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INHIBITION OF TRANSFER RIBONUCLEIC ACID METHYLATING ENZYMES BY CYTOTOXIC ANALOGS OF ADENOSINE*

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Abstract—Several purine derivatives have been found to inhibit tRNA methylases of either animal or bacterial origin. The experiments reported here show that arabinosyl adenine, 6-chloropurine ribonucleoside, 2,6-diaminopurine ribonucleoside, 6-methylaminopurine ribonucleoside, and 6,6′-dimethylaminopurine ribonucleoside are inhibitors of tRNA methylation. The ribonucleosides of methylaminopurine and dimethylaminopurine inhibit guanine tRNA methylation by competition with the methyl donor, *S*-adenosyl methionine, for the enzyme. 6-Chloropurine ribonucleoside inhibits the reaction non-competitively. All of these analogs, along with isopentenyladenosine, 6-furfurylamino purine ribonucleoside, and 7-deazaadenosine, which we showed earlier to be inhibitors of tRNA methylation, are cytotoxic and have shown some usefulness as antitumor agents.

THE tRNA methylating enzymes are widely distributed in living systems and represent a mechanism for rapid modification of transfer RNA *in vivo*. Since the ability of transfer RNA to function is influenced by the extent of its modification,[1–5] the tRNA methylases may be regarded as one of the factors that regulate the functional state of cells. Alteration in the specificities and levels of activity of the methylases has been observed after virus infection,[6,7] latent virus induction[8–10] in transformed cells[11,12] and in neoplasms.[6,13–17] Although control of tRNA methylase activity in living cells is, as yet, incompletely understood, at least one of the factors involved is the occurrence of varying levels of intracellular methylase inhibitors.[8,18]

Wainfan and Borek[19] observed that adenine and several of its analogs inhibited the activity of tRNA methylating enzymes. One of these analogs was isopentenyl adenosine (IPA), a naturally occurring minor component of tRNA that has been reported to exhibit cytokinin activity,[20,21] cytotoxicity and antitumor activity.[22,23] In addition, Gallo *et al.*[24,25] have found that, while very low concentrations of IPA could stimulate lymphocyte transformation, higher concentrations of this substance were inhibitory. In view of these varied and interesting effects it seemed of interest to us to investigate the possibility that other cytotoxic purine analogs might also be able to inhibit tRNA methylation.

EXPERIMENTAL

The methods used to prepare and assay the tRNA methylating enzymes have been described in detail in an earlier publication.[26]

* A preliminary report of some of these results was presented at the meeting of the Federation of American Societies for Experimental Biology, June 1972.

The assay system consisted of methyl-deficient tRNA from *Escherichia coli* K12W6 (λ), methyl-labeled *S*-adenosyl methionine as methyl donor, enzyme and inhibitor where used, all in a buffer concentration of 0·015 M Tris, 0·015 M $MgCl_2$, 0·007 M mercaptoethanol at pH 8. Incorporation of radioactive methyl groups into methyl-deficient tRNA in the presence and in the absence of inhibitors was compared. In this way, each preparation acted as its own control.

Enzyme sources. (1) *E. coli* strains. Cells of *E. coli* M3S and *E. coli* M3 ($\lambda C_1 857$) (a gift from Dr. Margaret Lieb) and *E. coli* K12AB1157 trm$^-$ (a gift from Dr. Malcolm Gefter) were prepared by growth in a tryptone–glucose medium.[26] *E. coli* B was purchased from Miles Laboratories, Inc. (2) Freshly frozen calf spleen.

Partial purification of enzymes. The crude enzyme preparations which represented, in each case, the 100,000 *g* supernatant of a disrupted cell extract were partially purified by fractional ammonium sulfate precipitation. Solid ammonium sulfate was added with stirring at 0°. When the desired percentage of $(NH_4)_2SO_4$ saturation was reached, the suspension was centrifuged at 12,000 *g* for 10 min at 3°.

Precipitates containing enzyme activity were resuspended in 0·01 M Tris. 0·01 M $MgCl_2$, 0·005 M mercaptoethanol at pH 8, in 0·2 M sucrose. Most preparations were dialyzed for 3 hr against 500 vol. of cold 0·001 M Tris, 0·001 M $MgCl_2$, 0·0005 M mercaptoethanol, pH 8, in 0·05 M sucrose before assay.

