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Chapter

Quantification of 5-fluorouracil incorporation into RNA of human and murine tumors as measured with a sensitive gas chromatography-mass spectrometry assay

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Analytical Biochemistry 231(1), 157-163, 1995

Abstract

5-Fluorouracil (5FU) can exert its cytotoxic activity either by inhibition of thymidylate synthase or incorporation into RNA. The extent and importance of the latter in tumors of patients is not clear, due to the lack of sensitive and reproducible methods. RNA from 5FU treated human WiDr colon tumor cells was isolated and [¹⁴C]-5FU incorporation into RNA was measured by traditional scintillation counting while that of non-radiolabelled 5FU was measured with the present, new method. For the latter purpose, isolated RNA was incubated with RNase, alkaline phosphatase, and uridine phosphorylase, resulting in a complete degradation of RNA, nucleotides and nucleoside to 5FU. 5FU was then measured with gas-chromatography coupled to mass-spectrometry (GC-MS). For both methods RNA incorporation was 0.4 pmol/hr/μg RNA at 25 μM 5FU while a similar time and concentration dependence was found. Reproducibility of the assay was more than 95%. In a murine colon tumor 5FU incorporation into RNA reached a peak of 10 pmol/μg RNA, which was retained until at least 72 hr at 2.5 pmol/μg. In tumors from patients treated with 500 mg 5FU/m² incorporation into RNA after 24 hr amounted to 1.0-1.5 pmol/μg RNA. In conclusion, a new, sensitive method was established to measure 5FU incorporation into RNA in clinical tumor specimens enabling to determine its clinical relevance.

Introduction

5-Fluorouracil (5FU) is one of the most widely used anticancer agents for treatment of colorectal cancer, breast cancer and head & neck cancer. The antitumor activity of 5FU has been associated with several mechanisms of action (1-3). For the treatment of advanced colorectal cancer 5FU is usually combined with a modulator such as leucovorin (LV) (1, 4). LV can increase and prolong 5FU induced inhibition of thymidylate synthase (TS) (5-7). The degree and retention of TS inhibition has been associated with the therapeutic efficacy, both in preclinical in vitro and in vivo models, but also for clinical studies (5, 6, 8). However, other modulators such as N-phosphon-L-acetyl-aspartate (PALA) are considered to exert their potentiating effect by increasing the incorporation of 5FU into RNA (1, 9) while an increase in dose or concentration will also enhance the incorporation of 5FU into RNA (10-13). A direct relation has been observed between the dose of 5FU and the antitumor effect (14, 15).

5FU incorporation into RNA leads to a disruption in maturation of nascent RNA (1, 2). Evaluation of the role of 5FU incorporation into RNA in patients has been hampered by the lack of suitable methods to determine 5FU incorporation into samples from

patients who received 5FU. Both in preclinical *in vitro* and *in vivo* models radiolabelled 5FU can be used to measure the extent of its incorporation into RNA as well as the effect of modulators (10-12, 16-18). However, in samples from patients treated with 5FU it proved to be difficult to measure 5FU incorporation into RNA. Degradation of RNA resulting in 5-fluorouridine-2'monophosphate (2'FUMP) and 5-fluorouridine-3'monophosphate (3'FUMP) which could be detected by HPLC, indicated that 5FU was indeed incorporated into RNA (19, 20). However, this approach suffered from low sensitivity and selectivity due to the limitations of HPLC sensitivity. Further degradation of the nucleotides to 5FU itself enabled the specific measurement of 5FU using the application of a gas chromatography method coupled to mass spectrometry (GC-MS) in combination with quantitative degradation of RNA to 5FU.

Materials and Methods

Chemicals

5FU and alkaline phosphatase type VII-S (APase) were obtained from Sigma (St Louis, MO, USA). 5FU-15N₂ was from Merck-Sharp and Dohme (Montreal, Canada) and pentafluorobenzylbromide was from Pierce Chemicals (Rockford, Ill.,USA). [6-¹⁴C]-5FU (56 mCi/mmol) was obtained from Amersham International (Buckinghamshire, UK). Uridine phosphorylase (UPase; E.C. 2.4.2.3.; 590 U/ml) isolated from *E. coli*, as described previously, was kindly provided by A. Komissarov (21). Before use the ammonium sulphate precipitated UPase was dialyzed over night against Tris/dithiothreitol (200 mM/ 2 mM). RNase A (50 U/mg) and RNase T1 (105 U/ml) were acquired from Boehringer Mannheim (Almere, The Netherlands). Fetal Calf Serum (FCS) was obtained from Gibco (New York, USA) and Dulbecco's Modified Eagle Medium (DMEM) was from Flow Laboratories (Irvine, Scotland). All other chemicals were of analytical grade. Solutions were made in water purified by a Millipore Reagent Q system (Millipore, Bedford, USA).

