Specific Inhibition of Transfer RNA Methylation and Modification in Tissues of Mice Treated with 5-Fluorouracil

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ABSTRACT

5-Fluorouracil was administered to normal mice and mice bearing a 7,12-dimethylbenz(a)anthracene-induced, transplantable mammary tumor. Base composition analysis of transfer RNA (tRNA) isolated from liver, normal mammary gland, and tumor showed that only the amounts of uridine and its 5-substituted derivatives (5-methyluridine, pseudouridine, and 5,6-dihydrouridine) were reduced in a dose- and time-dependent manner. The modified uridine derivatives were reduced in excess of the substitution of uridine by 5-fluorouridine. Therefore, we examined the tRNA methyltransferase activities in homologous and heterologous assay systems with α-ethionine-induced, partially methyl-deficient mouse liver tRNA and Mycoplasma hominis tRNA as substrates. The data showed a very rapid, dose-dependent, and specific inhibition of tRNA uracil-5-methyltransferase in dialyzed enzyme extracts prepared from the liver and tumor of mice treated with 5-fluorouracil.

The reduction in the amounts of pseudouridine and dihydrourid ine in tRNA was only partially due to analog incorporation, as shown by base composition analysis. Thus the drug appears to inhibit the biosynthesis of these compounds also.

5-Fluorouracil exhibited a preferential inhibitory effect on tRNA in terms of 5-fluorouridine incorporation and inhibition of uridine modification. This preferential inhibition may in part contribute to the antineoplastic actions of the drug.

INTRODUCTION

It has been shown that tRNA methyltransferase activities are highly elevated in malignant tumors compared to corresponding normal tissues (3, 20, 37, 43). These enzymes belong to the class of key enzymes that may provide potential targets for cancer chemotherapy, as defined by the molecular correlation concept of neoplasia (43, 44). The effects of methylation on tRNA structure and function are not completely understood (22, 28, 39, 40). m5U, a methylated component located exclusively at a specific position in Loop 4 of tRNA regardless of the species, has been shown to be involved in steric interaction stabilizing the 3-dimensional structure of tRNA (33). Some in vitro and in vivo studies have demonstrated the possible physiological role of m5U in tRNA. The protein-synthetic ability of mammalian phenylalanine-specific tRNAs in a rat liver in vitro protein-synthesizing system has been reported to be directly proportional to the m5U content (35). A m5U-deficient Escherichia coli mutant has been found to be at a distinct disadvantage when grown together with wild-type cells, although the mutant cells can grow normally when not competing with the wild-type cells (2). These results suggest that agents capable of inducing m5U deficiency in tumor tRNA might exhibit antineoplastic properties.

A major mechanism of action of the antineoplastic agent, FUra (9, 10), is the inhibition of the methylation of dUMP to dTMP, a 5,10-methylenetetrahydrofolate-dependent reaction catalyzed by thymidylate synthetase (6). We wondered whether FUra might also inhibit other biologically important reactions at Position 5 of uracil such as the posttranscriptional formation of m5U, pseudouridine, and hU in tRNA. We approached this question in 2 ways: (a) by analyzing the base composition of tRNA isolated from liver, mammary gland, and a 7,12-dimethylbenz(a)anthracene-induced mammary tumor of mice following treatment with various doses of FUra; and (b) by assaying in vitro tRNA methyltransferases extracted from control and FUra-treated mouse tissues.

These data show that the administration of FUra results in a pronounced decrease of the activity of tRNA uracil-5-methyltransferase in liver and tumor. Base composition analysis of tRNA isolated from FUra-treated tissues showed a reduction of the amounts of m5U, pseudouridine, and hU in excess of analog incorporation, suggesting that the enzymes catalyzing the biosynthesis of pseudouridine and hU are also inhibited following the administration of FUra. An abstract describing this work has appeared (41).

