PERGAMON

European Journal of Cancer 39 (2003) 2387–2394

European Journal of Cancer

www.ejconline.com

Superior antitumour activity of S-1 in tumours with a high dihydropyrimidine dehydrogenase activity

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Received 13 December 2002; received in revised form 24 March 2003; accepted 29 May 2003

Abstract

To elucidate the mechanism of the enhanced antitumour activity of S-1 (1 M tegafur, 0.4 M 5-chloro-2, 4-dihydroxypyridine, and 1 M potassium oxonate) in terms of the phosphorylation and degradation pathways of 5-fluorouracil (5-FU) metabolism, we investigated tumoral thymidylate synthase (TS) content, dihydropyrimidine dehydrogenase (DPD) activity, the TS inhibition rate (TS-IR), and 5-FU incorporated into RNA (F-RNA) in four human gastric cancer xenografts (MKN-28, MKN-74, GCIY and GT3TKB) and compared the results obtained with S-1 with those obtained with 5-FU and UFT (1 M tegafur, 4 M uracil). 5-FU was administered intraperitoneally (i.p.) to mice at a dose of 50 mg/kg, three times, on days 0, 4 and 8. S-1 and UFT were administered orally at doses of 10 and 24 mg/kg, respectively, once a day, for 9 consecutive days. Antitumour activity was evaluated as the maximum inhibition of tumour growth in each animal. S-1 showed a better antitumour activity than 5-FU and UFT in tumours with a high DPD activity (GCIY and GT3TKB). There were inverse correlations between the antitumour activity and both TS content and DPD activity in the 5-FU and UFT groups. However, no such correlations were observed in the S-1 group. In GCIY and GT3TKB xenografts, TS-IR was significantly higher in the S-1 group than in the 5-FU or UFT groups. In GT3TKB xenografts, the F-RNA level was significantly higher in the S-1 group than in the 5-FU or UFT groups. The superior cytotoxicity of S-1 appears to be attributable to both an increased inhibition of DNA synthesis and an enhanced blockade of RNA function against tumours with a high DPD activity.

Keywords: 5-Fluorouracil; UFT; S-1; Thymidylate synthase; Dihydropyrimidine dehydrogenase; Antitumour activity; Gastric cancer

1. Introduction

5-Fluorouracil (5-FU), an antitumour pyrimidine, was first synthesised in 1957. Standard regimens for gastrointestinal tumours include biomodulated 5-FU chemotherapy [1,2]. The mechanism of action of 5-FU involves the incorporation of fluorouridine 5′-triphosphate (FUTP) into RNA, resulting in the distortion of gene expression, or in the inhibition of thymidylate synthase (TS) by the active metabolite 5-fluorouridine monophosphate (FdUMP) [3,4]. Dihydropyrimidine dehydrogenase (DPD), which is both an initial and a rate-limiting catabolic enzyme of 5-FU, has been reported to play an important role in the pharmacokinetics of 5-FU [5,6]. In order to maintain stable plasma 5-FU levels and a superior antitumour activity, several oral fluoropyrimidine derivatives with DPD inhibitory activity have been developed [7–9,10].

UFT is a combination drug consisting of 1 M tegafur, a prodrug of 5-FU, and 4 M uracil. Uracil selectively inhibits the degradation by DPD of the 5-FU formed by conversion from tegafur [7,11]. Clinical efficacy of UFT has been confirmed in the West and in Japan [12,13].

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0959-8049/03/$ - see front matter © 2003 Elsevier Ltd. All rights reserved.
doi:10.1016/S0959-8049(03)00513-6

Two articles on S-1 published in 1996 extended the DPD-inhibiting fluoropyrimidine concept [9,14]. S-1 consists of 1 M tegafur, 0.4 M 5-chloro-2, 4-dihydroxypyridine (CDHP), and 1 M potassium oxonate (Oxo). CDHP is a potent inhibitor of DPD that is approximately 200 times more effective than uracil in inhibiting DPD *in vitro* [15]. Oxo is an inhibitor of orotate phosphoribosyltransferase (OPRT) and a protector against 5-FU-induced gastrointestinal toxicity [16]. Excellent antitumour activity has been reported for advanced gastric cancer in a phase II study [17].

