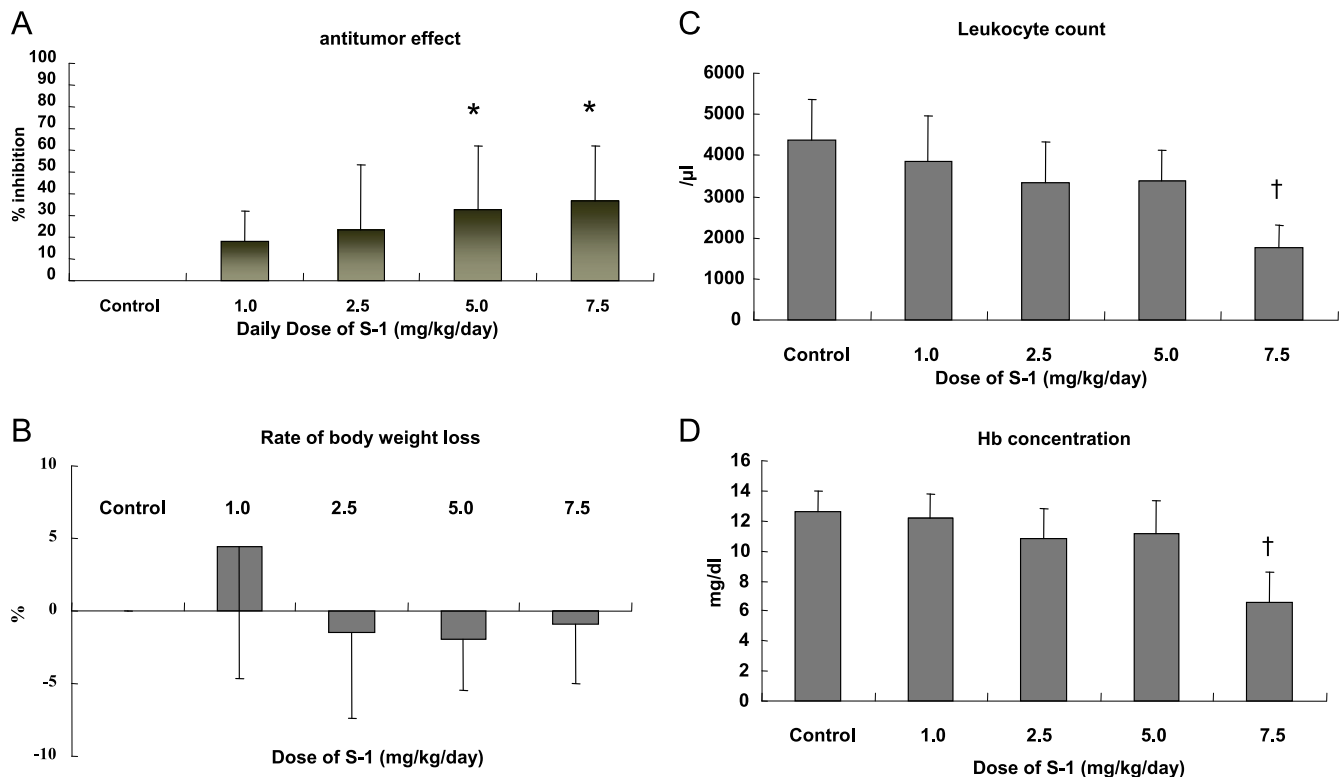


Figure 2. Determination of the optimal dose of S-1 in metronomic chemotherapy. Huh-7 subcutaneous tumor models were treated daily with either HPMC or different metronomic doses of S-1 (1.0, 2.5, 5.0, or 7.5 mg/kg per day) for 14 consecutive days. (A) Inhibition rates of tumor volumes (%) are expressed as mean \pm SD ($n = 10$ per group). Dosages of 5.0 and 7.5 mg/kg per day S-1 groups statistically inhibited tumor growth compared with the control group (* $P < .05$). (B–D) Toxicity parameters are represented as mean \pm SD. (B) Body weight (BW) changes on killing were calculated according to the following formula: BW change (%) = [(BW on sacrifice) – (BW on day 0)] \times 100. (C) Hb concentration. (D) Leukocyte count. Each different dose of S-1 did not show body weight loss. However, the only 7.5-mg/kg-per-day S-1 group represented severe myelosuppression, such as decreased Hb concentration or leukocyte count. † $P < .001$ by compared with the control group.