MATERIALS AND METHODS

Female BALB/c mice weighing 20 to 30 g were used in all experiments. The mammary tumor (T2363) was originally induced by 7,12-dimethylbenz(a)anthracene and serially transplanted s.c. into 8- to 10-week-old mice. The transplanted tumor grew for 30 to 40 days, at which time the mean tumor weight was 1 to 2 g. At this stage the tumor-bearing mice were treated with FUra. Four-day lactating mice were used as a source of normal mammary glands. Liver tissue was taken from both lactating and tumor-bearing mice. FUra was obtained in 10-ml ampuls for i.v. injection from Hoffman-La Roche Inc., Nutley, N. J., as an
aqueous solution containing 500 mg FUra, at a pH of about 9. All dilute FUra solutions were made from this stock solution by the addition of 0.9% NaCl solution to the desired concentration. Apparatus used for tissue and polycrylamide gel homogenization, chemicals and apparatus for gel electrophoresis, enzymes and chemicals for digestion of tRNA and labeling of the digests, and materials for thin-layer chromatography and liquid scintillation counting have been described earlier (31, 32). S-Adenosyl-L-[methyl-\(^3\)H]methionine (6.9 Ci/mmol) was purchased from New England Nuclear, Boston, Mass. E. coli B tRNA was obtained from Calbiochem, La Jolla, Calif. Partially methyl-deficient liver tRNA was isolated from ethionine-treated mice (see "Animal Treatment"). M. hominis tRNA was a generous gift from Dr. Jack Horowitz.

**Animal Treatment.** Two i.p. dose schedules of FUra were used for the treatment of mice (4 mice/group): (a) the animals received a single dose (20, 60, 180, 540, and 1620 mg/kg), and were sacrificed 24 hr after injection; (b) the animals received a triple dose of 500 mg/kg at 0, 6, and 12 hr for 1 to 3 days and were sacrificed 12 hr after the last injection. Control groups of mice received injections of 0.9% NaCl solution. Mice were sacrificed by cervical dislocation and bled before the removal of livers, mammary glands, and mammary tumors, which were pooled separately and stored at -80° prior to the extraction of tRNA. Fresh tissues were used for the preparation of tRNA methyltransferases. Some variation was observed when results of identical experiments run at different times after tumor transplantation were compared. Therefore, the same batch of tumor was used whenever a comparison of results was intended.

Normal mice were treated with dl-ethionine, 100 mg/kg, and adenine, 48 mg/kg, for 3 days to isolate partially methyl-deficient liver tRNA, as described previously (25).

**Isolation and Analysis of tRNA.** tRNA was isolated from 2 to 3 g of tissues, with phenol extraction at pH 4.5 and adsorption to DEAE-cellulose (34). tRNA was further purified by polycrylamide gel electrophoresis (5). The nucleoside composition of tRNA was analyzed by a chemical tritium derivative method (31, 32). 3H-Labeled nucleoside trialcohols of liver and tumor tRNA's were separated on No. 5502 cellulose thin layers (EM Laboratories) in Solvents A and B of Randerath et al. (32). Fluorouridine was converted to the trialcohol derivative by treatment with NaOH and NaBH\(_4\) (31) and used as a UV marker for chromatography (26). Data were analyzed by Student's t test.

**Isolation and Assay of tRNA Methyltransferases.** tRNA methyltransferases were isolated as described by Jackson and Pegg (14), with the following modifications. The enzyme extract was stored as \((\text{NH}_4)_2\text{SO}_4\) precipitate at -80°. Before use for assays, the extract was dissolved in 0.01 M Tris buffer, pH 7.5, containing 1 mM dithiothreitol and 0.1 mM EDTA, and then dialyzed at 4° for 2 consecutive 12-hr periods against 1000 volumes of the same buffer. Protein concentration was determined by the method of Lowry et al. (23). The standard assay mixture for \textit{in vitro} methylation contained, in a volume of 0.1 ml, 0.1 M Tris buffer (pH 8.8), 0.3 M ammonium acetate, 16 \(\mu\text{M}\) [methyl-\(^3\)H]S-adenosyl-L-methionine, 2 \(\mu\text{M}\) tRNA substrate, and a saturating amount of enzymes (1.3 mg of tumor protein and 1.8 mg of liver protein, respectively). Two tRNA substrates were used, liver tRNA of ethionine-treated mice and tRNA of \textit{M. hominis}. The reaction was carried out at 38° for 1 hr and was stopped by adding an equal volume of phenol saturated with 0.14 M sodium acetate, pH 4.5, and mixing. For the assay of total tRNA methylation, 0.1 mg \textit{E. coli} B tRNA in 25 \(\mu\text{l}\) H\(_2\)O was then added as a carrier. For the assay of individual methylated nucleosides, 16 \(\mu\text{g}\) carrier tRNA in 4 \(\mu\text{l}\) H\(_2\)O were added. The methylated tRNA was isolated from the aqueous phase by alcohol precipitation. The precipitate was dissolved in 0.14 M sodium acetate, pH 4.5: reprecipitated; and dried. For the determination of the individual methylated nucleosides, the RNA was then digested to nucleosides enzymatically as described earlier (31, 32). The digest (200,000 to 400,000 dpm) was separated by 2-dimensional thin-layer chromatography on cellulose. An UV marker of m\(^5\)U was applied with the digest for the location of its position on the chromatograms. Solvents for the first and second dimensions, respectively, were acetonitrile:2 n NH\(_4\)OH (4:1), and acetonitrile:0.1 n formic acid (5:1). The tank atmosphere was not saturated with solvent vapors before chromatography. Development in both dimensions was up to the top edge of the chromatogram. Radioactive spots on chromatograms were detected by fluorography (31). Radioactivities of the methylated nucleosides were counted after elution of the nucleosides as described for tritiated nucleoside trialcohols (31).

