Biochemical Pharmacology, Vol. 28, pp. 489–495.

0006-2952/79/0215-0489 $02.00/0

EFFECTS OF 5-FLUOROCYTIDINE ON MAMMALIAN TRANSFER RNA AND TRANSFER RNA METHYLTRANSFERASES*

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(*Received* 7 *April* 1978; *accepted* 27 *June* 1978)

Abstract—Effects of 5-fluorocytidine on mouse liver tRNA and mouse liver tRNA methyltransferases were investigated. tRNA isolated from drug-treated tissue was shown to contain 5-fluorocytidine and 5-fluorouridine. The amounts of all 5-substituted pyrimidine nucleosides such as 5-methylcytidine, 5-methyluridine, pseudouridine and 5,6-dihydrouridine were substantially reduced. The decreased methylation of tRNA was shown to result from decreased tRNA cytosine-5-methyltransferase and tRNA uracil-5-methyltransferase activities and capacities. Incorporation of 5-fluorouridine into tRNA, as well as the effects of 5-fluorocytidine administration on the modified uridine derivatives in tRNA, suggested the *in vivo* conversion of 5-fluorocytosine derivatives to 5-fluorouracil derivatives, as administration of the latter had been shown previously to cause the same effects. The inhibition of tRNA cytosine-5-methyltransferase after administration of 5-fluorocytidine resembles the effect of another cytidine analog, 5-azacytidine, which is known to cause lack of 5-methylcytidine in mouse liver tRNA and the inhibition of this particular tRNA methyltransferase.

Transfer RNA contains a large number of modified nucleosides which are synthesized by post-transcriptional modification reactions [1, 2]. The enzymes catalyzing these reactions are highly specific. No definite functions can as yet be assigned to most modified nucleosides in tRNA [1–3]. The biological functions of the modifying enzymes are therefore not understood. tRNA methyltransferase activities have quite consistently been found to be highly elevated in neoplastic tissues compared to corresponding normal tissues [4–8] and the increased enzyme activities were shown to correlate with tumour growth rates [4, 5, 9], suggesting that tRNA methyltransferases, and perhaps also other tRNA modifying enzymes, might provide potential targets for cancer chemotherapy.

Several cytotoxic analogs of adenine have been shown to inhibit the activity of tRNA-methylating enzymes *in vitro* [10, 11]. To our knowledge, none of these agents has been shown to reduce the methyl content of mammalian tRNA *in vivo*. Recently, we have demonstrated that several clinically useful antineoplastic pyrimidine analogs specifically alter tRNA modifications *in vivo* [12–14]. Thus, the administration of 5-azaCR† to mice was found to decrease the m^5C content of liver tRNA [12] while the administration of 5-FU [13] and 5-FUR [14] to mice decreased the m^5U, ψ and hU content of liver [13, 14], mammary gland [13] and mammary tumor [13] tRNA. 5-AzaCR was found to have no effect on the biosynthesis of m^5U, ψ and hU in tRNA; similarly, 5-FU and 5-FUR were shown to have no effects on the formation of m^5C in tRNA.

The molecular mechanism underlying the selective reduction of the m^5C content of tRNA by 5-azaCR [12]‡ and the selective reduction of the m^5U content of tRNA by 5-FU [13] appears to be the same, i.e. decreased activity and capacity of the corresponding tRNA pyrimidine-5-methyltransferases after drug administration [13].‡ The strict structural requirements of the effects of 5-azaCR [12],‡ 5-FU [13] and 5-FUR [14] on tRNA modification suggest a common mode of action by the 5-substituted pyrimidines on tRNA methylases.

5-FCR is, like 5-azaCR, a 5-substituted analog of cytidine and has been shown to have antitumour activity [15, 16] but thus far has found only limited clinical application. In view of the specific inhibitions of tRNA modification exerted by 5-azaCR [12]‡ and 5-FU [13], it seemed of interest to investigate if 5-FCR would also interfere with specific post-transcriptional modification reactions of tRNA. The question was studied by (1) analyzing the base composition of tRNA isolated from the livers of mice treated with 5-FCR and (2) assaying *in vitro* tRNA methyltransferases isolated from the livers of mice treated with 5-FCR.

*This work was supported by USPHS Grants CA-13591 and CA-10893 (P8) and American Cancer Society Faculty Research Award PRA-108.