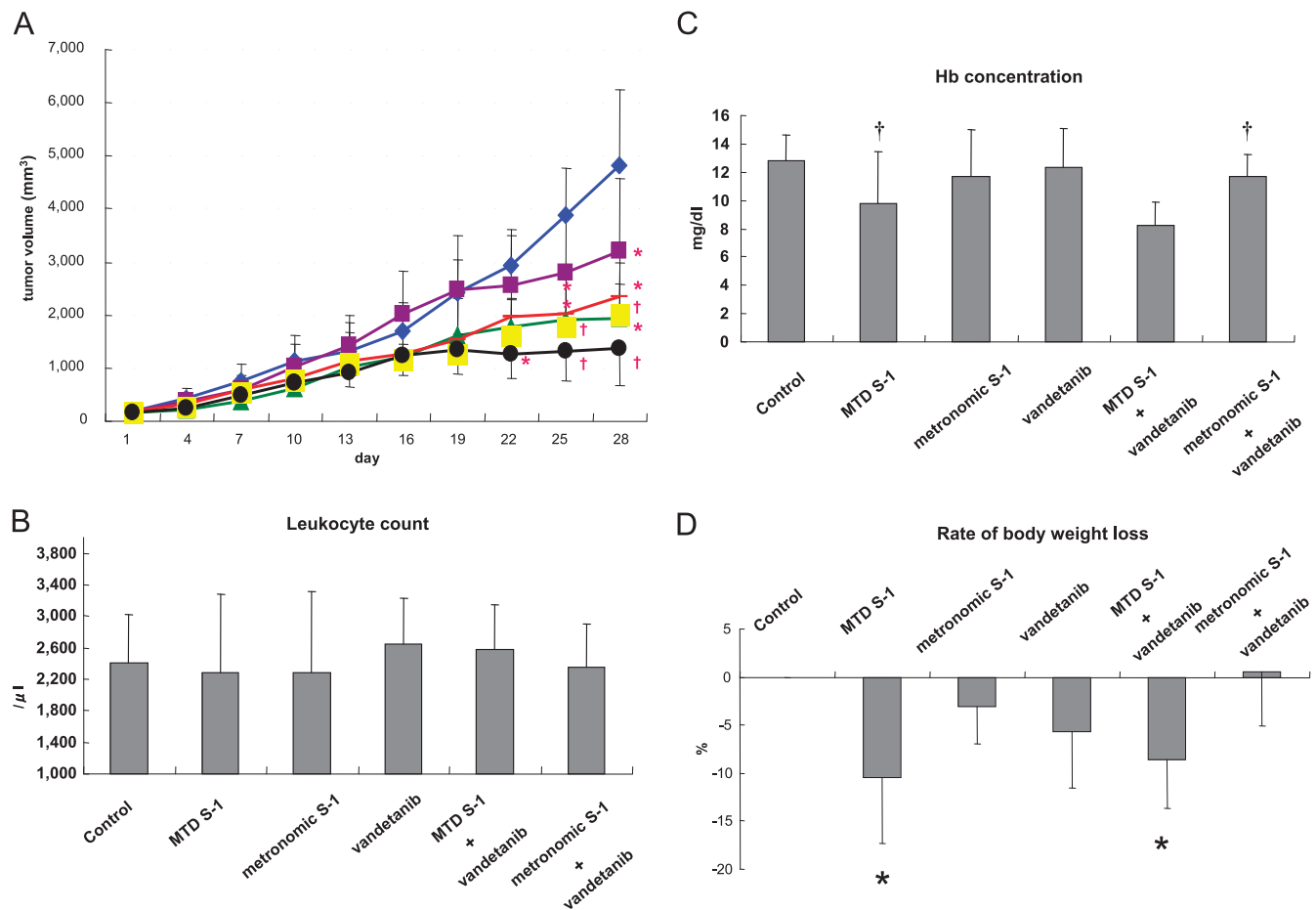


Figure 3. Therapeutic effects of metronomic S-1 chemotherapy in the Huh-7 subcutaneous tumor transplant model. (A) Tumor-bearing nude mice ($n = 10$ per group) were treated in the following six groups: 1) HPMC as the control group (blue); 2) MTD S-1 15 mg/kg per day for 1 week, followed by a 1-week break period (purple); 3) metronomic S-1 5 mg/kg per day for 2 weeks without break period (green); 4) vandetanib 25 mg/kg per day for 2 weeks (red); 5) MTD S-1 with vandetanib (yellow); or 6) metronomic S-1 with vandetanib (black). All treatments were performed for 4 weeks in total. Tumor volume changes are expressed as mean \pm SD. All treatments showed efficacy compared with the control group ($*P < .05$), and the metronomic S-1 therapy was more effective than the MTD S-1 treatment. The metronomic S-1 with vandetanib significantly inhibited tumor growth compared with the control group ($^{\dagger}P < .001$). (B–D) Toxicity parameters are expressed as mean \pm SD. (B) Hb concentration. (C) Leukocyte count. (D) Rate of body weight loss. MTD S-1 and the MTD S-1 with vandetanib showed severe body weight loss ($*P < .01$) and decreased Hb concentration ($^{\dagger}P < .05$) compared with the control group. Metronomic S-1 and the metronomic S-1 with vandetanib did not show any overt toxicities.

Evaluation of Survival Using Metronomic S-1 Chemotherapy in an Orthotopic Liver Transplant Model

The mean survival time in the control group was 28.9 ± 6.4 days. MTD S-1 did not prolong survival (mean survival time, 29.6 ± 3.9 days). In contrast, metronomic S-1 significantly prolonged survival (mean survival time, 34.3 ± 4.8 days). The mean survival time in the vandetanib group was 33.6 ± 5.0 days. MTD S-1 plus vandetanib treatment did not prolong survival times compared with vandetanib monotherapy (mean survival time, 37.6 ± 5.5 days). However, the metronomic S-1 plus vandetanib group provided the greatest prolonged survival times among all the treatment groups (mean survival time, 49.6 ± 11.5 days; Figure 4B).

Effect of Metronomic S-1 Chemotherapy Alone and in Combination with Vandetanib on Parameters of Tumor Angiogenesis

The results in Figure 5 show the MVD count in each treatment group. There was no significant difference in the MVD count be-

