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ORIGINAL ARTICLE

Sorafenib augments cytotoxic effect of S-1 in vitro and in vivo through TS suppression

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Abstract
Purpose Sorafenib, a multikinase and tyrosine-kinase inhibitor, has anti-tumor activity in patients with advanced renal cell carcinoma (RCC). Recently, we reported that S-1 was active and well tolerated for the treatment of cytokine-refractory metastatic RCC. Therefore, we hypothesized that S-1 might be a good candidate for combination therapy with molecular targeting agents. In this study, we examined the mechanisms underlying for the synergism between S-1 and Sorafenib for RCC treatment in vitro and in tumor-bearing murine models.
Methods Human RCC cell lines were used for the in vitro cell proliferation assay. ACHN and 786-O tumors were subcutaneously transplanted into NCr-nu/nu-mice. Mice were treated with S-1 and/or Sorafenib, and tumor growth and side effects were monitored.
Results Synergistic anti-proliferative effects of Sorafenib and S-1 were clearly demonstrated in ACHN and 786-O cell lines in vitro due to the suppression of TS and E2F-1 expression. In the NCr-nu/nu model, the synergistic anti-tumor effects of S-1 and Sorafenib were again clearly seen, indicating direct synergistic effects of each drug on tumor growth.
Conclusions Our results demonstrate the synergistic activity of S-1 and Sorafenib and provided the rationale for combination therapy with S-1 and Sorafenib for the treatment of patients with advanced RCC.

Keywords Sorafenib · S-1 · Cytotoxic effect · TS suppression

Introduction

Renal cell carcinoma (RCC) has a poor prognosis when diagnosed in its advanced stages. RCC accounts for 80–95% of kidney tumors, and −30% of RCC patients have metastatic disease at the time of diagnosis [1]. Clear cell carcinomas, which account for 75–85% of renal tumors, are characterized by loss of the von Hippel–Lindau tumor-suppressor gene, leading to overexpression of pro-angiogenic vascular endothelial growth factor (VEGF) and platelet-derived growth factor (PDGFβ) [2].

As a result, the treatment of metastatic RCC (MRCC) has recently evolved from being predominantly cytokine based to using drugs that target the dysregulated VEGF and PDGFβ pathways. Sorafenib (Nexavar; Bayer Healthcare Pharmaceuticals and Onyx Pharmaceuticals Inc) is a multikinase inhibitor targeting VEGF receptors (VEGFR) 1–3, the PDGFβ receptor, and Raf kinase [3]. Chang et al. reported that Sorafenib mediates the inhibition of both immature and mature tumor vessels, as measured by CD31 (endothelial cells) and aSMA (pericytes) staining, respectively, in immunoincompetent nude mice model [4]. And we previously reported that daily treatment with Sorafenib

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leads to increased TUNEL positivity as compared with no treated groups in tumor-bearing nude mice model, indicating that Sorafenib induced apoptotic cell death [5]. Those data indicated inhibition of VEGF- and PDGF-mediated survival of endothelial cells, and pericytes led to the observed tumor growth inhibition. In a randomized phase III trial comparing Sorafenib with placebo as a second-line therapy for RCC, the response rate of Sorafenib was 10%, and the ratio of stable disease was 74%. As a result, the median progression-free survival was 5.5 months in the Sorafenib group and 2.8 months in the placebo group [3].