5FU incorporation into RNA from cultured colon tumor cells

The source of the human colon WiDr cell line was described previously (16). The cell line was maintained in logarithmic growth at 37°C under a 5% CO₂ atmosphere in DMEM medium supplemented with 10% heat inactivated FCS. Cells were passaged every 3-4 days. During experiments 100 U/ml penicillin and 100 µg/ml streptomycin were added to the medium. For validation of the assay for 5-FU incorporation into RNA we exposed the cells to 5-FU under various conditions. Subconfluent cells were incubated for 2 and 4 hours in 600 ml tissue culture flasks ($\pm 30 \times 10^6$ /flask)(Greiner GmbH, Frickenhausen, Germany) with 25 and 50 µM 5-FU (final concentration)

either without addition of radioactivity or with ^{14}C labeled 5FU (2.45 $\mu\text{Ci}/\text{flask}$). After incubation the cells were harvested by trypsinization, washed, transferred to an Eppendorf vial, centrifuged and rapidly frozen in liquid nitrogen. Until RNA isolation the cell pellets were stored at -80°C .

5FU incorporation into RNA from murine and human tumors

The 5FU incorporation into RNA of murine tumors was measured at several time points after 5FU administration. Mice bearing colon carcinoma 26 (22) were treated with 80 mg 5FU/kg and tumors sized 100-200 mm^3 were removed at several time points after injection. Mice were killed by cervical dislocation, tumors were excised immediately and directly frozen in liquid nitrogen. The frozen tissue was pulverized with a micro-dismembrator as described previously (23), during which the tissues remain frozen preventing any degradation of nucleic acids. After transfer of the frozen powder to a chilled polypropylene tube, ice-cold saline was added to the powder, thoroughly mixed and centrifuged for 10 min at 4000 g at 4°C . The resulting pellet containing the nucleic acids was subsequently frozen and stored at -80°C . At the day of RNA isolation (part of) the pellet was solubilized in denaturation buffer and RNA was isolated as described below.

From five patients included in a study described elsewhere (5, 23) samples of primary colorectal cancer and/or liver metastases of colorectal cancer were analyzed for the incorporation of 5FU into RNA. These patients received an i.v. bolus injection of 5FU at a dose of 500 mg/m^2 about 24 hr prior to surgical resection of the primary tumor or of liver metastases; biopsy specimens of the tumor tissue were immediately frozen in liquid nitrogen and stored in liquid nitrogen or at -80°C . RNA was extracted from pulverized tissues after a similar procedure as described above for murine tumors. From several patients the frozen pulverized tissue was solubilized directly in the denaturation buffer and RNA was isolated. A comparison of these samples with RNA isolated from the 4000 g pellets did not reveal significant differences. From several patients the tumor biopsy specimen was divided into various parts which were pulverized and processed separately. In addition specimens of the pulverized tumor were split in separate parts, processed and assayed at different days.

RNA isolation

The RNA isolation was essentially performed as described previously (24). Precipitated nucleic acids from tissues or cell pellets were suspended in 5 ml denaturation solution consisting of 4 M guanidine-isothiocyanate, 25 mM sodium citrate, 0.5% N-lauroylsarcosine and 0.1 M β -mercaptoethanol. After addition of 0.5 ml 2 M sodium acetate (pH=4) the suspension was mixed thoroughly. Subsequently 5 ml water saturated phenol and 1 ml chloroform/iso-amylalcohol (49/1, v/v) was added.

