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ORIGINAL ARTICLE

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Reduction of 5-fluorouracil (5-FU) gastrointestinal (GI) toxicity resulting from the protection of thymidylate synthase (TS) in GI tissue by repeated simultaneous administration of potassium oxonate (Oxo) in rats

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Abstract *Purpose*: An important cytotoxic effect of 5-fluorouracil (5-FU) is the inactivation of thymidylate synthase (TS) (EC 2.1.1.45) activity by the formation of a ternary complex consisting of covalently bound 5-fluorodeoxyuridine 5′-monophosphate (FdUMP), TS and 5,10-methylenetetrahydrofolate (CH_2FH_4). The gastrointestinal (GI) toxicity of 5-FU is also caused by its phosphorylation in the GI tract. Potassium oxonate (Oxo) competitively inhibits pyrimidine phosphoribosyltransferase (EC 2.4.2.10), which converts 5-FU to 5-fluorouridine 5′-monophosphate (FUMP) in vitro. In this study the benefits of combining Oxo and tegafur (FT), which is a masked compound of 5-FU, in reducing the GI toxicity of 5-FU and in protecting the activity of TS in the normal GI tissues were evaluated. *Methods*: We administered orally a preparation of 1 *M* FT and 0.4 *M* 5-chloro-2,4-dihydroxypyridine (CDHP) with or without 1 *M* Oxo (called S-1 and FT + CDHP, respectively) or vehicle only (control) to rats for ten consecutive days and compared the toxicity, the histopathological findings and the free TS activity in the GI tissues of the treated rats. *Results*: During the experimental periods, the signs of toxicity, such as a decrease in body weight, diarrhea and death, were only observed in the rats treated with FT + CDHP. The histopathological findings in the ileum and colon samples from rats treated consecutively with S-1 on day 1, day 4, day 7 and day 10 were less frequent and more mild than in the samples from rats treated with FT + CDHP. Furthermore, the free TS activities in the ileum samples of rats given S-1 and FT + CDHP were significantly decreased compared with the activity in samples from the control rats throughout the experimental periods. The free TS activities in GI tissues of rats treated with S-1 were higher than the TS activities in tissues from rats treated with FT + CDHP daily from day 4 to day 10, although activities in S-1-treated rat were decreased to almost same low levels as in FT + CDHP-treated rats on day 1. *Conclusions*: Our results suggest that repeated simultaneous administration of Oxo and FT can effectively protect the activity of TS by decreasing FdUMP via FUMP from 5-FU in GI tissue, and may lead to a reduction in GI toxicity.

Key words S-1 · Potassium oxonate · Thymidylate synthase · Gastrointestinal toxicity · Ileum

Abbreviations *CDHP* 5-chloro-2,4-dihydroxypyridine · CH_2FH_4 5,10-methylenetetrahydrofolate · *CVI* continuous venous infusion · *DPD* dihydropyridine dehydrogenase · *FdUMP* 5-fluorodeoxyuridine 5′-monophosphate · *FT* tegafur · *FT + CDHP* 1 *M* tegafur/0.4 *M* 5-chloro-2,4-dihydroxypyridine · *5-FU* 5-fluorouracil · *GI* gastrointestinal · *HPMC* hydroxypropylmethyl cellulose · *Oxo* potassium oxonate · *S-1* 1 *M* tegafur/0.4 *M* 5-chloro-2,4-dihydroxypyridine/1 *M* potassium oxonate · T_{max} time of maximum concentration · *TS* thymidylate synthase

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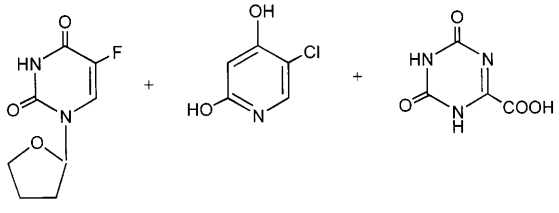
Introduction

5-FU has been used extensively in clinical practice for the treatment of solid tumors [1, 2, 3, 4], either as a

single agent or in combination with other drugs. However, low response rates to intravenous bolus doses of 5-FU and short durations of remission have been observed because of the rapid degradation of 5-FU by the enzyme dihydropyrimidine dehydrogenase (DPD) (EC 1.3.1.2) in the liver. Continuous venous infusion (CVI) of 5-FU has been found to increase the response rates of patients with gastric, colorectal and breast cancer [4, 5, 6, 7, 8, 9, 10, 11] and long-term CVI of 5-FU results in a higher response rate than intravenous bolus administration as an adjuvant chemotherapy of metastatic colorectal cancers [4]. The toxic side effects of 5-FU in patients depend on dose, schedule and route of administration of the drug. For bolus injection, bone marrow toxicity is the dose-limiting toxicity, whereas CVI is limited by gastrointestinal (GI) toxicity [12].