S-1 is an oral fluorinated pyrimidine that includes tegafur (FT), a prodrug of 5-FU [6]. Therefore, the main active anti-tumor compound is 5-FU, the nucleic acid analog of deoxythymidylic acid (dTMP), which causes arrest at the S phase of the cell cycle [6]. Unfortunately, 5-FU is rapidly catabolized by dihydropyrimidine dehydrogenase (DPD) in the liver. To solve this problem, the anti-tumor activity of S-1 is enhanced by the inclusion of 5-chloro-2,4-dihydroxypyridine (CDHP), an inhibitor of DPD, which biochemically modulates 5-FU [6]. S-1 also contains potassium oxonate (Oxo), another 5-FU modulator, which decreases the phosphorylation of 5-FU in the gastrointestinal tract resulting in reduced gastrointestinal side effects [7]. Therefore, S-1 maintains a high blood level of 5-FU for an extended time with fewer gastrointestinal side effects. The promising anti-tumor effects of S-1 have been demonstrated in clinical trials against a variety of solid tumors. The major drug-related adverse events recognized in such trials were myelosuppression and gastrointestinal toxicity, though most were tolerable and reversible. According to these findings, the commercial availability of S-1 for the treatment of patients with gastric cancer, colorectal cancer, non-small cell lung cancer, breast cancer, head and neck cancer, pancreatic cancer, and biliary tract cancer has been approved in Japan [8–13]. We reported that S-1 had a promising anti-tumor effect on cytokine-refractory MRCC [14]. In that study, S-1-treated patients showed no evidence of hand–foot skin reactions or hypertension, which are both characteristic of Sorafenib or Sunitinib and sometimes lead to dose reduction or interruption of therapy [3, 15]. S-1 was easily administered, and most patients could be treated as outpatients, resulting in good compliance. Molecular targeting agents such as Sorafenib rarely cause myelosuppression, so S-1 might be a good candidate for combination therapy with molecular targeting agents such as Sorafenib.

S-1 is hypothesized to provide improved clinical activity in combination with other systemic agents. However, there are currently no reported data concerning the safety or clinical activity of S-1 in combination with other agents in RCC. In the present study, synergistic anti-proliferative effects of Sorafenib and S-1 were observed in ACHN and 786-O cell lines mediated via the suppression of TS and E2F-1 expression. These synergistic anti-tumor effects were also clearly demonstrated in ACHN and 786-O NCr-nu/nu mouse model, indicating the direct synergistic effects of each drug on tumor growth.

Our results show the synergistic activity of S-1 and Sorafenib and may provide the rationale for combination therapy with S-1 and Sorafenib for the treatment of patients with advanced RCC.

Materials and methods

Cell culture and reagents

Human renal cancer ACHN (Eagle's Minimal Essential Medium, MEM) and 786-O (RPMI1640) were cultured in the indicated media (Sigma, St. Louis, MO, USA) containing 10% fetal bovine serum. The cell lines were maintained in a 5% CO_2 atmosphere at 37°C. S-1, 5-Fluorouracil (5-FU), and gimeracil were provided by TAIHO Pharmaceutical (Tokyo, Japan). Sorafenib was obtained from Wako (Osaka, Japan).

Antibodies

Anti-TS, anti-OPRT, and anti-DPD antibodies were provided by TAIHO Pharmaceutical. Anti-β-actin antibody was purchased from Sigma.

Cytotoxicity assays

Cytotoxicity assays were performed as previously described [16]. Briefly, ACHN, 786-O, and RENCA cells (2.5×10^3) were seeded into 96-well plates with media containing vehicle or 1 μM of Sorafenib. The following day, various concentrations of 5-FU and gimeracil (molecular ratio: 1:0.4) were added. After 72 h, surviving cells were stained using alamarBlue assay (TREK Diagnostic Systems, Cleveland, OH, USA) for 180 min at 37°C. The absorbance of each well was measured using a plate reader (ARVO^{TM} MX; Perkin Elmer Inc., Waltham, MA, USA).

Western blotting

Whole-cell extracts were prepared as previously described [17, 18]. The protein concentration was determined using a Protein Assay Kit (Bio-Rad, Hercules, CA, USA). Whole-cell extracts (30 μg) were separated on SDS–PAGE gels and transferred to polyvinylidine difluoride microporous membranes (GE Healthcare Bio-Science, Piscataway, NJ,

USA) using a semi-dry blotter. The blotted membranes were incubated for 1 h at room temperature with a primary antibody. Membranes were then incubated for 40 min at room temperature with a peroxidase-conjugated secondary antibody. The bound antibody was visualized using an ECL kit (GE Healthcare Bio-Science), and membranes were exposed to Kodak X-OMAT film.

RNA isolation, reverse transcription, and quantitative real-time PCR

Quantitative real-time PCR was performed as previously described [17], using the following primer pairs: 5′-GAA GCTTCTAGCTGGGGTCTG-3′ (Fw) and 5′-CACACAC ACATGCTCACACACAT-3′ (Rv) for E2F-1, 5′-TACC TGAATCACATCGAGCCACT -3′ (Fw) and 5′-GAAGA ATCCTGAGCTTTGGGAAA-3′ (Rv) for TS and 5′-GCT CATTTCCTGGTATGACAACG-3′ (Fw) and 5′-GGGTC TCTCTCTTCCTCTTGTGC-3′ (Rv) for GAPDH; SYBR Premix Ex Taq II (Takara Bio, Shiga, Japan) and a ABI 7900HT.