The final suspension was shaken vigorously and cooled on ice for 15 min. After centrifugation for 20 min at 4000 rpm at 4°C the upper layer containing the RNA was removed and mixed with an equal volume of 2-propanol. In order to precipitate the RNA this suspension was placed at -80°C for 2 hours, and subsequently centrifuged for 10 min at 4000 rpm and 4°C. The pellet containing RNA was resuspended in 1.8 ml denaturing solution and precipitated again with 2 ml 2-propanol. After centrifugation for 10 min at 4000 rpm and 4°C the RNA pellet was reconstituted in enzyme buffer (40 mM Tris, 1 mM MgCl₂, 0.1 mM ZnCl₂ and 40 mM KH₂PO₄, pH 7.4). For measurement of the concentration and purity 4 µl of the RNA suspension was diluted with milli-q water to 1 ml and the optical density (OD) was measured at 260 and 280 nm. Only samples with a OD ratio (OD260/OD280) larger than 1.6 were considered to be pure (25). The concentration in µg/µl of the undiluted sample was calculated by multiplying the OD260 by a factor 10. The samples for the [¹⁴C]-5FU incorporation were either (partially) counted directly, after addition of 5 ml Ultima Gold scintillation fluid (Packard, Tilburg, NL) for 5 min, or after subjection to enzymatic degradation similar to non-radiolabeled RNA.

RNA degradation

In order to degrade the RNA enzymatically, to 260 µl RNA suspension 20 µl RNase A/ RNase T1 (500 U/ml), 10 µl APase (1 U/ml) and 10 µl UPase (1:100 dilution) were added. In this composition the reaction mixture was optimal to degrade all RNA to mononucleotides and subsequently of 3'-FUMP to 5FU during an incubation period for 1 hour at 37°C. The incubation was terminated by heating the samples at 95°C for 3 min, cooled on ice and centrifuged for 10 min at 12000 rpm and 4°C. The supernatant was frozen at -20°C until extraction for GC-MS.

For validation and optimization of the degradation procedure we used various concentrations of the four enzymes under different conditions with either isolated RNA as a substrate, the nucleotides 2'FUMP and 3'FUMP, and the nucleoside fluorouridine (FUR). These nucleotides and nucleoside would be intermediate products of the reaction. In order to have a quantitative assay all intermediate products have to be completely converted to the base 5FU. 2'FUMP and 3'FUMP were synthesized from 2'UMP and 3'UMP as described previously for the synthesis of fluoronucleosides from uridine (26). Since we wanted to perform the degradation from RNA to 5FU in one assay mixture we had to validate the conditions enabling a complete degradation to 5FU. Since the various enzymes act under different optimal conditions we defined conditions under which all enzymes would act sufficiently to achieve complete degradation. For that purpose 2'FUMP and 3'FUMP were incubated under various conditions with APase, at a pH varying from 7.4 to 8; with an incubation time varying

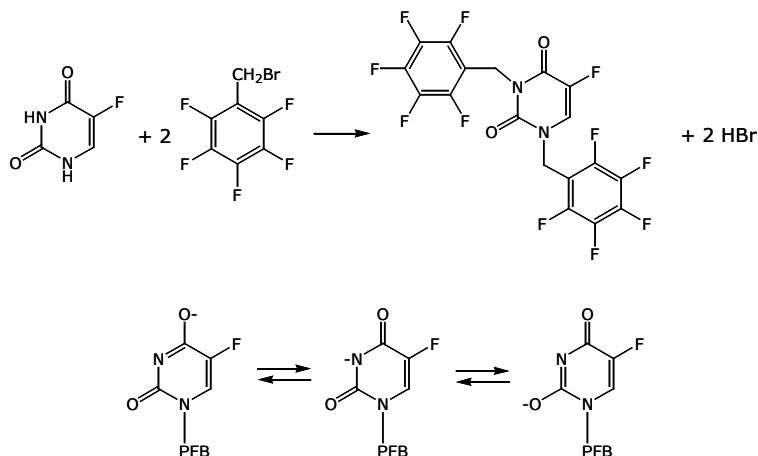


Figure 1. (Top) Derivatization reaction of 5FU with pentafluorobenzyl bromide (PFB-BR). (Bottom) The fragmentation product formed after negative chemical ionization (m/z -309 for 5FU and -311 for $5FU-^{15}N_2$)

