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Comparative Antitumor Activity and Intestinal Toxicity of 5′-Deoxy-5-fluorouridine and Its Prodrug Trimethoxybenzoyl-5′-deoxy-5-fluorocytidine

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N^4-Trimethoxybenzoyl-5′-deoxy-5-fluorocytidine (Ro 09-1390), a prodrug of the cytostatic 5′-deoxy-5-fluorouridine (5′-DFUR), was synthesized with the aim of reducing of the dose-limiting toxicity of 5′-DFUR, which is diarrhea. In mice bearing Lewis lung carcinoma, 5′-DFUR given po produced a substantial amount of 5-fluorouracil (5-FU) in the intestinal tract as well as in tumors, where the enzyme pyrimidine nucleoside phosphorylase, essential for conversion of 5′-DFUR to 5-FU, is predominantly located. With the oral administration of Ro 09-1390 only a small amount of 5-FU was formed in the intestine; however, the administration of Ro 09-1390 and 5′-DFUR at the same dose produced similar amounts of 5-FU in tumor tissues. These differences in metabolism were reflected in their toxicity and antitumor efficacy. The administration of 5′-DFUR resulted in damage to the intestinal mucosal membrane and diarrhea in normal mice, whereas Ro 09-1390 was much less toxic to the intestinal tract. As regards antitumor activity, Ro 09-1390 and 5′-DFUR at equivalent doses inhibited the growth of Lewis lung carcinoma to similar extents. Since Ro 09-1390 was much less toxic to the intestinal tract than 5′-DFUR, mice bearing Lewis lung carcinoma could be given Ro 09-1390 daily over a longer period and at a higher dose, resulting in a longer survival time.

Key words: Antitumor activity — Intestinal toxicity — Trimethoxybenzoyl-5′-deoxy-5-fluorocytidine (Ro 09-1390) — 5′-Deoxyfluorocytidine — 5′-Deoxy-5-fluorouridine

5′-Deoxy-5-fluorouridine (5′-DFUR) is an orally available cytostatic agent, that is currently being used clinically for the treatment of patients with stomach, colorectal and breast cancers.[1,2] The dose-limiting toxicity of this drug is intestinal toxicity, namely diarrhea.[2–4] 5′-DFUR is a prodrug that is converted to 5-fluorouracil (5-FU) by pyrimidine nucleoside phosphorylases.[5] The enzyme activity in mice bearing transplantable tumors was found to be predominantly localized in the tumors but not in normal tissues, except for the intestinal tract.[5,6] Consequently, 5′-DFUR was efficiently converted to 5-FU in the tumors and to some extent in the intestine after its oral administration.[7] The efficient conversion of 5′-DFUR to 5-FU in tumor tissue accounts for its potent antitumor activity.[5,6]

Aiming at the reduction of intestinal toxicity, we have attempted to design prodrugs of 5′-DFUR which would produce 5′-DFUR after they pass through the intestinal mucosal membrane. We have synthesized many compounds which could be convertible to 5′-DFUR. Among them, we have selected 5′-deoxyfluorocytidine (5′-DFCR)-related compounds from which 5′-DFUR is generated by the action of cytidine deaminase as candidate drugs for further evaluation. 5′-DFCR is not susceptible to pyrimidine nucleoside phosphorylase and is converted to 5′-DFUR by cytidine deaminase,[8] which is located mainly in the liver and only to a small extent in the intestinal tract of humans.[9] However, the expected improvement of intestinal toxicity in mice was not found with this compound, because 5′-DFCR is retained in the intestinal tract for a long period and so is converted to 5′-DFUR and 5-FU there. Thus, we have selected N^4-trimethoxybenzoyl-5′-deoxy-5-fluorocytidine (Ro 09-1390) as the best candidate among the various 5′-DFCR-related compounds synthesized. In the present study we compared the antitumor activity and intestinal toxicity of Ro 09-1390 in mice with those of 5′-DFUR, 5-FU and UFT (a mixture of tegafur and uracil). 5-FU is the active metabolite of Ro 09-1390, while UFT is most frequently prescribed for treatment of cancer patients in Japan among 5-FU derivatives. The results clearly indicate that Ro 09-1390 has a much higher safety margin than 5′-DFUR, 5-FU and UFT.

MATERIALS AND METHODS

Animals Male C57BL/6, BDF_1 (C57BL/6×DBA/2)F_1 and CDF_1 (BALB/c×DBA/2)F_1 mice, aged 4 weeks, were obtained from Japan SLC, Inc., Hamamatsu. The mice were used after at least one week of observation.

