Cancer Chemother Pharmacol (2005) 56: 648–652
DOI 10.1007/s00280-004-0985-9

ORIGINAL ARTICLE

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# Effects on DNA and RNA after the administration of two different schedules of 5-fluorouracil in colorectal cancer patients

Received: 26 May 2004 / Accepted: 24 September 2004 / Published online: 16 June 2005


**Abstract** *Purpose*: 5-Fluorouracil (5-FU) has two major mechanisms by which it exerts its anticancer activity. One mechanism operates through the inhibition of thymidylate synthetase (TS) by the active metabolite 5-fluorodeoxyuridine 5'-monophosphate. The other mechanism is the incorporation of 5-FU into RNA. Using tumor tissue specimens from colon carcinoma patients given 5-FU by two different modes of administration, we investigated the effects of 5-FU on DNA and RNA. *Experimental design*: Group A patients received 200 mg/day of 5-FU as a rapid infusion for 5 days preoperatively, and group B patients received 200 mg/day of 5-FU as a continuous infusion for 5 days preoperatively. Postoperatively, we analyzed the 5-FU concentration, 5-FU incorporation into RNA (F-RNA), and TS inhibition rate (TSIR) in normal tissue, cancerous tissue, and lymph nodes. *Results*: The F-RNA concentration in tumor tissue from group A patients was higher than in tissue from group B patients. The TS concentrations in tumor tissue were significantly higher than in non-tumor tissue in both groups. In lymph nodes, the TSIR of group A was 78.5% and that of group B was 55.2%, a significant difference. *Conclusion*: Bolus injection can be considered to be more effective with respect to RNA damage in tumor tissue. Especially in cases involving lymph node metastasis, bolus injection was effective with respect to DNA damage as well as RNA damage.

**Keywords** DNA · 5-Fluorouracil · Colorectal cancer

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## Introduction

While 5-fluorouracil (5-FU) has been used for the treatment of malignant diseases and remains the mainstay of chemotherapy for patients with colorectal cancer, its efficacy rate is limited to 21% [1]. Increased understanding of the mechanism of action of 5-FU has led to various injection strategies. The main mechanism of action of 5-FU is thought to be via the inhibition of thymidylate synthetase (TS) by the active metabolite 5-fluorodeoxyuridine 5′-monophospate (FdUMP) [2] and its incorporation into RNA as fluorouridine triphosphate (FUTP), resulting in the inhibition of RNA polymerase [3]. In vitro studies suggest that TS may be more significant when cells are subjected to a prolonged exposure to 5-FU, whereas the RNA effects may predominate when shorter exposures are used [4]. As regards clinical treatment, 5-FU is used in patients as an internal medicine as well as being administered by intravenous injection. In the present study, we used an experimental intravenous dose of 200 mg 5-FU per body. We administered 5-FU to colorectal cancer patients by either rapid (bolus) or continuous infusion (CI) and assayed the resulting 5-FU concentration, and F-RNA and the TS inhibition rate (TSIR) in normal tissue, cancerous tissue, and lesional lymph nodes in order to determine the differences in the effects of the two types of 5-FU administration and the differences in each tissue type. Additionally, we sought to determine whether 200 mg was a sufficient dose.

## Materials and methods

The subjects comprised 21 patients with colorectal cancer that had been resected at the Department of second Surgery, Fukuoka University Hospital. None of the patients had received chemotherapy or radiotherapy prior to surgery. The patients included 12 men and 9 women (mean age 62.1 ± 9.0 years). According to the

TNM staging system, the histological stages were: I (four patients), IIA (ten patients), IIIA (one patient), IIIB (three patients), and IIIC (three patients). The characteristics of the patients are shown in Table 1. The patients were randomly divided into two groups according to the following dosage regimens: 5-FU 200 mg/day as a rapid infusion (bolus) over 3–4 min on each day for the 5 days preceding surgery (group A); and 5-FU 200 mg/day as a CI for 5 days preoperatively (group B). On the day of operation, patients in group A received a rapid infusion of 5-FU just before transportation to surgery. The CI of the patients in group B was stopped just before transportation to surgery. Cancerous tissue, normal tissue samples and lymph nodes were obtained immediately after surgery, and each of these materials was stored at −80°C until analysis. This study was approved by the institutional review board at each participating center and eligible patients provided written informed consent.