from 30 to 120 min. Similarly we optimized the concentration, time and pH for the conversion of FUR to 5FU. Finally we combined the two enzymes in order to demonstrate quantitative degradation from the nucleotides to the base. The substrates and products of these reactions were analyzed using reversed phase HPLC. The HPLC system consisted of two M300 pumps (Gynkotek, Munchen, Germany), a Triathlon auto-sampler (Spark Holland, Emmen, the Netherlands), a microsphere C18 column (Chrompack, 100*4.6 mm, particle size 3 μ m), a diode-array detector set at 270 and 254 nm (Applied Biosystems, Foster City, CA, U.S.A.) and a 737 data acquisition system with pump control (AXXIOM, Calabasas, CA, U.S.A.). Elution with a flow of 1.0 ml/min was performed with a gradient of two buffers. Buffer A consisted of 60 mM potassiumdihydrogenphosphate and 5 mM tetrabutylammoniumhydrogensulfate (pH = 6.0) and Buffer B was 50% (v/v) acetonitrile. The gradient was started with 2% B from 0-2 min; from 2-12 min B was linearly increased to 40%; from 12-13 min B was decreased to 2% and maintained to 20 min. Retention times for 5FU, FUR and FUMP were 1.8, 3.4 and 11.9 min, respectively.

GC-MS extraction and measurement

The extraction for GC-MS was performed essentially as described before (23) with a slightly modified derivatization. Briefly, to 100 μ l enzyme degradation product 50 μ l 1 μ M $5FU-^{15}N_2$, 1 ml milli-q water and 100 μ l 2 M Tris (pH=6) were added. The solution was extracted twice with 4 ml diethylether/2-propanol (80/20, v/v). The organic fraction was blown to dryness under N_2 at 60°C. The residue was reconstituted in

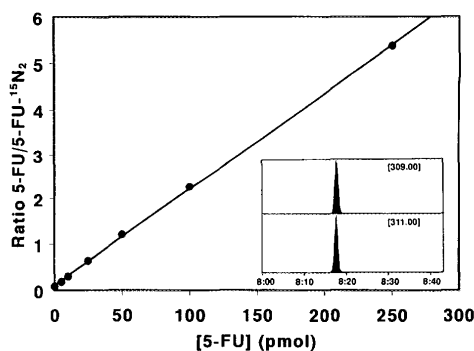


Figure 2. Typical example of a calibration line of 5FU (5 to 250 pmol) relative to 50 pmol of the internal standard 5FU-15N₂ (correlation coefficient of 0.9998). This line was not corrected for the blank, which was 0.013 ± 0.010 (mean \pm sd) of 16 separate assays. As sample limit we used a ratio of 0.033 (mean \pm 2 sd). The insert shows the selected ion recording of a standard of 5FU (upper part) and 5FU-15N₂ (lower part) in a ratio of 1 (50 pmol/50 pmol).

80 μ l acetonitrile and 10 μ l triethylamine and 10 μ l pentafluorobenzylbromide was added. The process of derivatization is shown in Fig. 1. The mixture was left at room temperature for 15 min, at which time-point the derivatization had reached a plateau. After addition of 400 μ l 0.1 M HCl the solution was extracted once with 1 ml hexane. The organic layer was blown to dryness under N₂ at 45°C and the residue was dissolved in 50 μ l hexane/propanon (3/1, v/v). From this sample 1 μ l was injected splitless, with an injector temperature of 320°C, into the GC-MS system (VG30-250, Fisons, Weesp, The Netherlands). Chromatographic separation was carried out on a CPSil19CB column (25 m * 0.25 mm I.D., film thickness 0.25 μ m) (Chrompack, Middelburg, The Netherlands) with an oven temperature gradient starting with 200°C for 1 min, increasing the temperature with 20°C/min to 320°C and maintaining this temperature for 2 min. The ions for 5-FU and 5FU-15N₂ (Fig. 1B; m/z -309 and m/z -311, respectively) were recorded with negative ionization detection and methane as the moderating gas (Fig. 2; insert). An excellent relationship was observed between 5FU and the ratio between 5FU and 5FU-15N₂ (Fig. 2) with a high signal/noise ratio. More details of the 5-FU measurement with GC-MS are described previously (23, 27, 28).