†Abbreviations used in the text are as follows: m^5U, 5-methyluridine (ribothymidine); hU, 5,6-dihydrouridine; ψ, pseudouridine; 4abu^{3}U, 3-(3-amino-3-carboxypropyl)uridine; I, inosine; m^1A, 1-methyladenosine; m^6A, N^6-methyladenosine; t^6A, *N*-[9-(β-D-ribofuranosyl)purin-6-yl carbamoyl] threonine; m^1G, 1-methylguanosine; m^2G, N^2-methylguanosine; m^2_2G, N^2, N^2-dimethylguanosine; m^7G, 7-methylguanosine; m^3C, 3-methylcytidine; m^5C, 5-methylcytidine; ac^4C, N^4-acetylcytidine; *N*′, a nucleoside trialcohol obtained by reduction of a nucleoside dialdehyde with sodium borohydride; 5-FC, 5-fluorocytosine; 5-FCR, 5-fluorocytidine; 5-FU, 5-fluorouracil; 5-FUR, 5-fluorouridine; 5-azaCR, 5-azacytidine; CR, cytidine; UR, uridine; AR, adenosine; GR, guanosine; FCR-tRNA, tRNA containing 5-FCR; and m^5C methylase, tRNA cytosine-5-methyltransferase. Analogous abbreviations apply to other tRNA methyltransferases. TCA, trichloroacetic acid; and S-Ado-Met, *S*-adenosyl-L-methionine.

‡ L.-J. W. Lu and K. Randerath, *Cancer Res.*, accepted for publication.

MATERIALS AND METHODS

Materials. CR and S-Ado-Met were obtained from Sigma Chemical Co., St. Louis, MO. 5-FCR and 5-FC were kindly supplied by Dr. W. E. Scott of Hoffmann-LaRoche, Nutley, NJ. [CH_3–^{3}H]-S-Ado-Met (6.9 to 12.2 Ci/m-mole) was purchased from New England Nuclear Corp., Boston, MA. *Escherichia coli* B tRNA and 5-FUR were obtained from CalBiochem, San Diego, CA. *Mycoplasma hominis* tRNA was a generous gift from Dr. Jack Horowitz of the Department of Biochemistry and Biophysics, Iowa State University, Ames, IA. Male BALB/Crgl mice (about 25 g), bred and maintained at the mouse colony of the Department of Cell Biology, Baylor College of Medicine, were used in all experiments unless specified otherwise.

Isolation and analysis of tRNA. To study the effect of 5-FCR administration on the base composition of tRNA, mice (four per group) were given i.p. injections of various doses of 5-FCR (10.0 to 100.0 mg/kg), once daily for 4 consecutive days, and were sacrificed 24 hr after the last injection. Control animals received 0.9% NaCl solution. tRNA was isolated from pooled liver tissue using phenol extraction at pH 4.5 and adsorption to DEAE-cellulose [17]. tRNA was purified further by polyacrylamide gel electrophoresis [18]. The nucleoside composition of tRNA was analyzed by a chemical tritium derivative method [19, 20]. 5-FCR and 5-FUR were converted to trialcohol derivatives by treatment with $NaIO_4$ and $NaBH_4$ [19] and used as u.v. markers for chromatography [13, 14].

The trialcohol derivative of 5-FCR co-chromatographed with the trialcohol derivative of m^{1}G on cellulose thin layers. In order to establish if the administration of 5-FCR had an effect on the amount of m^{1}G, and also to measure the incorporation of 5-FCR into tRNA, the spot containing m^{1}G′ and FC′ was eluted with 2 N NH_4OH [19], and an aliquot of the eluate was re-chromatographed on a silica gel sheet (No. 13181, EM Laboratories, Elmsford, NY). A corresponding aliquot containing U′, obtained from the same chromatogram, was added to the aliquot containing m^{1}G′ and FC′ prior to chromatography as an internal standard. The solvent used for rechromatography was $CHCl_3$–MeOH–HOAc (40:10:1.5, by vol.) for both dimensions. The radioactive spots were located with the aid of u.v. markers. The chromatographic mobilities of FC′ and m^{1}G′ relative to the mobility of U′ ($R_{U'}$) in the two dimensions, respectively, were 0.51 and 0.56 for m^{1}G′ and 0.71 and 0.72 for FC′. Each tRNA digest was applied to four cellulose thin-layer sheets and each eluate was re-chromatographed on silica gel in duplicate.

Data were analyzed by Student's *t*-test.