**RESULTS**

Fig. 1 shows a fluorogram of a 2-dimensional cellulose thin-layer chromatogram of tritium-labeled nucleoside trialcohols (31) obtained by digestion and labeling of tRNA from mammary tumors of mice treated with FUra. The same spot pattern was obtained from control tRNA, except for the absence of the FU' spot and an increased intensity of the m\(^4\)U', \(\psi\)', and hU' spots. FUrd' was well separated from the other compounds and cochromatographed with an authentic UV marker (12, 26).

When the base compositions of bulk tRNA's from mammary tumor and lactating mammary gland were compared (Table 1), the former exhibited a small but statistically significant elevation \((p < 0.01)\) of the degree of methylation, which was mainly due to an increase in the amounts of m\(^1\)G, m\(^2\)G, and m\(^3\)G. On the other hand, the amounts of hU and pseudouridine were slightly reduced in mammary tumor tRNA. Similar trends were observed when liver tRNA was compared with mammary gland tRNA (Table 1). In other words, mammary gland tRNA was found to be slightly undermethylated but not undermodified as compared to tRNA of liver and mammary tumor. Rat (30), monkey (20), and human (11) hepatoma tRNA's have been reported to be slightly less methylated than was liver tRNA.

Chart 1 shows the base compositions of liver (A) and tumor (B) tRNA's from tumor-bearing mice treated with different single doses of FUra. The incorporation of FUrd into tRNA was dose dependent for both tissues, but considerably more analog was incorporated into tumor tRNA. Thus, while for liver tRNA, incorporation was 0, 0.11, 0.27, and 0.70 mol % at 20, 60, 180, and 540 mg/kg, respectively, the corresponding values were 0.33, 0.36, 0.86, and 1.93 mol %, respectively, for tumor tRNA. A further increase in

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1251

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Table 1

Comparison of base compositions of tRNA's from normal liver, mammary gland, and mammary tumor

The tRNA's of normal liver and mammary gland were obtained from 4 lactating mice 4 days after delivery. tRNA of mammary tumor was obtained from 4 tumor-bearing mice. Base analysis was carried out by a chemical tritium derivative method after enzymatic digestion of the RNA's to nucleosides (31, 32). Base composition data were calculated from count rates of tritium-labeled nucleoside trialcohols.