tween the control and the MTD S-1 group (control 41.1 ± 9.2 , MTD S-1 35.8 ± 5.5 ; Figure 5B). However, tumor MVD was decreased in the metronomic S-1 group (17.2 ± 4.1) compared with the control group ($P < .001$) and the MTD S-1 group ($P < .001$; Figure 5B). Tumor MVD in mice treated with vandetanib was 13.7 ± 5.1 . In the MTD S-1 plus vandetanib group, the MVD count was 18.8 ± 7.4 . Metronomic S-1 plus vandetanib group showed the greatest reduction of tumor MVD ($P < .01$ compared with MTD S-1 plus vandetanib group, 8.2 ± 1.6 ; Figure 5B).

Detection of Proliferation and Apoptotic Cells in Tumor Tissues

To further investigate the mechanism of the observed antitumor effect, we examined the effect of metronomic S-1 and in combination with vandetanib on tumor cell proliferation and apoptosis (Figure 5). With respect to tumor cell proliferation, there were no differences between the control and all treated groups. The mean

number of apoptotic tumor cells (apoptotic index) measured in the control group was 6.2 ± 2.6 . The MTD S-1 group did not show any significant difference (6.1 ± 4.9). However, the metronomic S-1 and vandetanib groups showed a significant increase in the apoptosis index (26.0 ± 5.4 and 18.4 ± 8.8 , respectively, $P < .0001$). A significant increase in the tumor cell apoptosis index was also observed in the metronomic S-1 plus vandetanib group with 42 ± 3.5 ($P < .0001$).

Expression of VEGF and TSP-1 in Tumor Tissues

The results in Figure 6 show the expression of TSP-1 and VEGF in treated tumor tissues. The expression level of TSP-1 was significantly upregulated by approximately two- to three-fold in both the metronomic S-1 and the metronomic S-1 plus vandetanib treatment groups ($P < .05$ compared with the control group; Figure 6, A and B). With respect to expression levels of VEGF, there were no differ-

ences between the control and the MTD S-1 and metronomic S-1 groups (Figure 6, C and D). In contrast, the vandetanib and the metronomic S-1 plus vandetanib groups showed significantly upregulated the VEGF expression compared with the control group ($P < .05$; Figure 6, C and D). There was a significant difference between the vandetanib monotherapy group and the metronomic S-1 plus vandetanib treatment group ($P = .045$).