Tumor cells Lewis lung carcinoma and colon 26 adenocarcinoma cells were supplied by the Cancer Chemotherapy Center, Japanese Foundation for Cancer Research, Tokyo. Lewis lung carcinoma cells were maintained by continuous passage in syngeneic C57BL/6

mice, and a suspension of the tumor cells (10^6 cells) was inoculated subcutaneously into BDF_1 mice. Colon 26 cells were passaged *in vitro* with Eagle's minimum essential medium (MEM) containing 10% fetal calf serum, penicillin G (50 U/ml) and streptomycin sulfate (100 μg/ml). A suspension of the tumor cells was prepared by the trypsinization of the monolayered cells and 10^6 cells were inoculated subcutaneously into CDF_1 mice.

Cytostatics 5-Fluorouracil (5-FU) and UFT (a mixture of tegafur and uracil (1:4)) were purchased from Kyowa Hakko Co. (Tokyo) and Taiho Pharm. Co. (Tokyo), respectively. Doses of UFT are expressed as those of tegafur. 5′-Deoxy-5-fluorouridine (doxifluridine; 5′-DFUR) was synthesized by Hoffmann-La Roche (Basel) while 5′-deoxy-5-fluorocytidine (5′-DFCR) and N^4-trimethoxybenzoyl-5′-deoxy-5-fluorocytidine (Ro 09-1390) were synthesized by Nippon Roche K.K. (Tokyo) as described elsewhere.[10] Ro 09-1390 is easily hydrolyzed to 5′-DFCR in an artificial gastric juice (pH 1.2) but not in a solution at a pH greater than 4.0. Therefore, all the compounds were dissolved or suspended in 40 m*M* citrate buffer (pH 6.0) containing 5% gum arabic as a vehicle and administered by the po route.

Measurement of metabolites Fifteen days after the tumor inoculation, mice were administered 1 mmol/kg of 5′-DFUR or Ro 09-1390 by the po route. At the indicated time, the intestinal tract and tumor tissues were resected from mice bearing Lewis lung carcinoma or colon 26 after a single drug treatment. The drugs that remained in the intestinal tract were washed out with ice-cold saline. The tissues and blood were homogenized with 4 volumes of ice-cold saline and acetonitrile (1:1) by using a Hiscotron homogenizer. An equal volume of acetonitrile was added to the supernatant of the homogenate, and the drugs and their metabolites were extracted. The content of the active metabolite 5-FU in the extract was measured by a bioassay method.[11] The contents of Ro 09-1390 and its major metabolites 5′-DFCR and 5′-DFUR were measured by an HPLC method with a column of YMC-pack A-312 ODS (Yamamura Ltd., Tokyo). For the measurement of Ro 09-1390, 5′-DFCR and 5′-DFUR, elution buffers of acetonitrile and 0.1 *M* phosphate buffer (pH 6.5) were used at the ratio of 3:7 for the parent drug and 4:96 for the metabolites.

Measurement of toxicity to the intestinal tract The toxicity of the drugs was estimated by two different methods. The feces from normal mice receiving daily drug treatment were observed. The intestinal toxicity was based on the character of the feces or diarrhea and scored as follows; −: normal feces, +: slightly loose, ++: loose, and +++: diarrhea. At the end of the treatment, we measured the occult blood in the feces by using the test kit of Shionogi Pharma Co. (Osaka). As the other method for measuring the intestinal toxicity, we utilized a simple and convenient technique for examining the tissue pathologically as described elsewhere.[12] The intestinal tract of the treated mice was resected, and the contents were washed out by using a syringe. The tract was then fixed with phosphate-buffered saline solution containing 10% formalin. The duodenum at about 2 cm below the pylorus was cut into thin circular slices (0.3–0.5 mm thickness), and tissue damage was observed pathologically.

Measurement of tumor volume The body weight of the mice, and the length (a) and width (b) of the tumor were measured twice a week. The tumor volume was estimated by using the equation, $V=ab^2/2$.

Statistical analysis Differences in the growth and survival of tumor-bearing mice were compared by using the SAS life test. Differences were considered to be significant when the probability (P) value was <0.05.

