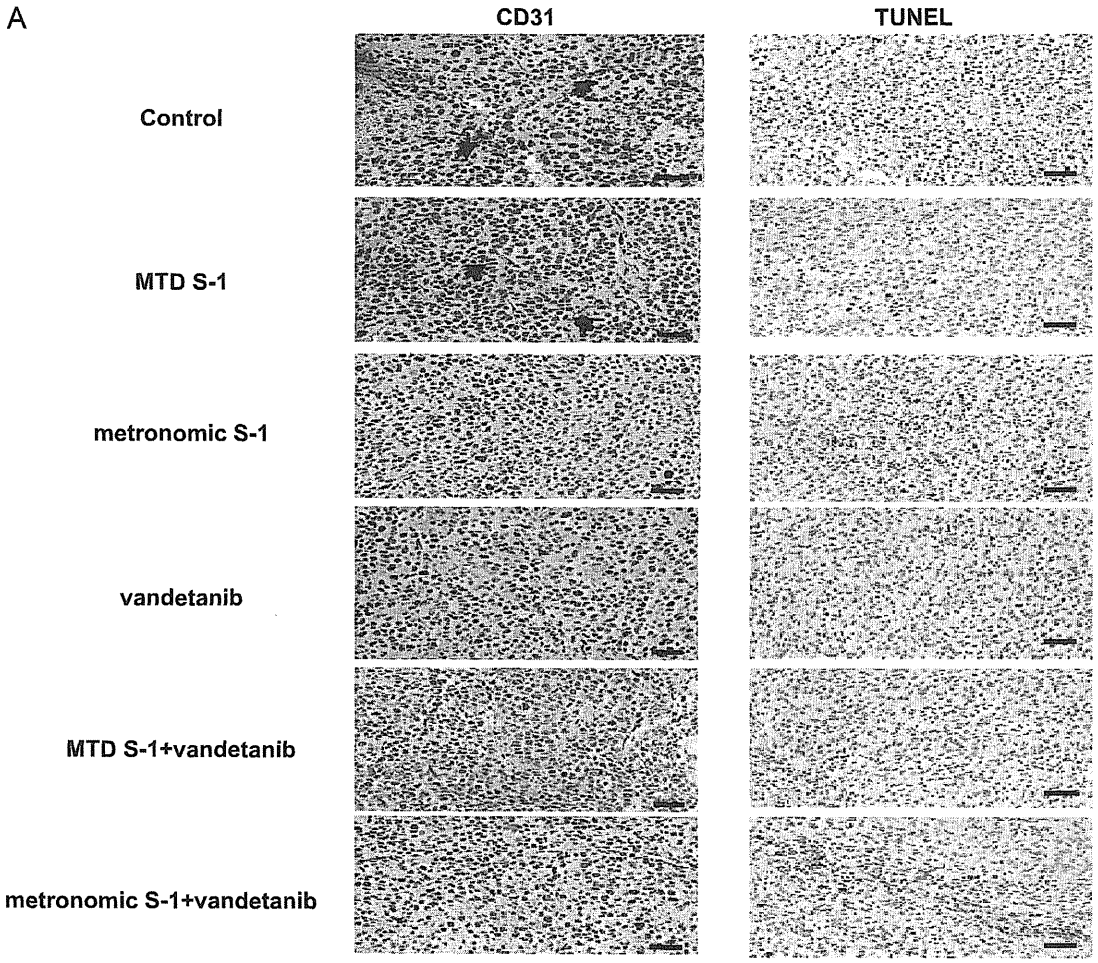
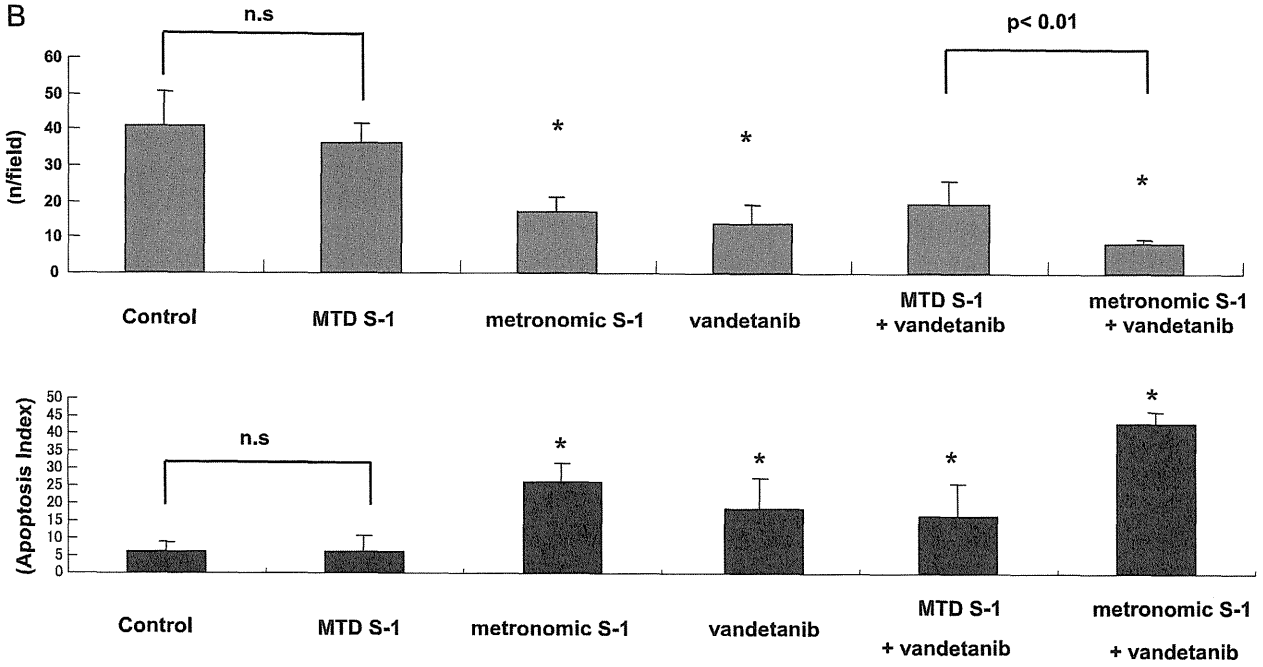