Recently, we have developed S-1, a new antitumor agent based on biochemical modulation of 5-FU, consisting of tegafur (FT), 5-chloro-2,4-dihydroxypyridine (CDHP) and potassium oxonate (Oxo) in a molar ratio of 1:0.4:1 (Fig. 1) [13, 14]. FT, which is a prodrug of 5-FU, acts as an effector, while both CDHP and Oxo, which do not have intrinsic antitumor activity, act as modulators. CDHP competitively inhibits DPD activity, leading to the prolonged retention of 5-FU concentrations in the blood, mimicking the CVI of 5-FU [15], and Oxo competitively inhibits pyrimidine phosphoribosyltransferase, which catalyzes the phosphorylation of 5-FU [16].

It is thought that one of the important cytotoxic effects of 5-FU is the inactivation of TS [2], which is essential for DNA synthesis [17]. Inactivation occurs through the formation of a ternary complex, consisting of covalently bound FdUMP, TS and CH_2FH_4 [18, 19, 20]. In the present study, to evaluate the beneficial effects of Oxo coadministered with FT, we compared the body weight changes, the histopathological findings in the GI tract and the activity of free TS in GI tissues of rats treated consecutively with S-1 with those of FT + CDHP-treated rats.



	FT		CDHP		Oxo
S-1	1	:	0.4	:	1
FT+CDHP	1	:	0.4	:	0

Fig. 1 Chemical structure of the components of S-1 and FT + CDHP

Materials and methods

Drugs

FT, CDHP and Oxo were synthesized by Taiho Pharmaceutical Co. (Tokyo, Japan). [6-^{3}H]-FdUMP (551 GBq/mmol) was enzymatically synthesized from [6-^{3}H]5-fluorodeoxyuridine (New England Nuclear, Boston, Mass.). All other chemicals used were highest grade standard commercial products. S-1 was prepared by mixing FT, CDHP and Oxo at a molar ratio of 1:0.4:1. FT + CDHP was prepared by mixing FT and CDHP at a molar ratio of 1:0.4. S-1 and FT + CDHP were dissolved in 0.5% (w/v) hydroxypropylmethylcellulose (HPMC) solution. Since only FT acts as an effector in both S-1 and FT + CDHP, the dosage is indicated as the FT dose in those preparations.

Animals and treatments

Donryu strain male SPF rats were purchased at 6 weeks of age from Nihon SLC Co., and were randomized into two groups, group A and group B. All in vivo experiments were performed under specific pathogen-free conditions in our laboratory. Rats had free access to tap-water and commercially available chow (CE-2, Clea Japan).

Groups of 20 rats were given S-1 or FT + CDHP at a dose of 20 mg/kg per day once daily for a maximum of 10 days. HPMC solution (0.5%, w/v) was administered to 20 control rats in the same manner. Rats were observed for signs of toxicity and weighed daily. Five rats from each treatment group were killed on day 1, day 4, day 7 and day 10 under ether anesthesia within 6 h of the last treatment. From group A rats, the ileum, the upper side of the cecum (about 20 cm), and colon were removed and used for histopathological study. From group B, the ileum was removed and used to determine the free TS activities. All removed tissues were rinsed with physiological saline and stored at −80 °C until used.

Pathological evaluation of injury to the GI tract

Removed ileum and colon tissues of group A rats were soaked in 10% formaldehyde and then stained for pathological observation by standard methods. After staining with hematoxylin-eosin, the degrees of bleeding, necrosis, glandular expansion, decrease in glandular tubes, loss of cilia, and a degenerative appearance were scored under a light microscope as − (none), + (slight), + + (medium), and + + + (severe). The incidence of GI toxicity was expressed as the number of rats out of five with grade + to + + + GI injury.

Preparation of enzyme solution

The frozen tissues were slowly thawed and pulverized in a microdismembrator (Braun, Melseungen, Germany) in a three times volume of homogenate buffer (200 m*M* Tris-HCl, 20 m*M* 2-mercaptoethanol, 100 m*M* NaF and 15 m*M* cytidine 5′-monophosphate, pH 7.4). During the procedure, all enzyme suspensions were kept on ice (4 °C). Suspensions were centrifuged at 105,000 *g*, and 50 µl of supernatant used for protein assay (Bio-Rad assay, using the method of Bradford [21]). Supernatants were diluted with homogenate buffer to a protein concentration of 5 mg/ml and then aliquoted as enzyme solutions.