## Assay of 5-FU in tissue

An internal standard (IS, 5-chlorouracil), silica gel and acetonitrile were added to the tissue sample and the mixture was homogenized in an ice bath and centrifuged. The removed upper layer was evaporated to dryness, followed by the dissolution of the residue in ethanol. The solution thus obtained was subjected to column chromatography on silica gel (10 × 17.8 mm, 125–250 μm). The fraction eluted with acetone was evaporated to dryness, and the residue was dissolved in the mobile phase and analyzed by high performance liquid chromatography (HPLC) with ultraviolet detection at 264 nm. Chromatographic separation was achieved at 10°C using a Develosil 60-3 column (4.6 × 100 mm; Nomura Chemical Company, Japan). Elution was carried out isocratically at a flow rate of 0.9 ml/min. A 150-μl aliquot of the sample was injected onto the chromatographic system. Aliquots of control tissue spiked with known concentrations of 5-FU were treated in a similar manner to the experimental samples. The solutions obtained were used as standard solutions to prepare a calibration curve for the determination of 5-FU concentrations in the tissue.

**Table 1** Characteristics of patients in each group

| | Group A | Group B |
|---|---|---|
| Age (years) | 61.8 ± 7.3 | 62.4 ± 10.9 |
| Sex | | |
| Male | 8 | 4 |
| Female | 2 | 7 |
| Histological stage | | |
| I | 1 | 3 |
| IIA | 6 | 4 |
| IIIA | 0 | 1 |
| IIIB | 1 | 2 |
| IIIC | 2 | 1 |

## Assay of TS in tissue

TS forms a ternary complex with FdUMP and methylene tetrahydrofolic acid (mTHF) in vivo and in vitro. After 5-FU treatment, which results in the formation of FdUMP, TS, which is naturally present in the body, is divided into two types, complex and free TS, depending on whether a ternary complex is formed or not. In addition the TS complex can be dissociated into free TS through treatment. The use of this property of TS enabled us to determine the levels of [$^3$H]-FdUMP binding to TS contained in the tissue ($TS_{free}$ and $TS_{total}$).

The tissue sample was homogenized in 50 mmol/l phosphate buffer (pH 7.4). After centrifugation, the supernatant was collected and divided into two aliquots which were used to assay [$^3$H]-FdUMP bound to TS present as a complex and free TS in the supernatant ($TS_{total}$) and [$^3$H]-FdUMP bound to free TS in the supernatant ($TS_{free}$), respectively.

For the determination of $TS_{total}$ levels, the supernatant was incubated at 25°C with an equal volume of 50 mmol/l Tris buffer (pH 8.0) in order to dissociate TS from the complex already contained in the supernatant. After the addition of [$^3$H]-FdUMP and mTHF, the mixture was incubated at 25°C again to allow a ternary complex containing [$^3$H]-FdUMP to be formed, and the complex was isolated and the radioactivity measured. For the determination of $TS_{free}$ levels, an equal volume of 50 mmol/l Tris buffer was added to the supernatant, followed by the addition of [$^3$H]-FdUMP and mTHF, and the mixture was incubated at 25°C to allow the complex to be formed. The complex was isolated and the radioactivity determined in a manner similar to that for $TS_{total}$. From the $TS_{free}$ and $TS_{total}$ levels, the TSIR was calculated by the following equation: $TSIR\ (\%) = (1-TS_{free}/TS_{total}) \times 100$.