Discussion

Our results add to an expanding body of literature reporting the therapeutic benefit of metronomic chemotherapy, especially when it is combined concurrently with a targeted antiangiogenic drug [5,12,13]. Moreover, to our knowledge, this is the first preclinical report of using S-1 in a metronomic dosing and administration schedule for HCC preclinical model. Also noteworthy is that we undertook a comparative

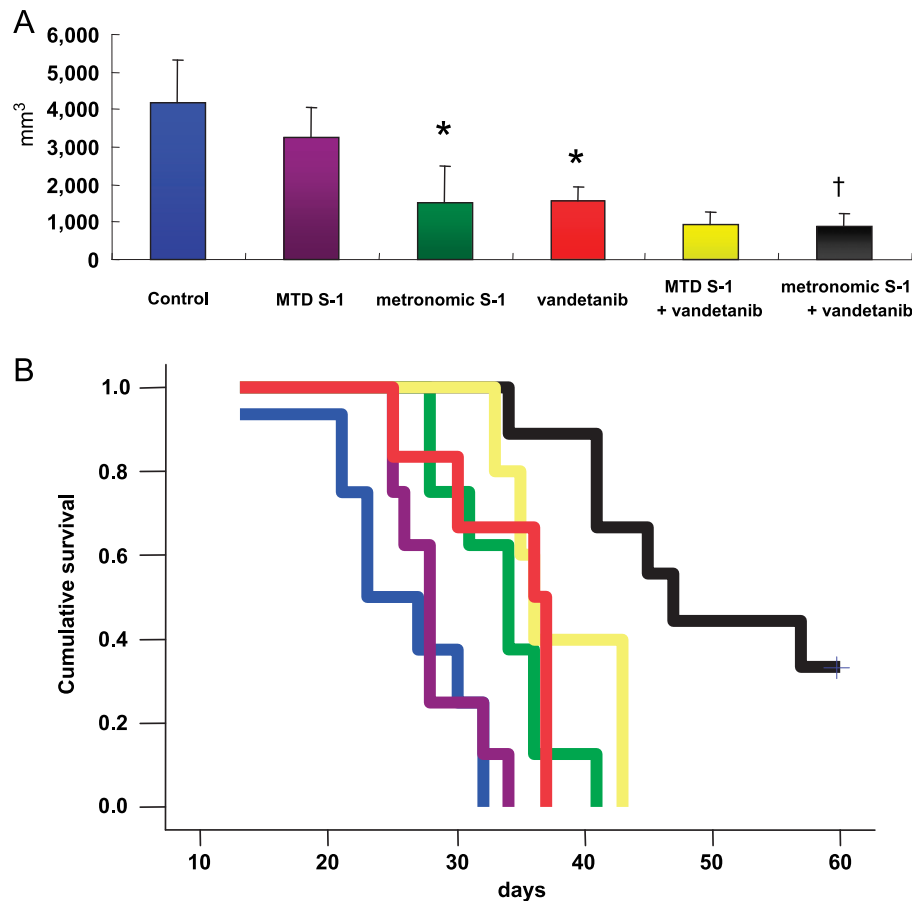
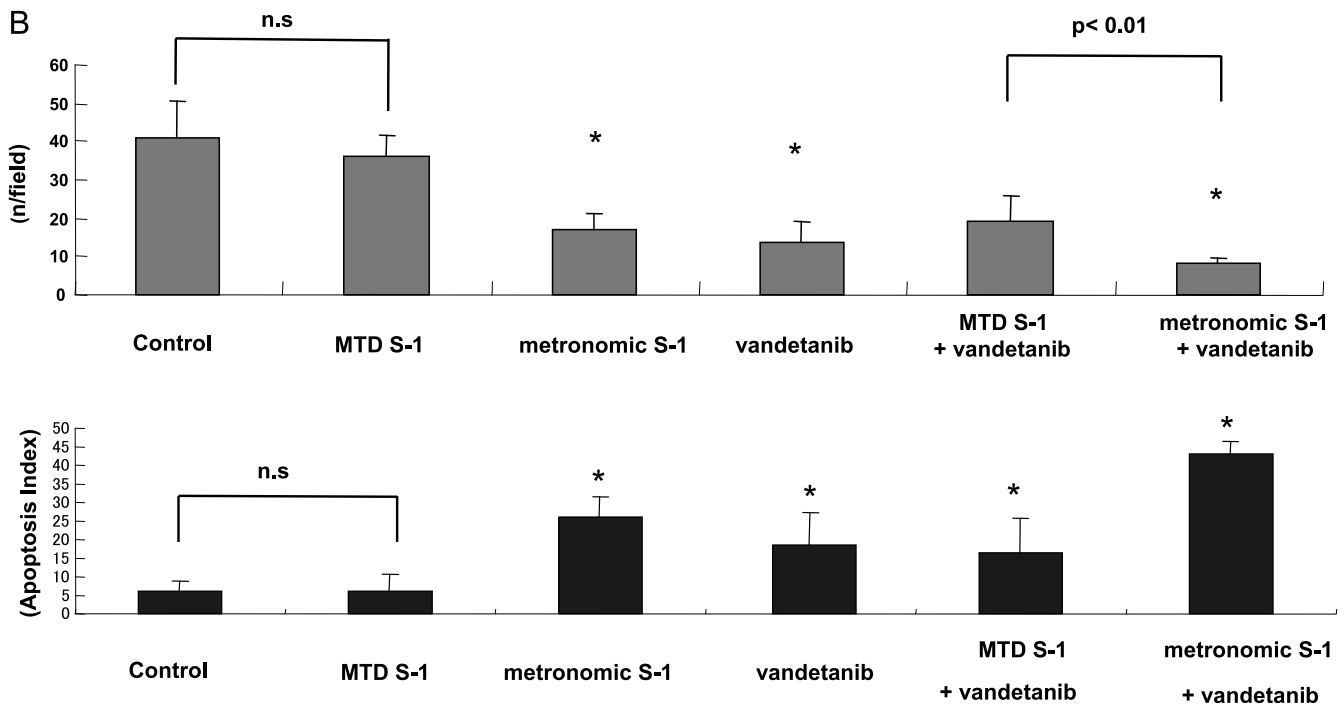
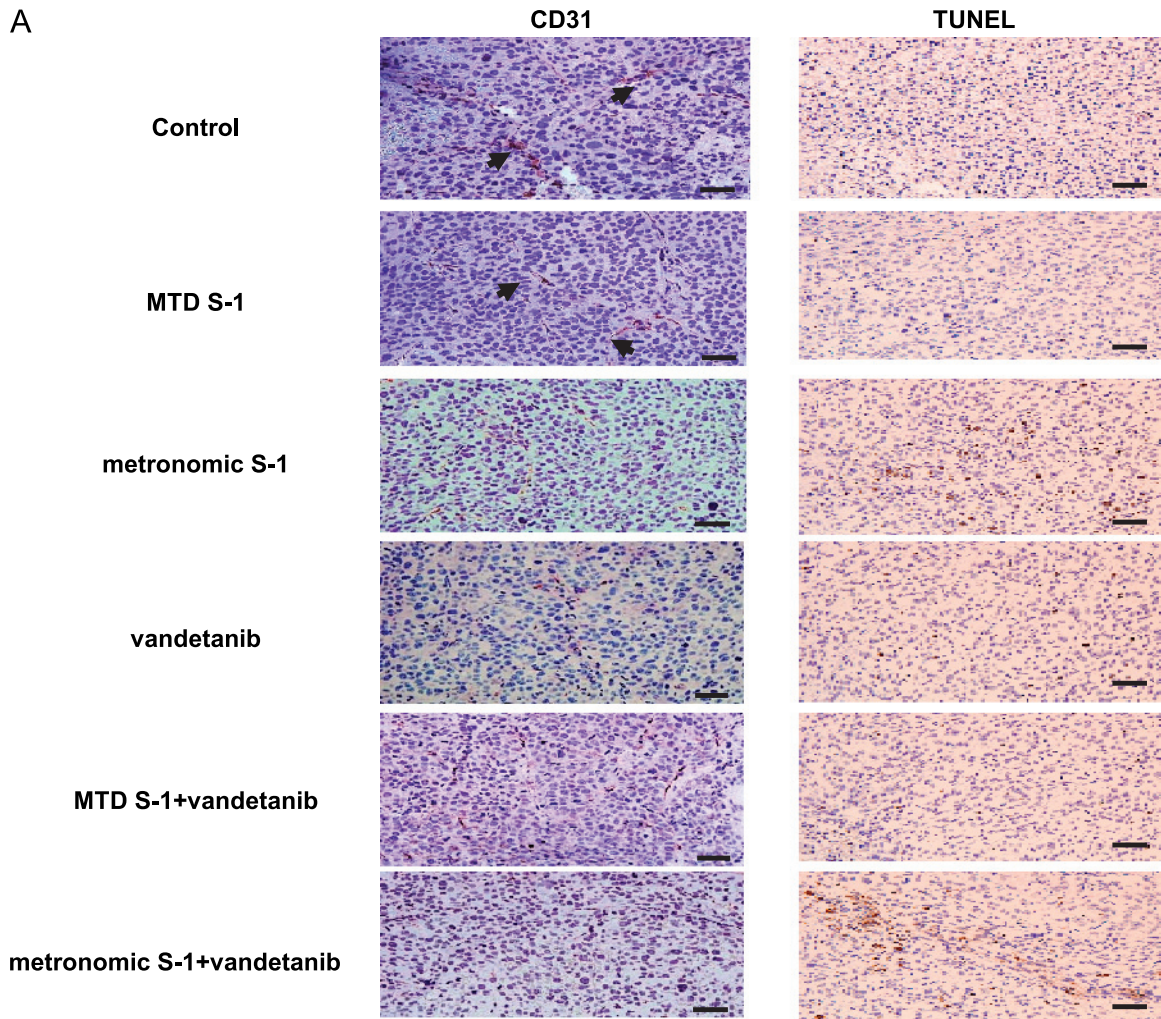