Animals

Female BALB/c (H-2^d) mice (6–8 weeks old) and Female NCr-nu/nu mice (6–8 weeks old) were obtained from Charles River Laboratories Japan (Yokohama, Japan).

Chemical for in vivo study

For the in vivo study, Sorafenib was dissolved in Cremophor EL/ethanol (50:50) solution and diluted as described previously [4]. Sorafenib control mice were received Cremophor EL/ethanol (50:50) solution as vehicle 1 and S-1 control animals received 0.5% (w/v) aqueous solution of hydroxypropylmethylcellulose as vehicle 2 [19].

Challenge with tumor cells

ACHN and 786-O (5.0×10^7) cells were inoculated subcutaneously into the shaved lateral flanks of the mice. After 5 days, 20 mice were randomly divided into four groups. These groups were as follows: Sorafenib (10 mg/kg) plus S-1 (10 mg/kg), Sorafenib (10 mg/kg) plus vehicle 2, vehicle 1 plus S-1 (10 mg/kg), or vehicle 1 plus vehicle 2. Sorafenib, S-1, or vehicle control was administered orally, once per day, for 28 days. The size of primary tumors was determined every 2–3 days using calipers. Tumor volume was calculated using the formula: $V = (A \times B^2)/2$, where V is the volume (mm^3), A is the long diameter (mm), and B is the short diameter (mm).

Statistics

The statistical significance of the data was determined using the unpaired two-tailed Student's t test. A P value of <0.05 was considered statistically significant.

Results

Additive anti-proliferative effects of 5-FU and Sorafenib in renal cancer cells

Tegafur, which is a component of S-1, is metabolized to 5-FU in the liver and exerts anti-tumor effects. We first examined the cytotoxic effects of a combination of 5-FU and Sorafenib in ACHN and 786-O cells. ACHN cells possess wild-type von Hippel–Lindau gene, whereas 786-O cells harbor a von Hippel–Lindau mutation. The effect of combined treatment with 5-FU and Sorafenib on the proliferation of the renal cancer cell lines was tested in triplicate. The proliferation of ACHN cells was suppressed by 5-FU and gimeracil in a dose-dependent manner. When low and non-toxic dose of Sorafenib (1 μM) were combined with 5-FU and gimeracil, the anti-proliferative effect was augmented, suggesting that Sorafenib plus 5-FU and gimeracil has a synergistic cytotoxic effect on ACHN cells (Fig. 1a). Similar results were obtained with 786-O cells (Fig. 1b).

Downregulation of TS expression and activity in renal cell cancer cells of Sorafenib

We next investigated whether Sorafenib might affect the expression of TS, OPRT, and DPD, which are major determinants of the sensitivity of cells to 5-FU [20]. We first examined the abundance of these enzymes in ACHN and 786-O cells by immunoblotting. Expression of DPD was detected in MiaPaca-2 cells (positive control, data not shown) but not in ACNH or 786-O cells. In contrast, TS and OPRT were detected in both cell lines. Treatment with Sorafenib (1 μM) for up to 48 h resulted in a time-dependent decrease in the amount of TS, whereas OPRT remained unaffected (Fig. 2a, b). And negative DPD means that cells are sensitive to 5-FU [20]. However, we tried to make cells more sensitive to 5-FU using Sorafenib. A reduced level of TS expression in tumors has been associated with a higher response rate to 5-FU-based chemotherapy [21, 22]. Thus, our data suggest that the suppression of TS expression by Sorafenib might increase the sensitivity of renal cancer cells to 5-FU.