## Assay of 5-FU in tissue RNA

### *Purification of RNA*

Tissue samples were homogenized in chilled water, shaken after the addition of chilled 10% trichloroacetic acid, and centrifuged. After the supernatant was discarded, the precipitate was mixed and centrifuged once with chilled 10% trichloroacetic acid, once with 70% ethanol, once with 95% ethanol and twice with a mixture of ethanol and ether (3:1). After each centrifugation, the supernatant was discarded to remove the soluble components from the precipitate. The precipitate obtained finally was allowed to react with 0.3 mol/l KOH at 37°C for 16–20 h, and the reaction mixture was centrifuged after the addition of 60% $HClO_4$. The supernatant obtained was neutralized with 3 mol/l KOH. The $KClO_4$ precipitate produced was removed to obtain a mononucleotide solution.

**Table 2** Effect of 5-FU on TS, TSIR, F-RNA, and 5-FU concentrations (means ± SD). In tumor tissue, in group A the F-RNA concentration was higher than in group B. In lymph node, in group A the TSIR was higher than in group B

| Parameter | Group A | Group B | *P* value |
|---|---|---|---|
| Tumor tissue | | | |
| TS (pmol/g) | 10.9 ± 10.5 | 10.6 ± 10.5 | 0.91 |
| TSIR | 77.9 ± 12.6 | 75.7 ± 12.8 | 0.714 |
| F-RNA (ng/mg) | 147.9 ± 88.6 | 52.2 ± 25.2 | 0.003 |
| 5-FU (ng/mg) | 280.6 ± 174.6 | 41.9 ± 20.5 | 0.003 |
| Normal tissue | | | |
| TS (pmol/g) | 2.8 ± 1.9 | 2.3 ± 1.9 | 0.494 |
| TSIR | 58.1 ± 23.9 | 60.7 ± 15.2 | 0.746 |
| F-RNA (ng/mg) | 53.8 ± 43.8 | 15.3 ± 10.6 | 0.011 |
| 5-FU (ng/mg) | 42.6 ± 29.2 | 11.7 ± 19.3 | 0.015 |
| Lymph node | | | |
| TS (pmol/g) | 4.1 ± 5.4 | 5.1 ± 4.9 | 0.626 |
| TSIR | 78.5 ± 11.8 | 55.2 ± 15.6 | 0.024 |
| F-RNA (ng/mg) | 30.4 ± 37.6 | 4.3 ± 0.8 | 0.056 |
| 5-FU (ng/mg) | 51.5 ± 86.2 | 3.9 ± 0.2 | 0.162 |

### *Assay of 5-FU*

A mixture of mononucleotide solution and an IS ($1,3^{-15}$ $N_2$-5-FU) solution was hydrolyzed with an equal volume of concentrated hydrochloric acid for 20 h at 100°C. After the reaction, the hydrolyzed solution was evaporated to dryness, and the residue was extracted with ethyl acetate and subjected to column chromatography on silica gel to obtain the 5-FU fraction. After labeling with 3.5-di-(trifluoromethyl)-benzyl bromide, the 5-FU was desalted, and the solvent removed. The residue was then dissolved in hexane to prepare the sample solution. This sample solution was analyzed by gas chromatography-mass spectrometry (GC-MS). The GC conditions were as follows: the carrier gas was helium at a flow rate of 1.0 ml/min at the outlet of column; the injector temperature was 250°C and the interface temperature 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 wad injected into the GC-MS in splitless mode. The mass spectrometer was operated in 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 150 eV. The 5-FU and IS 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 QC samples.

### *Determination of RNA content in the mononucleotide solution*

With an aliquot of mononucleotide solution, the concentrations of RNA were determined by the orcinol method using bread yeast-derived RNA as the reference standard. From the RNA concentrations obtained and the results of GC-MS, the amount of 5-FU incorporated into the RNA (ng/mg-RNA) was calculated. Each item was measured twice, and the mean value was used for analysis.

