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TUBERCIDIN AND ISOPROPYLUREIDOPURINE—INHIBITORS OF RIBOTHYMIDINE SYNTHESIS *IN VITRO*

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Abstract—Two purine analogs, 7-deazaadenosine (tubercidin) and isopropylureidopurine (IPUP), have been found to inhibit the uracil tRNA-methylating enzymes of *Escherichia coli*. Conversion of isopropylureidopurine to its ribonucleoside derivative rendered it ineffective as a methylation inhibitor. The uracil tRNA-methylating enzyme system, which specifically catalyzes the biosynthesis of ribothymidine in transfer RNA, is relatively resistant to inhibition by a number of adenine derivatives that were previously shown to interfere with the activity of enzymes that catalyze the methylation of guanine in tRNA. Included amongst the substances that inhibited the enzymatic transfer of methyl groups to tRNA guanine, but at comparable concentrations did not significantly affect uracil tRNA methylation, were kinetin riboside, 6-methylaminopurine riboside, isopentenyladenosine and 6-isoamylureidopurine riboside. Tubercidin and IPUP, however, inhibited both guanine and uracil tRNA-methylating enzymes. These results indicate that the structural requirements for inhibitors of the various base specific tRNA-methylating enzymes are not identical. It, therefore, seems possible that the relative activities of these enzymes can be selectively altered by drugs and that they may be separately regulated *in vivo*.

The enzymes that catalyze the formation of ribothymidine in tRNA are specific for the transfer of methyl groups to uracil in the tRNA polymer. The analogous enzymes that catalyze the methylation of the other base moieties in tRNA are also base specific [1, 2]. Species specific and tissue specific differences in kind as well as of levels of these enzymes have been observed [3]. In addition, total levels of tRNA-methylating enzymes found in extracts of foetal and of cancerous tissues have been reported to be higher than those found in normal adult animal tissues [4–10]. Although the control of the tRNA-methylating enzymes *in vivo* remains only incompletely understood, one of the factors involved is known to be the presence of varying amounts of naturally occurring inhibitors [11–14].

Recent work in this laboratory and in others [15–18] has shown that guanine tRNA-methylating enzymes can be inhibited *in vitro* by a number of purine analogs that are chemically related to adenine. Some of these compounds also have either growth stimulatory activity in plant culture *in vitro* or cytotoxic activity against cultured animal cells [16, 17, 19–21]. There are considerable structural differences possible amongst the purine derivatives that inhibit guanine tRNA methylation, since N^6 substituents with either hydrophilic or hydrophobic properties were included in this group.

The low levels of uracil tRNA methylases in most normal animal tissues make study of this system very difficult [4, 8, 9, 22]. Nevertheless, most tRNAs, except for initiator tRNA from eukaryotes, contain one residue of ribothymidine at position 23 from the 3-OH end [10, 23]. It, therefore, seemed interesting to look for substances that might inhibit the enzymes that catalyze ribothymidine formation in tRNA. We have tested some of the compounds that inhibit guanine tRNA methylases for their ability to affect the activity of uracil tRNA-methylating enzymes, by making use of a quantitative assay for the uracil tRNA methylases of enteric bacteria, which is simple and specific [24]. This assay method depends upon the utilization of ribothymidine-deficient tRNA from trm$^-$ mutants of *Escherichia coli* [25, 26]. This tRNA is a monospecific substrate for the uracil tRNA methylases from homologous sources. The results of these studies are presented here. Information gained from the study of inhibitors in this bacterial system will serve as a guide in further experiments involving uracil tRNA-methylating enzymes of more complex organisms.

EXPERIMENTAL

Bacterial strains. Cells of *E. coli* M3S, *E. coli* K12-25 trm$^-$ (derived from *E. coli* K12Ab1157) were grown with aeration at 37° in a medium consisting of 10 g tryptone, 7 g NaCl and 1 g yeast extract/liter, supplemented with 0·9 g glucose. *E. coli* M3S was provided by Dr. Margaret Lieb and *E. coli* strains K12Ab1157 and K12-25 trm$^-$ were gifts from Dr. M. L. Gefter. The preparation and properties of these strains have been studied and described by Yang, *et al.* [25].