<table>
<thead>
<tr>
<th>Nucleoside</th>
<th>Liver</th>
<th>Mammary gland</th>
<th>Mammary tumor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uridine</td>
<td>14.04 ± 0.10</td>
<td>14.04 ± 0.10</td>
<td>14.18 ± 0.08</td>
</tr>
<tr>
<td>Adenosine</td>
<td>17.44 ± 0.18</td>
<td>17.57 ± 0.16</td>
<td></td>
</tr>
<tr>
<td>Cytidine</td>
<td>25.91 ± 0.06</td>
<td>25.34 ± 0.09</td>
<td></td>
</tr>
<tr>
<td>Guanosine</td>
<td>26.70 ± 0.28</td>
<td>27.54 ± 0.16</td>
<td></td>
</tr>
<tr>
<td>m5U</td>
<td>0.58 ± 0.01</td>
<td>0.56 ± 0.01d</td>
<td></td>
</tr>
<tr>
<td>hU</td>
<td>3.05 ± 0.05</td>
<td>2.71 ± 0.02c</td>
<td></td>
</tr>
<tr>
<td>Pseudouridine</td>
<td>4.30 ± 0.07</td>
<td>3.64 ± 0.11f</td>
<td></td>
</tr>
<tr>
<td>3-(3-Amino-3-carboxypropyl)-uridine</td>
<td>0.48 ± 0.02</td>
<td>0.58 ± 0.10</td>
<td></td>
</tr>
<tr>
<td>m1A</td>
<td>1.22 ± 0.02d</td>
<td>1.28 ± 0.02d</td>
<td></td>
</tr>
<tr>
<td>N-[9-(β-D-Ribofuranosyl)purin-6-y-carbamoyl]threonine</td>
<td>0.39 ± 0.02</td>
<td>0.32 ± 0.02b</td>
<td></td>
</tr>
<tr>
<td>Inosine</td>
<td>0.30 ± 0.01</td>
<td>0.30 ± 0.01</td>
<td></td>
</tr>
<tr>
<td>3-Methylcytidine</td>
<td>0.38 ± 0.01</td>
<td>0.35 ± 0.03</td>
<td></td>
</tr>
<tr>
<td>m3C</td>
<td>1.79 ± 0.02</td>
<td>1.80 ± 0.08</td>
<td></td>
</tr>
<tr>
<td>N4-Acetylcytidine</td>
<td>0.14 ± 0.01</td>
<td>0.12 ± 0.01d</td>
<td></td>
</tr>
<tr>
<td>m5G</td>
<td>0.61 ± 0.02</td>
<td>0.54 ± 0.08</td>
<td></td>
</tr>
<tr>
<td>m2G</td>
<td>1.12 ± 0.02</td>
<td>1.36 ± 0.05c</td>
<td></td>
</tr>
<tr>
<td>m1G</td>
<td>0.67 ± 0.01</td>
<td>0.74 ± 0.02b</td>
<td></td>
</tr>
<tr>
<td>m1G</td>
<td>0.90 ± 0.03</td>
<td>1.07 ± 0.02f</td>
<td></td>
</tr>
</tbody>
</table>

Total 100.00 100.00 100.00

Modified nucleosides 15.91 ± 0.13 15.37 ± 0.24d

Methylated nucleosides 7.27 ± 0.09 7.70 ± 0.06c

a Mean ± S.D. obtained from 4 chromatographic analyses of the labeled tRNA digest derived from pooled tissue samples of each group of animals.
b p < 0.01 when compared with corresponding values of mammary gland.
c p < 0.001 when compared with corresponding values of mammary gland.
d p < 0.05 when compared with corresponding values of mammary gland.

Chart 1. The effect of FUra on the base composition (mol %) of tRNA's from liver (A) and tumor (B) isolated 24 hr after treatment with various single doses of FUra. The data are the means ± S.D. of 4 chromatographic analyses. For abbreviations, refer to Footnote 4 and legend of Fig. 1. ψ, pseudouridine; FU, 5-fluorouridine; acC, W-acetylcytidine, m3C, 3-methylcytidine.

the FUra dose did not result in higher incorporation into either tRNA (Chart 1). All the modified nucleosides except the modified uridine derivatives remained unaffected over the dose range used. There was a dose-dependent reduction in the amounts of uridine derivatives modified in Position 5 (m5U, pseudouridine, and hU), leveling off at 540
mg/kg. 3-(3-Amino-3-carboxypropyl)uridine, a uridine derivative modified in Position 3, was not affected at all in a statistically significant way. $U_{\text{total}}$ in tumor and liver tRNA's was not altered significantly (not shown in Chart 1). There was no dose-related effect of FURA on modified uridine derivatives (m$^1$A, $N$-[9-(β-D-ribofuranosyl)purin-6-yl-carbamoyl]threonine, inosine, m$^3$G, m$^2$G, and m$^7$G) and on modified cytosine derivatives (3-methylcytidine, m$^5$C, and N$^4$-acetylcystidine) (data shown in part in Chart 1). Similar specific effects on mouse liver tRNA have been described previously for treatment with FURd (26).