Figure 4. Assessment of therapeutic effects in KYN-2 liver transplant model. Tumor-bearing nude mice were treated in the following six groups: 1) HPMC as the control group (blue); 2) MTD S-1: 15 mg/kg per day for 1 week, followed by 1 week break period (purple); 3) metronomic S-1: 5 mg/kg per day for 2 weeks without break period (green); 4) vandetanib 25 mg/kg per day for 2 weeks (red); 5) MTD S-1 with vandetanib (yellow); or 6) metronomic S-1 with vandetanib (black). (A) Inhibition of tumor growth for KYN-2 liver transplant model. All treatments were performed 4 weeks in total. There was no significant difference between the control and the MTD S-1 groups. The metronomic S-1 group contributed to obvious inhibitory effect of tumor growth ($*P < .05$ compared with the control and the MTD S-1 groups). The metronomic S-1 with vandetanib treatment group showed the greatest inhibitory effect of tumor growth among all the groups ($†P < .001$). (B) Survival of mice treated with MTD S-1 or metronomic S-1 and in combination with vandetanib ($n = 10$ per group). Treatment was continued until mice were moribund, and days of life were recorded. Survival data were compared for significance with the log-rank test. MTD S-1 did not prolong survival compared with the control group. In contrast, metronomic S-1 prolonged survival compared with the control and MTD S-1 groups. The metronomic S-1 with vandetanib group provided the most effective therapy with longest survival times among all the groups ($P < .001$).