Inhibitors. The purine analogs tested as inhibitors were obtained from the following sources: kinetin ribonucleoside from Nutritional Biochemical Corp.; isopentenyladenosine, 6-methylaminopurine ribonucleoside and 2,6-diaminopurine sulfate from Sigma Chemical Co.; 2,6-diaminopurine ribonucleoside from Cyclo Chemical Co.; arabinosyl adenine was a gift from Squibb & Co. Isopentenyl adenosine and arabinosyl adenine were dissolved in either 0·15% Tween 80 in buffer or in a small amount of dimethyl sulfoxide (DMSO). When DMSO was used, the final concentration of this solvent in the incubation tubes was about 4 per cent. Neither Tween nor DMSO at the concentrations used was found to inhibit the activity of methylases.

RESULTS

Earlier studies by Wainfan and Borek[19] showed that two cytotoxic derivatives of adenosine, i.e. 7-deazaadenosine (tubercidin) and N-6 isopentenyladenosine, inhibited tRNA methylation. Since a number of adenosine analogs are cytotoxic,[27,28] we have investigated the possibility that others of these derivatives might share the ability to act as methylase inhibitors.

The addition of a 2-amino group to adenosine yields 2,6-diaminopurine ribonucleoside. We have studied the effect of this compound on the rate of tRNA methylation catalyzed by enzymes from *E. coli*. The results, shown in Table 1, indicate that this compound causes a significant decrease in the rate of this enzymatic reaction. The effect is also observed with 2,6-diaminopurine sulfate. When the 100,000 *g* supernatant of extracts of *E. coli* M3S was permitted to catalyze methyl group transfer to methyl-deficient tRNA to saturation level, the product contained methyl guanine and methyluracil (ribothymidine) in the proportions of 0·24:1.[10] Addition of $2{\cdot}6 \times 10^{-3}$ M 2,6-diaminopurine sulfate in the presence of $5{\cdot}8 \times 10^{-9}$ M *S*-adenosyl methionine to such an incubation mixture resulted in a product in which methyl guanine and ribothymidine were present in the ratio 0·09:1. It is thus seen that 2,6-diaminopurine and its ribonucleoside inhibit more strongly the enzymes which

TABLE 1. INHIBITION OF BACTERIAL tRNA METHYLATING ENZYMES BY 2,6-DIAMINOPURINE RIBONUCLEOSIDE*

Enzyme source	SAM concn. ($\times 10^{-9}$ M)	2,6-Diamino-purine ribonucloeside ($\times 10^{-3}$ M)	Counts/min incorporated	Inhibition (%)
E. coli M3 ($\lambda C_1 857$)	2·2	0	2605 ± 85	
(extract)	2·2	2·1	975 ± 55	61
E. coli K12AB1157 trm$^-$	1·7	0	485 ± 55	
(extract)	1·7	1·8	155 ± 5	67
E. coli K12AB1157 trm$^-$	3·3	0	1210 ± 35	
[40–60 per cent saturated	3·3	0·9	240 ± 20	80
$(NH_4)_2SO_4$ ppt of extract]	3·3	1·7	135 ± 85	89
	6·6	0	2140 ± 85	
	6·6	0·9	660 ± 5	69

* Typical incubation tube contained 75 μg methyldeficient tRNA, $^{14}CH_3$-*S*-adenosyl methionine (sp. act. 55 mCi/m-mole) in amounts indicated above, inhibitor, and enzyme (at about a one-half saturating level)[7] in a final buffer concentration of 0·015 M Tris, 0·015 M $MgCl_2$, 0·007 M mercaptoethanol, pH 8. The volume of the incubation mixture was 0·3–0·5 ml. Samples were incubated for 30–40 min at 35° and then the reaction was terminated by addition of 0·2 ml of 0.15 M hydroxylamine, pH 7·5. After incubation for 10 min at 35°, samples were chilled in an ice bath and RNA and protein were precipitated by addition of 4 ml of 0·5 M NaCl in 75% ethanol. Samples were washed and assayed for radioactivity incorporated into RNA as described earlier.[26] Enzyme extracts contained 5 μg/ml of DNAase.[7]