Enzyme extracts. Frozen cells of *E.coli* were disrupted by grinding in the cold with three times their weight of wet Alumina A-305 and extracted with 0·01 M Tris, 0·01 M $MgCl_2$, 0·005 M mercaptoethanol buffer, pH 8 (SMB), containing 5 μg/ml of DNase. The supernatant, from centrifugation at 8000 *g* at 5° for 20 min, was centrifuged at 100,000 *g* for 70 min at 0°. The supernatants from this second centrifugation were used as the enzyme preparations.

Ribothymidine-deficient tRNA. The tRNA was prepared from *E.coli* K12-25, a trm^- mutant, [25, 26] by a method based on the procedure of Fleissner and Borek [27]. Details of the preparation of tRNA from these mutants and the demonstration of the ability of this tRNA to act as a monospecific substrate for the quantitative assay of uracil tRNA methylases of *E. coli* and *Aerobacter aerogenes* have been described in an earlier publication [24].

Enzyme assays. The assays measured the enzymatic transfer of $^{14}CH_3$ from $^{14}CH_3$-*S*-adenosyl methionine (SAM) to ribothymidine-deficient tRNA. Each incubation tube contained the following materials in a final buffer concentration of 0·01 M Tris, 0·005 to 0·01 M $MgCl_2$, 0·005 M mercaptoethanol, pH 8·0: 75–125 μg ribothymidine-deficient tRNA, $^{14}CH_3$-SAM, in varying amounts as indicated blow, enzyme preparation and inhibitors. The volume was 0·40–0·50 ml. Enzyme quantities were kept well below saturation level, so that the rate of the reaction was limited by the amount of enzyme present [28]. After 30–45 min of incubation at 35°, the reaction was terminated by the addition of 0·2 ml of 1·5 M hydroxylamine, pH 7·5. The incubation at 35° was continued for 10 min longer, and then the tubes were rapidly cooled in an ice bath. RNA was precipitated and washed as described earlier [17]. Radioactivity as $^{14}CH_3$ was assayed in a scintillation counter. Incorporation of $^{14}CH_3$ was assayed simultaneously in the presence and absence of the compounds being tested as inhibitors. Each preparation was thus able to act as its own control. Inhibitors were dissolved in 0·15% Tween 80. The amounts of Tween 80 used were previously shown not to interfere with the activity of bacterial tRNA methylases [17]. Nevertheless, equivalent amounts of Tween 80 were present in all control samples.

Inhibitors. Isopentenyladenosine, kinetin riboside and 6-methylaminopurine riboside were purchased from Sigma Chemical Co. Tubercidin was obtained from CalBiochem. Formycin was a gift from Dr. M. E. Balis. All of the ureidopurine derivatives were synthesized in the laboratory of Dr. G. B. Chheda by Dr. C. I. Hong [29].

RESULTS

In earlier publications, it has been reported that adenine, adenosine, 7-deazaadenosine, and a number of N^6 substituted derivatives of these compounds are inhibitors of guanine tRNA-methylating enzymes [13, 16–18, 20]. We have taken advantage of the availability of a simple specific assay for uracil tRNA-methylating enzymes to test some of these analogs for their ability to inhibit uracil tRNA methyltransferases.

As shown in Table 1, tubercidin (7-deazaadenosine), can inhibit uracil tRNA-methylating enzymes. At similar concentration levels, neither the structurally related compound [7-amino-3-(β-D ribofuranosyl)-pyrazolo 4,3-d pyrimidine] nor kinetin riboside (6-furfuryla-

Table 1. Inhibition of Uracil tRNA Methylase by Purine Nucleosides*

Inhibitor	Concn inhibitor (mM)	$^{14}CH_3$ incorporated (cpm)	Per cent inhibition
None		925 ± 15	
Tubercidin	0·55	700 ± 40	24
None		1275 ± 45	
Tubercidin	0·45	945 ± 65	26
Tubercidin	0·85	745 ± 40	44
None		750 ± 80	
Tubercidin	0·45	570 ± 5	24
Formycin	0·4	980 ± 85	0
Formycin	1·0	740 ± 20	2
Adenine	1·0	660 ± 10	12
None		1945 ± 75	
Kinetin riboside	0·5	2055 ± 135	0
Adenosine	0·4	1930 ± 45	1
Adenosine	0·7	1804 ± 130	7
None		1700 ± 85	
Kinetin riboside	1·8	1625 ± 30	4