A
CD31
TUNEL
Control
MTD S-1
metronomic S-1
vandetanib
MTD S-1+vandetanib
metronomic S-1+vandetanib


B
n.s
p< 0.01
(n/field)
60
50
40
30
20
10
0
*
Control
MTD S-1
metronomic S-1
vandetanib
MTD S-1
+ vandetanib
metronomic S-1
+ vandetanib
(Apoptosis Index)
50
45
40
35
30
25
20
15
10
5
0
n.s
*
Control
MTD S-1
metronomic S-1
vandetanib
MTD S-1
+ vandetanib
metronomic S-1
+ vandetanib

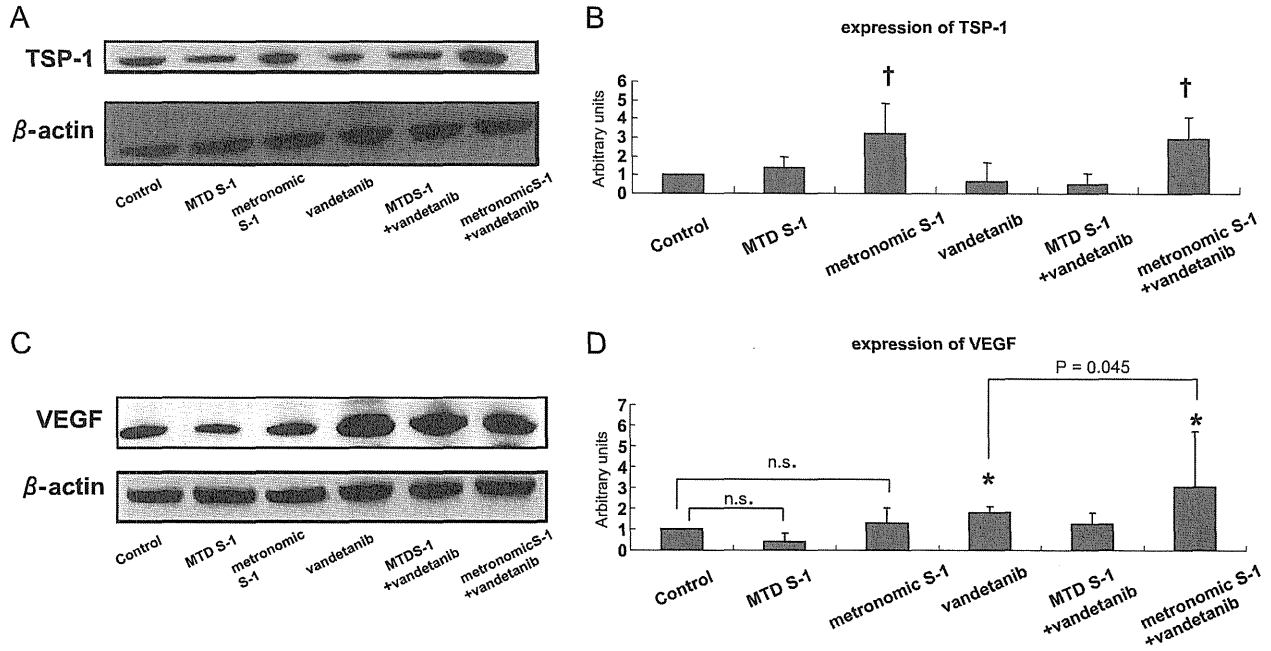


Figure 6. Western blot analysis of TSP-1 and VEGF. The band intensities of both TSP-1 and VEGF in treatment groups were measured and calibrated with each protein in control group and β-actin. (A and B) The metronomic S-1 and metronomic S-1 with vandetanib groups showed strongly upregulated the expression 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 antiangiogenic 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, ×200. The density of CD31-positive vessels (arrow) and TUNEL in a tumor field are represented as mean ± SD ($n = 30$ per group). (A) Representative sections for each treatment are shown. Bar, 10 μm. (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.

References

[1] Parkin D, Bray F, Ferlay J, and Pisani P (2001). Estimating the world cancer burden Globocan 2000. *Int J Cancer* **94**, 153–156.

[2] Llovet J, Di Bisceglie A, Bruix J, Kramer B, Lencioni R, Zhu A, Sherman M, Schwartz M, Lotze M, Talwalkar J, et al. (2008). Design and endpoints of clinical trials in hepatocellular carcinoma. *J Natl Cancer Inst* **100**, 698–711.

[3] Kim JJ and Tannock IF (2005). Repopulation of cancer cells during therapy: an important cause of treatment failure. *Nat Rev Cancer* **5**(7), 516–525.

[4] Kerbel R and Kamen B (2004). The anti-angiogenic basis of metronomic chemotherapy. *Nat Rev Cancer* **4**, 423–436.

