Enhancement of the anti-tumor activity of S-1 by low-dose cisplatin in mice bearing the sarcoma-180 model

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The combination of S-1, consisting of 1 mol/l tegafur, 0.4 mol/l 5-chloro-2,4-dihydroxypyridine and 1 mol/l potassium oxonate, plus low-dose cisplatin has showed promising anti-tumor activities in experimental and clinical studies. The aim of this study was to investigate the mechanism of this combination chemotherapy. Mice bearing sarcoma-180 cells were divided into groups of seven animals each – Group A: no treatment; Group B: 5-fluorouracil (5-FU) 10 mg/kg continuous i.p. infusion; Group C: S-1 10 mg/kg p.o.; Group D: cisplatin 0.2 mg/kg i.p.; Group E: B + D; Group F: C + D. Treatments were given for 5 consecutive days, and then anti-tumor activity, the concentration of 5-FU, the thymidylate synthase inhibition rate (TSIR) and the level of 5-FU incorporated into RNA (F-RNA) in tumor tissue were evaluated. Anti-tumor activity in Group F was higher than in any other group. A significantly higher concentration of 5-FU in tumor was detected in the S-1-treated groups (C and F) than in the 5-FU-treated groups (B and E). No differences in TSIR were observed between the groups treated with 5-FU or S-1 with or without cisplatin; however, the F-RNA level in Group F was about 1.24 times significantly higher than that in Group C. Group F showed the highest anti-tumor activity, with increasing intratumoral levels of 5-FU and F-RNA, but not that of TSIR. These results suggested that the superior anti-tumor activity obtained by S-1 + cisplatin might be associated with an incorporation of 5-FU into RNA. *Anti-Cancer Drugs* **16:1109–1114 © 2005 Lippincott Williams & Wilkins.**

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Introduction

The combination chemotherapy of protracted continuous i.v. infusion (c.i.v.) of 5-fluorouracil (5-FU) and low-dose cisplatin has been widely used for gastrointestinal cancer patients [1–10]. Response rates of this combination chemotherapy were 36–55% in esophagus cancer [1,2], 47% in hepatocellular cancer [3], 35–66% in gastric cancer [4–9] and 57% in colorectal cancer [10], with acceptable toxicities. S-1, consisting of 1 mol/l tegafur, 0.4 mol/l 5-chloro-2,4-dihydroxypyridine (CDHP) and 1 mol/l potassium oxonate (Oxo), is an oral anti-cancer drug, which was developed to keep the 5-FU level in blood and tumor tissue high by a dihydropyrimidine dehydrogenase inhibitor, CDHP, and to reduce gastrointestinal toxicities by inhibiting activation of 5-FU in gastrointestinal mucosa by Oxo [11]. Recently, preliminary studies of the combination of S-1 and cisplatin in patients with gastric cancer have indicated favorable response rates, i.e. 48–74% [12–15], which appear to be as good as, or better than, those of 5-FU c.i.v. plus low-dose cisplatin, i.e. 35–66% [4–9]. Furthermore, an oral form of S-1 is advantageous in terms of quality of life.

Previously, our colleagues reported that the combination of S-1 and low-dose cisplatin showed a greater anti-tumor effect in mice bearing sarcoma-180 (S-180) than the combination of 5-FU and low-dose cisplatin [16]. Experimental studies using mice bearing Colon 26 liver metastasis and nude mice bearing human gastric cancer cells also suggested that the anti-tumor activity of S-1 was enhanced greatly by low-dose cisplatin that did not have an obvious anti-tumor effect alone [17,18].

The mechanism of the combination of 5-FU c.i.v. and low-dose cisplatin is still open to debate. For instance, Shirasaka *et al.* [19] and Scanlon *et al.* [20] suggested cisplatin functioned as a modulator of 5-FU, whereas, contrary to this theory, Fujishima *et al.* [22] and Esaki *et al.* [23] suggested 5-FU acted as a modulator of cisplatin.

In this experimental study, we investigated the concentration of 5-FU, thymidylate synthase (TS) inhibition rate (TSIR) and 5-FU [more specifically, fluorouridine-5′-triphosphate (FUTP)] incorporated into RNA (F-RNA) in tumor tissue following the administration of S-1 with or without low-dose cisplatin, and compared them with those by 5-FU with or without cisplatin, in order to elucidate the mechanism of S-1 + low-dose cisplatin.