RESULTS

Metabolites in the intestine, blood and tumor 5′-DFUR is known to be converted to 5-FU, while the major

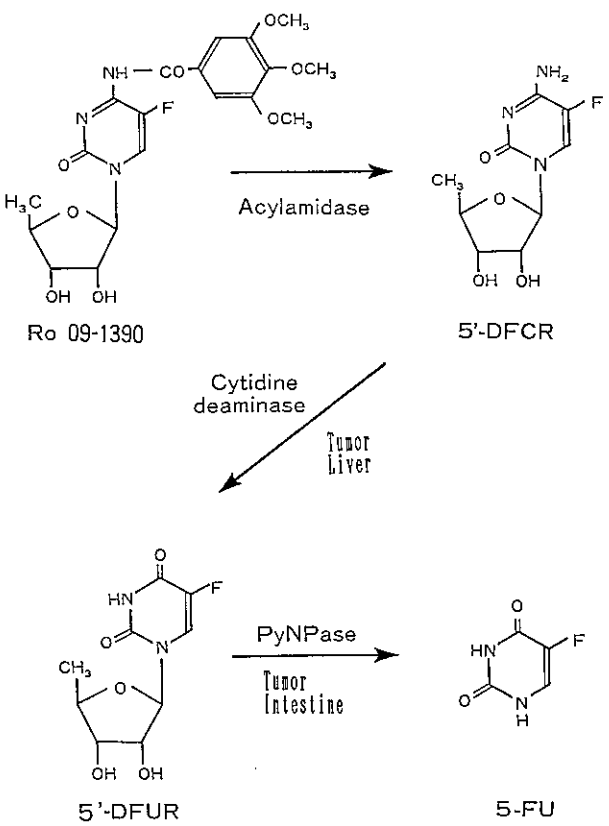

Fig. 1. Metabolic pathway of Ro 09-1390.

metabolites of Ro 09-1390 are 5′-DFCR, 5′-DFUR and 5-FU (Fig. 1). We measured the contents of these metabolites in the small intestine, blood and tumors after oral administration of 5′-DFUR and Ro 09-1390 to mice implanted with Lewis lung carcinoma (Fig. 2) and colon 26 (Fig. 3) two weeks before. As Fig. 2 shows, a substantial amount of 5′-DFUR was converted to 5-FU in the intestinal tract, but most of the 5′-DFUR remained unchanged and some was later taken up by the tumor tissues after its intestinal absorption. On the other hand,

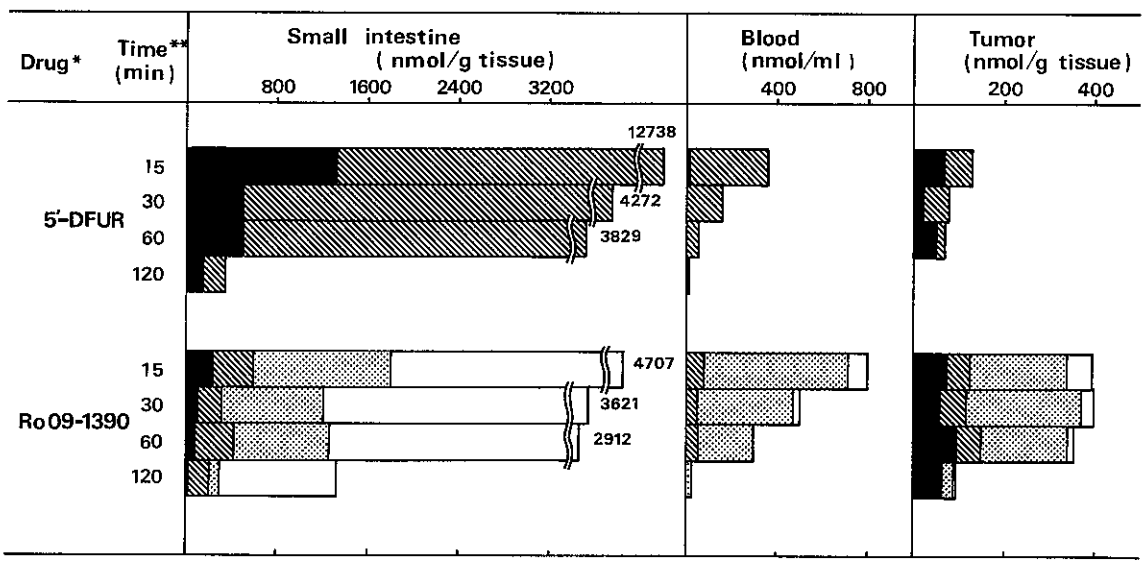

Fig. 2. Metabolites of Ro 09-1390 and 5′-DFUR in the intestine, blood and tumors of mice bearing Lewis lung carcinoma. BDF_1 mice were inoculated with Lewis lung carcinoma (10^6 cells, sc), and 2 weeks later, the mice were orally given Ro 09-1390 and 5′-DFUR at 1 mmol/kg. ▒, 5-FU; ▨, 5′-DFUR; ⁘, 5′-DFCR; □, Ro 09-1390. *, 1 mmol/kg po; **, time after drug administration.