* Each incubation tube contained: 75 μg ribothymidine-deficient tRNA, 0·07 μC $^{14}CH_3$-*S*-adenosylmethionine (sp. act. 55 mCi/m-mole) inhibitor dissolved in 0·01 M Tris, 0·01 M $MgCl_2$, 0·005 M mercaptoethanol, pH 8, containing 0·15% Tween 80 and enzyme preparation. Total volume was 0·45 ml. Final concentration of pH 8 buffer was 0·01 M Tris, 0·01 M $MgCl_2$ and 0·005 M mercaptoethanol.

mino purine riboside) was effective as an inhibitor of this enzyme. The SAM concentration in these experiments was 2·8 μM. Kinetin riboside is a plant cytokinin [19] which inhibits guanine tRNA methylases of both bacterial and animal origin [17]. We reported earlier that, in a mixture of tRNA-methylating enzymes, adenosine preferentially inhibited guanine tRNA methylation as compared with uracil tRNA methylation [16]. Our tests with the selective assay for uracil tRNA-methylating enzymes confirm these findings, since concentrations of adenosine which had been found to give 50 per cent inhibition of bacterial guanine tRNA methylases [20] gave no significant levels of inhibition in this system (Table 1).

Experiments were carried out with other purine derivatives that we previously found to be guanine tRNA methylase inhibitors [17, 20, 30,. 31]. At SAM concentrations of 1·6 to 2·2 μM, neither the plant cytokinins 6-isoamylureidopurine riboside, 6-allylureidopurine riboside and isopentenyladenosine, nor the cytoxic drugs 6-methylaminopurine riboside, 6-chloropurine riboside and 6-methylmercaptopurine riboside caused significant inhibition of the uracil tRNA methylase reaction.

In contrast, isopropylureidopurine, which is both a cytokinin and an inhibitor of guanine tRNA methylation [30, 31] is also able to inhibit the enzymatic transfer of methyl groups to uracil in tRNA (Table 2). It should be noted that addition of ribose at the 9 position of the purine moiety to yield 6-isopropylureidopurine riboside (IPUPR) (Fig. 1) rendered it ineffective as a uracil tRNA methylase inhibitor in this assay system (Table 2).

The apparent K_m for SAM in the uracil tRNA methylase reaction was found to be about $1{\cdot}6 \times 10^{-5}$M (Fig. 2). Increases in the concentration of SAM resulted in decreased inhibition of the reaction by IPUP (Table 2). It was not clear from these results, however, whether the relationship between SAM and IPUP is strictly competitive. Inhibition of uracil tRNA methylase by tubercidin was also decreased when the concentrations of SAM were raised, but in this case the relationship appeared to be more complex than one of simple competitive inhibition. More precise determinations of these parameters will have to await studies with highly purified enzymes.

DISCUSSION

Work in this laboratory showed earlier that various adenosine analogs which are cytokinins, both naturally occurring and synthetic, are guanine tRNA methylase inhibitors [17, 30]. These included N^6-alkyl substituted adenosine derivatives, such as isopentenyladenosine and kinetin riboside, and also 6-ureidopurines, such as 6-isoamylureidopurine riboside and 6-isopropylureidopurine [29]. Several cytotoxic analogs of adenosine, with demonstrated antitumor activity, were alo found by us and by other workers to inhibit *in vitro* the methylation of guanine in tRNA, [16, 18, 20]. Because of the difficulties involved in studying uracil tRNA methylation, relatively little is known as yet about the nature of substances which can affect this enzymatic reaction. The development of a simple specific assay for uracil tRNA methylases has made the search for inhibitors easier [24]. We have tested a

Table 2. Comparison of isopropylureidopurine and isopropylureidopurine riboside as inhibitors of uracil tRNA methylase*