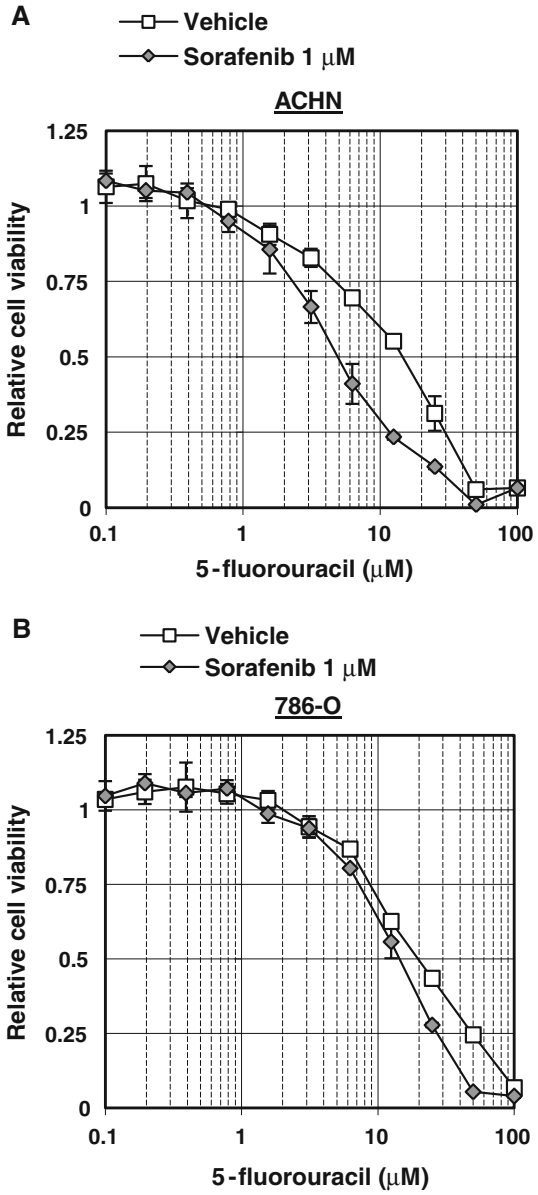

Fig. 1 Effect of the combination of 5-FU and Sorafenib on renal cancer cell growth in vitro. ACHN (**a**) and 786-O (**b**) cells were seeded into 96-well plates along with media containing vehicle or 1 μM Sorafenib. The following day, various concentrations of 5-FU and gimeracil were added. After incubation for 72 h, cell survival was analyzed by cytotoxicity assay. Cell survival in the absence of 5-FU and gimeracil corresponds to 1. All values are representative of at least three independent experiments. *Boxes* mean, *bars* ± SD

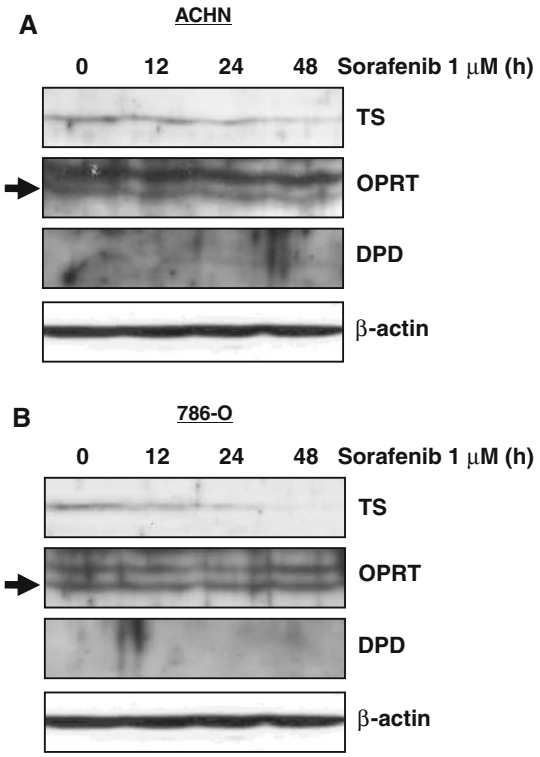

Fig. 2 Effects of Sorafenib on the expression of TS, OPRT, and DPD in renal cancer cells. ACHN (**a**) and 786-O (**b**) cells were treated with 1 μM of Sorafenib for the indicated time. Cells were then harvested, and whole-cell extracts were subjected to SDS–PAGE. Western blotting was performed using the indicated antibodies. *β*-actin is shown as a loading control

Effects of Sorafenib on E2F-1 and TS mRNA abundance in renal cancer cells

It is known that the transcription factor E2F-1 regulates the expression of the TS gene [23]. We, therefore, examined the possible effects of Sorafenib on E2F-1 expression in renal cancer cells. We determined the amounts of E2F-1 and TS mRNA in ACHN and 786-O cells at various time points after exposure to Sorafenib by reverse transcription and quantitative real-time PCR analysis. As shown in Fig. 3a, b, Sorafenib induced a time-dependent decrease in the amount of E2F-1 and TS mRNA in both cells lines, suggesting that the downregulation of TS expression by Sorafenib occurs at the transcriptional level and may be due to the suppression of E2F-1 expression.

