In-vitro differential metabolism and activity of 5-fluorouracil between short-term, high-dose and long-term, low-dose treatments in human squamous carcinoma cells

Baoli Qin[a], Risa Tanaka[a], Hiroshi Ariyama[a], Yoshihiro Shibata[a], Shuji Arita[a], Hitoshi Kusaba[a], Eishi Baba[a], Mine Harada[a] and Shuji Nakano[a]

Although continuous infusion of 5-fluorouracil (5-FU) has been clinically demonstrated to be superior to bolus administration, the mechanistic difference between the treatments still remains unclear. Here, we investigated *in vitro* whether there were any differences in the metabolism and activity of 5-FU between these schedules. To simulate bolus and infusional treatments of 5-FU, HST-1 human squamous carcinoma cells were treated with short-term, high-doses and long-term, low-doses so that the area under the curve (AUC) of 5-FU became equivalent between both schedules, and compared the cytotoxicity, fluorinated RNA (F-RNA) levels, 5-fluorodeoxyuridine monophosphate (FdUMP) content and thymidylate synthase (TS) activity. F-RNA and FdUMP were measured by capillary gas chromatography–mass spectrometry and competitive ligand-binding assay, respectively. The [^{3}H]FdUMP binding site in TS was determined as an index of the amount of TS using the radio-binding assay. Long-term, low-dose treatment of 5-FU was found to be 1.3–1.7 times more cytotoxic than the short-term, high-dose treatment. F-RNA content increased as the AUC of 5-FU was increased and was 2–4 times significantly higher in the cells treated with the long-term, low-dose than those with the short-term, high-dose schedule, indicating that the levels of F-RNA are AUC and schedule dependent. In contrast, there were no significant differences in FdUMP levels, TS activity and TS inhibition rate between the schedules. These data suggest that the superior activity of 5-FU administered long-term, low-dose over short-term, high-dose could be explained by more 5-FU incorporated into RNA during a long-term, low-dose exposure, thus providing a strategic rationale for the clinical advantage of continuous infusion over bolus administration. *Anti-Cancer Drugs* 17:439–443 © 2006 Lippincott Williams & Wilkins.

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[a]First Department of Internal Medicine and Department of Biosystemic Science of Medicine, Graduate School of Medicine, Kyushu University, Fukuoka, Japan.

Correspondence to S. Nakano, First Department of Internal Medicine and Department of Biosystemic Science of Medicine, Graduate School of Medicine, Kyushu University, 3-1-1 Maidashi, Higashi-Ku, Fukuoka 812-8582, Japan.
Tel: +81 92 642 5226; fax: +81 92 642 5247;
e-mail: sn@intmed1.med.kyushu-u.ac.jp

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Introduction

5-Fluorouracil (5-FU) is effective either singly or in combination with other anti-cancer drugs against a variety of human solid tumors. This drug has multiple mechanisms of action including (a) inhibition of thymidylate synthase (TS) by 5-fluorodeoxyuridine monophosphate (FdUMP), (b) incorporation of 5-fluorouridine triphosphate (FUTP) into RNA, and (c) incorporation of 5-fluorodeoxyuridine triphosphate (FdUTP) into DNA, thereby disrupting DNA synthesis as well as RNA/DNA function. Because of these differential mechanisms, 5-FU exhibits clinically different maximal tolerated doses and different dose-limiting toxicity according to the administration schedules (e.g. bolus or infusion) [1]. Continuous infusion of 5-FU has been shown to be clinically superior to bolus administration in anti-tumor activity [2–4]. However, the mechanistic differences between these treatments still remain to be clarified. It has been shown *in vitro* that short-term treatment with 5-FU produced resistance via decreased incorporation of FUTP into RNA, while repeated prolonged exposure to 5-FU produced resistance via rapid recovery from TS activity [5], suggesting that 5-FU administered in bolus and continuous fashions may preferentially cause RNA- and DNA-directed (inhibition of TS) cytotoxicity, respectively. Clinically, TS inhibition in cancer tissues has been shown to be significantly higher in patients treated with infusional 5-FU than those treated with the bolus administration [6]. However, these experiments were not conclusive because the RNA- and DNA-directed parameters indicative of 5-FU activity had not been directly measured and compared between these schedules.