Preparation of tRNA methyltransferases. tRNA methylase preparations were isolated from livers of mice by the procedure of Jackson and Pegg [21]. The enzyme extract prepared from 65% $(NH_4)_2SO_4$ precipitates was dialyzed exhaustively and stored in portions at −80°. The enzyme preparations were thawed only once before use. Protein concentration was determined by the procedure of Lowry *et al.* [22]. Control enzyme was isolated from livers of mice given 0.9% NaCl solution. To study the dose-dependent effect of 5-FCR on tRNA methylases, mice (four animals per group) were given a single i.p. injection of 10.0, 50.0 or 100.0 mg/kg of 5-FCR, 3 hr prior to enzyme isolation. To study the time course of the effect of 5-FCR on the enzymes, mice (five animals per group) were given one injection of 18 mg/kg of 5-FCR for various intervals prior to enzyme isolation.

tRNA substrates. The substrates were: (1) *E. coli* B tRNA, (2) *Mycoplasma hominis* tRNA (for the assay of tRNA m^{5}U methylase [13]), (3) FCR-tRNA and (4) normal mouse liver tRNA. FCR-tRNA was prepared from livers of mice (BALB/Crgl or Swiss mice) that had been given various doses of 5-FCR; the base composition of the tRNA was analyzed as described above.

tRNA methyltransferase assays. Unless specified otherwise, the standard incubation mixture (100 μl) contained 0.3 M NH_4OAc, 0.15 M Tris–HCl, pH 8.8, and 20 μM [CH_3–^{3}H]-S-Ado-Met (2.5 to 6.9 Ci/m-mole) [23]. For determining enzyme activity (rate of methylation), 50 μg *E. coli* B tRNA and 250 μg protein were incubated for 30 min at 38°. For determining enzyme capacity (extent of methylation), 4 μg *E. coli* B tRNA and 1.3 mg protein were incubated for 2 hr. The reaction was stopped with 1.0 M NaCl, 0.14 M NaOAc buffer, pH 4.5, pre-saturated with phenol. For analysis of total methylation, tRNA was precipitated by the addition of 10% TCA. Precipitates were collected on glass fiber filter (Whatman, GF/A, 2.4 cm diameter), and counted in toluene containing 0.4% 2,5-diphenyloxazole (PPO) in a liquid scintillation counter. For the analysis of per cent distribution of methyl label in methylated bases, tRNA was precipitated by the addition of 3 vol. alcohol, and was digested enzymatically to nucleosides [19]. The labeled nucleosides were separated on silica gel thin layers with acetonitrile–conc. NH_4OH (4:1, by vol.) in the first dimension and acetonitrile–2 M HCOOH (10:1, by vol.) in the second dimension. Radioactive spots were located by fluorography [24], unless otherwise specified, and their identity was established by co-chromatography with authentic u.v. markers. Radioactivity in each spot was evaluated by elution with 2 N NH_4OH (95 per cent elution efficiency), followed by liquid scintillation counting in xylene containing 0.3% PPO (by wt) and 15.8% Triton X-100 (by vol.). Results were expressed as per cent of total cpm, unless otherwise specified. To analyze the activity and capacity of individual base methylases, the radioactivity incorporated into each methylated nucleoside was calculated by multiplying the per cent methyl incorporated into the particular nucleoside, after chromatographic separation, by the total TCA-precipitable radioactivity incorporated into tRNA.

Inhibitor studies. 5-FCR, 5-FC, 5-FUR and CR were added to tRNA methylase assays *in vitro* to test whether the nucleosides would inhibit specifically tRNA m^{5}C methylase activity and to establish structure–activity relationships. The enzyme activity was determined according to the standard assay conditions described above. At pH 7.5, relatively more m^{5}C (70 per cent of total methyl label in m^{5}C) was formed compared to pH 8.8 (50 per cent of radioactivity in m^{5}C). Thus, the effects of nucleosides on tRNA m^{5}C methylase were also studied at pH 7.5.

RESULTS AND DISCUSSION

5-FCR resembles 5-azaCR in that both are structural analogs of CR. Biotransformation of 5-FCR to 5-FUR has been suggested to be responsible for the decreased dTTP pool in cultured lymphocytes treated with 5-FCR [25]. In view of the effects of 5-azaCR [12] and 5-FU [13] on the biosynthesis of m^5C and m^5U, respectively, in tRNA, it was of interest to investigate if 5-FCR would inhibit the modifications of both CR and UR in tRNA.