Determination of free TS activity

For the ligand binding assay to determine the free TS activity, 300 µl of enzyme solutions were used with [6-^{3}H]-FdUMP as the substrate. To control for non-specific binding of [6-^{3}H]-FdUMP to protein, 300 µl of 5 mg/ml bovine serum albumin (BSA) was also

prepared. The FdUMP ligand-binding assay was performed by the addition of 1.54 pmol/10 µl [6-^{3}H]-FdUMP to the sample solution and 90 µl cofactor solution (50 m*M* potassium phosphate buffer, pH 7.4, 20 m*M* 2-mercaptoethanol, 100 m*M* NaF, 15 m*M* cytidine 5′-monophosphate, 2 m*M* tetrahydrofolic acid, 9 m*M* sodium ascorbate, 9 m*M* formaldehyde, 1.6 m*M* magnesium chloride, 2% BSA). The mixture was incubated at 37 °C for 1 h, and incubation stopped by the addition of 100 µl 2% BSA and 2 ml 1 *N* perchloric acid. The denatured protein pellet was solubilized with 1 ml of a 1:1 mixture of Soluene-350 (Packard, Meriden, Conn.) and 2-propanol, and 0.3 ml 30% H_2O_2 solution was added to the solubilized samples for decolorization. Solubilized samples were added to 13 ml scintillation cocktail (Hionic Fluor; Packard) and radioactivity measured with a liquid scintillation counter (Tri-Carb 1500; Packard) with quenching corrected automatically using an external standard.

Statistical analysis

Data storage and statistical analyses were performed using the commercially available software Statistical Analysis System (SAS Institute, SAS Proprietary Software, Cary, N.C.). Comparison between groups was performed by Dunnett's multiple range test or Tukey's multiple comparison test.

Results

Effect of S-1 and FT + CDHP on body weight

The daily mean body weights of the rats measured throughout the study are shown in Fig. 2. Only slight reductions in body weight gain occurred in S-1-treated rats compared with vehicle-treated rats. In contrast, the body weights of FT + CDHP-treated rats decreased. They were significantly lighter than vehicle-treated rats ($P < 0.01$) by day 3. On day 7, 50% of the FT + CDHP-treated rats had diarrhea and all FT + CDHP-treated rats had diarrhea from day 8, with one rat dying on day 10.

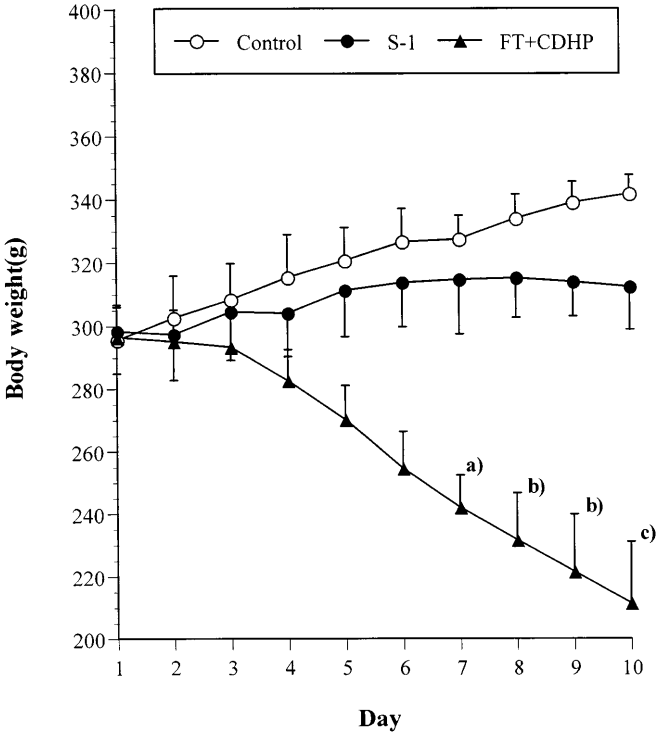


Fig. 2a–c Effect of daily administration (20 mg/kg) of S-1 and FT + CDHP on the body weight of rats. The graph shows the mean body weights and standard deviation (*bars*) of rats treated with vehicle, S-1 or FT + CDHP during the experimental period. Each value is the mean and standard deviation of body weights of 20 rats (day 1), 15 rats (days 2, 3, 4), 10 rats (days 5, 6, 7) and 5 rats (days 8, 9, 10). Five rats had diarrhea on days 7, 8 and 9. One rat died and all the remainder had diarrhea on day 10. Significant differences compared to vehicle-treated rats were observed in the FT + CDHP- ($P < 0.01$) and S-1-treated rats ($P < 0.05$) after day 3 and day 9, respectively. **a** Ten rats (five had diarrhea); **b** five rats (all had diarrhea); **c** four rats (one died and the remainder had diarrhea)