The potent antitumour effect of S-1 has been explained pharmacokinetically in a rodent model and clinically [14,18,19]. Our previous study found better antitumour activity for S-1 than for 5-FU alone or UFT, in tumours with a high DPD activity [20]. However, there have been no reports on correlations between the effect of S-1 and both the degradation and phosphorylation pathways in the treatment of gastrointestinal carcinomas. Since tumour sensitivity to 5-FU is closely related to TS and DPD activity [6,21–23], to clarify the differences between the antitumour activity and mechanism of action of 5-FU, UFT, and S-1, we investigated the relationship between the antitumour activity of these drugs and TS content, DPD activity, TS inhibition rate (TS-IR), and 5-FU incorporated into RNA (F-RNA).

2. Materials and methods

2.1. Animals

Male BALB/cA nude mice (age 4 weeks; weight 18–20 g) were purchased from CLEA Japan, Co. Ltd (Tokyo, Japan), and fed a sterilised pellet diet and autoclaved water *ad libitum*. The animals were housed under specific-pathogen-free conditions in a laminar flow rack.

2.2. Drugs

5-FU was obtained from Kyowa Hakko Kogyo, Co., Ltd. (Tokyo, Japan) and dissolved in 0.9% NaCl solution. Tegafur, CDHP, Oxo, and uracil were provided by Taiho Pharmaceutical Co. Ltd., Tokyo, Japan. We prepared S-1 by mixing tegafur, CDHP, and Oxo at a molar ratio of 1:0.4:1, and UFT by mixing tegafur and uracil at a molar ratio of 1:4 [14,18]. S-1 was dissolved in 0.5% (w/v) hydroxypropylmethylcellulose (HPMC) solution, and UFT was suspended in a 0.5% (w/v) HPMC solution. The doses of both drugs are expressed as the dose of tegafur, because the active component of both drugs is tegafur. [6-^{3}H]-FdUMP (16.9 Ci/mmol) was obtained from Moravek Biochemicals Inc. (Bera, CA, USA). [6-^{14}C]-5-FU (56 mCi/mmol) was purchased from American Radiolabeled Chemicals Co. (St Louis, MO, USA), and nicotinamide adenine dinucleotide phosphate (NADPH) was obtained from the Sigma Chemical Co. (St Louis, MO, USA).

2.3. Tumour inductions

Four human gastric cancer cell lines (GCIY, GT3TKB, MKN-28, MKN-74) were obtained from the Riken Cell Bank (Institute of Physical and Chemical Research, Saitama, Japan) and cultured in Roswell Park Memorial Institute (RPMI)-1640 medium containing 10% fetal bovine serum (GIBCO/BRL, Rockville, MD, USA) at 37 °C under 5% CO_2. Cells in the logarithmic growth phase were detached, and 1×10^6 cells were subcutaneously transplanted with a trocar needle into the dorsal flank of each mouse. Tumour volume [(major axis)×(minor axis)^2×1/2] was measured twice a week. When the tumour volume reached 100 mm^3, the mice were sacrificed, and tumours were harvested for further experiments. All animal experiments were carried out in accordance with the Guidelines for the Welfare of Animals in Experimental Neoplasia.

2.4. Antitumour activity of 5-FU in human gastric cancer xenografts

When the tumour volume reached approximately 100 mm^3, the tumour-bearing mice were randomly allocated to drug groups of 10 animals each. Six of the mice in each group were used to determine antitumour activity, and the other four were used to measure enzyme activity and F-RNA, as described below. To evaluate the antitumour effect, each drug was administered at the maximal tolerated dose (MTD) in nude mice as previously described in Refs. [18,20]. 5-FU was administered intraperitoneally (i.p.) at a dose of 50 mg/kg, three times, on days 0, 4 and 8. S-1 and UFT were administered orally at doses of 10 and 24 mg/kg, respectively, once a day for 9 consecutive days [18]. A 0.5% (w/v) HPMC solution was administrated orally to the control group, once a day for 9 consecutive days. Tumour volume was measured every 2 days after the start of drug administration, and relative tumour volume (RTV) was calculated as (mean tumour volume during treatment)/(mean tumour volume at the start of treatment). The following formula was used to calculate antitumour effect: tumour growth inhibition rate (TGIR%) = (1−mean RTV of treatment group/mean RTV of untreated group)×100. The maximum value was used as the value of the antitumour effect.