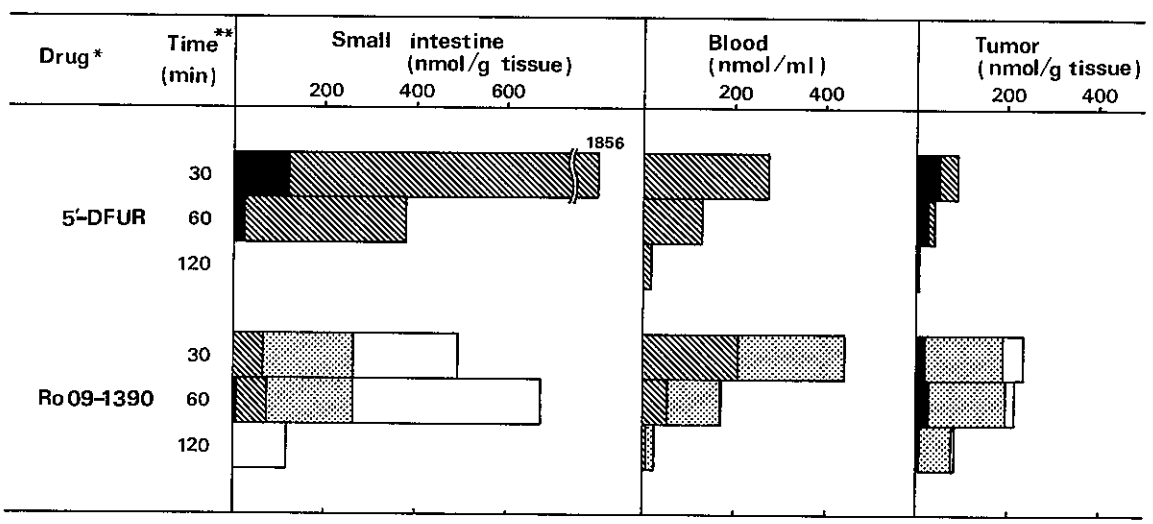

Fig. 3. Metabolites of Ro 09-1390 and 5′-DFUR in the intestine, blood and tumors of mice bearing colon 26. CDF_1 mice were inoculated with colon 26 adenocarcinoma (10^6 cells, sc), and 2 weeks thereafter the mice were orally given Ro 09-1390 or 5′-DFUR at 1 mmol/kg. ▒, 5-FU; ▨, 5′-DFUR; ⁘, 5′-DFCR; □, Ro 09-1390. *, 1 mmol/kg po; **, time after drug administration.

the mice that were administered Ro 09-1390 had lower amounts of 5-FU and 5′-DFUR in the intestine, but 5-FU was produced in tumor tissues to a somewhat greater degree than 5′-DFUR. In blood, the major component was 5′-DFUR after 5′-DFUR administration and 5′-DFCR after Ro 09-1390 administration, and only a minute amount of 5-FU was detected in either case. Similar results were also obtained in mice bearing colon 26 adenocarcinoma (Fig. 3).

When the levels of 5-FU found in various parts of the gastrointestinal (GI) tract after the administration of 5′-DFUR and Ro 09-1390 were compared, more 5′-DFUR was converted to 5-FU throughout the GI tract up to the large intestine, compared with Ro 09-1390 (Fig. 4).

Intestinal toxicity The intestinal toxicities caused by the oral administration of 5′-DFUR, 5-FU and Ro 09-1390 in mice are compared in Fig. 5. The toxicities were based on the character of the feces and occult blood in the feces. Treatment of BDF_1 mice with 5′-DFUR daily for 7 days by the po route caused diarrhea or loose feces and the degree of occult blood observed in the feces was greater at larger doses. The intestinal toxicity caused by 5′-DFUR was observed approximately 6 days after the start of the treatment, and 5′-DFUR was toxic to the intestinal tract at doses over 1 mmol/kg/day. 5-FU given by the po route was also toxic at a dose of 0.4 mmol (52 mg)/kg/day, a sublethal dose. On the other hand, Ro 09-1390 was only slightly toxic at a much higher dose of 6 mmol/kg/day, indicating that even if a six-fold greater molar amount is administered it is safer than 5′-DFUR in terms of the intestinal toxicity.

