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INHIBITION OF METHYLATION OF NUCLEAR RIBONUCLEIC ACID IN L1210 CELLS BY TUBERCIDIN, 8-AZAADENOSINE AND FORMYCIN

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Abstract—The adenosine analogs tubercidin (7-deazaadenosine), formycin (7-amino-3-[β-D-ribofuranosyl]pyrazolo[4,3-d]pyrimidine) and 8-azaadenosine were examined for their effects on the synthesis and methylation of nuclear RNA in L1210 cells *in vitro*. Total RNA and DNA synthesis was affected to the greatest extent by tubercidin ($IC_{50} = 7 \times 10^{-6}$ M) and to an insignificant degree by 8-azaadenosine and formycin; however, the effects of the latter two drugs, but not of tubercidin, were potentiated by 2′-deoxycoformycin, an inhibitor of adenosine deaminase. In the presence of 2′-deoxycoformycin, RNA synthesis was inhibited by 40 per cent at 1×10^{-4} M 8-azaadenosine and by 50 per cent at 2×10^{-4} M formycin, while DNA synthesis was inhibited less extensively. Alkaline hydrolysis of nuclear RNA labeled with [^{14}C]uridine and L-[*methyl*-^{3}H]methionine showed preferential inhibition of base methylation in mononucleotides, but not of 2′-*O*-methylation in dinucleotides, for all three drugs. This differential effect persisted to varying degrees in >18S and 4S nuclear RNA separated by electrophoresis. The reduction in base methylation in 4S RNA was associated with seven of the eight methylated nucleosides in 4S RNA separated by two-dimensional thin-layer chromatography. These results indicate that tubercidin, 8-azaadenosine and formycin can preferentially inhibit the base methylation of nuclear RNA relative to its synthesis.

The antitumor activities of several adenosine analogs, such as 8-azaadenosine and formycin, are potentiated to varying degrees by the adenosine deaminase inhibitor, dCF* [1]. The synergism observed with these therapeutic combinations arises from blockade of the catabolism of 8-azaadenosine and formycin via adenosine deaminase [1–3]. In contrast, the pyrrolopyrimidine analog, tubercidin, does not serve as a substrate for adenosine deaminase [2–4], and hence its toxicity is not altered by dCF [1].

The modes of action of tubercidin and formycin are believed to be associated with their anabolism to the nucleoside 5′-triphosphate [5, 6], and their subsequent incorporation into RNA [7, 8]. Both analogs impair the processing, but not the transcription, of 45S precursor rRNA to 28S and 18S rRNA [9, 10], while formycin preferentially inhibits the synthesis of cytoplasmic 4S and 5S RNA [10]. These results suggest that processes associated with the processing of RNA, such as methylation, may be involved, in part, in their mechanism of action. In this regard, tubercidin, but not formycin, inhibited guanine and uracil tRNA methyltransferases in *Escherichia coli* [11].

The present study was designed to investigate the effects of tubercidin, formycin and 8-azaadenosine on the synthesis and methylation of nRNA, and the influence of dCF on their specificities of action.

MATERIALS AND METHODS

Materials. Tubercidin, 8-azaadenosine, formycin and dCF were obtained from the Drug Synthesis and Chemistry Branch, National Cancer Institute. [5-^{3}H-*methyl*]Thymidine (20 Ci/mmole), L-[*methyl*-^{3}H]methionine (80 Ci/mmole), and [U-^{14}C]uridine (464 mCi/mmole) were purchased from the New England Nuclear Corp., Boston, MA. m^1A, m^3C, m^5C, m^1G, m^2G, m^2_2G, m^7G and m^5U were obtained from P–L Biochemicals, Milwaukee, WI.

Animals. L1210 cells were inoculated, i.p., into BALB/c × DBA/2 F_1 mice at an inoculum of 10^5 cells/0.1 ml Hanks' balanced salt solution. Cells were harvested 6 days after inoculation and were further diluted with incubation medium.