2.5. Collection of tumours

Four tumour-bearing mice were killed before drug administration, and four mice per group in the 5-FU, UFT and S-1 groups were killed 6 h after the final drug

administration [20]. Tumour fragments were collected and stored at −80 °C pending measurement of TS content, DPD activity and F-RNA.

2.6. TS content and TS inhibition rate

TS content was determined as the amount of [6-^{3}H]-FdUMP binding sites in the 105 000g supernatant (cytosol) of tumour tissue homogenates, based on the method described by Spears and colleagues [23], with minor modifications [16,24]. The samples for TS total were prepared by causing the ternary complex present in the cytosol to be fully dissociated to unbound TS at pH 8.0 during the preincubation period. In the case of TS-free samples, pre-incubation for the dissociation process was omitted. TS total and TS-free samples were incubated with [6-^{3}H]-FdUMP in the presence of 5,10-methylenetetrahydrofolate for 20 min at 30 °C, and radioactivity in the acid-insoluble fraction was measured with a liquid scintillation counter. Protein concentration was determined using a protein assay kit (Bio-Rad, Richmond, CA, USA). TS content is expressed as pmol/mg of protein, and the TS inhibition rate (TS-IR) was calculated as TS-IR (%) = (1−free TS/total TS)×100.

2.7. DPD activity

DPD activity was measured according to the procedures of Takechi and colleagues [25], with minor modifications [24]. Briefly, enzyme solution obtained from a tumour was incubated with reaction mixture containing 2 mM dithiothreitol, 5 mM $MgCl_2$, 20 μM [6-^{14}C]-FU (56 nCi) and 100 μM NADPH at 37 °C for 10 min or 30 min. The reaction was suspended by boiling. The mixture was centrifuged at 1500*g* for 10 min, and the supernatant was then incubated with 0.36 M KOH at room temperature for 30 min. The solution was then mixed with 0.36 M $HClO_4$ and centrifuged at 1500*g* for 10 min. An aliquot of supernatant was applied to a thin-layer chromatography plate (silica gel 60 F254, Merck, Darmstadt, Germany), which was then developed with a mixture of 99% ethanol and 1 M ammonium acetate (5:1, v/v). The plate was then read on an imaging analyser (Bio-Rad), and the densities of 5-FU and the degradation products were calculated. DPD activity was expressed as pmol/mg protein/min.

2.8. F-RNA levels

Isolation and quantification of the 5-FU incorporated into the RNA fraction was based on the method of Uchida and colleagues [26]. RNA fractions were extracted from the tissues and separated according to the method of Schneider [27]. To isolate 5-FU, RNA fractions were heated to 100 °C in 6 M HCl and hydrolysed for 24 h. Finally, the quantity of 5-FU was determined by using a gas chromatograph–mass spectrometer as previously described [14,16].

2.9. Statistical analysis

Statistical analysis was performed on a personal computer with Stat View V. 5.0 software (SAS Institute, Inc., Cary, NC, USA). To evaluate the correlations between two variables, a linear regression analysis was performed, and the Pearson's correlation coefficient was calculated. Statistical differences between two unpaired groups were evaluated using the Mann–Whitney U-test. Statistical differences between two corresponding groups were evaluated using the Wilcoxon's signed rank test. A P value of less than 0.05 was considered to be statistically significant.

3. Results

3.1. Antitumour activities of 5-FU, UFT and S-1 against human gastric cancer xenografts

Changes in RTV after drug administration and data for antitumour activity expressed as maximum TGIR are summarised in Fig. 1 and Table 1. MKN-74 xenografts were the most sensitive to 5-FU and UFT, and GT3TKB xenografts were the most resistant. By contrast, GT3TKB xenografts were the most sensitive to S-1, and MKN-28 xenografts were the most resistant. In all xenografts, S-1 showed a superior antitumour activity to UFT. There were no significant differences in the antitumour activity of the drugs in MKN-28 and MKN-74 xenografts. However, the antitumour activity was significantly higher in the S-1 groups of the cell lines GCIY and GT3TKB than in the 5-FU or UFT groups.