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Materials and methods

Drugs

5-FU was provided by Kyowa Hakko Kogyo (Tokyo, Japan), cisplatin was purchased from Nippon Kayaku (Tokyo, Japan) and S-1 was provided by Taiho Pharmaceutical (Tokyo, Japan).

Tumor cells

S-180 cells grown in mice were selected because this cell line has confirmed sensitivity to 5-FU, S-1 and cisplatin as we reported before [16,24].

Animals

Six-week-old ddY male mice (average weight 30 g) were purchased from Chiyoda Kaihatsu (Tokyo, Japan). They were maintained under specific pathogen-free conditions.

Treatment schedule

The mice were inoculated s.c. with 5×10^6 S-180 cells into the lateral region of the abdomen. These mice were allocated to six groups of seven animals each and the treatment was started 3 days after tumor transplantation (day 0) – A: no treatment, serving as a control; B: continuous i.p. infusion of 5-FU 10 mg/kg/day (days 3–7) via an implanted micropump (model 1007D; Alzet, Cupertino, California, USA); C: oral S-1 10 mg/kg/day (days 3–7); D: i.p. cisplatin 0.2 mg/kg/day (days 3–7); E: continuous i.p. infusion of 5-FU 10 mg/kg/day concurrently with i.p. cisplatin 0.2 mg/kg/day (days 3–7); F: oral S-1 10 mg/kg/day concurrently with i.p. cisplatin 0.2 mg/kg/day (days 3–7).

On day 8, the mice were weighed before being sacrificed, and the tumor was isolated and weighed. Then all samples were promptly stored at −80°C to measure the concentration of 5-FU, TSIR and level of F-RNA. The anti-tumor activity and body weight change, as an indicator of toxicity, were assessed as follows: treatment/control (T/C) ratio = (mean tumor weight of the treatment group)/(mean tumor weight of the control group) and body weight change (g) = (body weight on day 8) – (body weight before treatment) – (tumor weight).

5-FU concentration in tumor

The concentration of 5-FU in tumor was determined by HPLC according to the previous report [25]. Briefly, the tumor sample was homogenized in acetonitrile containing the internal standard (IS) substance, 5-bromouracil, and centrifuged. The supernatant fluid was evaporated to dryness, dissolved in dehydrated ethanol and absorbed into the silica gel column. The column was eluted with acetone and the 5-FU fraction was collected. After acetone was evaporated, the fraction was dissolved in the mobile phase (ethyl acetate:*n*-hexane:formic acid: water = 50:50:0.5:0.3). This solution was injected into the HPLC apparatus.

F-RNA level

The F-RNA level in tumor was determined by gas chromatography/mass spectrometry (GC-MS) according to the previous report [26]. Briefly, the homogenized tumor mixed with 10% trichloroacetic acid was centrifuged. The precipitate was washed, dissolved in 3N KOH and incubated at 37°C for 20 h to hydrolyze into mononucleotides. The solution to which 60% $HClO_4$ was added was centrifuged and the supernatant was obtained after neutralization with 3N KOH. This was diluted with 12NHCl containing 1,3-$^{15}N_2$-5-fluorouracil as IS and maintained at 100°C for 20 h. After washing the solution with chloroform and centrifugation, the aqueous phase was dried up. This residue was dissolved in 1 mol/l phosphate buffer (pH 4.0) and extracted with ethyl acetate. The upper layer was evaporated and the residue was reconstituted with ethanol. This fluid was absorbed into the silica gel column which was eluted with acetone and the 5-FU fraction was collected. This solution was injected into the GC-MS.

TSIR

The method of TS measurement reported by Spears *et al.* [27] was partially modified [28]. Briefly, the tumor sample was homogenized in cytidine-5′-monophosphate buffer (pH 7.4) containing 2-mercaptoethanol and centrifuged to separate cytosol.

Total TS

The cytosol, mixed with 2-mercaptoethanol-containing buffer at pH 8.1, was incubated at 25°C for 3 h. After adding [^{3}H]FdUMP (5-fluoro-2′-deoxyuridine-5′-monophosphate)/FH_4 (tetrahydrofolate)/BSA reaction fluid, the mixture was further incubated for 20 min. The radioactivity level in the centrifuged supernatants which were added to dextran-coated charcoal was measured using a scintillator.