In this study, we investigated the mechanisms whereby bolus or infusional schedules of 5-FU administration cause cytotoxic effects by directly measuring the parameter including 5-FU incorporated into RNA (F-RNA), FdUMP and TS inhibition using HST-1 human squamous carcinoma cells.

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Materials and methods

Cell culture and chemicals

A clonally isolated subline of HST-1 human tongue squamous carcinoma cells [7] was maintained in DMEM (Nissui Pharmaceutical, Tokyo, Japan) supplemented with 10% heat-inactivated FBS (Gibco/Life Technologies, Grand Island, New York, USA), 100 IU/ml penicillin and 100 μg/ml streptomycin (Gibco/Life Technologies) at 37°C in a humidified atmosphere of 5% CO_2/95% air. 5-FU was kindly provided by Kyowa Hakko (Tokyo, Japan). Immediately before use, 5-FU was dissolved in a culture medium. [^{3}H]6-FdUMP was purchased from Moravek Biochemicals (Brea, California, USA), FdUMP·Na and FH_4 from Sigma (St Louis, Missouri, USA), and TS (derived from *Lactobacillus casei*) from Biopure (Boston, Massachusetts, USA). All other reagents were the highest available grade.

TS assay

We measured cytosolic FdUMP and [^{3}H]FdUMP binding sites present in TS using a reported method with some modifications [8,9]. In brief, HST-1 cells were seeded into tissue culture dishes (100 mm) at a density of 4×10^6 cells and incubated overnight. Then the cells in 10 dishes were exposed to each schedule of 5-FU. Subsequently, the cells were mechanically detached by a scraper and cells from 10 dishes were collected together. The collected samples were homogenized and sonicated in 2 ml of 50 mmol/l KH_2PO_4 buffer, pH 7.4, containing 20 mmol/l 2-mercaptoethanol (ME), 100 mmol/l NaF and 15 mmol/l cytidylate at 4°C. The cytosol then was prepared by centrifugation and 1 ml of the cytosol mixed with 2 ml of 1 mol/l acetic acid was used for the FdUMP assay. The mixture was centrifuged and the supernatant was lyophilized. The acid extract then was loaded on a DEAE cellulose column (Wako Pure Chemical Industry, Osaka, Japan) to separate FdUMP and 2′-deoxyuridine-5′-monophosphate (dUMP). FdUMP was measured by a competitive ligand-binding assay.

To determine the TS_{total}, 50 μl of the cytosol was added to 50 μl 50 mmol/l Tris containing 100 mmol/l NaF, 15 mmol/l cytidine 5′-monophosphate and 100 mmol/l 2-ME (pH 8.0), and the resulting solution incubated for 3 h at 25°C in order to dissociate FdUMP from the complex. After incubation, 9.5 pmol [^{3}H]FdUMP (0.15 mCi) and 25 μl of a solution containing 2 mmol/l tetrahydrofolic acid, 16 mmol/l sodium ascorbate and 9 mmol/l formaldehyde were added, followed by incubation for 20 min at 25°C. To this solution was added 1 ml of a cold slurry comprising 3.3 g activated charcoal in 100 ml 0.1 mol/l HCl containing 3% dextran and 2% BSA, and the suspension was allowed to stand for at least 40 min in an ice-water bath. The [^{3}H]FdUMP-bound ternary complex was collected in the supernatant by centrifugation, 850 μl of which was transferred to a scintillation vial and mixed with 8 ml Scintisole EX-H, and the radioactivity determined with a liquid scintillation counter. The same procedure was used for purified *L. casei* TS with previously quantitated [^{3}H]FdUMP binding sites as the standard protein. Thus, the sum of [^{3}H]FdUMP binding sites in samples, TS_{total}, was calculated from the standard curve based on *L. casei* TS. TS_{free} was determined in the same manner as TS_{total}, except without incubation for 3 h at 25°C. Further, the inhibition rate of TS activity with FdUMP was calculated using the formula $[1-(TS_{free}/TS_{total})] \times 100$.