Synergistic anti-tumor effects of Sorafenib and S-1 in a nude mouse model

Athymic nude mice were subcutaneously implanted with ACHN and 786-O tumors (5×10^7 cells/mouse) on Day 0, and treatment with Sorafenib (10 mg/kg) and/or S-1 (10 mg/kg) was initiated on Day 4, when the tumors were established. As shown in Fig. 4a, b, daily treatment with Sorafenib plus S-1 resulted in greater inhibition of ACHN and 786-O tumor growth than either Sorafenib alone or S-1 alone. These findings suggest that combined treatment with S-1 and Sorafenib has a synergistic anti-tumor effect in vivo, which is consistent with the in vitro results.

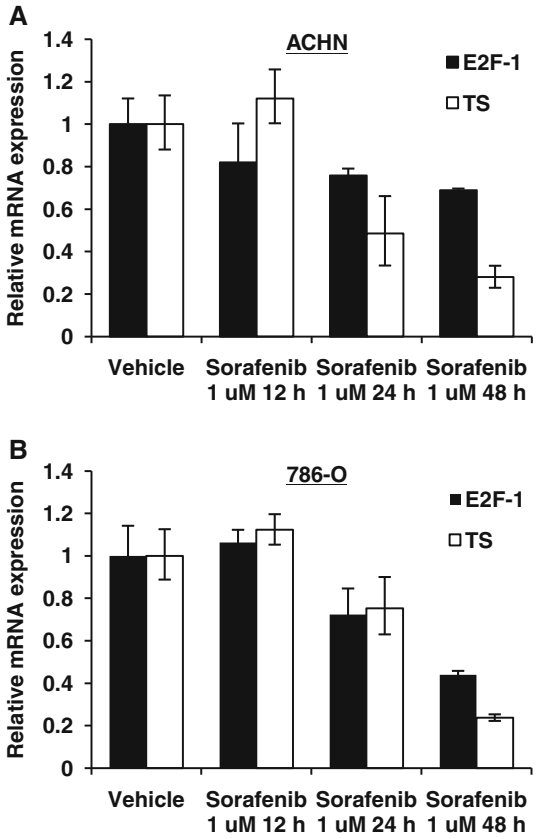

Fig. 3 Effects of Sorafenib on the amount of E2F-1 and TS mRNA in renal cancer cells. ACHN (**a**) and 786-O (**b**) cells were treated with 1 μM of Sorafenib for the indicated time. After extraction of total RNA and synthesis of cDNA, quantitative real-time PCR was performed using primers and probes for E2F-1, TS, and GAPDH. The E2F-1 and TS transcript levels were normalized to GAPDH. All values represent at least three independent experiments. The levels of E2F-1 and TS transcript from cells treated with vehicle are defined as 1. *Boxes* mean, *bars* ± SD