Free TS

The cytosol, mixed with the same buffer at pH 8.1, was directly followed by adding the reaction fluid. The mixture was incubated for 20 min and radioactivity was measured in the same manner as above. Free TS and TSIR were calculated by the following formulas: free TS = (apparent free TS – 0.13 × total TS)/0.87 and TSIR (%) = (TS total – TS free)/TS total × 100.

Statistical analysis

Differences between data were evaluated using the Mann–Whitney's *U*-test with the level of significance set at $P < 0.05$. All data were analyzed by the SPSS II software package for Windows (release 11.0; SPSS, Tokyo, Japan).

Results

Anti-tumor activity

Figure 1 shows anti-tumor activity as the mean T/C ratio ± SD. Group D (cisplatin alone) showed weak anti-tumor activity with a T/C ratio of 0.84. The T/C ratio in

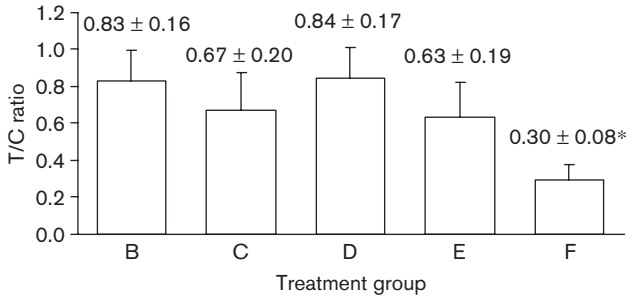
Group B (5-FU alone) was 0.83 and that in Group C (S-1 alone) was 0.67, with no statistical difference between the two groups. When cisplatin was added to 5-FU (Group E), the mean T/C ratio of 0.63 did not prove to be significantly different compared to the mean T/C of 0.83 in mice treated with 5-FU alone (Group B). However, Group F (S-1 + cisplatin) resulted in a mean T/C ratio of 0.30, which was found to be significantly lower than 0.67 obtained in Group C ($P = 0.004$) and 0.63 obtained in Group E ($P = 0.012$).

Levels of 5-FU, TSIR and F-RNA in tumor

As Fig. 2 shows, the S-1-treated groups (Groups C and F) showed 6- to 11-fold higher intratumoral concentrations of 5-FU than the 5-FU-treated groups (Groups B and E) with statistical significance (Group C or F versus B or E, $P < 0.001$). The results of TSIR are shown in Fig. 3. There was a trend toward enhancement of the mean TSIRs in the S-1-treated groups (Group C, 66%; Group F, 71%) as compared with the 5-FU-treated groups (Group B, 50%; Group E, 55%), although the differences were not statistically significant. When cisplatin was added to S-1, the mean TSIR (71% in Group F) did not increase statistically significantly compared to that of 66% in Group C (S-1 alone). As well as this result, the mean TSIR of 50% in Group B (5-FU alone) was not significantly enhanced by the addition of cisplatin (55% in Group E).

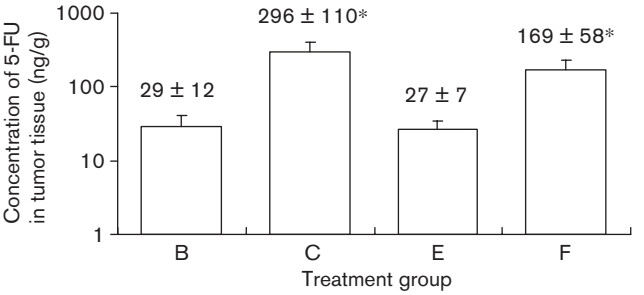
Figure 4 shows the mean levels of intratumoral F-RNA. Seemingly, the levels of F-RNA in Group B, C and E were not so different when considering SDs. However, the mean levels of F-RNA in the S-1-treated groups (Group C, 109 ng/mg RNA; Group F, 135 ng/mg RNA) were significantly higher than those in the 5-FU-treated groups (Group B, 82 ng/mg RNA; Group E, 87 ng/mg RNA) ($P = 0.002$, Group C versus B; $P < 0.001$, Group F versus E). F-RNA was produced significantly more by combining S-1 with cisplatin (Group F) and the mean F-RNA was increased from 109 ng/mg RNA in Group C (S-1 alone) to 135 ng/mg RNA in Group F ($P = 0.011$). On the contrary, no difference in the amount of F-RNA between Group B (5-FU alone) and Group E (5-FU + cisplatin) was observed.