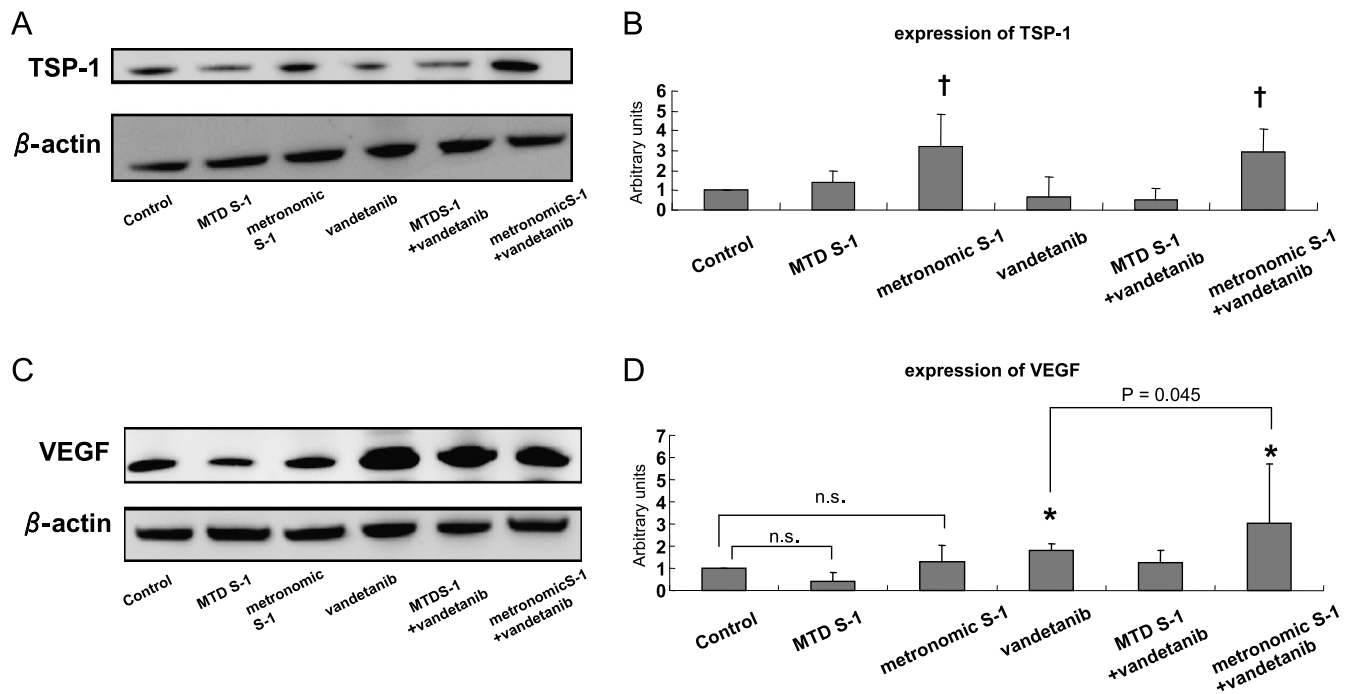


Figure 6. Western blot analysis of TSP-1 and VEGF. The band intensities of both TSP-1 and VEGF in treatment groups were measured and calibrated with each protein in control group and β -actin. (A and B) The metronomic S-1 and metronomic S-1 with vandetanib groups showed strongly upregulated the expression of TSP-1 ($^{\dagger}P < .001$ compared with the control group). (C and D) The expression of tumor VEGF was increased by the vandetanib and the metronomic S-1 with vandetanib groups. There were no differences between the control and the MTD S-1, metronomic S-1 group ($*P < .05$ compared with the control group). There was a significant difference between the vandetanib and metronomic S-1 with vandetanib group ($P = .045$).

analysis of the effects of S-1 given in a more conventional MTD schedule with metronomic S-1, and our results consistently showed the metronomic dosing/schedule was superior to the MTD protocol, both in terms of increased antitumor efficacy and reduced toxicity. Importantly, in this regard, the metronomic protocol we used involved a cumulative dose over time that was 30% less than the corresponding MTD protocol. Below we discuss a number of different aspects of our results and some of the translational/clinical implications.

Antiangiogenic Effects Mediated by Metronomic S-1 Chemotherapy

Previous studies during the last decade have indicated that metronomic chemotherapy regimens using cytotoxic agents inhibit tumor growth by various mechanisms, namely, antiangiogenic effects, direct tumor cell targeting effects, or anticancer immune responses [4,19,20]. Our results with metronomic S-1 would seem to confirm the anti-

angiogenic effect findings. First, we found that exposure of 5-FU in a metronomic-type protocol *in vitro* brought about a greater antiproliferative effect at distinctly low concentrations not only of 5-FU on two different tumor cell lines but also, especially, HUVECs, compared with an MTD-like exposure. This is similar to the results of other studies such as that of Bocci et al. [16] using paclitaxel or the active metabolite of cyclophosphamide. Second, we found reduced MVD and increased number of apoptotic tumor cells in mice treated with the metronomic S-1 schedule but not the MTD protocol. Third, we observed an increased expression of TSP-1, which has been reported previously using other cytotoxic drugs administered in a metronomic fashion *in vivo*, including cyclophosphamide [21]. Fourth, we noted that a tumor cell line (KYN-2) that is intrinsically resistant *in vitro* to high concentrations of 5-FU—the major metabolite of S-1—nevertheless responds to metronomic S-1 *in vivo* but not to MTD S-1, suggesting that a target other than the tumor cell population *per se* is likely involved in the *in vivo*