Fig. 6 shows sections of the small intestine of mice treated with 5-FU (0.4 mmol/kg), 5′-DFUR (3 mmol/kg) and Ro 09-1390 (3 mmol/kg) daily for 7 days. 5′-DFUR caused severe damage to the intestinal villi. It changed the overall appearance of the villi from smooth to rough, and shortened them by 30–40%. Ro 09-1390 was less toxic and caused no significant damage to the villi.

Antitumor activity The antitumor activity of Ro 09-1390, 5′-DFUR, UFT and 5-FU was examined in mice bearing advanced Lewis lung carcinoma. In the experiment shown in Table I, drugs were administered daily for ten days to mice that had been implanted with the tumor

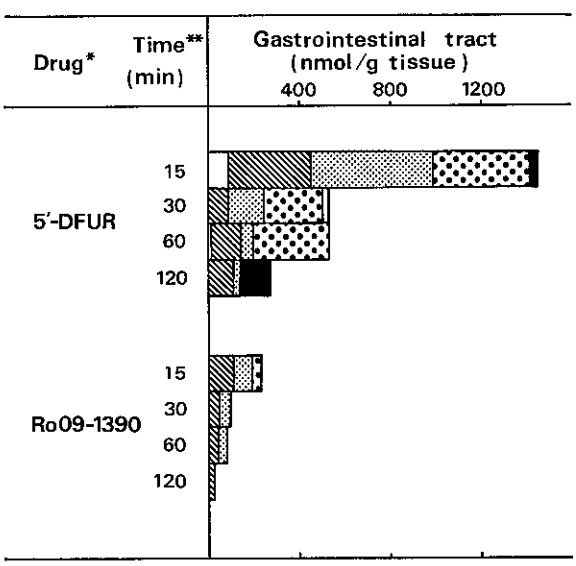

Fig. 4. The concentration of 5-FU in various parts of the gastrointestinal tract of mice bearing Lewis lung carcinoma. The experiment was the same as in Fig. 2, but the gastrointestinal tract was subdivided as follows; ▭, stomach; ▨, duodenum; ▩, jejunum; ▩, ileum; ▩, large intestine. *, 1 mmol/kg po; **, time after drug administration.

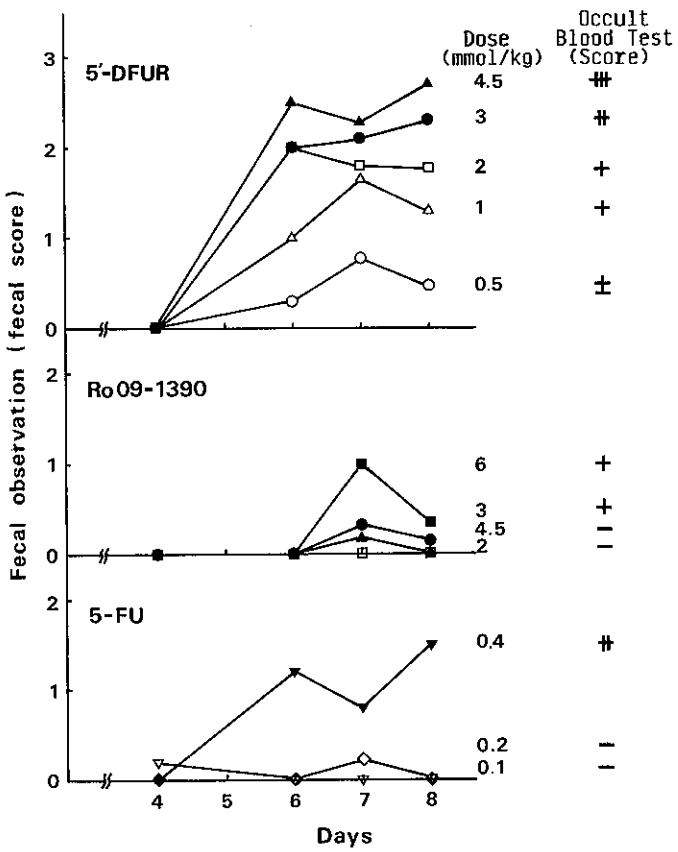

Fig. 5. Intestinal toxicity of 5′-DFUR, Ro 09-1390 and 5-FU (po) in mice. Drugs at various doses as indicated were given to BDF_1 mice daily for 7 days by the po route. The character of the feces was observed daily, while the feces were examined for occult blood on day 8. Doses of drugs (mmol/kg): ■, 6; ▲, 4.5; ●, 3; □, 2; △, 1; ○, 0.5; ▼, 0.4; ◇, 0.2; ▽, 0.1.