Base composition analysis of tRNA after administration of 5-FCR showed not only 5-FCR but also 5-FUR to be present in tRNA (Fig. 1B), indicating the deamination of 5-FCR to 5-FUR in mammalian tissue. The incorporation of 5-FCR and 5-FUR into tRNA was dose dependent (Fig. 2). At the highest dose studied, the incorporation of 5-FCR reached about one in three CR residues, causing a marked reduction of the amount of CR in tRNA. Thus, the CR content of tRNA after 100.0 mg/kg of 5-FCR was 18.99 ± 0.19 mole per cent compared to 25.15 ± 0.33 mole per cent of control tRNA. The extensive incorporation of 5-FCR into tRNA is in sharp contrast to the negligible incorporation of 5-azaCR into tRNA [12]. As shown also in Fig. 2, 5-FCR administration inhibited not only the formation of m^5C (decrease of up to 50 per cent), in analogy to the action of 5-azaCR [12], but also the formation of m^5U (40 per cent), ψ (20 per cent) and hU (10 per cent), in analogy to the action of 5-FU and 5-FUR [13, 14]. There were no changes in UR_{total} (= UR + all modified derivatives of uridine), AR_{total}, CR_{total} and GR_{total}.

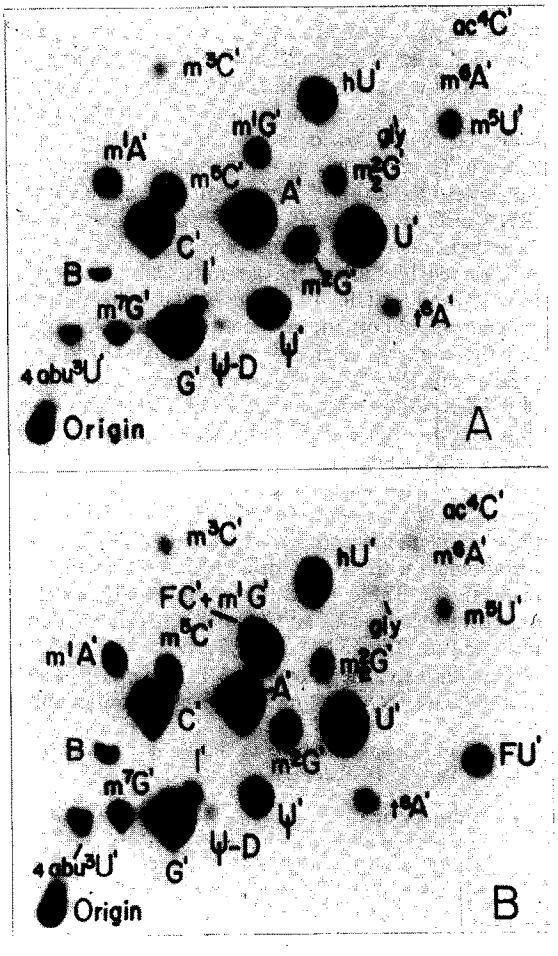

Fig. 1. Fluorograms of cellulose thin-layer maps obtained by digestion of tRNA to nucleosides and tritium labeling. Panel A: tRNA isolated from livers of control mice; panel B: tRNA isolated from livers of mice given one daily i.p. injection of 50.0 mg/kg of 5-FCR for 4 consecutive days. *N'*, nucleoside trialcohols. FC', trialcohol of 5-FCR. B, background spot (not derived from RNA).

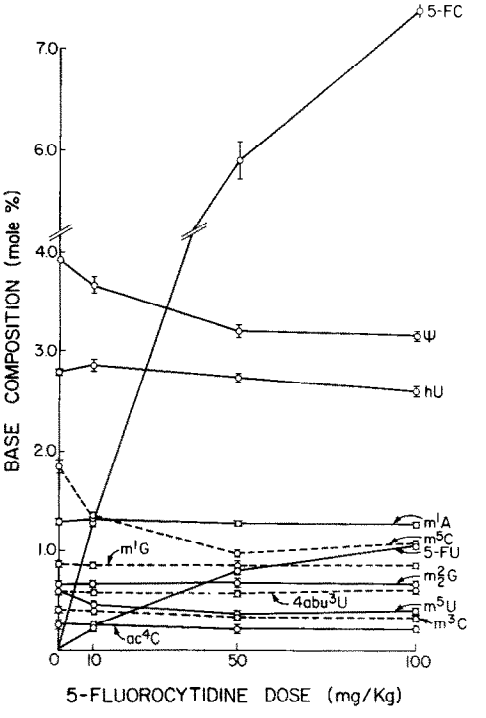

Fig. 2. Dose-dependent effect of 5-FCR on the base composition (mole per cent) of tRNAs. Mice were given one daily i.p. injection of the indicated dose of 5-FCR for 4 consecutive days. tRNA was isolated 24 hr after the last injection. Data are the means ± S. D. of four chromatographic analyses. 5-FC, 5-FCR incorporated into tRNA.