Fig. 1



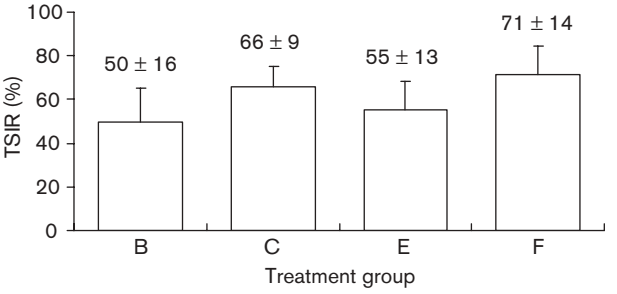
Anti-tumor activity as the T/C ratio after treatment in S-180-bearing mice (mean ± SD of seven mice). Group B, 5-FU alone; Group C, S-1 alone; Group D, cisplatin alone; Group E, 5-FU + cisplatin; Group F, S-1 + cisplatin. *$P = 0.004$ (versus Group C) and $P = 0.012$ (versus Group E).

Fig. 2



Intratumoral concentration of 5-FU after treatment in S-180-bearing mice (mean ± SD of seven mice). Group B, 5-FU alone; Group C, S-1 alone; Group E, 5-FU + cisplatin; Group F, S-1 + cisplatin. *$P < 0.001$ (Group C or F versus B or E).

Fig. 3



TSIRs after treatment in S-180-bearing mice (mean ± SD of seven mice). Group B, 5-FU alone; Group C, S-1 alone; Group E, 5-FU + cisplatin; Group F, S-1 + cisplatin.

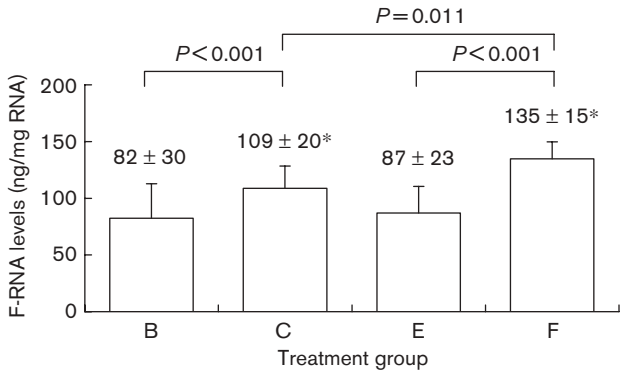
Body weight change

Figure 5 shows the body weight change on day 8. Decrease of the mean body weights in mice treated with S-1 (Groups C and F) was observed; however, there was no statistical difference in body weight change among the groups.

Discussion

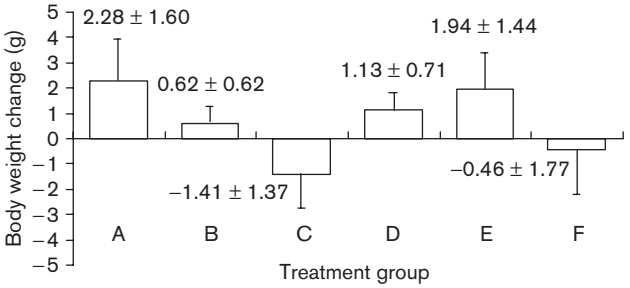
We investigated the mechanism of combined chemotherapy of S-1 + low-dose cisplatin. As a result, we observed, in mice bearing the S-180 model, a significantly

Fig. 4



Level of F-RNA after treatment in S-180-bearing mice (mean ± SD of seven mice). Group B, 5-FU alone; Group C, S-1 alone; Group E, 5-FU + cisplatin; Group F, S-1 + cisplatin.

Fig. 5



Body weight change measured on day 8 after starting treatment in S-180-bearing mice (mean ± SD of seven mice). Group A, no treatment; Group B, 5-FU alone; Group C, S-1 alone; Group D, cisplatin alone; Group E, 5-FU + cisplatin; Group F, S-1 + cisplatin.

greater anti-tumor effect of combined S-1 + cisplatin than 5-FU + cisplatin or monotherapy of 5-FU, cisplatin or S-1. This effect was associated with an increase of 5-FU incorporated into RNA in tumor, but not with level of TS inhibition.