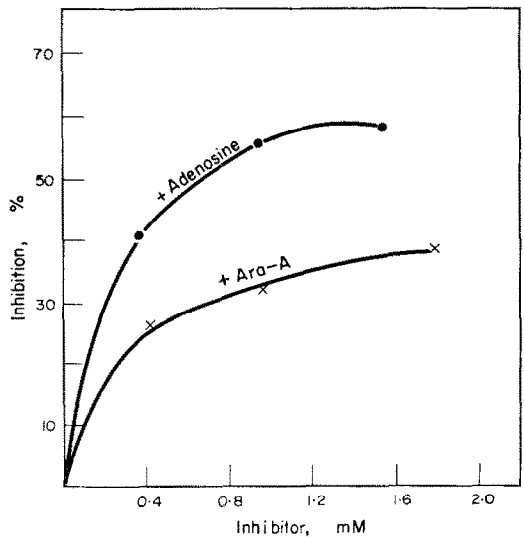

FIG. 1. Inhibition of bacterial tRNA methylases by adenosine and by arabinosyl adenine. Inhibitors were dissolved in dimethyl sulfoxide (DMSO) and then diluted with 0·01 M Tris, 0·01 M $MgCl_2$, 0·005 M mercaptoethanol, pH 8, before addition to the enzyme assay tubes. Final concentration of DMSO in all samples, including controls was 0·04 ml/0·5 ml of incubation mixture. ^{3}H-methyl-*S*-adenosyl methionine (sp. act. 1·2 Ci/m-mole) was used in these experiments.

methylate guanine in tRNA as compared with the enzymes which catalyze methylation of uracil in tRNA. Our earlier studies showed that adenine, adenosine, and N6-furfurylamino purine ribonucleoside also acted as preferential inhibitors of guanine tRNA methylases.[19,26]

Substitution of the ribofuranosyl group of adenosine by an arabinofuranosyl ring yields arabinosyl adenine (Ara-A). Figure 1 shows a comparison of the effects of various concentrations of adenosine and of Ara-A on the rate of tRNA methylation catalyzed by enzymes from *E. coli* K12AB1157 trm^-. It can be seen that under these conditions a given concentration of Ara-A is somewhat less effective than adenosine as a tRNA methylase inhibitor. The effect of Ara-A on the reactions in this bacterial system was found to be dependent upon the relative concentrations of *S*-adenosyl methionine and the inhibitor. Some sample results are shown in Table 2.

TABLE 2. EFFECT OF *S*-ADENOSYL METHIONINE CONCENTRATION ON INHIBITION OF *E. coli* tRNA METHYLASES BY ARABINOSYL ADENINE

Ara-A concn. ($\times 10^{-3}$ M)	SAM ($\times 10^{-10}$ M)	Counts/min incorporated		Inhibition %
		Control	Plus Ara-A	
1·4	2·5	1005 ± 25	325 ± 100	57
1·4	3·6	1895 ± 5	1360 ± 135	28
1·85	1·8	570 ± 40	225 ± 90	61
1·85	2·8	800 ± 95	395 ± 85	51
1·85	4·2	1650 ± 30	1315 ± 65	21
1·8	3·5	955 ± 70	595 ± 10	37

N^6-methyladenosine (Met AR) and N^6,$N^{6\prime}$-dimethyladenosine (Dimet AR) were able to inhibit tRNA methylation catalyzed by enzymes of both bacterial and animal origin. Inhibition of *E. coli* enzymes did not exceed 25 per cent and was somewhat variable, but the tRNA methylating enzymes of calf spleen were found to be very sensitive to both of these compounds (Table 3). The tRNA methylase activity of partially purified calf spleen extracts is about 95 per cent* specific for tRNA guanine. Studies of the effects of Met AR and Dimet AR in this system indicated that both these compounds are competitive inhibitors of guanine tRNA methylase. Lineweaver–Burk plots of data obtained in experiments where the *S*-adenosyl methionine concentrations were varied are presented in Figs. 2 and 3. The K_m for *S*-adenosyl methionine was found to be about $2{\cdot}5 \times 10^{-10}$. The K_i with either Met AR or Dimet AR was

* Quantitative assays for ^{14}C-methyl incorporated into methyl-deficient tRNA resulted in values of 95 per cent ± 5 per cent of total cpm in methylguanine. The data from two experiments were as follows:

	Expt. No. 1	Expt. No. 2
MeA	0	9
MeC	0	0
MeG	244	296
MeU	0	22

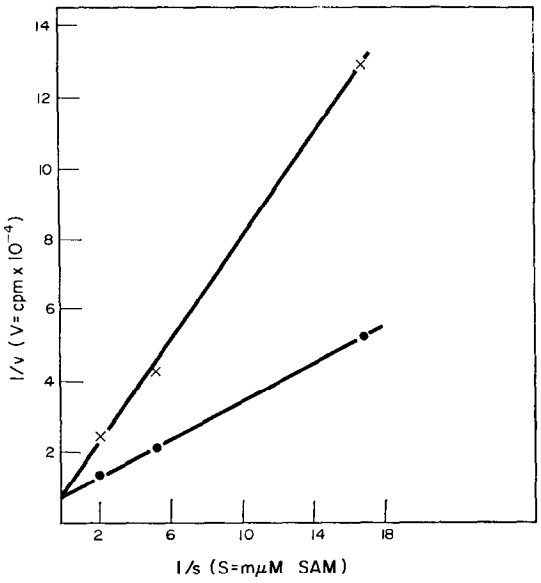

FIG. 2. Competitive inhibition of guanine tRNA methylation by N^6-methylaminopurine ribonicleoside. Enzyme used was the redissolved and dialyzed 60 per cent saturated ammonium sulfate precipitate of calf spleen extract. Inhibitor concentration was $2{\cdot}6 \times 10^{-3}$ M.

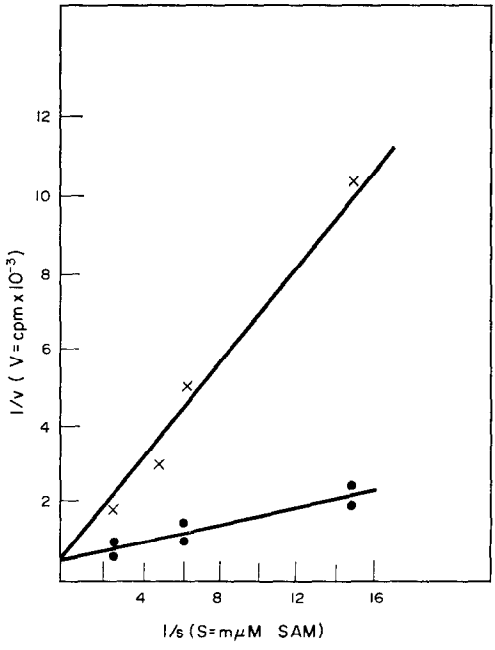

FIG. 3. Competitive inhibition of methylation by N^6, $N^{6\prime}$-dimethylaminopurine ribonucleoside. Enzyme used was the redissolved and dialysed 40–60 per cent saturated ammonium sulfate precipitate of calf spleen extract. Inhibitor concentration was $2{\cdot}6 \times 10^{-3}$ M.

TABLE 3. INHIBITION OF tRNA METHYLASES BY N^6-METHYLAMINOPURINE RIBONUCLEOSIDE AND BY N^6,$N^{6'}$-DIMETHYLAMINOPURINE RIBONUCLEOSIDE

Inhibitor	Enzyme source	Inhibitor ($\times 10^{-3}$ M)	SAM ($\times 10^{-9}$ M)	Counts/min incorporated		Inhibition (%)
				Control	Plus inhibitor	
Met AR	*E. coli*	2·4	18	2770 ± 60	2330 ± 105	16
Met AR	*E. coli*	2·3	13	6430 ± 370	4800 ± 265	25
Met AR	Calf spleen	2·9	14	1170 ± 20	730 ± 5	38
Met AR	Calf spleen*	2·3	0·19	4854 ± 55	2310 ± 35	52
Dimet AR	*E. coli*	1·3	13	2490 ± 65	2010 ± 5	19
Dimet AR	*E. coli*	2·3	18	2770 ± 60	2190 ± 160	21
Dimet AR	Calf spleen	2·0	2·2	1130 ± 25	770 ± 5	32
Dimet AR	Calf spleen	2·2	13	1285 ± 55	755 ± 30	41
Dimet AR	Calf spleen*	2·3	0·19	1840 ± 100	565 ± 5	69

* Sixty per cent saturated ammonium sulfate precipitate, redissolved and dialyzed.

2×10^{-9}. When crude 100,000 *g* supernatants without further fractionation were used as enzyme preparations, the competitive nature of the inhibitions was also apparent. However, higher K_m values were observed. K_i values were substantially the same as those observed after fractional ammonium sulfate precipitation and dialysis. The results obtained with these two compounds are very similar to those we reported previously for experiments with 6-furfurylamino purine ribonucleoside (KR).[26]