Few mammalian undermodified tRNAs are currently available [12, 26] to serve as substrates for studying mammalian tRNA modifying enzymes. Since base composition anlysis of tRNAs isolated from livers of mice treated with 5-FCR demonstrated deficiency of m^5C (Fig. 2), this undermethylated tRNA was tested for its ability to act as a homologous substrate *in vitro* for tRNA methylases extracted from livers of control mice. The ratio of radioactivity incorporated into m^5C to the radioactivity in all other methylated nucleosides, was 0.45:1 for control tRNA and 2.49:1 for tRNA isolated from livers of mice treated for 4 days with 50.0 mg/kg of 5-FCR (m^5C content, 0.97 mole per cent, Fig. 2). Thus, m^5C-deficient tRNA isolated from livers of mice treated with 5-FCR was a good homologous substrate for tRNA m^5C methylase *in vitro*, suggesting that the fraudulent tRNA would also be a substrate for the normal enzyme *in vivo*.

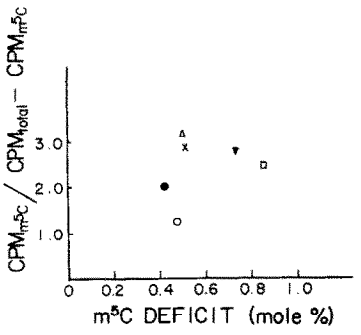

Fig. 3. Relationship between m^{5}C deficiency of tRNA and its capacity to serve as a substrate for tRNA m^{5}C methylase *in vitro*. Incorporation of 5-FCR (mole per cent) into the tRNA samples was as follows: (○) 1.28 ± 0.01; (□) 5.89 ± 0.20; (▼) 7.43 ± 0.06; (●) 1.31 ± 0.01; (X) 3.24 ± 0.03; (△) 4.66 ± 0.07, Symbols ○, □ and ▼: tRNA isolated from BALB/Crgl mice treated with one daily dose of 10.0, 50.0 and 100.0 mg/kg, respectively, of 5-FCR for 4 days. Symbols ●, X and △: tRNA isolated from Swiss mice treated with two daily injections of 10.0, 50.0 and 100.0 mg/kg, respectively, of 5-FCR for 3 days. Enzyme preparation from normal mouse liver was used for measuring the extent of methylation. Each value represents the average of two assays.

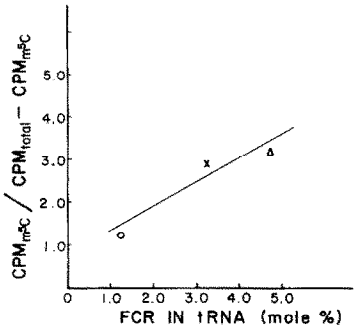

Fig. 4. Correlation of 5-FCR incorporation into tRNA with its capacity to serve as a substrate for tRNA m^{5}C methylase *in vitro*. tRNAs were the same as described in Fig. 3. m^{5}C content (mole per cent) of tRNA was as follows: (○) 1.35 ± 0.03; (X) 1.31 ± 0.03; and (△) 1.32 ± 0.02. Enzyme preparation from normal mouse liver was used for measuring the extent of methylation. Each value represents the average of two assays.

tRNA preparations isolated from livers of mice treated with different dose schedules of 5-FCR were compared for their ability to act as substrates for tRNA m^{5}C methylase *in vitro*. As shown in Fig. 3, there was a poor correlation between the ability of tRNA to serve as a substrate for tRNA m^{5}C methylase *in vitro* and the degree of its m^{5}C deficiency. On the other hand, if tRNAs having similar m^{5}C contents but containing different amounts of 5-FCR were compared, it was found that tRNAs containing more 5-FCR were the better substrates for tRNA m^{5}C methylase *in vitro* (Fig. 4). This may be due to the presence of 5-FCR in place of CR residues which may cause alterations of the tertiary structure of tRNA. This may also provide an explanation for the observation that the m^{2}G content was 1.50 ± 0.02 mole per cent after four daily treatments with 50.0 mg/kg of 5-FCR compared to 1.35 ± 0.03 mole per cent for control tRNA.