[5] Munoz R, Man S, Shaked Y, Lee C, Wong J, Francia G, and Kerbel R (2006). Highly efficacious nontoxic preclinical treatment for advanced metastatic breast cancer using combination oral UFT-cyclophosphamide metronomic chemotherapy. *Cancer Res* **66**, 3386–3391.

[6] Man S, Bocci G, Francia G, Green S, Jothy S, Hanahan D, Bohlen P, Hicklin D, Bergers G, and Kerbel R (2002). Antitumor effects in mice of low-dose (metronomic) cyclophosphamide administered continuously through the drinking water. *Cancer Res* **62**, 2731–2735.

[7] Fukushima M, Satake H, Uchida J, Shimamoto Y, Kato T, Takechi T, Okabe H, Fujioka A, Nakano K, Ohshimo H, et al. (1998). Preclinical antitumor efficacy of S-1: a new oral formulation of 5-fluorouracil on human tumor xenografts. *Int J Oncol* **13**, 693–698.

[8] Koizumi W, Narahara H, Hara T, Takagane A, Akiya T, Takagi M, Miyashita K, Nishizaki T, Kobayashi O, Takiyama W, et al. (2008). S-1 plus cisplatin *versus* S-1 alone for first-line treatment of advanced gastric cancer (SPIRITS trial): a phase III trial. *Lancet Oncol* **9**, 215–221.

[9] Kato H, Ichinose Y, Ohta M, Hata E, Tsubota N, Tada H, Watanabe Y, Wada H, Tsuboi M, and Hamajima N (2004). A randomized trial of adjuvant chemotherapy with uracil-tegafur for adenocarcinoma of the lung. *N Engl J Med* **350**, 1713–1721.

[10] Watanabe T, Sano M, Takashima S, Kitaya T, Tokuda Y, Yoshimoto M, Kohno N, Nakagami K, Iwata H, Shimozuma K, et al. (2009). Oral uracil and tegafur compared with classic cyclophosphamide, methotrexate, fluorouracil as postoperative chemotherapy in patients with node-negative, high-risk breast cancer: National Surgical Adjuvant Study for Breast Cancer 01 Trial. *J Clin Oncol* **27**, 1368–1374.