TABLE 4. INHIBITION OF CALF SPLEEN GUANINE tRNA METHYLASE BY 6-CHLOROPURINE RIBONUCLEOSIDE

Expt.	6-ClPR concn. ($\times 10^{-3}$ M)	SAM ($\times 10^{-10}$ M)	Counts/min incorporated		Inhibition %
			Control	Plus inhibitor (%)	
1*	2·3	0·65	1185 ± 100	855 ± 65	28
	2·3	1·8	2910 ± 5	1970 ± 65	32
	2·3	4·6	4825 ± 90	3325 ± 70	31
2*	2·1	1·6	805 ± 60	475 ± 25	41
	2·1	4·9	2235 ± 85	1650 ± 210	37
3†	2·4	1·7	1110 ± 60	895 ± 10	20
	3·6	1·7	1110 ± 60	585 ± 5	47
4‡	1·2	2·5	1535 ± 20	1365 ± 5	12
	2·4	2·5	1535 ± 20	1185 ± 35	23

* Redissolved and dialyzed 40–60 per cent saturated ammonium sulfate precipitate of calf spleen extract used as enzyme in experiments 1 and 2.

† Crude extracts were used in experiment 3.

‡ Enzyme used in experiment 4 was the 80 per cent saturated ammonium sulfate precipitate of calf spleen extract, redissolved and dialyzed.

6-Chloropurine was also tested as an inhibitor of spleen enzyme. This purine analog inhibited the rate of the reaction as shown in Table 4. The extent of inhibition appeared to be dependent upon the 6-CIPR concentration and not upon *S*-adenosyl methionine concentration. It was therefore concluded that this compound in contrast to Met AR and Dimet AR, was a noncompetitive inhibitor of calf spleen guanine tRNA methylase.

DISCUSSION

After the primary structure of tRNA molecules is transcribed directly from the DNA, enzymatic modification of selected bases in tRNA occurs.[29] Methyl groups are added to bases in tRNA at sites which are species specific.[30] A deficiency of methylated bases in tRNA has been shown to result in ambiguity in response to messenger RNA,[2,3] and decreased rates of enzymatic charging with amino acids for protein synthesis have been observed when either an excess or a deficient number of methyl groups is present.[4,5] Alteration of even a single base in a tRNA molecule can seriously impair its function.[1]

Although control of tRNA methylating enzyme activities in living cells is, as yet, incompletely understood, at least one of the factors involved appears to be the occurrence of varying levels of intracellular methylase inhibitors.[8,18] The presence of substances which specifically inhibit particular tRNA methylating enzymes may be important in maintaining the relatively constant species-specific methylated base content of tRNA, under normal conditions. The levels and specificities of the tRNA methylating enzymes have been observed to change during growth, differentiation and development, and in response to hormones.[18,31] In addition, alterations in the activity of these enzymes have been found in cells carrying a latent virus,[10] after certain virus infections,[6–7] in transformed cells,[11,12] and in many neoplastic tissues.[6,13–17]

The findings that we have presented here and in earlier reports indicate that adenine and adenosine, which are themselves cytotoxic, and a number of cytotoxic adenosine analogs have in common the ability to inhibit tRNA methylating enzymes. This group includes, among others, compounds with either hydrophilic or hydrophobic substituents on the N6 group of adenosine. Comparatively high concentrations of the analogs were required in order to demonstrate tRNA methylase inhibition *in vitro*. Since most of these drugs compete with *S*-adenosyl methionine (SAM) for the methylating enzymes, their ability to act as methylase inhibitors must be subject to the influence of the other intracellular reactions in which each of these compounds including SAM participates.[27,28] It seems possible that, under appropriate conditions, concentrations of SAM and of adenine or its derivatives in the intracellular pools could attain proportionate levels such as to affect tRNA methylase activities.

Although the drugs discussed here have been reported to have antitumor activity, their usefulness in cancer chemotherapy has been relatively limited. This seems to be, in part, related to their metabolic conversion to inosine and related compounds, by removal of the substituents at the 6 position of the purine ring[27,28,32] It may be pertinent that LePage recently reported that Ara-A was inhibitory only to experimental tumors with very low levels of adenosine deaminase,[33] and that Ara-A is susceptible to deamination by that enzyme.[33,34] As we have shown earlier, inosine itself does not inhibit tRNA methylation.[19]

When these observations are considered together with the findings of Tritsch *et al.*[35]

that methionine can reverse the cytotoxic effects of isopentenyl adenosine on a carinoma cell line, it appears possible that the cytotoxicity of adenine and some of its derivatives is due, at least in part, to the ability of these compounds to alter the levels and patterns of cellular tRNA methylation. Additional work will be necessary to determine whether this mechanism has a significant role *in vivo*.

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