The lack of m^{5}C and m^{5}U in tRNA (decreases of up to 50 and 40 per cent, respectively) from drug-treated mice could not be fully accounted for by random analog incorporation (23 per cent replacement of CR by 5-FCR and 6 per cent replacement of UR by 5-FUR). This suggested possible drug-induced alterations of tRNA methyltransferases. The results in Table 1 demonstrated specific marked inhibitions of tRNA m^{5}C (14 per cent of control) and m^{5}U (27 per cent of control) methylase, 12 hr after administration of 18 mg/kg of 5-FCR. There were no consistent changes of tRNA m^{1}A, m$^2_2$G, m^{2}G, m^{1}G and m^{7}G methylase activities and capacities (Table 1 and Figs. 5 and 6). The decrease in tRNA m^{5}C methylase after 5-FCR administration was dose dependent (Fig. 5) and time dependent (Fig. 6). As previously shown, the administration of both 5-FU [13] and 5-FUR [14] to mice led to a dose-dependent decrease of both the m^{5}U content of tRNA [13, 14] and the capacity of tRNA m^{5}U methylase [13]. The present data (Fig. 2 and Table 1) suggest a similar effect of 5-FCR administration on tRNA m^{5}U methylase.

It thus appears that the sequence of events leading to tRNA alterations after 5-FCR administration is as follows: (1) partial deamination of 5-FCR to 5-FUR (Fig. 1B); (2) incorporation of 5-FCR and 5-FUR into tRNA; (3) interference of 5-FCR and 5-FUR or their metabolites with tRNA m^{5}C and m^{5}U methylases, respectively (Table 1 and Figs. 5 and 6), by an as yet unknown mechanism; and (4) decreased formation of m^{5}C and m^{5}U in tRNA (Fig. 2).

Qualitatively, the effects of 5-FCR (Fig. 6) and 5-azaCR * on tRNA m^{5}C methylase were similar, but the

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Table 1. Distribution of methyl label in nucleosides after *in vitro* methylation of *Mycoplasma hominis* tRNA by enzymes from livers of normal mice and mice treated with 18 mg/kg of 5-FCR for 12 hr

Enzyme source	Radioactivity distribution *						
	m^{1}A	m^{5}C	m$^2_2$G	m^{2}G	m^{1}G	m^{7}G	m^{5}U
Control enzyme	34.32 ± 0.21	54.08 ± 0.70	24.65 ± 1.16	24.60 ± 0.38	2.54 ± 0.07	13.89 ± 1.16	4.45 ± 0.17
5-FCR-treated enzyme	38.78 ± 0.73†	7.46 ± 0.09†	21.39 ± 0.96	22.79 ± 0.73	2.19 ± 0.11	14.85 ± 0.48	1.21 ± 0.17†

* Extent of methylation was measured. Each value represents mean ± S. D. for triplicate analyses. Results are expressed as: $\frac{cpm_{Me-N}}{cpm_{total\ Me-N} - cpm_{(m^1C+m^5U)}} \times 100\%$, where Me–*N* is a methylated nucleoside.

† $P < 0.001$ (compared with control).

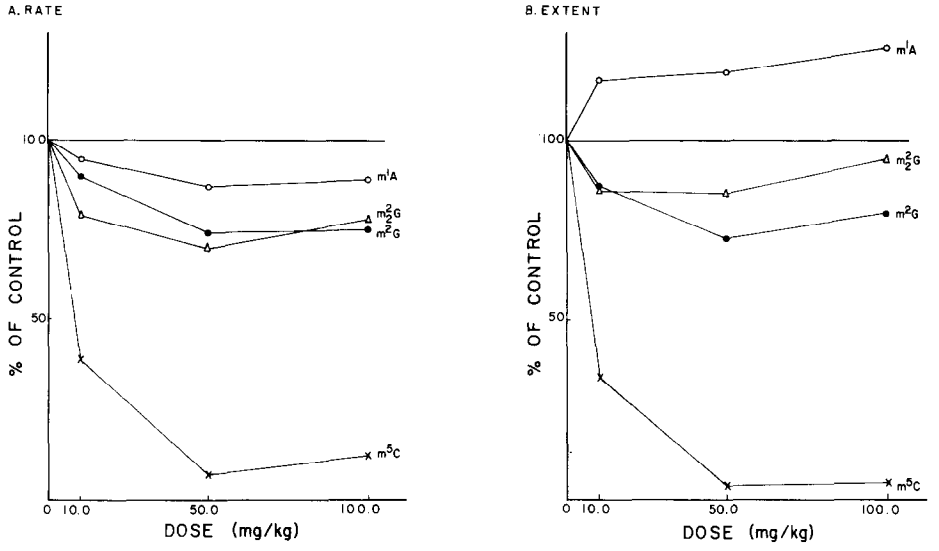

Fig. 5. Effects of a single dose of 5-FCR on activities (A) and capacities (B) of individual mouse liver tRNA methylases. The radioactivity incorporated into each methylated nucleoside was derived by multiplying the percentage of radioactivity in each methylated nucleoside (duplicate assays) by the total TCA-precipitable radioactivity incorporated into tRNA (triplicate assays).