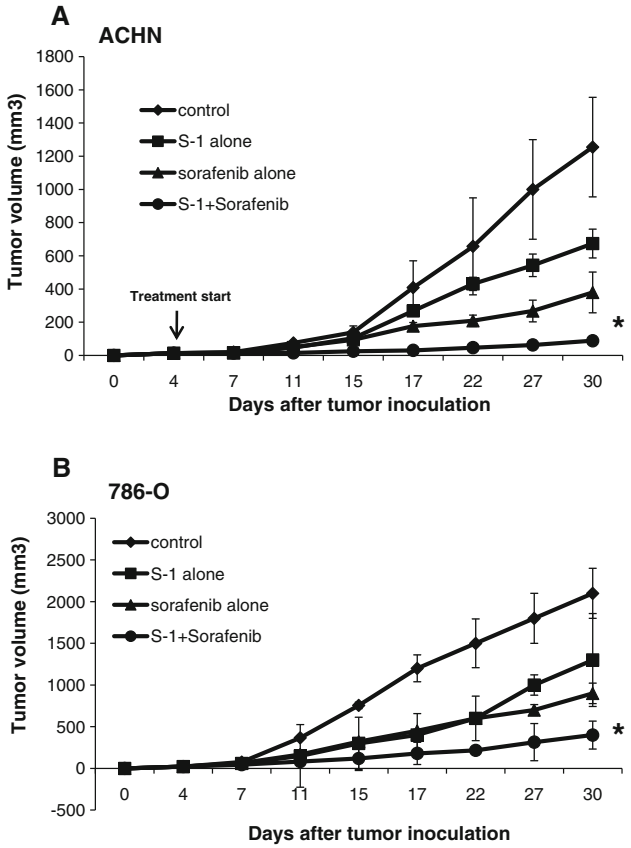

Fig. 4 Synergistic anti-tumor effects of S-1 and Sorafenib in a nude mouse model. Athymic nude mice ($n = 5$ per group) were subcutaneously implanted with ACHN and 786-O tumors on Day 0 and treated with S-1 and/or Sorafenib on Day 4, when the tumors were established ($^*P < 0.05$ compared with the other 3 groups). Data are representative of three separate experiments

Combination therapy with Sorafenib and S-1 does not increase the risk of side effects compared with the Sorafenib or S-1

We next assessed the side effects in the four groups by measuring weight loss. To exclude variations due to weight of tumor, non-tumor-bearing mice were treated with drugs and vehicles according to the protocol outlined above. No difference in the body weight was observed between the mice treated with Sorafenib (10 mg/kg) and/or S-1 (10 mg/kg) (Fig. 5). These data suggest combination therapy with Sorafenib and S-1 does not increase the risk of side effects compared with the Sorafenib or S-1.

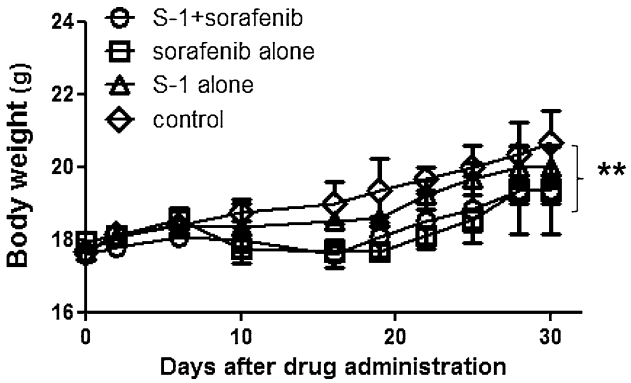

Fig. 5 No difference was observed in body weight between the four groups. Athymic nude mice ($n = 5$ per group) were treated with S-1 and/or Sorafenib on Day 0 [**not specific among the mice treated with Sorafenib (10 mg/kg) and/or S-1 (10 mg/kg)]. Data are representative of three separate experiments

Discussion

5-FU, first synthesized 40 years ago, is still one of the most widely used agents for the treatment of gastrointestinal cancers. The oral xuoropyrimidine derivative, S-1, was developed on the basis of biochemical modulation by CDHP, a dihydropyrimidine dehydrogenase inhibitor, and

Oxo, a protector against 5-FU-induced gastrointestinal toxicity. The anti-tumor effects of S-1 monotherapy have already been demonstrated in a variety of solid tumors. The response rate for advanced gastric cancer, colorectal cancer, non-small cell lung cancer, breast cancer, head and neck cancer, pancreatic cancer, and biliary tract cancer in the phase II trials conducted in Japan was 44.2, 39.5, 22.0, 41.7, 28.8, 37.5, and 35.0%, respectively [8–11]. We previously reported that S-1 was active and well tolerated for the treatment of cytokine-refractory MRCC [14]. In that study, we suggested that S-1 might be a good candidate for combination therapy with molecular targeting agents such as Sorafenib. Therefore, we undertook this study. Our results show that treatment with S-1 or Sorafenib as a single agent reduced the volume of tumor xenografts generated from two different renal cancer cell lines (Fig. 4). Moreover, we show that the combination of S-1 and Sorafenib exerts a synergistic anti-tumor effect in renal cancer cells accompanied by an acceptable decrease in body weight (Figs. 4, 5).