Incubations. Incubations of L1210 cells were carried out at 37° in a shaking water bath at 100 r.p.m. and consisted of: (1) 25 ml of L-methionine-free RPMI 1630 medium, 5×10^7 cells, 200 μCi [^{3}H]methionine (80 Ci/mmole) and 5 μCi [^{14}C]uridine (464 mCi/mmole) or (2) 5 ml of RPMI 1630 medium, 1×10.7 cells, 1 μCi [^{14}C]uridine (464 mCi/mmole) and 5 μCi [^{3}H]thymidine (20 Ci/mmole). Cells were preincubated with dCF for 15 min before further incubation for 30 min with

* Abbreviations used are: nRNA, nuclear RNA; SDS, sodium dodecylsulfate; TCA, trichloroacetic acid; formycin, 7-amino-3-[β-D-ribofuranosyl]pyrazolo[4,3-d] pyrimidine; dCF. 2′-deoxycoformycin[(R)-3-(2-deoxy-β-D-erythropentofuranosyl)-3, 6, 7, 8-tetrahydroimidazo [5, 4-*d*] [1,3]diazepin-8-ol], m^1A, 1-methyladenosine; m^3C, 3-methylcytidine; m^5C, 5-methylcytidine; m^1G, 1methylguanosine; m^2G, N^2-methylguanosine; m^2_2G, N^2,N^2-dimethylguanosine; m^7G, 7-methylguanosine; m^5U, 5-methyluridine; IC_{50}, median inhibitory concentration; and SAH, *S*-adenosylhomocysteine.

either tubercidin, 8-azaadenosine or formycin. The period of labeling was 1 hr.

Total RNA and DNA synthesis. Incorporation of [^{14}C]uridine and [^{3}H]thymidine into total RNA and DNA, respectively, was measured as described previously [12].

RNA extraction. After incubation, cells were centrifuged at 400 *g* for 20 min and washed once with incubation medium. Nuclei were prepared as described previously [12], except that cells were swelled in 10 mM magnesium acetate (pH 5.1). This change in swelling medium was found to markedly arrest the activity of intracellular RNase, and thereby minimize hydrolysis of nRNA before extraction. nRNA was extracted from nuclei by vortexing vigorously for 2 min with 3 ml of 0.1% SDS : 0.14 M NaCl : 0.025 M sodium acetate (pH 5.1) and 3 ml of phenol mixture [phenol–*m*-cresol–water (7:2:2), v/v] containing 0.1% 8-hydroxyquinoline. The emulsion was clarified by centrifugation at 12,000 *g* for 10 min, and the upper aqueous phase was removed and precipitated with 3 vol. of 2% potassium acetate in 95% ethanol at −20° overnight.

Electrophoresis. nRNA was resolved by electrophoresis in cylindrical polyacrylamide gels (0.4 × 7 cm) containing: 8% (w/v) acrylamide, 0.32% (w/v) diallyltartardiamide, 6 M urea, 0.1% (w/v) SDS, 0.2% (w/v) ammonium persulfate, 0.04% (v/v) *N,N,N'N'*-tetramethylenediamine, 0.4 M Tris–acetic acid (pH 7.2), 0.02 M sodium acetate and 0.002 M EDTA. RNA samples containing one A_{260} unit were mixed with sample buffer to give a final concentration of: 0.04 M Tris–acetic acid (pH 7.2), 0.02 M sodium acetate, 0.002 M EDTA, 0.02% bromphenol blue and 20% (w/v) sucrose (RNase-free). Gels were electrophoresed at 4 mA/gel at 4°. Gels were sectioned into 2 mm slices, dissolved in 2% (w/v) periodic acid at 37° for 15 min, mixed with 10 ml Aquasol (New England Nuclear Corp.) and counted in a Searle Mark III liquid scintillation spectrometer. Gels were stained with 2% methylene blue dissolved in 15% acetic acid for 1 hr and destained by diffusion in 15% acetic acid.