time course of the effects was different for the two drugs. Maximal inhibition of tRNA m^5C methylase was observed 12 hr after the administration of 5-FCR (18 mg/kg), compared to 4–7 hr after the same dose of 5-azaCR.* A gradual recovery was detectable 24 hr after 5-FCR treatment, compared to 7–12 hr after 5-azaCR administration.* At 72 hr after a single dose of 18 mg/kg of 5-azaCR, tRNA m^5C methylase was about 75 per cent of control* compared to about 20 per cent of control for the same dose of 5-FCR (Fig. 6). The exact reason for the extremely prolonged effect of 5-FCR on tRNA m^5C methylase in comparison with that induced by 5-azaCR* is not clear at the present time; it may be speculated that both the chemical stability of 5-FCR and the high level of incorporation are involved. 5-AzaCR is known to be chemically unstable [27] and its incorporation into mouse liver tRNA is negligible [12]. Because of the extensive incorporation of 5-FCR into tRNA, and perhaps also into other stable RNAs such as ribosomal RNAs, FCR-RNA itself may serve as a depot of 5-FCR whose release in the course of normal degradation of the RNAs in the cell may in part be responsible for the prolonged inhibition of tRNA m^5C methylase.

In view of the selective *in vivo* inhibition of tRNA modifications exerted by 5-azaCR [12],* 5-FU [13], 5-FUR [14] and 5-FCR (present work), it is appropriate to ask whether the nucleoside analogs themselves are the inhibitors *in vivo* or whether metabolic activation is required. This question was examined by assaying the tRNA methylase activities of control liver *in vitro* in the presence of various nucleoside analogs. The presence of 5-FCR led to decreased total methylation (TCA-precipitable radioactivity) which was solely due to decreased formation of m^5C in *E. coli* B tRNA substrate (Fig. 7). On the other hand, CR had no effect on total methylation, or on the distribution of radioactivity in m^5C, m^2_2G, m^2G and m^1A in the tRNA substrate (Fig. 7). Neither 5-FUR nor 5-FC (10 mM) had an effect on the radioactivity incorporated into these nucleosides at pH 7.5. Since 5-FC did not show this effect, it appears that the ribose moiety is required for inhibition to take place *in vitro*. When 20 mM CR was added to the assay mixture containing various concentrations of 5-FCR, it was found that CR could not reverse the inhibitory effect of 5-FCR on tRNA m^5C methylase activity (Fig. 7). CR or 5-FCR (10 mM)

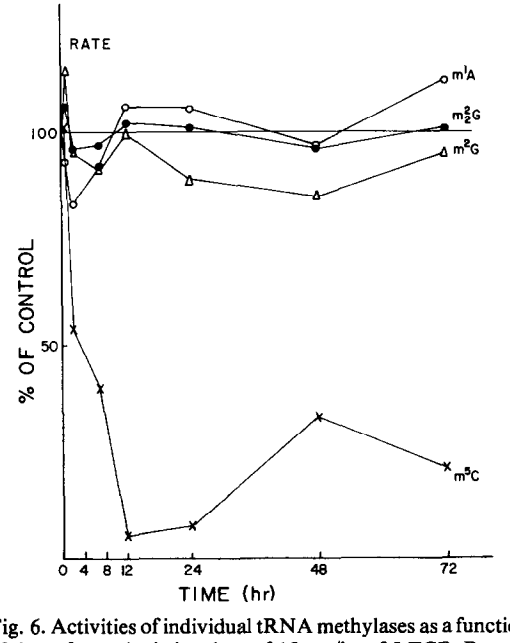

Fig. 6. Activities of individual tRNA methylases as a function of time after a single i.p. dose of 18 mg/kg of 5-FCR. Results were obtained as described in Fig. 5.