3.2. Correlation between antitumour activity and TS and DPD levels

In all xenografts, measurements of TS content and DPD activity were possible (Table 1). MKN-74 showed the lowest TS content and DPD activity before drug administration, and GT3TKB showed the highest. Changes in TS content and DPD activity after drug treatment are summarised in Table 2. DPD remained unchanged after drug treatment. TS content tended to increase after drug treatment, however, the difference was not statistically significant. In the comparisons between TS content and DPD activity and antitumour activities of 5-FU, antitumour activities showed inverse correlations with both TS content and DPD activity (Figs. 2a and 3a). Similarly, there were inverse correlations between the tumour response to UFT and TS content and DPD activity (Figs. 2b and 3b). However,

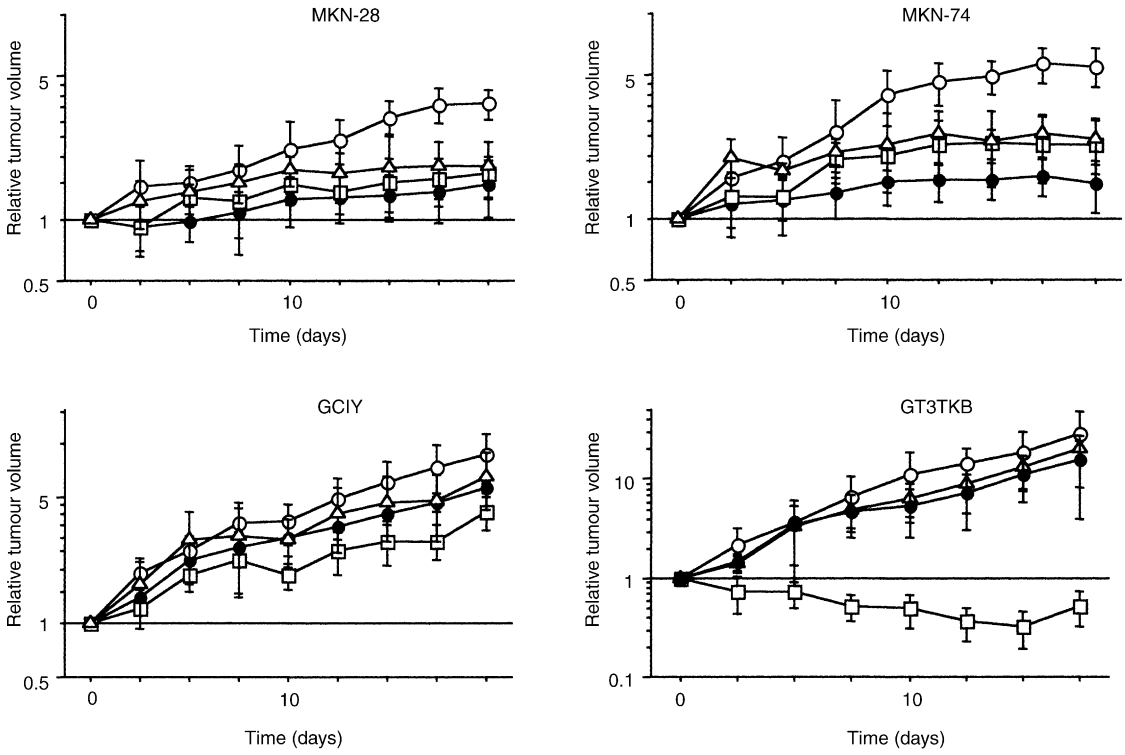

Fig. 1. Antitumour activity of 5-fluorouracil (5-FU), 1M tegafur, 4M uracil (UFT), and 1M tegafur, 0.4M 5-chloro-2,4-dihydroxypyridine, 1M potassium oxonate (S-1) in human gastric cancer xenografts. ○, Control; ●, 5-FU; △, UFT; □, S-1. Each value represents the mean and standard deviation of relative tumour volumes (RTVs) from six tumours. Antitumour activity was significantly higher in the S-1 groups in GCIY and GT3TKB cells than in the 5-FU or UFT groups.

no significant correlations were observed between the antitumour activities and TS content or DPD activity in the S-1 group (Figs. 2c and 3c).