Thin-layer chromatography. 4S RNA was isolated by polyacrylamide gel electrophoresis. 4S RNA was sliced from the appropriate section of the gel and sectioned into 2 mm slices. RNA was extracted from four gel slices with 1 ml of RNA extraction buffer (0.1% SDS : 0.014 M NaCl : 0.025 M sodium acetate, pH 5.1) and continuous vortexing at room temperature for 1 hr. The gel was removed by centrifugation at 16,000 *g* for 2 min in an Eppendorf centrifuge, and the RNA was precipitated at −20° for 2 hr. Enzymatic digestion was carried out for 18 hr at 37° in 20 μl of 0.05 M Tris–HCl (pH 8.0) : 5 mM $MgCl_2$ containing: 6 μg RNase A, 5 μg calf intestine alkaline phosphatase (1000 units/mg) and 10 μg snake venom phosphodiesterase. Samples were freeze dried and reconstituted with 20 μl of a standard mixture containing the eight methylated nucleosides at a concentration of 0.4 mg/ml. An aliquot of 5 μl was spotted on 0.25 mm silica gel plates containing fluorescent indicator (EM Laboratories, Elmsford, NY), and the eight methylated nucleosides were separated by two-dimensional thin-layer chromatography with acetonitrile–concentrated NH_4OH (4:1, v/v) for the first dimension and acetonitrile–2 N HCOOH (10:1, v/v) for the second dimension [13].

RESULTS

Initial experiments were designed to assess the specificity of the effects of tubercidin, 8-azaadenosine and formycin on total RNA and DNA synthesis in the presence and absence of 1×10^{-6} M dCF (Fig. 1). Only tubercidin demonstrated a marked inhibitory effect on both macromolecules in the absence of the adenosine deaminase inhibitor where the IC_{50} was approximately 7×10^{-6} M (Fig. 1, panel A). In contrast, both 8-azaadenosine and formycin were dependent on the blockade of adenosine deaminase for the expression of their inhibitory effects (Fig. 1, panels B and C). In the presence of dCF, 1×10^{-5} M and 1×10^{-4} M 8-azaadenosine inhibited RNA synthesis by 40 per cent and DNA synthesis by only 10–15 per cent. Similarly, 1×10^{-4} M

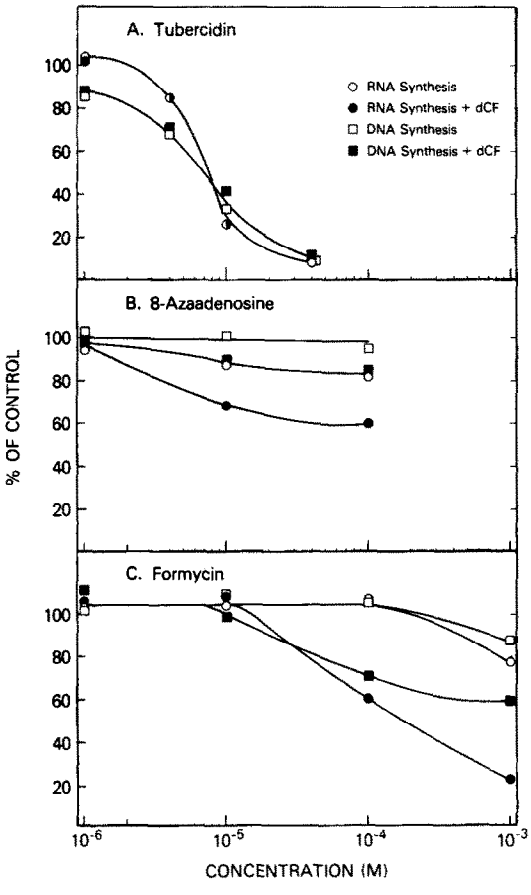

Fig. 1. Dose–response of RNA and DNA synthesis by tubercidin, 8-azaadenosine and formycin. L1210 cells (1×10^{7} cells/flask) were preincubated with (●, ■) or without (○, □) 1×10^{-6} M dCF, and incubated for 30 min with tubercidin (A), 8-azaadenosine (B) or formycin (C). Cells were then double-labeled for 1 hr with [^{14}C]uridine (○, ●) and [^{3}H]thymidine (□, ■) and TCA-precipitable radioactivity was determined as described under Materials and Methods. Each value is the mean of two to three determinations.