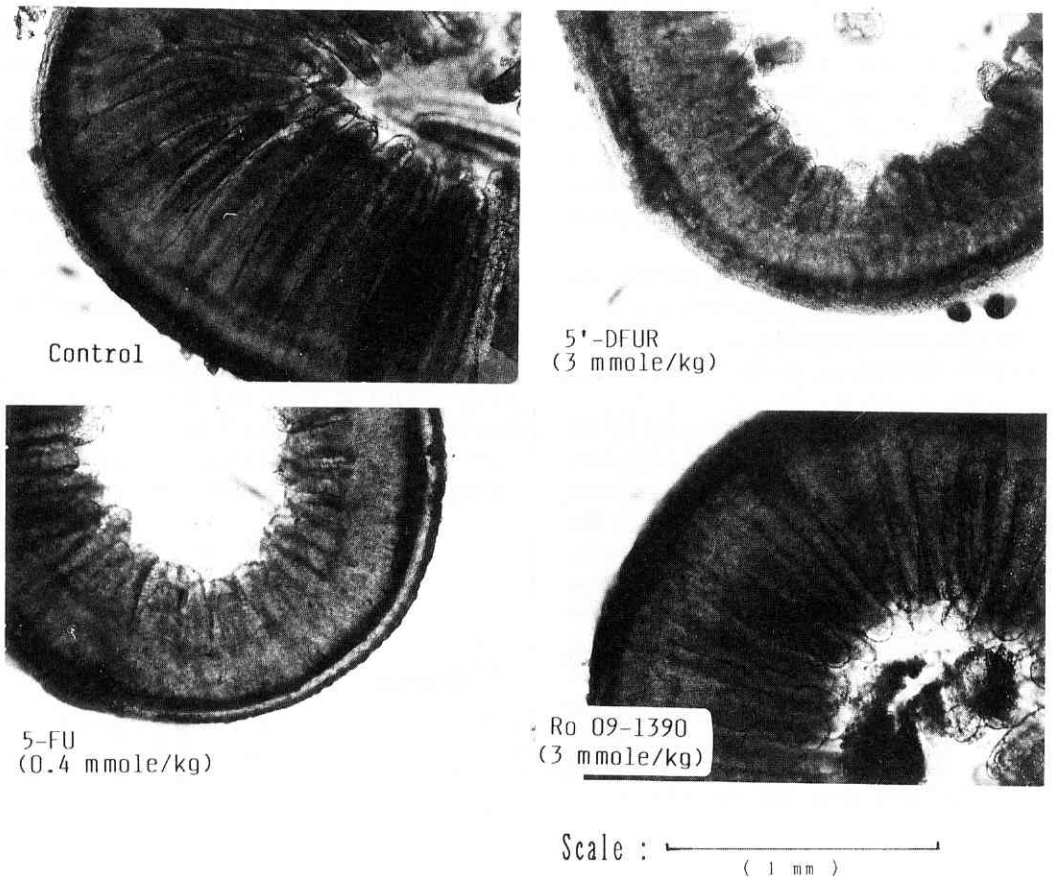

Fig. 6. Morphology of the small intestinal villi of mice administered with 5′-DFUR, Ro 09-1390 and 5-FU. Drugs were given to BDF_1 mice daily for 7 days by the po route.

Table I. Antitumor Activities of Fluorinated Pyrimidine Derivatives on Lewis Lung Carcinoma in Mice

Drugs	Dose (mmol/kg/day)	Tumor vol. change (mm^3: day 20–day 10)	Survival (day 20)	Body wt. change (g: day 20–day 10)	Intestinal toxicity[a)]
Control		6,900	4/6	5.5	−
Ro 09-1390	0.5	1,920	6/6	0.7	−
	1.0	500	6/6	2.1	−
	2.0	−130	6/6	1.0	−
	3.0	−920	4/6	−3.1	+
5′-DFUR	0.5	2,500	6/6	0.4	±
	1.0	1,050	6/6	1.5	+
	2.0	−330	5/6	−3.5	++
5-FU	0.2	4,990	5/5	1.7	−
	0.4	850	3/5	−6.0	+++
UFT	0.1	4,500	5/5	1.3	−
	0.2	760	1/5	−6.2	+++

a) Fecal observation (fecal score).