### Statistical analysis

Student's *t*-test was used to compare the 5-FU, F-RNA concentrations, and TSIR. For the clinicopathological features of the patients, *P* values were calculated by the chi-square test and Student's *t*-test, with $P < 0.05$ considered significant.

## Results

### 5-FU concentrations

The 5-FU concentration in tumor was 280.6 ng/mg and in non-tumor regions was 42.6 ng/mg in group A. These concentrations were significantly higher than those in group B. However, in the lymph nodes the 5-FU concentration was 51.5 ng/mg in group A and 3.9 ng/mg in group B (the difference was not significant; Table 2). In group A, the 5-FU concentration in tumor was significantly higher than in non-tumor tissues (Table 3), but in lymph nodes the concentration was not different from

**Table 3** Comparison between groups (means ± SD). In both groups, the F-RNA concentration in tumor tissue was higher than in normal tissue

| | 5-FU | | TS | | F-RNA | |
|---|---|---|---|---|---|---|
| | Concentration (ng/ml) | *P* value | Concentration (pmol/g) | *P* value | Concentration (ng/mg) | *P* value |
| Group A | | | | | | |
| Normal tissue | 42.6 ± 29.2 | | 2.8 ± 1.9 | | 53.8 ± 43.8 | |
| Tumor tissue | 280.6 ± 174.6 | 0.001 | 10.9 ± 10.5 | 0.038 | 147.9 ± 88.6 | 0.035 |
| Lymph nodes | 51.5 ± 86.2 | 0.758 | 4.1 ± 5.4 | 0.166 | 30.4 ± 37.6 | 0.409 |
| Group B | | | | | | |
| Normal tissue | 11.7 ± 19.3 | | 2.3 ± 1.9 | | 15.3 ± 10.6 | |
| Tumor tissue | 41.9 ± 20.5 | 0.109 | 10.6 ± 10.5 | 0.042 | 52.2 ± 25.2 | 0.001 |
| Lymph nodes | 3.9 ± 0.2 | ND | 5.1 ± 4.9 | 0.089 | 4.3 ± 0.8 | 0.005 |

that in normal tissue. In group B, there were no differences among the three types of tissue.

### F-RNA concentrations

In tumor tissue, the F-RNA concentration in group A was 147.9 ng/mg and in group B was 52.2 ng/mg. In normal tissue, the F-RNA concentration in group A was 53.8 ng/mg and in group B was 15.3 ng/mg (in both groups the differences between tumor and normal tissue were significant). In lymph node tissue, the F-RNA concentration in group A was 30.4 ng/mg and in group B was 4.3 ng/mg (no significant difference). In group A, the F-RNA concentration in tumor tissue was significantly higher than in non-tumor tissues.

### TS concentrations

There were no significant differences between groups A and B in tumor tissue, normal tissue, and lymph nodes. On the other hand, the TS concentrations in tumor tissue were significantly higher than in non-tumor tissues in both groups.

### TSIR

In tumor tissue and normal tissue, there were no significant differences, but in lymph nodes, the TSIR in group A was 78.5% and in group B was 55.2% ($P = 0.024$). In group A, the TSIR in lymph nodes was the highest among the three tissue types, whereas in group B, the TSIR in lymph nodes was the lowest among the three tissue types.

## Discussion

Doses ranging from 300 to 500 mg have been used in most previous studies on this topic. The present study is the first in which TS and F-RNA have been investigated after the administration of two different schedules of 5-FU at 200 mg. Even though the dose was only 200 mg, 5-FU uptake in the tissues was sufficient. Furthermore, differentiation according to two means of administration led to different results. Volume may reduce side effects, especially when 5-FU is used in bolus doses.