Table 1. DEAE Sephadex-urea chromatography of alkaline hydrolysates of nRNA from L1210 cells treated with tubercidin, 8-azaadenosine and formycin*

Treatment	RNA fraction (10^3 d.p.m.) −2 ^{3}H	−2 ^{14}C	−3 ^{3}H	−3 ^{14}C	−5 ^{3}H	−5 ^{14}C
Control	268 ± 28	110 ± 6	186 ± 19	4.15 ± 0.91	20.6 ± 1.7	0.55 ± 0.16
			% of control			
Tubercidin						
4×10^{-6} M	59	99	77	53	47	0
4×10^{-5} M	9	16	14	2	8	0
dCF + 8-azaadenosine						
1×10^{-5} M	45	62	57	42	29	26
1×10^{-4} M	50	61	61	43	35	0
dCF + formycin						
1×10^{-4} M	28	73	59	21	26	0
1×10^{-3} M	14	38	32	10	14	0

* Values for pooled controls (with and without dCF) represent the means ±S.E. of four determinations. Values for incubations with the lower concentration of each drug represent the mean of two determinations, and at the higher concentration of each drug a single determination. L1210 cells were incubated with tubercidin, dCF plus 8-azaadenosine, or dCF plus formycin followed by labeling with L-[*methyl*-^{3}H]methionine and [^{14}C]uridine as described under Materials and Methods. The fractions −2, −3 and −5 denote the net charge of mono-, di- and tetranucleotides of alkaline hydrolysates of nRNA eluted by DEAE Sephadex chromatography depicted in Fig. 2.

and 1×10^{-3} M formycin inhibited RNA synthesis by 40–80 per cent, whereas DNA was reduced 30–40 per cent. dCF alone at a concentration of 1×10^{-6} M, which was used throughout all the experiments, did not affect RNA or DNA synthesis.

To further examine the effects of these adenosine analogs on RNA, nRNA was double-labeled with L-[*methyl*-^{3}H]methionine and [^{14}C]uridine, and the alkaline hydrolysates were chromatographed on DEAE Sephadex (Table 1, Fig. 2). The methylation of the mononucleotide fraction (−2 charge) representing base methylation was preferentially reduced by all three drugs vs [^{14}C]uridine incorporation (Table 1). This differential effect was not evident in either the dinucleotide fraction (−3 charge) representing 2′-*O*-methylation or in the tetranucleotide fraction (−5 charge) representing 5′-cap structures. In addition, alkaline hydrolysates from cells treated with dCF plus formycin showed a methylated fraction representing 1.7 per cent of the total incorporated ^{3}H which eluted before the mononucleotide (−2) peak (Fig. 2, panel D). This fraction was not further characterized, but possibly represents m^{7}G monophosphate from the 5′-cap

Table 2. Effects of tubercidin, 8-azaadenosine and formycin on the methylation and synthesis of nRNA from L1210 cells*

Treatment	RNA fraction (% of control) Total ^{3}H	Total ^{14}C	>18S ^{3}H	>18S ^{14}C	4S RNA ^{3}H	4S RNA ^{14}C
Tubercidin						
4×10^{-6} M	57 ± 2	85 ± 2	50 ± 5	75 ± 8	43 ± 3	61 ± 8
4×10^{-5} M	16 ± 3	8 ± 2	15 ± 3	15 ± 3	14 ± 4	2 ± 1
dCF + 8-azaadenosine						
1×10^{-5} M	47 ± 2	62 ± 2	45 ± 4	70 ± 9	38 ± 6	40 ± 10
1×10^{-4} M	42 ± 3	63 ± 4	32 ± 3	58 ± 10	30 ± 8	40 ± 12
dCF + formycin						
1×10^{-4} M	33 ± 2	55 ± 2	52 ± 7	57 ± 4	25 ± 1	41 ± 4
1×10^{-3} M	27 ± 1	35 ± 1	22 ± 6	32 ± 8	14 ± 3	12 ± 2