If the decrease in the amount of each modified uridine derivative were simply due to random incorporation of FURd into the positions of uridine in pre-tRNA during transcription, then the percentage of decrease in each modified uridine derivative would equal the percentage of substitution of $U_{\text{total}}$ by FURd. However, Chart 2 shows that in liver (A) and tumor (B) tRNA's the percentage of decrease of each modified uridine derivative was much larger than the percentage of substitution at each dose; the decrease was largest for m$^5$U.

Preferential incorporation of FURd into the positions that are normally occupied by modified uridine derivatives has been suggested to explain the reduction of m$^5$U in the tRNA from FURA-treated E. coli (1). This mechanism implies that the total decrease in modified uridine derivatives should not exceed the amount of FURd incorporated into tRNA.

Following treatment with any FURA dose, the data (calculated from Chart 1 and Table 2) showed that the decrease in modified uridine derivatives was larger than was FURd incorporation, i.e., $0.44 \pm 0.04$ versus $0.27 \pm 0.03$ mol% (in host liver at 180 mg/kg, $p < 0.01$), $1.07 \pm 0.11$ versus $0.86 \pm 0.08$ mol% (in mammary tumor at 180 mg/kg, $p < 0.05$), and $1.38 \pm 0.05$ versus $0.55 \pm 0.01$ mol% (in mammary gland at $3 \times 500$ mg/kg, $p < 0.001$). Analogous results were obtained for liver tRNA from normal mice treated with FURA, 150 and 300 mg/kg (Chart 3). The reduction in the sum of (pseudouridine + hU) was still larger than the incorporation value (Chart 3). Thus, these data indicate a probable inhibition by FURA of enzymatic modification reactions responsible for converting uridine to m$^5$U, pseudouridine, and hU during tRNA maturation, in addition to a physical replacement of uridine by FURd during tRNA transcription.

When tumor-bearing mice were treated with FURA, 500 mg/kg, 3 times/day for either 1, 2, or 3 days to achieve different degrees of FURd incorporation, uridine and its modified derivatives were still the only nucleosides affected by FURA (data not shown). When the percentage of decrease of these nucleosides was plotted against the percentage of substitution, m$^5$U, pseudouridine, and hU showed larger decreases in tumor tRNA than in liver tRNA, even when compared at the same level of FURd incorporation (Chart 4). During treatment time, from 1 to 3 days, the substitution increased, and the amount of m$^5$U, pseudouridine, and hU decreased (Chart 4).
Table 2

Effects of FUra on the base compositions of tRNA's from liver and mammary gland of lactating mice and from mammary tumor of tumor-bearing mice

Mice (4/group) received FUra, 500 mg/kg, at 0, 6, and 12 hr. Tissues were isolated at 24 hr.

<table>
<thead>
<tr>
<th></th>
<th>% substitution of (U_{\text{total}})</th>
<th>% mU decrease</th>
<th>% hU decrease</th>
<th>% pseudouridine decrease</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td>7.75 ± 0.04</td>
<td>19.36 ± 1.61</td>
<td>15.08 ± 0.98</td>
<td>17.41 ± 1.00</td>
</tr>
<tr>
<td>Mammary gland</td>
<td>3.07 ± 0.09^a^</td>
<td>24.14 ± 3.45^b^</td>
<td>17.43 ± 1.64^b^</td>
<td>16.36 ± 0.47^b^</td>
</tr>
<tr>
<td>Mammary tumor</td>
<td>12.83 ± 0.33^c^</td>
<td>24.00 ± 0.80^c^</td>
<td>24.73 ± 1.07^c^</td>
<td>29.77 ± 1.02^c^</td>
</tr>
</tbody>
</table>

^a^ Mean ± S.D., significantly smaller than the corresponding value of liver; \(p < 0.001\). The values were obtained from 4 chromatographic analyses of the labeled tRNA digest.

^b^ Not significantly different from the corresponding values of liver.

^c^ Significantly different from the corresponding values of liver and mammary gland; \(p < 0.01\).

Table 2 shows results of an experiment comparing FUra effects on tRNA's in liver, normal mammary gland, and mammary tumor. Percentages of both substitution and decrease of mU, pseudouridine, and hU were much greater in tumor tRNA than in tRNA of normal mammary gland. tRNA modification in 2 different normal tissues, mammary gland and liver, was affected by FUra to similar extents, although the substitution in tRNA of mammary gland was only 40% of the substitution in liver tRNA (Table 2).