3.3. Correlation between antitumour activities and TS-IR after drug administration

Comparisons of TS-IR in each xenograft after drug administration are shown in Table 2. No significant

Table 1
Sensitivity to 5-FU, UFT, and S-1 and TS content and DPD activity of human gastric cancer xenografts

	MKN-28	MKN-74	GCIY	GT3TKB
TGIR[a]				
5-FU	63.8±10.9	71.9±12.5	34.2±5.6	15.2±7.8
UFT	50.5±11.4	55.7±9.9	55.7±9.9	11.8±4.3
S-1	54.6±14.3	58.3±11.2	69.2±8.5[b,c]	95.1±3.1[b,c]
TS content[a] (pmol/mg protein)	0.258±0.067	0.094±0.044	0.311±0.141	2.621±2.241
DPD activity[a] (pmol/mg protein/min	20.3±7.3	6.0±4.6	277.8±70.2	287.8±14.3

5-FU, 5-fluorouracil; UFT, 1M tegafur, 4M uracil; S-1, 1M tegafur, 0.4M 5-chloro-2,4-dihydroxypyridine, 1M potassium oxonate; TS, thymidylate synthase; DPD, dihydropyrimidine dehydrogenase; TGIR, tumour growth inhibition rate.

[a] Values represent the means and standard deviations of results from six tumours.

[b] Significantly different from the 5-FU treatment group at $P<0.005$.

[c] Significantly different from the UFT treatment group at $P<0.005$.

Table 2
TS content, DPD activity, TS inhibition rate levels and F-RNA levels of human gastric cancer xenografts after administration of 5-FU, UFT and S-1

	MKN-28	MKN-74	GCIY	GT3TKB
TS content (pmol/mg protein)[a]				
5-FU	0.294±0.059	0.552±0.184	1.038±0.38	7.263±1.281
UFT	0.395±0.149	0.325±0.018	1.415±0.319	4.846±1.351
S-1	0.528±0.054	0.234±0.038	1.293±0.283	6.967±0.959
DPD activity (pmol/mg protein/min)[a]				
5-FU	21.7±9.2	8.8±2.9	305.3±111.2	255.6±93.0
UFT	18.6±8.8	6.0±4.6	259.4±55.0	284.7±124.6
S-1	20.4±12.4	9.5±5.3	338.5±115.3	269.5±114.4
TS inhibition rate (%)[a]				
5-FU	72.5±20.9	63.5±14.5	45.9±5.4	29.6±7.0
UFT	68.9±3.3	77.2±6.5	56.2±2.8[b]	22.4±7.2
S-1	59.6±15.4	68.7±8.3	75.3±2.5[b,c]	53.4±2.6[b,c]
F-RNA level (ng/mg RNA)[a]				
5-FU	404.7±135.8	181.0±91.8	101.0±36.7	118.3±29.4
UFT	321.7±67.4	274.0±78.5	76.3±17.5	106.7±25.7
S-1	391.7±86.4	224.7±43.5	128.7±42.5	237.0±118.7[b,c]

5-FU, 5-fluorouracil; UFT, 1M tegafur, 4M uracil; S-1, 1M tegafur, 0.4M 5-chloro-2,4-dihydroxypyridine, 1M potassium oxonate; TS, thymidylate synthase; DPO, dihydropyrimidine dehydrogenase.

[a] Values represent the means and standard deviations of results from four tumours.

[b] Significantly different from the 5-FU treatment group at $P<0.005$.

[c] Significantly different from the UFT treatment group at $P<0.005$.