Measurement of 5-FU incorporated into RNA

F-RNA was assayed using gas chromatography–mass spectrometry (GC-MS) with some modifications as described previously [10]. In brief, collected treated samples were homogenized in 2 ml water, mixed with 5 ml cold 5% trichloroacetic acid (TCA) and centrifuged. The resulting precipitate was washed twice by mixing with each of 5 ml cold 5% TCA, 70% ethanol, 95% ethanol and ethanol:diethylether (3 : 1) solvent, followed by centrifugation and discarding the supernatant. The final precipitate was dissolved in 0.3 mol/l KOH and incubated overnight at 37°C to hydrolyze RNA to mononucleotide. After neutralizing with $HClO_4$ and desalting, a portion of the mononucleotide solution was used for the determination of the concentration of RNA in terms of the color reaction between mononucleotide and orcinol and standard RNA from bakers yeast. To 1.4 ml of the residual mononucleotide solution was added 100 μl of 1 μg/ml [$^{15}N_2$]5-FU as internal standard, and this solution was mixed with an equivalent volume of 12 mol/l HCl and hydrolyzed in a closed tube for 20 h at 100°C. After cooling and washing the reaction solution with $CHCl_3$, the solution was evaporated to dryness under a stream of nitrogen. The residue was reconstituted with 1 mol/l phosphate buffer (pH 4.0) and extracted with ethyl acetate. The extract was purified by preparative silica gel column chromatography. The residue of the fraction containing 5-FU and internal standard was dissolved in 30 μl acetonitrile, and reacted with 10 μl ditrifluorobenzyl bromide in the presence of triethylamine for 20 min at room temperature. Then 50 μl ethyl acetate followed by 450 μl *n*-hexane was added to the reaction mixture, and a precipitate formed in the solution. After centrifugation of the solution, the supernatant was transferred to a new tube. The solvent was removed under a stream of nitrogen and the residue was reconstituted with 200 μl *n*-hexane as the sample solution for GC-MS.

GC-MS analysis was carried out with a system consisting of a Hewlett Packard 5890 (Hewlett Packard, Tokyo, Japan) gas chromatograph and a JEOL Automass JMS-AM150 (JEOL, Tokyo, Japan) mass spectrometer. To the gas chromatograph was connected a DB-1 fused silica capillary column, 30 m × 0.25 mm internal diameter (J & W Scientific, Folsom, California, USA). The GC conditions were as follows: the carrier gas was helium at a

flow rate of 1.0 ml/min at the outlet of the column, the injector temperature was 250°C, the interface temperature was 250°C and the oven temperature was maintained at 100°C for 1 min, then programmed to increase at 20°C/min to 300°C and maintained at 300°C for 10 min. An aliquot (1 μl) of the sample solution was injected into the GC-MS in splitless mode. The mass spectrometer was operated under negative-ion chemical ionization mode. Isobutane was introduced into the ion source at about 0.5 Torr as reagent gas. The source temperature was 150°C and the ionization energy was 150 eV. 5-FU and internal standard were monitored with ions of m/z 355 and 357, respectively. The analysis was based on an established procedure. The apparatus for analysis was regularly checked with quality control samples. We calculated the mean ± SD values from the analytical data for each item to be used as the supplemental data for validation.