To obtain direct evidence for an effect of FUra on modifying enzymes, we determined tRNA methyltransferase activities of extracts from control and drug-treated tissues. With \(M.\ hominis\) or homologous methyl-deficient tRNA as the substrate, tumor enzyme catalyzed methylation faster than did liver enzyme, but both reactions plateaued at the same level in about 60 min. The protein amount required to reach the methylation plateau was much smaller for tumor enzyme (0.4 mg) than for liver enzyme (1.2 mg). For the standard assays of individual methyltransferases, an enzyme amount in excess of that required for saturation was used, and incubations were for 1 hr at 38°C, as described in "Materials and Methods."

Chart 5 shows the distribution of methyl groups accepted by partially methyl-deficient liver tRNA during \textit{in vitro} methylation by enzyme extracts from liver (A) and mammary tumor (B) isolated 24 hr after treatment with single doses of FUra. Values, average of 2 assays.

![Chart 5](chart.png)

Chart 6 shows the distribution of methyl groups accepted by partially methyl-deficient liver tRNA during \textit{in vitro} methylation by enzyme extracts from liver (A) and mammary tumor (B) isolated 24 hr after treatment with single doses of FUra. Values, mean of 4 assays ± S.D.

Heterologous \(M.\ hominis\) tRNA, which has been shown to be highly deficient in mU (15), was also used as a substrate for assaying tRNA methyltransferase activities in liver (Chart 6A) and mammary tumor (Chart 6B). Again, only mU formation was affected in a dose-dependent manner following treatment with FUra. Since the mU yield represented 10 to 15% of total methylation in \textit{Mycoplasma} tRNA as compared to only 1 to 2% in liver tRNA, the decrease of
m^2U formation was reflected in a relative increase of other methylated bases when the data were expressed as percentage of total methylation (Chart 6). If the m^2U yield was excluded from the calculations, the other methylated bases did not change significantly, as shown in Chart 6 for m^3C.

The methylation patterns produced by liver and mammary tumor enzyme extracts were similar in the homologous and heterologous assay systems. The yields of methylated bases by either enzyme extract in decreasing order were m^3C > m^2G > m^5U > m^3G > m^5G = m^4A for Mycoplasma tRNA and m^3C > m^1G > m^5G > m^2G > m^1A > m^4U for partially methyl-deficient liver tRNA.

Chart 7 illustrates the time course of inhibition of tRNA uracil-5-methyltransferase after a single i.p. dose of FUra, 180 mg/kg. The enzyme activity in liver and tumor was reduced very rapidly after treatment, and over 90% of the activity was lost 6 hr after treatment. The enzyme activity had recovered very little after 24 hr, indicating prolonged inhibition.

Thus, the in vitro methylation assays demonstrated specific, rapid, and prolonged decrease of the activity of tRNA uracil-5-methyltransferase in liver and mammary tumor following treatment with FUra. In addition, base composition analysis of tRNA from FUra-treated tissues indicated that the reduction in the amounts of pseudouridine and hU also was due at least in part to inhibition of posttranscriptional modification reactions.

**DISCUSSION**

A specific deficiency of m^3C in mouse liver tRNA can be induced by the antileukemic agent, 5-azacytidine (24). In this report, we find that another anticancer agent, FUra, is capable of the inhibition of the formation of another methylated base, m^4U, in tRNA without affecting other methylated bases. This inhibition is due not only to the physical substitution of uridine by FUrd in tRNA (4, 27) but also to the in vivo inhibition of tRNA uracil-5-methyltransferase after FUra treatment. This conclusion was supported by 2 lines of evidence: (a) base analysis of tRNA from FUra-treated tissues indicated a specific reduction in the amount of m^2U (Chart 1); and (b) assays of tRNA uracil-5-methyltransferase activity in drug-treated tissues indicated specific inhibition of the enzyme. The latter effect was demonstrated by in vitro methylation assays with homologous and heterologous m^4U-deficient tRNA's as substrates (Charts 5 and 6).