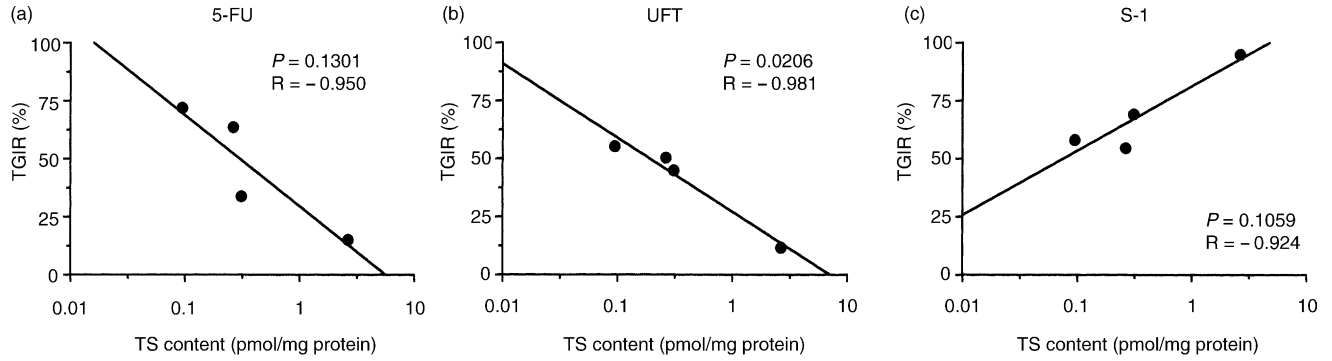

Fig. 2. Correlation between thymidylate synthase (TS) content and the antitumour activity in four human gastric cancer xenografts. In the 5-fluorouracil (5-FU) and 1M tegafur, 4M uracil (UFT) groups, TS content was inversely correlated to antitumour activity. However, there was no correlation in the 1M tegafur, 0.4M 5-chloro-2,4-dihydroxypyridine, 1M potassium oxonate (S-1) group.

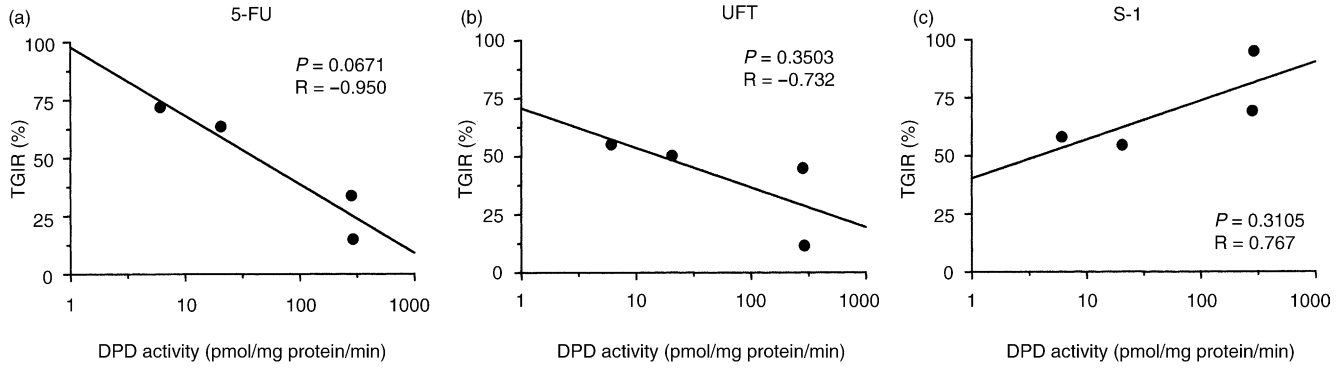

Fig. 3. Correlation between dihydropyrimidine dehydrogenase (DPD) activity and the antitumour activity in four human gastric cancer xenografts. In the 5-fluorouracil (5-FU) and 1M tegafur, 4M uracil (UFT) groups, DPD activity was inversely correlated to antitumour activity. However, there was no correlation in the 1M tegafur, 0.4M 5-chloro-2,4-dihydroxypyridine, 1M potassium oxonate (S-1) group.

differences in TS-IR were observed among the drugs in MKN-28 and MKN-74 xenografts. However, TS-IR in the GCIY and GT3TKB xenografts was significantly higher in the S-1 groups than in the 5-FU or UFT groups. Correlations between the TS-IR and antitumour activity of each drug are shown in Fig. 4. TS-IR was correlated with antitumour activity in the 5-FU and UFT groups (Fig. 4a and b). However, there was no correlation between the antitumour activity and TS-IR in the S-1 group (Fig. 4c).