Cell survival assay

To avoid the density-dependent inhibition of cell growth, an appropriate number of cells (1×10^4) was seeded into 60-mm culture dishes (Falcon 3002; Oxnard, California, USA) such that control cultures did not reach confluence at the time of harvest. The cells were allowed to attach to the bottom overnight. In the low AUC, cells were exposed to 1 μg/ml of 5-FU for 24 h and 24 μg/ml of 5-FU for 1 h, while in the high AUC, cells were exposed to 5 μg/ml of 5-FU for 24 h and 120 μg/ml of 5-FU for 1 h. After drug treatment, the medium was replaced with fresh medium and the cells were cultured for 10 days with medium change every 3 days. The control dishes were cultured in the medium without drug. The cell number was counted using a Coulter counter (Model ID; Hialeah, Florida, USA). The percentage of survival was calculated by dividing the number of cells in the drug-treated culture by the number of cells in the culture not exposed to drug. The data include results from three separate experiments.

Statistical analysis

For statistical analysis, Student's *t*-test was used to compare the differences in F-RNA contents, FdUMP levels, TS inhibition rate and growth-inhibition rate between the two different schedules of 5-FU. Differences were considered statistically significant at $P < 0.05$.

Results

Detection of RNA incorporation

To simulate the bolus and infusional treatments of 5-FU, we treated HST-1 cells either with a high dose of 5-FU for a short-term or a low dose of 5-FU for a long-term, so that the AUC became equivalent between both treatment schedules. Exposure to 5-FU for 24 h caused an IC_{50} at the concentration of 1.0 μg/ml. Therefore, in the low AUC, cells were treated with either 1 μg/ml for 24 h or 24 μg/ml for 1 h, while in the high AUC, cells were treated with either 5 μg/ml for 24 h or 120 μg/ml for 1 h. When the cells were treated with a low AUC, F-RNA contents in the cells treated with 1 μg/ml for 24 h exposure and 24 μg/ml for 1 h exposure were 264 and 67.1 ng/mg RNA, respectively. When the cells were treated with a high AUC, F-RNA contents in the cells treated with 5 μg/ml for 24 h exposure and 120 μg/ml for 1 h exposure were 759 ± 62.9 and 375 ± 62.9 ng/mg RNA, respectively. F-RNA contents increased as AUC of 5-FU increased and were 2–4 times significantly higher in the cells treated with low-dose, long-term exposure than those treated with high-dose, short-term exposure, indicating that the levels of F-RNA is AUC and schedule dependent (Table 1).

Measurements of FdUMP, TS activity and TS inhibition rate

Although the amounts of FdUMP, total TS and free TS were not detected in the cells treated with a low AUC, except total TS in a long-term schedule, these parameters were available in the cells treated with a high AUC. As shown in Table 1, FdUMP levels in the cells treated with 5 μg/ml for 24 h exposure and 120 μg/ml for 1 h exposure were 20.1 ± 19.4 and 4.56 ± 3.56 ng/mg RNA, respectively. FdUMP levels of long-term exposure appeared higher than those of short-term exposure. There was no statistical difference, however. The total TS levels in the cells treated with 5 μg/ml for 24 h exposure and 120 μg/ml for 1 h exposure were 4.68 ± 4.02 and 3.08 ± 4.38 pmol/g, respectively. Free TS levels in the cells treated with 5 μg/ml for 24 h exposure and 120 μg/ml for 1 h exposure were 0.96 ± 0.74 and 0.95 ± 0.64 pmol/g, respectively. Thus, TS inhibition rates in the cells treated with 5 μg/ml for 24 h exposure and 120 μg/ml for 1 h exposure were 75.4 ± 14.4 and 74.9 ± 14.9%, respectively. Consequently, FdUMP levels, TS activity and TS inhibition rate all increased as the AUC of 5-FU increased, but no significant differences were found between low-dose, long-term exposure and high-dose, short-term exposure, suggesting that FdUMP levels, TS activity and TS inhibition rate are AUC dependent, but not schedule dependent.