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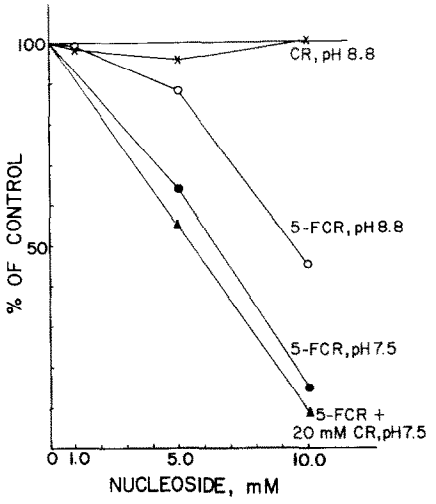

Fig. 7. Effects of 5-FCR, CR and a combination of 5-FCR and CR, on activity of tRNA m^5C methylase, isolated from normal mouse liver, assayed *in vitro* with *E. coli* B tRNA as the substrate. The effects of the nucleosides were studied at pH values of 7.5 and 8.8. The ratio of the radioactivity incorporated into an individual methylated nucleoside to the radioactivity in the sum of (m^1A, m_2^2G and m^2G), was obtained from triplicate enzyme assays and one chromatographic analysis for each assay. The methylation of adenine and guanine was not affected by the addition of these nucleosides (data not shown).

had no effect on the capacity of tRNA m^5C methylase assayed under extent conditions.

When the enzyme preparation was pretreated with 10 mM 5-FCR at either 0° (8 hr) or 38° (15 min), followed by exhaustive dialysis to remove 5-FCR, tRNA m^5C methylase activity was normal. Our data are consistent with the hypothesis that 5-FCR binds reversibly to tRNA m^5C methylase, thereby inhibiting the transfer of methyl groups from S-Ado-Met to CR moieties in tRNA. Examination of the kinetics of this inhibition would require the purification of the enzyme, which has not been accomplished thus far. Whether 5-FCR itself is the inhibitor *in vivo* is still an open question. The high concentration of 5-FCR (> 1 mM) required for inhibition of tRNA m^5C methylase makes it improbable that such conditions could be encountered *in vivo*, as this compound is probably very rapidly metabolized to nucleotides.

In Table 2, the effects of 5-FCR, 5-FU, 5-FUR and 5-azaCR on mouse liver tRNA have been summarized. Although there are differences, these drugs have much in common. Perhaps the most striking difference is the extensive incorporation of 5-FCR into tRNA and the lack of incorporation of 5-azaCR. All four drugs cause the inhibition of modification reactions at position 5 of the pyrimidines in tRNA, 5-FU and 5-FUR being specific inhibitors of uracil modifications and 5-azaCR being a specific inhibitor of the methylation of cytosine. 5-FCR affects all these reactions, which in part appears to be due to biotransformation of this compound to 5-FUR derivatives. It is clear from these data that of the four drugs investigated by us, 5-FCR is the most powerful agent in terms of both qualitative and quantitative effects on tRNA. The prolonged inhibition of

Table 2. Summary of effects of 5-FCR, 5-FU, 5-FUR and 5-azaCR on tRNA and tRNA modification*

Analog	Incorporation	Inhibitory effects on m^5C	m^5U	ψ	hU
5-FCR	++++† ++‡	++++	+++	++	+
5-FU and 5-FUR	++	—	++++	+++	+
5-AzaCR	ND§	++++	—	—	—

* Based on the present and previously reported [12–14] work.
† Incorporation of 5-FCR.
‡ Incorporation of 5-FUR.
§ Not detected.

tRNA m^5C methylase by 5-FCR demonstrated in the present paper is also noteworthy in this connection.

In 1958, Danenberg *et al.* [28] discovered that 5-FU and its derivatives 5-fluoro-2′-deoxyuridine, 5-FUR and 5-fluoro-orotic acid, all inhibit the formation of thymine in DNA. In the same year, Cohen *et al.* [29] demonstrated that the nucleotide derivative of 5-FU, 5-fluoro-2′-deoxyuridylic acid, is a powerful inhibitor of thymidylate synthetase, an enzyme catalyzing the N^5, N^{10}-methylene-tetrahydrofolate-dependent methylation of the 5-position of dUMP to dTMP [30]. Our work has demonstrated that 5-fluoropyrimidines and 5-azaCR (and perhaps other 5-azapyrimidines) interfere with post-transcriptional methylation and modification reactions taking place also at the 5-position of pyrimidines in tRNA, i.e. reactions that formally resemble the reaction catalyzed by thymidylate synthetase. It thus appears that 5-fluoropyrimidines and 5-azapyrimidines have as their common targets a variety of enzymes responsible for modification reactions involving the 5-position of pyrimidines in both nucleic acid precursors and nucleic acids.

Acknowledgements—We thank Dr. D. Medina for generous help with the supply of BALB/Crgl mice used in this study. We are grateful to Drs. E. Randerath and J. S. Harris for discussions. Ms. G. H. Chiang provided skillful technical assistance in the base composition studies.

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