Effect of S-1 and FT + CDHP on pathological findings in ileum and colon samples

The histopathological findings in the ileum and colon samples of group A rats are shown in Table 1. There were no abnormal findings in the samples from rats given vehicle only throughout the experiment. In FT + CDHP-treated rats, a slight karyorrhexis in cryptal epithelial cells was apparent in the ileum and colon samples as early as day 1, with a slight decrease of goblet cells and dilatation of crypts apparent in samples of both ileum and colon on day 4. More severe findings were observed in proportion to increasing FT + CDHP-doses. In contrast, a slight decrease in goblet cells in ileum samples and a slight dilatation of crypts in colon samples were noted in S-1-treated rats on day 4 and moderate grade findings were observed day 10. The histopathological findings in rats that had received S-1 were consistently less severe than in rats that had received FT + CDHP.

Effects of S-1 and FT + CDHP on the activity of TS

The relative changes in the activity of TS in the ileum tissue of rats that had received S-1, FT + CDHP or vehicle only for several consecutive days are shown in Fig. 3. The free TS activities in S-1- and FT + CDHP-treated rats were significantly lower than in vehicle-treated rats throughout the experimental period. On day 1 the free TS activity in rats that had received S-1 did not significantly differ from the activity in rats that received FT + CDHP (8.58 ± 0.65 and 7.64 ± 1.46 pmol/g wet tissue, respectively). However, on day 4, day 7 and day 10 the free TS activities in S-1-treated rats were significantly increased compared with the activities in FT + CDHP-treated rats. The free TS activity in FT + CDHP-treated rats remained low at about 8 pmol/g wet tissue throughout the experimental period. In S-1-treated rats, the activity was temporarily decreased to 8.58 pmol/g wet tissue on day 1, but had recovered to the levels observed on day 1 in untreated rats by day 4 and levels of about 11 pmol/g wet tissue were

Table 1 Histopathological findings in ileum and colon tissue of rats of group A during the experimental period. Drugs were administered once daily consecutively for a maximum of 10 days. On days 1, 4, 7 and 10 ileum and colon tissue were removed 6 h after each treatment and pathological examination was accomplished as described in Materials and methods. The values shown are the numbers of rats with that toxicity grade (– negative, + slight, + + moderate, + + + severe)