Cytotoxicity assay of 5-FU

When the cells were treated with a low AUC, the inhibition rates of cell growth after 10 days in the cells treated with 1 μg/ml for 24 h exposure and 24 μg/ml for 1 h exposure were 75 ± 7 and 43 ± 16%, respectively. When the cells were treated with a high AUC, the inhibition rates in the cells treated with 5 μg/ml for 24 h exposure and 120 μg/ml for 1 h exposure were 96 ± 3 and 72 ± 5%, respectively. These data indicated that the activity of 5-FU increased as the AUC of 5-FU increased and were 1.3–1.7 times significantly more cytotoxic in the cells treated with low-dose, long-term exposure than those treated with high-dose, short-term exposure, indicating that the cytotoxicity of 5-FU is AUC and schedule dependent (Table 2).

Table 1 F-RNA, FdUMP content and TS activity in HST-1 cells treated with two different schedules of 5-FU

Dose intensity	Treatment schedule	F-RNA (ng/mg RNA)		FdUMP (ng/mg RNA)		TS activity					
						TS total (pmol/g)		TS free (pmol/g)		Inhibition rate (%)	
Low AUC[a]	1 μg/ml (24 h exposure)	264	3[c]	ND[b]		2.4		ND		–	
	24 μg/ml (1 h exposure)	67.1		ND		ND		ND		–	
High AUC	5 μg/ml (24 h exposure)	759 ± 62.9	3[c]	20.1 ± 19.4	NS	4.68 ± 4.02	NS	0.96 ± 0.74	NS	75.4 ± 14.4	NS
	120 μg/ml (1 h exposure)	375 ± 62.9		4.56 ± 3.56		3.08 ± 4.38		0.95 ± 0.64		74.9 ± 14.9	

In the low AUC, cells were treated with either 1 μg/ml for 24 h or 24 μg/ml for 1 h, while in the high AUC, cells were treated with 5 μg/ml for 24 h or 120 μg/ml for 1 h. The values are means ± SD of three independent experiments. NS: not significant.
[a]The experiment was done once at this concentration, because the parameters other than F-RNA could not be detected.
[b]ND: not detected.
[c]$P < 0.05$ by Student's *t*-test.

Table 2 Cytotoxicity of 5-FU in HST-1 cells treated with two different schedules

Dose intensity	Treatment schedule	Actual no. of cells/dish (× 104)		Survival fraction		Growth inhibition rate (%)	
	No treatment	50.4 ± 3.82		1		0	
Low AUC	1 μg/ml (24 h exposure)	12.5 ± 3.54	a	0.25 ± 0.07	a	75 ± 7	a
	24 μg/ml (1 h exposure)	28.7 ± 8.08		0.57 ± 0.16		43 ± 16	
High AUC	5 μg/ml (24 h exposure)	2.0 ± 1.41	b	0.04 ± 0.03	b	96 ± 3	b
	120 μg/ml (1 h exposure)	14.3 ± 2.31		0.28 ± 0.05		72 ± 5	

In the low AUC, cells were treated with 1 μg/ml for 24 h and 24 μg/ml for 1 h, while in the high AUC, cells were treated with 5 μg/ml for 24 h and 120 μg/ml for 1 h. The values are means ± SD of three independent experiments.
[a]$P < 0.05$ by Student's *t*-test.
[b]$P < 0.01$ by Student's *t*-test.

Discussion

Although continuous infusion of 5-FU has been clinically demonstrated to be superior to bolus administration [2–4], the mechanistic difference between the treatments has yet to be determined. Therefore, by simulating bolus and continuous infusion *in vitro*, we investigated the mechanisms of growth inhibition by directly measuring the parameters including 5-FU incorporated into RNA (F-RNA), FdUMP and TS inhibition. We found that both F-RNA content and cytotoxicity of 5-FU increased with elevation of the AUC of 5-FU and that the cells treated with a low dose of 5-FU for a long-term exposure exhibited 2–4 times significantly more F-RNA content and 1.3–1.7 times significantly higher cytotoxicity, respectively, than those administered with the high-dose, short-term exposure. These data, together with the fact that the extent of 5-FU-mediated TS inhibition did not differ significantly between the two different schedules, suggest that increases of 5-FU activity on continuous versus short-term exposure might be explained by the increase of F-RNA content. This increase of F-RNA might be conceivable because 5-FU is a cell cycle-specific drug that exhibits its anti-tumor activity through incorporation into DNA and RNA during both G_1 and S phases. Upon short-term exposure, substantial numbers of cells will not enter the G_1/S phase, thus evading 5-FU-induced DNA and RNA damage [11].