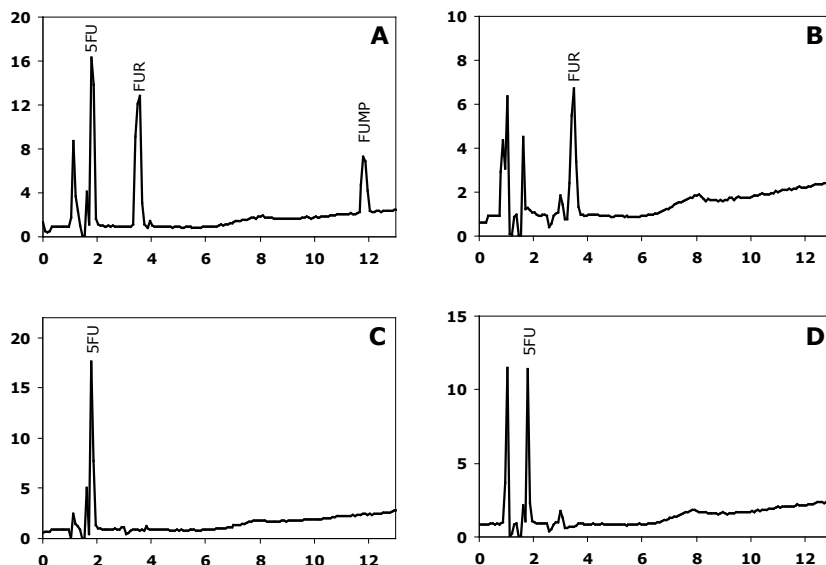


Figure 3. HPLC chromatograms showing standards of the nucleotides 2'FUMP and 3'FUMP (not separated), the nucleoside FUR and the base 5FU (A). Degradation of 2'FUMP and 3'FUMP to FUR when incubated with APase (B); degradation of FUR to 5FU when incubated with UPase (C); and complete degradation of 2'FUMP and 3'FUMP to 5FU when incubated with APase and UPase (D). The conditions used for these assays are the final conditions as described in Materials and Methods.

Results

For validation of the method we first determined whether the enzyme mixture would quantitatively degrade 2'FUMP and 3'FUMP and FUR to 5FU. As we aimed to perform RNA degradation to the bases in one reaction mixture, an optimal pH, enzyme and co-factor concentration had to be established. However, alkaline phosphatase has a higher pH optimum than the other enzymes; in addition to that some of the co-factors might interfere with each other (e.g. ZnCl_2 and phosphate). In order to prevent precipitation of the latter, buffers had to be made just before use. Fig. 3 shows the quantitative degradation of 2'FUMP and 3'FUMP to 5FU; using HPLC no nucleotide could be detected after incubation with APase. UPase degraded all FUR quantitatively to 5FU. The combination of both enzymes showed a complete disappearance of the nucleotide,

Table 1. Comparison of 5-FU incorporation determined by GC-MS and ¹⁴C scintillation counting.

Exposure time (hrs)	Concentration (μM)	5-FU incorporation	
		GC-MS	¹⁴ C
2	25	0.81 ± 0.10	0.79 ± 0.17
2	50	1.45 ± 0.47	1.31 ± 0.31
4	25	1.56 ± 0.26	1.50 ± 0.27
4	50	2.69 ± 0.55	2.88 ± 0.90

Values are means of 3 separate experiments and expressed as pmol/μg RNA ± s.d. No growth inhibition was observed during the period of the experiment.

while no nucleoside was detectable after incubation. Only 5FU could be recovered. Incubation of RNA isolated from either tumor cells or tumor tissues with RNase and T1 completely degraded RNA as determined by gelelectrophoresis (data not shown). The next step in the validation was a comparison of [6-¹⁴C]-5FU incorporation into RNA with that of non-labeled 5FU as determined with the GC-MS method. WiDr cells were exposed to the drug for 2 and 4 hr at 25 and 50 μM 5FU. For both assays RNA was isolated similarly. Quantitation of RNA in 30 samples demonstrated that the RNA concentration in the cell lines varied between 5-15 μg RNA/10⁶ cells. [¹⁴C]-radiolabeled RNA as measured either directly by scintillation counting after solubilization of the RNA, or by degradation of the labeled RNA to the bases, did not reveal differences between both methods. This latter observation again confirmed that the degradation procedure indeed quantitatively degraded RNA to the bases. Table 1 summarizes the final result of a comparison between both methods. An excellent correlation was observed between both methods, demonstrating that 5FU incorporated into RNA can be reliably measured after enzymatic degradation by using GC-MS. The sensitivity of the GC-MS assay is dependent on the ratio of the internal standard compared to that of the unknown quantity (this ratio should be between 0.1 and 10; see Fig. 2), but was also dependent on the yield of isolated RNA. Samples containing 0.1 pmol 5FU/μg RNA are considered to be the limit of the total assay, including RNA degradation. The method was subsequently used to measure 5FU incorporation into RNA from colon tumors obtained from mice treated with 5FU. Table 2 demonstrates that 5FU incorporation into RNA of murine tumors is highest at 2 hr after injection of the compound and that it is retained for at least 10 days after injection (data not shown). Preliminary measurement of 5FU incorporation into RNA from tumor samples derived from patients treated with 5FU also revealed that 5FU incorporated into RNA could be measured in tumor samples from patients.