Base analysis also indicated that the reduction in the amounts of pseudouridine and hU was not due to random analog incorporation (Charts 2 and 3). As preferential incorporation of FUrd into positions destined to become modified to pseudouridine and hU after transcription was excluded (Chart 3), the data strongly suggest that the drug also inhibited the enzymatic synthesis of these 2 nucleosides. We did not present direct evidence for the latter conclusion, however, because there is no enzymatic system available yet for the assay of mammalian enzymes catalyzing the formation of pseudouridine and hU in tRNA.

After FUra treatment, mammary tumor tRNA was altered to a much greater extent than were the tRNA's of the mammary gland and liver in terms of the inhibition of uridine modification and the substitution of U_{num} by FUrd (Chart 2; Table 2). This phenomenon may be related to a higher tRNA turnover rate in tumor tissue (20). No definite correlation between the degree of the inhibition of uridine modification and the level of substitution was found when different tissues were compared (Chart 4; Table 2). This also suggests that the inhibition of uridine modification was not the direct consequence of FUrd incorporation.

Both homologous and heterologous assay systems revealed a distinct dose-dependent reduction of tRNA uracil-5-methyltransferase activity, although the in vitro methylation patterns of these 2 tRNA substrates were very different (Charts 5 and 6).

Unexpectedly, m^2G was formed only in *M. hominis* tRNA but not in partially methyl-deficient liver tRNA although the latter substrate is very deficient in m^2G (25). It thus appears that the m^2G enzyme was present in the extracts, but partially methyl-deficient liver tRNA was a poor substrate in our assay conditions. The formation of m^2G in the homologous assay system may require different methylating conditions or particular stimulating factor(s). tRNA methylation in vitro is known to vary greatly with assay conditions (8, 21, 29).

The reduced activity of tRNA uracil-5-methyltransferase was observed with enzyme extracts that had been exhaustively dialyzed. This and the persistence of the effect suggest a possible mechanism resembling the inhibition of thymidylate synthetase by 5-fluorodeoxyuridine 5'-monophosphate (6) which is covalently bound to the enzyme (7, 36). Our data are consistent with the hypothesis that a still unidentified drug metabolite binds tightly, and perhaps covalently, to the tRNA uracil-5-methyltransferase. This is
in contrast to inhibition by ethionine, which affects all methylated bases in mammalian tRNA (8, 25, 42) and is reversible by dialysis (42). In vitro assays of tRNA uracil-5-methyltransferase activity in the presence of 2 mM FUra (W-C. Tseng, unpublished experiments) indicated that the drug per se was a weak inhibitor at a concentration 1000-fold greater than that of the tRNA substrate (m5U yield, 82.94 ± C. Tseng, unpublished experiments) indicated that the drug in contrast to inhibition by ethionine, which affects all biochemical functions (13, 18, 38).

The inhibition of thymidylate synthesis, resulting in the inhibition of DNA synthesis, is considered to be a major effect of FUra (6, 9). However, FUra and its ribo- and deoxyribonucleoside derivatives have been shown to cause alterations of other cellular components such as chromosomes (see Ref. 9) and RNA. It seems unlikely that oncogenic transformation induced by FUra and 5-fluoro-2-deoxyuridine in vitro can result from inhibition of DNA synthesis per se (16). In addition, thymidine cannot reverse the effects of FUra in some prokaryotic and eukaryotic cells (see Refs. 9 and 27). The cytotoxicity caused by a short exposure of Novikoff hepatoma cells to a highly toxic concentration (10 μM) of FUrd in culture can be reversed by uridine but not by thymidine and thus may be related to effects of the drug on RNA (45).

tRNA methyltransferases may provide potential targets for cancer chemotherapy since neoplastic tissues exhibit highly elevated activities of these enzymes (3, 20, 37, 43), which appear to be linked with neoplastic progression (43). Therefore, the effect of FUra on tRNA uracil-5-methyltransferase described in this communication may contribute to the antitumor actions of this drug. Whether the effects of FUra on the formation of pseudouridine and Hu in tumor tRNA are significant for the cytotoxic actions of the drug is not known at present, inasmuch as the activities of the corresponding enzymes in normal and neoplastic tissues have not yet been examined. Bacterial tRNA’s lacking pseudouridine and Hu have been found to be deficient in various biochemical functions (13, 18, 38).

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