Day	Tissue	Findings	Toxicity grade											
			Control group				S-1-treated group				FT + CDHP-treated group			
			–	+	+ +	+ + +	–	+	+ +	+ + +	–	+	+ +	+ + +
1	Ileum	Karyorrhexis in cryptal epithelial cells	5	0	0	0	5	0	0	0	1	4	0	0
	Colon	Karyorrhexis in cryptal epithelial cells	5	0	0	0	5	0	0	0	4	1	0	0
4	Ileum	Decrease in goblet cells	5	0	0	0	1	4	0	0	2	3	0	0
		Dilatation of crypt	5	0	0	0	5	0	0	0	3	2	0	0
		Karyorrhexis in cryptal epithelial cells	5	0	0	0	5	0	0	0	0	5	0	0
		Swelling of nucleus of epithelial cells	5	0	0	0	5	0	0	0	0	5	0	0
		Atrophy of mucosa	5	0	0	0	5	0	0	0	4	1	0	0
	Colon	Decrease in goblet cells	5	0	0	0	5	0	0	0	0	5	0	0
		Dilatation of crypt	5	0	0	0	4	1	0	0	2	3	0	0
		Karyorrhexis in cryptal epithelial cells	5	0	0	0	5	0	0	0	4	1	0	0
7	Ileum	Decrease in goblet cells	5	0	0	0	0	5	0	0	0	0	5	0
		Dilatation of crypt	5	0	0	0	3	2	0	0	0	5	0	0
		Karyorrhexis in cryptal epithelial cells	5	0	0	0	0	5	0	0	0	0	5	0
		Swelling of nucleus of epithelial cells	5	0	0	0	0	5	0	0	0	0	5	0
		Atrophy of mucosa	5	0	0	0	5	0	0	0	0	5	0	0
	Colon	Decrease in goblet cells	5	0	0	0	4	1	0	0	0	2	1	2
		Dilatation of crypt	5	0	0	0	5	0	0	0	0	3	1	1
		Karyorrhexis in cryptal epithelial cells	5	0	0	0	5	0	0	0	1	3	1	0
		Swelling of nucleus of epithelial cells	5	0	0	0	1	4	0	0	0	1	4	0
		Atrophy of mucosa	5	0	0	0	5	0	0	0	0	2	1	2
10	Ileum	Decrease in goblet cells	5	0	0	0	0	0	5	0	0	0	4	0
		Dilatation of crypt	5	0	0	0	5	0	0	0	1	3	0	0
		Karyorrhexis in cryptal epithelial cells	5	0	0	0	0	5	0	0	0	1	3	0
		Swelling of nucleus of epithelial cells	5	0	0	0	0	5	0	0	0	0	4	0
		Atrophy of mucosa	5	0	0	0	5	0	0	0	0	3	1	0
	Colon	Decrease in goblet cells	5	0	0	0	4	1	0	0	0	0	2	2
		Dilatation of crypt	5	0	0	0	5	0	0	0	0	1	2	1
		Karyorrhexis in cryptal epithelial cells	5	0	0	0	3	2	0	0	0	4	0	0
		Swelling of nucleus in epithelial cells	5	0	0	0	0	5	0	0	0	0	3	1
		Atrophy of mucosa	5	0	0	0	5	0	0	0	0	0	4	0

maintained after day 10. [6-^{3}H]-FdUMP did not show any binding to BSA (data not shown).

Discussion

One of the important cytostatic effects of 5-FU is mediated through the formation of the phosphorylated metabolite, FdUMP, which together with CH_2FH_4 binds to TS to form a stable ternary complex, resulting in significant enzyme inhibition [18, 19, 20]. The GI toxicity of 5-FU is also related to its phosphorylation in the GI tract [22], which is strongly inhibited by Oxo [16]. In this investigation, we examined the beneficial effects of Oxo coadministered with 5-FU derivatives, FT by comparing toxic signs, histopathological findings in the GI tract and relative changes in free TS activities in GI tissues after daily administration of S-1 or FT + CDHP for 10 days.

As a sign of toxicity, only slight reductions in body weight gain were observed in S-1-treated rats compared with vehicle-treated rats, while FT + CDHP treatment decreased body weight gain after day 3. Death or intestinal disorders such as diarrhea were observed only in

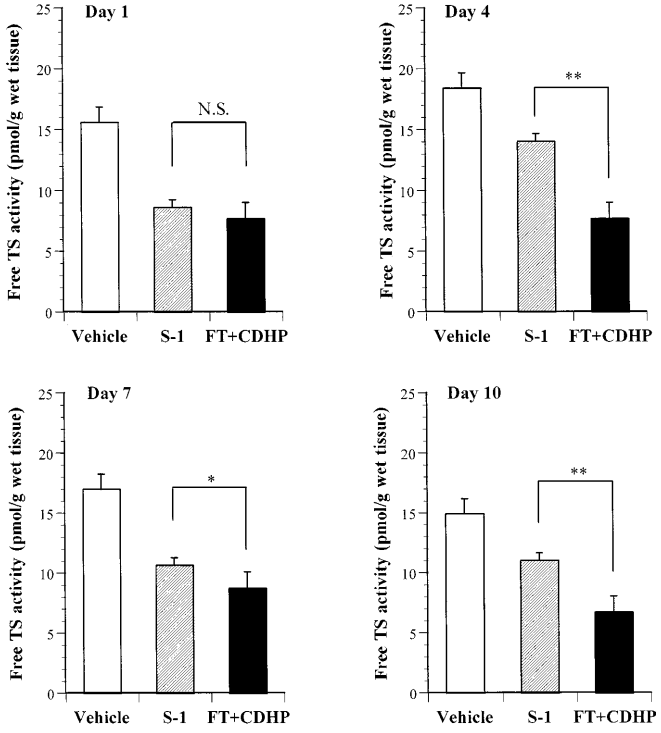


Fig. 3 The relative changes in the free TS activities in rat ileal tissue after daily administration of vehicle (*open bars*), S-1 (*hatched bars*) and FT + CDHP (*black bars*). The data were determined on day 1, day 4, day 7 and day 10 as described in Materials and methods. The values are the means and standard deviation (*bars*) of individual samples from five rats (the day-10 data were derived from four rats, as one had died). There are significant difference between the vehicle-treated group and drug-treated groups ($P < 0.01$) throughout the experimental period. $*P < 0.05$, $**P < 0.01$, S-1-treated group vs FT + CDHP-treated group (*N.S.* not significant)