10 days before. Ro 09-1390 inhibited the growth of the tumor over a wider dose range than the other drugs and regressed the tumor size at higher doses of 2 and 3 mmol/kg/day. Concerning toxicity, Ro 09-1390 caused weight gain reduction and loose feces at the highest dose. 5′-DFUR was somewhat less effective in terms of antitumor activity at equimolar doses and more toxic to the intestinal tract than Ro 09-1390 was. 5-FU and UFT had antitumor activity only at toxic doses. When comparing therapeutic indices, Ro 09-1390 is the most effective antitumor agent among the compounds tested. The values of the therapeutic index, the minimum dose causing intestinal toxicity/the minimum dose inhibiting tumor growth by 50% (ED_{50}), of Ro 09-1390, 5′-DFUR, 5-FU and UFT were >6, 2, 1 and 1, respectively. The values of another parameter of therapeutic effectiveness, the minimum dose causing weight gain reduction/ED_{50}, were >6, 4, 1 and 1, respectively.

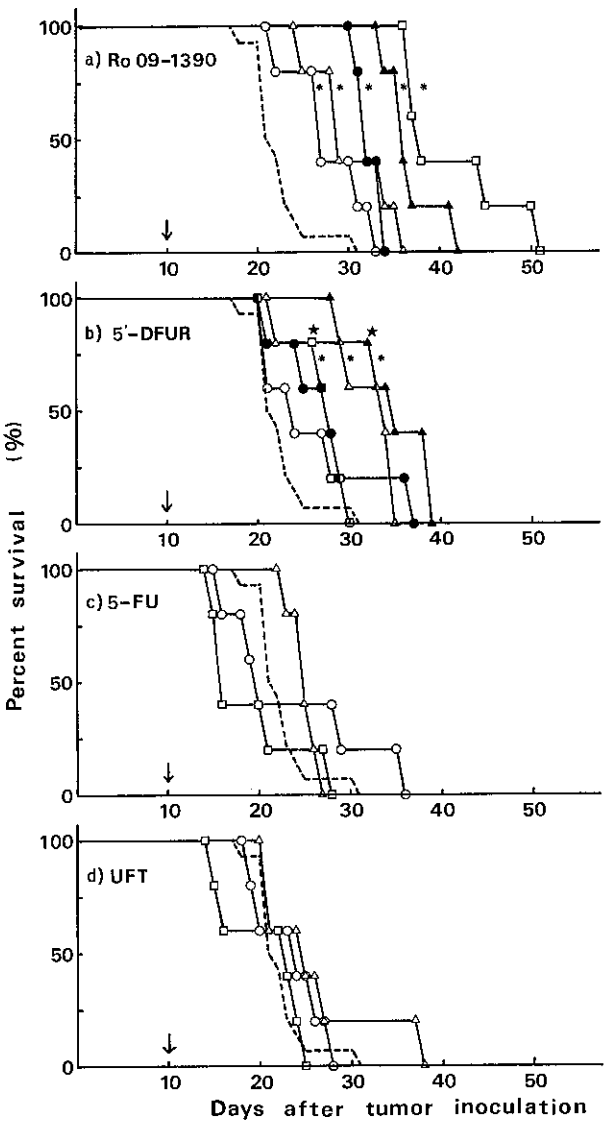

Fig. 7. Increase of survival by 5′-DFUR, Ro 09-1390, 5-FU, and UFT in mice bearing advanced Lewis lung carcinoma. BDF_1 mice were inoculated with Lewis lung carcinoma (10^6 cells, sc) on day 0. Thereafter, the mice were daily given drugs by the po route, beginning at day 10, until they died. Dose of drugs were as follows (mmol/kg). a) and b): □, 2.0; ▲, 1.33; △, 0.89; ●, 0.59; ○, 0.4. c): □, 0.3; △, 0.15; ○, 0.08. d): □, 0.2; △, 0.1; ○, 0.05. ·····, Control (vehicle). ★ Doses at which the mice showed loose feces or diarrhea. ↓ Start of drug treatment. * $P<0.05$.