Figure 5. MVD and apoptosis in tumors tissues. The sections of tumors from the KYN-2 liver transplant model were stained by anti-CD31 antibody and Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end labeling (TUNEL). Original magnification, $\times 200$. The density of CD31-positive vessels (arrow) and TUNEL in a tumor field are represented as mean \pm SD ($n = 30$ per group). (A) Representative sections for each treatment are shown. Bar, 10 μ m. (B) There was no significant difference in MVD between the control and the MTD S-1 groups. Tumor vessel numbers were reduced by metronomic S-1. The metronomic S-1 with vandetanib group showed the most inhibitory effect of tumor vessel count among all the groups ($*P < .001$ compared with the control group and the MTD S-1 group). The MTD S-1 group did not show any significant difference in the number of tumor cell apoptosis index (6.1 ± 4.9). However, the metronomic S-1 and vandetanib groups significantly increased in the number of apoptosis index, respectively (26.0 ± 5.4 and 18.4 ± 8.8 , $P < .0001$). A significant increase of tumor cell apoptosis index was also observed in the metronomic S-1 with vandetanib group with 42 ± 3.5 ($P < .0001$).

antitumor activity that was observed using metronomic S-1. Fifth, O'Reilly et al. [22] have reported that the antiangiogenic effect mediated by endogenous antiangiogenic factors induces increased apoptosis of tumor cells, likely a secondary effect due to decreased MVD, whereas proliferation of tumor cells was not affected. Similarly, tumor apoptotic cell numbers were increased, whereas proliferation of tumor cells was not inhibited by metronomic S-1 chemotherapy in our study. On the basis of all of the aforementioned data and information, the antitumor effect of metronomic S-1 chemotherapy was likely to be mainly through antiangiogenesis mediated by inhibiting the proliferation of endothelial cells and inducing the expression of TSP-1, although some additional mechanisms cannot be entirely excluded. The mechanism of antiangiogenesis of metronomic S-1 chemotherapy is thought to be quite different from that of vandetanib. Inhibiting VEGFR by vandetanib resulted in increased VEGF production in tumor tissues, paradoxically, whereas metronomic S-1 chemotherapy did not increase VEGF production. Ebos et al. [23] reported that this difference of production of VEGFs influenced to achieving malignant potential of cancer cells. Also, at this point, metronomic chemotherapy is thought to be a promising strategy of long-term treatment of cancer.

Translational/Clinical Implications of the Metronomic S-1 + Vandetanib Preclinical Results

There are several potentially important implications of our results with respect to how they might conceivably be exploited for the future treatment and management of HCC patients. It is well known that there are no effective chemotherapy regimens for the treatment of advanced HCC using conventional chemotherapy regimens. One reason for this is the frequent underlying liver dysfunction [2]. As a consequence, using MTD given in conventional schedules is often contraindicated because of possible excessive toxicity. However, chemotherapy drugs given in a metronomic, less toxic fashion may be an alternative strategy to circumvent this problem. In this regard, there is conflicting evidence regarding the clinical benefit of metronomic UFT, another 5-FU prodrug, at least in the postoperative adjuvant use for HCC [24]. However, some aspects regarding the negative clinical findings should be taken into consideration. One is the dosing. The daily dose used in the aforementioned adjuvant study was less than the dose used for a positive phase 3 adjuvant UFT clinical trial for non-small cell lung cancer patients [9]. The second is the benefit that might be gained by using an antiangiogenic drug in combination with metronomic UFT. For example, a recent report by Tang et al. showed that neither metronomic UFT nor antiangiogenic drug therapy alone had overt antitumor activity in a model of locally advanced HCC, whereas these drugs when combined showed significant antitumor activity [25]. Also, in our study, combining with vandetanib resulted in enhanced antitumor effects for S-1 chemotherapy; nevertheless, MTD S-1 monotherapy did not show any effective antitumor effects. VEGFR is related to chemoresistance for tumor endothelial cells through surviving [26]. Inhibiting VEGFR by vandetanib might have contributed to enhanced chemosensitivity for tumor endothelial cells. And EGFR is associated with resistance to 5-FU [15]. Inhibiting EGFR by vandetanib might have enhanced chemosensitivity to 5-FU. In addition, it is notable in our study that not only the combination with vandetanib but also metronomic S-1 monotherapy showed significant antitumor effects. Because S-1 may be superior to UFT in antitumor effect by virtue of its biochemical modulators [7], S-1 might be an even more suitable agent for metronomic chemotherapy.

In summary, we have demonstrated preclinically that metronomic S-1 chemotherapy showed effective therapeutic outcomes without overt toxicity for treatment of HCC, mainly by suppressing tumor angiogenesis, and the activity of which is amplified by concurrent combination with vandetanib. Metronomic S-1 and the concurrent combination treatment with an antiangiogenic agent might be a promising treatment strategy for HCC.

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