3.4. F-RNA levels after drug administration

Comparisons of F-RNA level after drug treatment in each xenograft are shown in Table 2. There were no significant differences in F-RNA levels for the drugs used in MKN-28 and MKN-74 xenografts. In GCIY xenografts, the F-RNA level in the S-1 group was slightly higher than in the 5-FU- or UFT-treated group, however, the differences were not statistically significant. In GT3TKB xenografts, the F-RNA level after

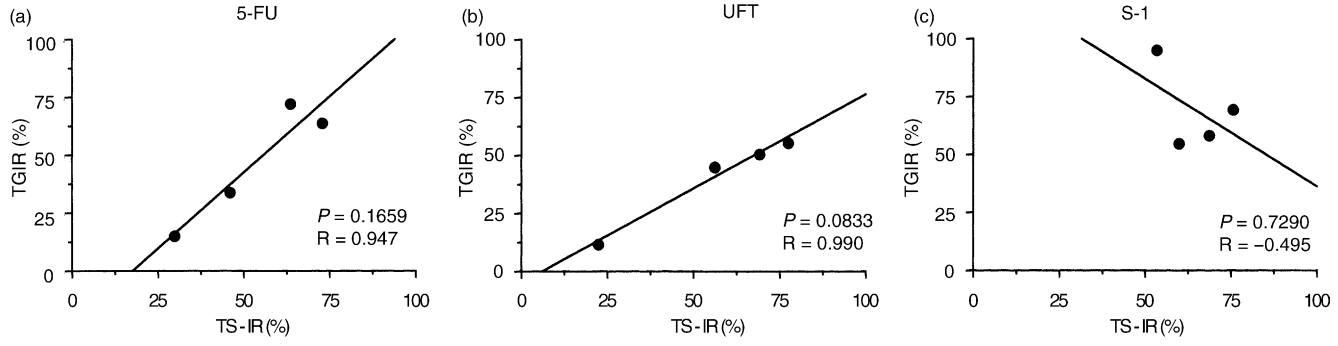

Fig. 4. Correlation between thymidylate synthase inhibition rate (TS-IR) and the antitumour activity of each drug. In the 5-fluorouracil (5-FU) and 1M tegafur, 4M uracil (UFT) groups, TS-IR was correlated to antitumour activity, but there was no correlation between the antitumour activity and TS-IR in the 1M tegafur, 0.4M 5-chloro-2,4-dihydroxypyridine, 1M potassium oxonate (S-1) group.

S-1 administration was significantly higher than that after 5-FU or UFT administration. There was no correlation between the antitumour activity of each drug and the F-RNA levels (data not shown).

4. Discussion

We used human gastric cancer xenografts to investigate the relationship between tumour enzyme activities and the antitumour activity of fluoropyrimidine derivatives that have different levels of DPD inhibition. To confirm the effects of the DPD inhibitors, we compared the relationship between tumour DPD activity and tumour sensitivity to 5-FU (with no DPD inhibition), to UFT (with mild DPD inhibition), and to S-1 (with strong DPD inhibition). In tumours with low DPD activity (MKN-28 and MKN-74), there were no significant differences in the antitumour effects for 5-FU, UFT and S-1.

However, the antitumour activity of UFT and S-1 was more potent against tumours with a high DPD activity (GCIY and GT3TKB) than was 5-FU. The antitumour activity was significantly enhanced in the S-1 group. Comparison of the antitumour effect and tumour DPD activity of each drug revealed an inverse correlation between the tumour sensitivity and DPD activity in the 5-FU and UFT groups. However, no such correlation was observed in the S-1 group. Fujii and colleagues reported that the antitumour activity of UFT was increased by inhibition of DPD activity by uracil [7], but we found no significant difference between the antitumour activities of 5-FU and UFT, and thus the DPD-inhibiting effect of uracil appeared to be limited. By contrast, the antitumour effect of S-1 was much more potent in tumours with a high DPD activity. We suggest that using a potent DPD inhibitor such as CDHP might circumvent the resistance to 5-FU that occurs at high levels of DPD activity, in addition to bringing a pharmacokinetic benefit resulting from the inhibition of 5-FU degradation.