Table 2. Incorporation of 5FU into RNA from murine and human colon carcinoma.

Time (hr)	Murine carcinoma Colon 26	Human colon tumors
2	10.1 ± 0.42	-
24	8.99 ± 1.51	1.21 ± 0.19
72	2.52 ± 0.16	-

Values (as pmol of 5FU incorporated per μg RNA) are means \pm SD of 3 murine tumors or from 5 patients. Mice were treated with 80 mg 5FU/kg and tumors were excised at the indicated time points as described previously. Patients received a bolus injection of 500 mg 5FU/m² and biopsy specimens were obtained at surgery 24 hr after injection.

Repeated measurements of the same sample (dividing one sample after pulverization into three samples, dissolving them in the denaturation buffer, but with the rest of the procedure performed at different days) revealed a variation of less than 5%. Intratumor variation was determined by taking four separate parts from the same tumor; these samples were pulverized separately and from each sample RNA was isolated and 5FU incorporation was quantitated. The amount of 5FU incorporation in these separate samples was 0.47, 0.35, 0.24 and 0.37 pmol/ μg RNA, indicating a relatively small variation (about 25%; mean \pm SD, 0.36 \pm 0.09 pmol/ μg RNA) in separate parts of the tumor. Comparison of samples from mice and patients taken at the same time demonstrated that in mice the extent of 5FU incorporation into RNA is much higher than in patients.

Discussion

This paper demonstrates that reliable and sensitive measurement of 5FU incorporation into RNA of tumor samples from patients is possible without the use of radiolabelled drug. In preclinical model systems the assay was shown to be comparable to values found with a traditional method, measurement of incorporation of labelled 5FU into RNA of colon tumor cells. When recalculating the incorporation in pmol 5FU per 10⁶ cells/hr similar values (6 pmol/hr/10⁶ cells) were obtained compared to that described previously in this cell line (16). In addition a similar time and concentration dependence was observed compared to studies described in these cell lines, but also compared to that in other cell lines and tumors (10, 13, 18, 29). In tumors of animals 5FU incorporated into RNA was retained for at least three days and possibly longer. Also this pattern was comparable to that described previously by above-mentioned authors in other model systems using radiolabelled 5FU.

Despite the combination of various sequential techniques, isolation of RNA,

subsequent degradation to 5FU and derivatization, the assay appeared to have a good reproducibility. The most crucial step, degradation of RNA was complete and quantitative. A direct quantitative comparison with a previously described method (19), by measurement of the 2'FUMP and 3'FUMP was not possible. By using this method the degraded 2'FUMP and 3'FUMP was detectable at the chromatogram, but peaks could not be quantitated reliably. Therefore this method was not found to be suitable for routine use. With the present method we also observed an excellent reproducibility in the same tumor sample. The variation within several parts of the tumor is in line with the observed variation in the 5FU concentration of a tumor. Also in tumor samples from the same patient we observed a similar variation in the 5FU tissue concentration compared to the 5FU incorporation into RNA (23).

The method described in the present paper represents a major advance in the study on the mechanism of action of 5FU in human beings. At least two hypotheses have been put forward considering the mechanism of action of 5FU in humans, inhibition of TS and incorporation of 5FU into RNA. In addition it has been hypothesized that incorporation of 5FU into RNA of normal tissues would be related to the toxicity caused by the drug (1). The quantitative measurement of 5FU in tumor samples from patients demonstrates that 5FU is still present in RNA until at least 24 hr after injection of a therapeutic dose. The present method will allow to study the retention and modulation of 5FU incorporation into RNA of tumors of patients being treated with 5FU. In addition this will allow to differentiate the various mechanisms of actions of 5FU both in tumors and normal tissues.

In conclusion, we have developed a new methodology to determine 5FU incorporation into RNA of biopsy specimens of both animals and patients.

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