As for the mechanism of a combination chemotherapy of 5-FU c.i.v. and low-dose cisplatin, apart from the formation of DNA interstrand cross-links by cisplatin, Shirasaka *et al.* [19] and Scanlon *et al.* [20] demonstrated that cisplatin acted as a biochemical modulator of 5-FU, i.e. the covalently bound ternary complex is increased and the anti-tumor activity of 5-FU is enhanced. However, Johnston *et al.* [29] reported that when a human colon carcinoma cell line was treated with 5-FU, no notable differences in the fraction of FdUMP-bound TS enzyme could be observed in the absence or presence of cisplatin.

In our experiment, TSIR was not increased by adding cisplatin to 5-FU or S-1, despite the high intratumoral level of 5-FU in the S-1- treated groups. We removed tumors 24 h after the last administration of S-1 in the S-1-treated groups; therefore, there might be a possibility of failing to detect the difference in TSIR between the groups treated with S-1 + cisplatin and S-1 alone. However, considering the results of the experiment using the same model as ours where there was no difference in TSIR at 2 h after 5-FU + cisplatin compared with 5-FU alone [30] and also our findings, we think cisplatin in combination of S-1 may not contribute to the increase and persistence of TSIR.

However, Fujishima *et al.* [22] and Esaki *et al.* [23] suggested 5-FU modulated the effect of cisplatin, i.e. 5-FU incorporated into RNA may inhibit synthesis of glutathione-S-transferase, and downregulate the excision cross-complementing gene 1 and gamma-glutamylcysteine synthetase gene which repair platinum–DNA adducts. Consequently, the anti-tumor activity of cisplatin is enhanced by 5-FU.

Although levels of F-RNA were higher in the S-1-treated groups than in the 5-FU treated groups, and especially when mice were treated with the combination of S-1 + cisplatin, levels of F-RNA were higher than in any other group. We suspected that this finding may be attributed to the high concentration of 5-FU in tumor tissue after treatment with S-1. Similar results were indicated by Takechi *et al.* [31]. They showed that after the administration of S-1 or UFT (a combination of tegafur and uracil at a molar ratio of 1:4) at equitoxic doses to rats bearing Yoshida sarcoma, S-1 showed a higher amount of 5-FU incorporated into the RNA in tumor tissue than UFT. In the present study, although the F-RNA level did not increase by adding cisplatin to 5-FU, it increased significantly when combining cisplatin with S-1. As far as we know, there was no report showing that cisplatin enhanced incorporation of 5-FU into RNA and the mechanism has not been defined by this study.

One hypothesis is that cisplatin inhibition of ribonucleotide reductase, which catalyzes a rate-limiting reaction of converting 5-fluorouridine diphosphates to 5-fluorodeoxyuridine diphosphates [32,33], together with a high concentration of 5-FU in tumors after S-1 administration might encourage anabolism of 5-FU to FUTP and, consequently, F-RNA is produced more.

However, we do not know whether increasing incorporation of 5-FU into RNA in a combination of S-1 + cisplatin

is observed in other cancer cell lines or animal models in the same way, because the F-RNA level following the administration of fluoropyrimidine is different depending on the type of cancer cell and levels of fluoropyrimidine-metabolizing enzymes in tumor tissue [34].

As preliminary studies of a combination chemotherapy of S-1 + low-dose cisplatin showed promising outcomes [12–15] which seem to be superior to those of 5-FU c.i.v. + low-dose cisplatin, we think some other mechanisms underlie the combination chemotherapy of S-1 + low-dose cisplatin. Our results suggest that the superior anti-tumor activity obtained by S-1 + cisplatin might be associated with incorporation of 5-FU into RNA.

Conclusion

A significantly higher anti-tumor activity was observed in S-180-bearing mice treated with a combination of S-1 + cisplatin than in any other groups including a combination of 5-FU + cisplatin. The concentration of 5-FU in tumor tissue was significantly higher in the S-1-treated groups than in the 5-FU-treated groups, although no differences in TSIRs were observed between the groups treated with 5-FU or S-1 with or without cisplatin. Meanwhile, intratumoral F-RNA was increased significantly by adding cisplatin to S-1 compared with S-1 alone. This change was not found between the groups treated with 5-FU with or without cisplatin. This result suggests that the incorporation of 5-FU into RNA might play an important role in the enhancement of anti-tumor activity in combination chemotherapy of S-1 + low-dose cisplatin.

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