FT + CDHP-treated rats after day 7. Furthermore, in vehicle-treated rats no pathological injury was found in ileum or colon samples throughout the experimental period, but such injury was found in S-1- and FT + CDHP-treated rats. However, the histopathological findings in the ileum and colon samples after administration of S-1 were less severe than after FT + CDHP administration. The severity of the pathological findings coincided with that of the clinical signs such as reduction in body weight gain in the same animals.

Furthermore, to investigate the reason for the differences in the toxicological findings and signs, we evaluated the relative changes in free TS activity during the experimental period, although the increase in total TS activity following 5-FU administration [23, 24] is less than spectacular. Some antineoplastic agents cause morphological changes in the small intestine, especially the ileum, that lead to anorexia and diarrhea [25], and high concentrations of FdUMP have been observed in the ileum after 5-FU administration [22]. We therefore focused on the ileum to determine the relative changes in free TS activity after administration of S-1, FT + CDHP or vehicle. The free TS activities in the ileum of both S-1- and FT + CDHP-treated rats were significantly lower than in the samples from vehicle-treated rats throughout the study, which suggests that the ternary complexes were formed in the GI tissues of both S-1- and FT + CDHP-treated animals.

On day 1 S-1 decreased the free TS activity in GI tissue to levels as low as those observed in FT + CDHP-treated rats in spite of containing Oxo. We estimate that Oxo could not completely have inhibited the phosphorylation of 5-FU as high concentrations and prolonged retention of 5-FU were observed in the blood after administration of S-1, because S-1 contained CDHP which competitively inhibits 5-FU degradation. The time of maximum concentration (T_{max}) of 5-FU in the blood after administration of S-1 was found to be about 2 h [13, 14] and FdUMP has been observed immediately after 5-FU administration [26]. The T_{max} of Oxo in small and large intestines after oral administration of Oxo are about 2 h and 8 h, respectively [16]. It is possible that concentrations 2 h after a single administration of S-1 are not sufficient to allow Oxo to distribute over the entire ileum tissue, so 5-FU phosphorylation could not totally be inhibited, because the ileum is the distal site of the small intestine. In other words, it is possible that the TS in the ileum tissue had already been exposed to FdUMP before enough Oxo was present to form the ternary complexes after a single administration of S-1. We suggest that this is the reason why the free TS activity in ileum tissue decreased on day 1 in S-1-treated rats to similar levels as in tissue from FT + CDHP-treated rats.

In subsequent days, the free TS activities in FT + CDHP-treated rats remained low at about 8 pmol/g wet tissue throughout the experimental period, whereas in S-1-treated rats, levels had recovered to untreated levels by day 4 and were maintained at levels of around 11 pmol/g wet tissue after day 7. Thus, free TS activities were significantly higher in S-1-treated rats compared to FT + CDHP-treated rats on day 4, day 7 and day 10. It is possible that Oxo would be sufficiently distributed throughout the ileum tissue by repeated administration so that some of the previously administered Oxo still remains [16] at the next administration.

It is also necessary for the cytostatic effects of 5-FU to maintain the TS inhibition [27], so we considered that the reason FT + CDHP-treated rats showed more severe toxicity than S-1-treated rats was because the free TS activities in the FT + CDHP-treated rats were significantly lower than in S-1-treated rats during the experimental period. Thus, the simultaneous Oxo treatments with FT were then able to protect the free TS activity, which allowed inhibition of the conversion of 5-FU to FdUMP. We conclude that repeated simultaneous administration of Oxo with FT is an effective treatment to protect free TS activity by decreasing FdUMP via FUMP from 5-FU in GI tissue.

These results suggest that Oxo reduces 5-FU cytotoxicity in the GI tract resulting in a reduction in intestinal disorders as a result of continuous protection of

free TS activity in GI tissue over several days. In conclusion, our results indicate that repeated simultaneous administration of Oxo with FT protected free TS activity in GI tissue by decreasing the formation of the ternary complex that results from inhibition of 5-FU phosphorylation, leading to reduced 5-FU cytotoxicity in the GI tract.

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