[11] Llovet J, Ricci S, Mazzaferro V, Hilgard P, Gane E, Blanc J, de Oliveira A, Santoro A, Raoul J, Forner A, et al. (2008). Sorafenib in advanced hepatocellular carcinoma. *N Engl J Med* **359**, 378–390.

[12] Klement G, Baruchel S, Rak J, Man S, Clark K, Hicklin D, Bohlen P, and Kerbel R (2000). Continuous low-dose therapy with vinblastine and VEGF receptor-2 antibody induces sustained tumor regression without overt toxicity. *J Clin Invest* **105**, R15–R24.

[13] Pietras K and Hanahan D (2005). A multitargeted, metronomic, and maximum-tolerated dose "chemo-switch" regimen is antiangiogenic, producing objective responses and survival benefit in a mouse model of cancer. *J Clin Oncol* **23**, 939–952.

[14] Wedge S, Ogilvie D, Dukes M, Kendrew J, Chester R, Jackson J, Boffey S, Valentine P, Curwen J, Musgrove H, et al. (2002). ZD6474 inhibits vascular endothelial growth factor signaling, angiogenesis, and tumor growth following oral administration. *Cancer Res* **62**, 4645–4655.

[15] Wang H, Jiang H, Zhou M, Xu Z, Liu S, Shi B, Yao X, Yao M, Gu J, and Li Z (2009). Epidermal growth factor receptor VIII enhances tumorigenicity and resistance to 5-fluorouracil in human hepatocellular carcinoma. *Cancer Lett* **279**, 30–38.

[16] Bocci G, Francia G, Man S, Lawler J, and Kerbel R (2003). Thrombospondin 1, a mediator of the antiangiogenic effects of low-dose metronomic chemotherapy. *Proc Natl Acad Sci USA* **100**, 12917–12922.

[17] Kamat A, Kim T, Landen CJ, Lu C, Han L, Lin Y, Merritt W, Thaker P, Gershenson D, Bischoff F, et al. (2007). Metronomic chemotherapy enhances the efficacy of antivascular therapy in ovarian cancer. *Cancer Res* **67**, 281–288.

[18] Zhang X and Lawler J (2007). Thrombospondin-based antiangiogenic therapy. *Microvasc Res* **74**, 90–99.

[19] Ghiringhelli F, Menard C, Puig P, Ladoire S, Roux S, Martin F, Solary E, Le Cesne A, Zitvogel L, and Chauffert B (2007). Metronomic cyclophosphamide regimen selectively depletes $CD4^+CD25^+$ regulatory T cells and restores T and NK effector functions in end stage cancer patients. *Cancer Immunol Immunother* **56**, 641–648.

[20] Pasquier E, Kavallaris M, and André N (2010). Metronomic chemotherapy: new rationale for new directions. *Nat Rev Clin Oncol* **7**, 455–465.

[21] Bocci G, Nicolaou K, and Kerbel R (2002). Protracted low-dose effects on human endothelial cell proliferation and survival *in vitro* reveal a selective antiangiogenic window for various chemotherapeutic drugs. *Cancer Res* **62**, 6938–6943.

[22] O'Reilly M, Boehm T, Shing Y, Fukai N, Vasios G, Lane W, Flynn E, Birkhead J, Olsen B, and Folkman J (1997). Endostatin: an endogenous inhibitor of angiogenesis and tumor growth. *Cell* **88**, 277–285.

[23] Ebos J, Lee C, Munoz W, Bjamason G, Christensen J, and Kerbel R (2009). Accelerated metastasis after short-term treatment with a potent inhibitor of tumor angiogenesis. *Cell Press* **15**, 232–239.

[24] Hasegawa K, Takayama T, Ijichi M, Matsuyama Y, Imamura H, Sano K, Sugawara Y, Kokudo N, and Makuuchi M (2006). Uracil-tegafur as an adjuvant for hepatocellular carcinoma: a randomized trial. *Hepatology* **44**, 891–895.

[25] Tang T, Man S, Lee C, Xu P, and Kerbel R (2010). Impact of metronomic UFT/cyclophosphamide chemotherapy and antiangiogenic drug assessed in a new preclinical model of locally advanced orthotopic hepatocellular carcinoma. *Neoplasia* **12**, 264–274.

[26] Tran J, Master Z, Yu J, Rak J, Dumont D, and Kerbel R (2002). A role for survivin in chemoresistance of endothelial cells mediated by VEGF. *Proc Natl Acad Sci USA* **99**, 4349–4354.