Next, we examined the mechanism underlying the anti-tumor effects in vitro study. Synergy between Sorafenib and 5-FU leading to tumor inhibition was observed in all cell lines (Fig. 1). A synergistic anti-tumor effect has previously been shown for S-1 and gefitinib, an epidermal growth factor receptor inhibitor, in non-small cell lung cancer cell lines [20]. In that study, TS downregulation was induced by gefitinib within 48 h, leading to the speculation that downregulation of TS by gefitinib may be responsible for the synergistic anti-tumor effects seen with combined treatment with S-1 and gefitinib. Thus, we considered the possibility that inhibition of TS by Sorafenib was behind these synergistic effects. We found that Sorafenib inhibits TS expression in renal cancer cells (Fig. 2). Downregulation of TS expression enhances the efficacy of 5FU, possibly as a direct result of a decrease in the amount of the protein target of 5FU available [24]. The amount of TS in neoplastic cells increases after exposure to 5FU, maintaining the amount of the free enzyme in excess of that of the enzyme bound to 5FU [25–27]. Such an increase in TS expression and activity has been viewed as a mechanistic driver of 5FU resistance in cancer cells [28–30]. Downregulation of TS by Sorafenib might thus contribute to the reversal of 5FU-induced increase in TS expression, resulting in enhancement of 5FU-induced apoptosis. In addition, prolonged inhibition of TS triggers apoptosis by inducing an imbalance in the deoxyribonucleoside pool and the consequent disruption of DNA synthesis and repair [31, 32]. The depletion of TS by Sorafenib might also contribute directly to the combined proapoptotic action with 5FU.

Downregulation of TS by Sorafenib was accompanied by a reduction in the amount of E2F1, suggesting that this effect results from attenuated E2F1-dependent transcription of the TS gene (Fig. 3). For other anti-cancer agents, Cisplatin and Docetaxel are reported to increase the expression of E2F-1. And 5-FU is reported not to affect the expression of E2F-1 [21, 33, 34]. The E2F family of transcription factors plays a major role in cell cycle control by regulating a group of genes involved in cell cycle progression and DNA replication. The transcriptional activity of the E2F family is regulated at many levels, but mainly through association with Rb family proteins [35, 36]. Many genes reported to be involved in angiogenesis, such as FLT-1, KDR, and angiopoietin 2 have potential E2F1-binding sites in their promoter. Although the mechanism responsible for the regulation of TS and E2F1 remains unclear, Tanizaki et al. reported that inhibition of the PI3K-AKT pathway contributes, at least in part, to the downregulation of TS [24]. Activation of PI3K-AKT signaling has been found to result in E2F1 accumulation [37, 38], supporting the notion that inhibition of such signaling leads to the downregulation of E2F1 and TS. However, it has been reported that Sorafenib cannot inhibit the PI3K-AKT pathway [39], although Pillai et al. reported that depletion of E2F1 prevents VEGF-induced angiogenic tubule formation [40]. In that study, chromatin immunoprecipitation assays showed that E2F1 associates with these promoters and the recruitment of E2F1 is enhanced upon VEGF stimulation with the concomitant dissociation of Rb, leading to the transcriptional activation of these promoters. Those results suggest that the Rb-E2F pathway contributes to the expression of VEGF receptors, thus facilitating angiogenesis, and might promote the growth and progression of tumors in response to aberrant signaling events. This suggests that a reduction in the amount of E2F1 mediated by Sorafenib might affect the expression of genes involved in other aspects of tumor growth and progression, such as angiogenesis (i.e., VEGF and VEGFR). However, to prove it surely, we consider the further experiments are needed.

In conclusion, we have shown that the combination of S-1 and Sorafenib exerts a synergistic anti-tumor effect in renal cancer cells mediated by the inhibition of TS and that this combination was accompanied by an acceptable decrease in body weight. Our observations provide a rationale for the clinical evaluation of combination chemotherapy with S-1 and Sorafenib. And we should consider the safety of the combination therapy before assessing clinical benefit, because the toxicity of various combinations of targeted agents for RCC has been reported [41].

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