Fig. 7 shows the antitumor activity of the same antitumor agents in terms of the increase of survival. In this experiment the agents were given orally to mice bearing Lewis lung carcinoma from day 10 after the tumor implantation, and the treatment was continued daily as long as the mice survived. Ro 09-1390 significantly increased the survival of the mice at all the doses tested (0.4 to 2.0 mmol/kg) without showing intestinal toxicity or loose feces. On the other hand, 5′-DFUR was effective at limited doses ranging from 0.59 to 1.33 mmol/kg but caused weight loss at 2 mmol/kg and intestinal toxicity at 1.33 mmol/kg. 5-FU and UFT showed only marginal activity even at the optimal doses. The values of the therapeutic index, the maximum dose increasing the survival (ED_{max})/the minimum dose increasing the survival by 20% (ED_{20}), of Ro 09-1390 and 5′-DFUR were 5 and 2.25, respectively, while 5-FU and UFT were ineffective.

DISCUSSION

5′-DFUR was reported to have a tumor-selective action.[5] Its antitumor efficacy was higher than those of 5-FU, 2′-dFUrd (2′-deoxy-5-fluorouridine) and tegafur in various tumor models, particularly in terms of therapeutic indices.[5, 13, 14] However, at higher doses 5′-DFUR caused intestinal toxicity in mice,[12] and diarrhea was the dose-limiting factor in clinical trials.[3, 4] 5-FU is generated from 5′-DFUR by pyrimidine nucleoside phosphorylase, mainly by uridine phosphorylase in mice and by thymidine phosphorylase in humans.[5, 15, 16] Both in man and mouse, pyrimidine nucleoside phosphorylases are more abundant in tumors than in normal tissues, except for the intestinal tract. Consequently, 5′-DFUR is effectively converted to 5-FU in tumors and in the intestinal tract after its oral administration.[7, 17] This tissue-specific conversion of 5′-DFUR to 5-FU accounts for the tumor-selective action and the dose-limiting factor, diarrhea, of orally administered 5′-DFUR in clinical trials.

Although diarrhea limited the dose of 5′-DFUR that could be given orally in clinical trials, it was not limiting

when the drug was given by the iv route.[18,19] In our preclinical study, 5′-DFUR given at 2.5 mmol/kg for a week by the po route but not by the iv route caused diarrhea (data not shown). These results suggest that the 5-FU that was formed from 5′-DFUR by pyrimidine phosphorylase in the intestine caused the toxicity. The present study confirmed the mechanism of the intestinal damage caused by 5′-DFUR. Ro 09-1390, which was converted into 5′-DFUR after it enters the bloodstream, caused little intestinal toxicity, whereas 5′-DFUR which was substantially converted into 5-FU in the intestinal tract caused marked intestinal toxicity, including diarrhea.

Ro 09-1390 is converted to 5′-DFCR by acylamidase-like enzyme activity and then to 5′-DFUR by cytidine deaminase. Therefore, its efficacy and toxicity must be related to the activities of these enzymes. Acylamidase activity, which was detected by the conversion of N^4-behenoyl-cytidine arabinoside to cytidine arabinoside (Ara-C), exists in the liver of many mammalian species.[20,21] On the other hand, the activity of cytidine deaminase (EC. 3.5.4.5), which was detected by the conversion of Ara-C to uridine arabinoside (Ara-U), varies from species to species.[21-23] The enzyme activity was high in mouse, monkey and human, but scarcely existed in rats and dogs.[21,23] Ro 09-1390 would be converted to 5′-DFUR in humans, as in mice, but might not be effectively converted in rats and dogs. In the present study we detected a substantial amount of 5′-DFCR in the blood and tumor tissues. The antitumor efficacy might be associated primarily with the enzyme activity in tumor tissues.

Ro 09-1390 was less toxic than 5′-DFUR even in terms of lethality and weight loss, though the antitumor activities of these drugs were similar. Ro 09-1390 was mainly circulated in the body as 5′-DFCR, and blood levels of 5′-DFUR in Ro 09-1390-treated mice were lower than those in 5′-DFUR-treated mice. In preliminary experiments, 5′-DFCR was found to be more than 10 times less cytotoxic to mouse tumor and bone marrow cells than 5′-DFUR was.

The intestinal toxicity of 5′-DFUR can be ascribed to its efficient conversion to 5-FU by pyrimidine nucleoside phosphorylase in the intestinal tract. The molecular design of an analog of 5′-DFUR, based on differences of enzyme activities among organs, made it possible to synthesize a compound with lower intestinal toxicity than the parent compound 5′-DFUR in an animal tumor model. Unique tissue distribution patterns of enzymes may result in various toxicities and these patterns can be used in the design of new cytostatics, derived from existing drugs, which offer tumor-selective activation and reduction of the toxicity in particular organs.

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