Meta-analysis data have demonstrated that 5-FU CI is superior to 5-FU bolus in terms of tumor response and survival, even though the magnitude of the benefit in terms of survival is small. However, the details of the main kinetic pathway of 5-FU remain unclear [5]. Bolus administration of 5-FU inhibits RNA synthesis, whereas CI may be more cytotoxic via the inhibition of TS. Administration by CI rather than by bolus infusion permits the delivery of more drug and limits toxicity due to myelosuppression to hand-foot syndrome. It is well known that CI allows higher doses of 5-FU to be administered than bolus infusion [6].

The different schedules of 5-FU administration used here may both lead to resistance via different mechanisms. The present data provide evidence supporting the contention that the mechanism of action of 5-FU depends on the schedule of administration. TS is an essential enzyme in DNA synthesis. TS catalyses the reductive methylation of deoxyuridine monophosphate (dUMP) to deoxythymidine monophosphate (dTMP), with reduced 5,10-methylenetetrahydrofolate ($CH_2THF$) as the methyl donor. This reaction provides the sole de novo source of thymidylate which is necessary for DNA replication and repair. TS protein functions as a dimer, both submits of which contain a nucleotide-binding site and a binding site for $CH_2THF$. The 5-FU metabolite FdUMP binds to the nucleotide-binding site of TS forming a stable ternary complex with the enzyme and $CH_2THF$, thereby blocking the binding of the normal substrate dUMP and inhibiting dTMP synthesis [7, 8].

TS expression has been measured in many investigations which have shown an improved response to 5-FU-based therapy in patients with low tumoral TS expression [9–11]. On the other hand, treatment with 5-FU has been shown to acutely induce TS expression in tumors [12, 13]. In the present study, no significant differences in TS expression in tumor tissue, normal tissue or lymph node between groups A and B were noted. However, the lymph node TSIR in group A was 78.5% and in group B was 55.2%, a significant difference ($P = 0.024$). Therefore, in patients with lymph node metastasis, bolus 5-FU treatment may more effective. We did not investigate whether the lymph nodes included cancerous tissue. Thus, the results in lymph nodes may have been affected by the presence of other tissues. In the present study, we investigated treatment with 200 mg 5-FU. As regards bolus administration, it is thought that 5-FU did not reach effective levels of concentration. More studies of 5-FU concentrations should be carried out in the future.

FUTP, a metabolite of 5-FU, is incorporated into RNA, thereby disrupting RNA function. 5-FU misincorporation can result in toxicity against RNA at several levels. F-RNA inhibits ribosomal RNA induction, the transcription of transfer RNA, and the splicing of premessenger RNA [14–16]. With these types of inhibition, cellular metabolism and viability are disrupted. In this study, the levels of F-RNA were markedly increased in tumor tissues treated by bolus injection of 5-FU compared to tissue treated by CI injection. In addition F-RNA levels in normal tissues and lymph nodes increased as well. On the other hand, the F-RNA levels in tumor were higher than the F-RNA levels in other tissue types. Kubota et al. [17] have reported that induction of ribonucleotide reductase (RNR), a key enzyme in the metabolism of fluorouridine diphosphate to fluorodeoxyuridine, occurs only with administration by CI. Regarding RNA disturbance, it is thought that as the concentration of F-RNA increases, the disturbance also

increases. Therefore, bolus injection is thought to be more effective in terms of leading to RNA damage. The 5-FU concentration in tumor was also higher than in the normal tissues, regardless of the injection method. Side effects would be expected to be suppressed with continuous injection in particular. Meanwhile, the F-RNA concentrations in lymph nodes were lower than in tumor tissue. In the treatment of lymph node metastasis, antitumor effects do not occur by RNA mechanisms. It is a primary goal of research to discover a more effective injection method. Therefore, it continues to be necessary to examine different concentrations, methods of administration, and relationships between various enzymes.

**Acknowledgements** We would like to give special thanks to the Pharmacokinetic Research Laboratories of the Pharmaceutical Research Institute, Kyowa Hakko Kogyo Company, for guidance with the material assays.

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