Inhibitor			$^{14}CH_3$ incorporated (cpm)		
IPUP (mM)	IPUPR (mM)	SAM concn (μM)	Control	Plus inhibitor	Per cent inhibition
2·3		1·5	1100† ± 35	585 ± 20	47
		1·4	730 ± 45	360 ± 15	51
2·3		3·4	1200 ± 30	945 ± 50	22
		6·8	2765 ± 5	2255 ± 5	18
0·65		2·0	630 ± 25	520 ± 55	18
1·3		2·0	630 ± 25	444 ± 10	30
	2·1	1·5	2595 ± 60	2445 ± 75	6
	0·7	1·5	1325 ± 80	1230 ± 15	5
	1·5	1·5	1325 ± 80	1425 ± 75	0
	2·5	1·5	1325 ± 80	1365 ± 65	0
	2·2	1·5	1010 ± 40	1000 ± 30	0
2·2		2·4	1835 ± 140	1345 ± 135	27
	2·2	2·4	1835 ± 140	1715 ± 70	6
2·4		2·7	3895 ± 120	2895 ± 90	28
	2·7	2·7	3895 ± 120	3775 ± 360	3

* Incubation conditions were identical with those described for Table 1, except that SAM concentrations were varied as indicated. In some experiments, 125 μg ribothymidine deficient tRNA was used.

† Value represents cpm incorporated in 30 min. In 15 min, 554 cpm was incorporated.

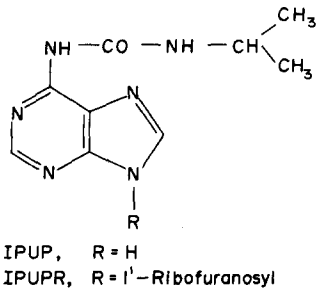

Fig. 1. Chemical structure of isopropylureidopurine and its ribonucleoside.

number of the compounds found earlier to be guanine tRNA methylase inhibitors and discovered that two purine analogs, i.e. tubercidin (7-deazaadenosine) and 6-isopropylureidopurine (IPUP), can inhibit uracil tRNA methylation. Under the same conditions, a number of structurally related compounds that inhibit guanine tRNA methylation do not interfere significantly with the transfer of methyl groups to uracil tRNA. It appears, therefore, that the structural requirements for inhibitors of guanine tRNA-methylating enzymes and of uracil tRNA-methylating enzymes are not identical. Perhaps these two enzymes are regulated by different kinds of metabolites *in vivo*. To date, however, no chemically defined substance which can inhibit specifically uracil tRNA-methylating enzymes has been identified.

The finding of substances that can selectively or preferentially inhibit any of the base specific tRNA-methylating enzymes may prove to be useful in maintaining the normally constant methylated base content of tRNA, which has been found to be altered in neoplasia and after certain virus infections [4, 8, 10, 22].

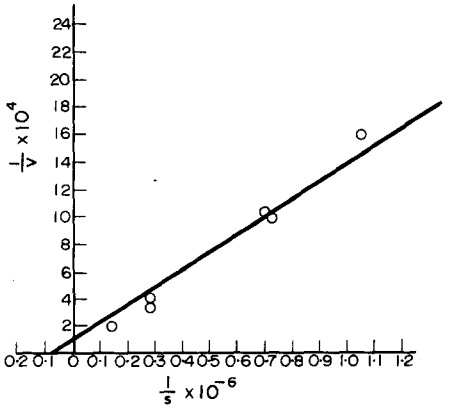

Fig. 2. Incubation conditions were the same as those described for Table 1, except that 100 μg RNA was used, and the final volume of the incubation mixture was 0·5 ml. S = SAM concentration is moles/liter. V = counts/min incorporated.

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ADDENDUM

In an earlier paper, "Inhibition of Transfer Ribonucleic Acid Methylating Enzymes by Cytotoxic Analogs of Adenosine," by Elsie Wainfan and Barbara Landsberg, *Biochem. Pharmac.* **22,** 493 (1973) K_m for SAM in the N^2-guanine tRNA methylase of calf spleen was stated to be $2{\cdot}5 \times 10^{-10}$. and the K_i for methylaminopurine riboside and dimethylaminopurine riboside to be 2×10^{-9}. These numbers are in error. The apparent K_m for this enzyme is $2{\cdot}5 \times 10^{-7}$ M. In the presence of $2{\cdot}6 \times 10^{-3}$ M methylaminopurine riboside or dimethylaminopurine riboside, the K_m is increased to $2{\cdot}0 \times 10^{-6}$. K_i was calculated to be $0{\cdot}4 \times 10^{-3}$ M.