It has been widely accepted that F-RNA is an important element of 5-FU cytotoxicity since there is a good correlation between the cytotoxicity and incorporation of 5-FU into RNA [12]. Decreased drug incorporation into RNA has been observed in 5-FU-resistant cells [13,14]. In addition, it has been suggested that measurement of F-RNA levels together with the determination of 5-FU concentration and TS inhibition rate should be considered as good parameters for the evaluation of anti-tumor efficacy of 5-FU and its analogs both in experimental and in clinical settings [15]. 5-FU has been shown to be extensively incorporated into both nuclear and cytoplasmic RNA species, interfering with normal RNA processing and function [12]. Nuclear 'run-on' transcription analysis revealed that 5-FU inhibited RNA transcription [16]. 5-FU can be incorporated into RNA, which will lead to non-DNA damage-directed effects such as disturbances at the transcriptional and post-transcriptional level [17]. The mechanism whereby 5-FU modulates the expression of mRNA transcripts remains unclear. Misincorporation of 5-FU into RNA may potentially affect many RNA processes important for mRNA function. It is quite possible that the RNA-directed cytotoxicity of 5-FU is due to the combination of many actions against RNA functions which simply overwhelm the cells, resulting in death [12].

It has been shown *in vitro* that short-term treatment with 5-FU produced resistance via decreased incorporation of FUTP into RNA, while repeated prolonged exposure to 5-FU produced resistance via rapid recovery from TS activity [5], suggesting that 5-FU administered in bolus

and continuous fashions may preferentially cause RNA- and DNA-directed (inhibition of TS) cytotoxicity, respectively. In addition, TS inhibition in cancer tissues has been shown to be significantly higher in patients treated with infusional 5-FU than those treated with the bolus administration [6]. Contrary to these studies, we have clearly shown here that long-term treatment did not increase the TS inhibition rate as compared to short-term treatment. Rather, long-term treatment enhanced the incorporation of 5-FU into RNA. Although FdUMP levels, total TS, free TS and TS inhibition rate all increased with elevation of the AUC of 5-FU, no significant differences in these parameters could be observed between low-dose, long-term and high-dose, short-term exposures, suggesting that these parameters are AUC dependent, but not schedule dependent. The mechanism of the lack of differences in these parameters between low-dose, long-term and high-dose, short-term treatments remains to be fully elucidated. The kinetics of 5-FU in murine tumor models *in vivo* and human carcinoma cells *in vitro* has shown that FdUMP levels were highest at the earliest 0.5 h, with rapid losses within 1 h after treatments [13,18,19]. In our study, the cells were treated with a low dose for a 1-h exposure and a high dose for a 24-h exposure. By catabolism and binding to free TS, newly produced FdUMP may be eliminated rapidly, resulting in no significant differences in FdUMP levels and subsequent TS inhibition rates between these schedules.

In conclusion, low-dose, long-term treatment of 5-FU exhibited higher growth-inhibition rates as well as higher levels of F-RNA than the high-dose, short-term treatment. Because the extent of 5-FU-mediated TS inhibition did not differ significantly between the two different schedules, superior activity of 5-FU administered as a low-dose, long-term exposure over high-dose, short-term exposure could be explained by more 5-FU incorporated into RNA during long-term exposure. Our data provide a strategic rationale for the clinical advantage of continuous infusion of 5-FU over bolus administration.

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