5-FU is well known to have different mechanisms of action, namely, blocking of RNA function by FUTP and inhibition of DNA synthesis by FdUMP and TS [3,4]. To clarify the mechanism of increased sensitivity to fluoropyrimidines caused by the DPD inhibitors, we investigated TS content, TS-IR and F-RNA in the tumours. In the 5-FU and UFT groups, there was an inverse correlation between the antitumour activity and TS content in the tumours, and a positive correlation between antitumour activity and TS-IR. These results suggest that the main mechanism of action of 5-FU and UFT in nude mice is inhibition of DNA synthesis. However, there was no correlation between tumour sensitivity to S-1 and TS content or TS-IR. Significant enhancement of the antitumour activities by S-1 treatment occurred in GCIY and GT3TKB xenografts, and the highest sensitivity to S-1 occurred in GT3TKB, which showed an extremely high TS content. In tumours with both a low TS content and a low DPD activity, no significant differences in the TS-IR or F-RNA levels were observed between the UFT and S-1 groups. These results suggest that the degree of DPD inhibition has no impact on the enhancement of antitumour effect in tumours with both a low TS content and a low DPD activity. In contrast, in tumours with a high DPD activity, TS-IR was significantly higher in the S-1 group than in the 5-FU or UFT group; a marked increase in TS-IR occurred in GCIY, which has a low TS content. This indicates that increasing the TS inhibition enhanced the inhibition of DNA synthesis, possibly by accelerated phosphorylation of 5-FU, resulting in increased FdUMP levels in the tumour owing to inhibition of 5-FU degradation.

TS content or TS-IR should be the major determinant of 5-FU sensitivity when DPD is successfully inhibited. However, there was no correlation between the sensitivity to S-1 and TS content or TS-IR in GT3TKB. Thus, we further evaluated the RNA dysfunction by measuring F-RNA. S-1 gave a significant increase in F-RNA level in GT3TKB xenografts. On the basis of these findings, we speculate that the enhanced antitumour effect of S-1 in GCIY may be due to enhanced inhibition of DNA synthesis, whereas the significant antitumour effect of S-1 in GT3TKB is attributable not only to increased inhibition of DNA synthesis, but also to enhanced blocking of RNA function, as indicated by the increased F-RNA level. Since GT3TKB has a high TS activity, the increase in the FdUMP level was insufficient to increase TS inhibition, in comparison with GCIY, and blocking of RNA function by the increased level of FUTP may have been the predominating mechanism. However, these results came from an experimental model using athymic nude mice. The significance of the results to clinical chemotherapy should be interpreted with caution. It has been reported that the serum thymidine level is approximately 10 times higher in mice than in humans [28]. In addition, the pharmacokinetic behaviour of S-1 in the nude mouse is different from its behaviour in the nude rat and human [18,19]. The pharmacokinetic behaviour of S-1 in the nude mouse resembles bolus injections of 5-FU rather than continuous infusions. When 5-FU is injected as a bolus, blocking of RNA function is considered to be its major mechanism of action [4]. Therefore, it is possible that the dysfunction of RNA is more predominant than TS inhibition following S-1 treatment in this experimental model.

We confirmed the superior antitumour activity of S-1 in a rodent model. Our results suggest that this superior activity is attributable to enhanced inhibition of DNA synthesis and, in part, to blockade of RNA function. This is the first paper to describe the role of both the degradation and phosphorylation pathways in the tumour sensitivity to S-1. The results suggest that tumour TS content and DPD activity are good markers

for predicting enhanced cytotoxicity and for understanding the mechanism of action of S-1. In tumours with a comparably low DPD activity level, inhibition of DPD does not increase cytotoxicity, even if tumour DPD activity is further reduced. The DPD level does not play a role in the cytotoxicity of 5-FU; the TS level was the major determinant. However, a significant increase in the antitumour effect of S-1 is expected in tumours with a high DPD activity. DPD level plays an important role, and TS inhibition becomes even more important as a predictive parameter. Furthermore, tumour TS activity may provide useful information for determining which pathway, DNA inhibition or RNA dysfunction, is predominant after S-1 administration. S-1 is considered the most potent anticancer drug for advanced gastric cancer, alone or in combination with other cytotoxic agents, such as cisplatin [17,29]. Investigation of the enzymes related to metabolism of 5-FU, such as OPRT, ribonucleotide reductase, and thymidine phosphorylase may provide useful information to clarify the mechanism of action of S-1 in greater detail [30].

Acknowledgements

We would like to express our gratitude to Naoko Sasaki and Sachiko Fujiwara for their assistance in completing this manuscript. This work was supported, in part, by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports and Technology.

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