* Values represent the means ± S.E. of three to six determinations. L1210 cells were incubated with tubercidin, dCF plus 8-azaadenosine, or dCF plus formycin followed by labeling with L-[*methyl*-^{3}H]methionine and [^{14}C]uridine as described under Materials and Methods. Nuclear RNA was isolated and electrophoresed in 8% polyacrylamide-urea gels as depicted in Fig. 3. Radioactivity in >18S and 4S nRNA was calculated from the radioactivity present at the top of the gel and the 4S region, respectively.

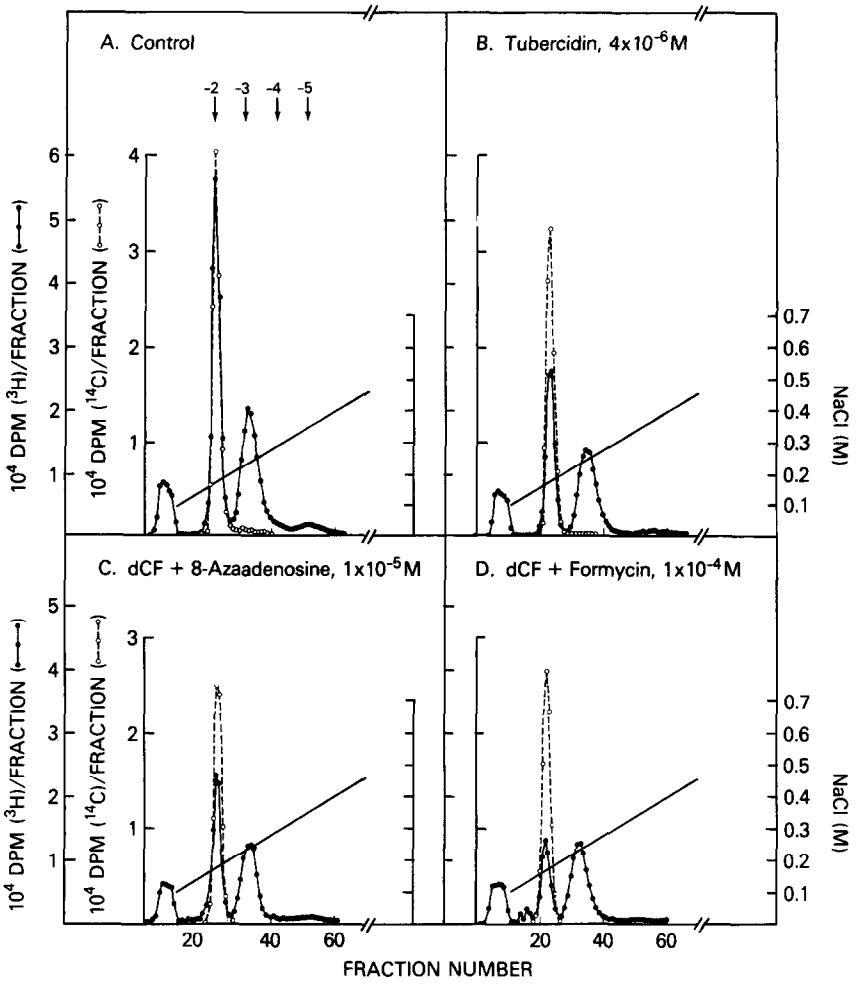

Fig. 2. DEAE Sephadex-urea chromatography of alkaline hydrolysates of nRNA. L1210 cells (5×10^7 cells/flask) were preincubated for 15 min with (C and D) or without (A and B) 1×10^{-6} M dCF and incubated for 30 min with 4×10^{-6} M tubercidin (B), 1×10^{-5} M 8-azaadenosine (C) or 1×10^{-4} M formycin (D). Cells were then double-labeled for 1 hr with 200 μCi L-[*methyl*-^{3}H]methionine and 5 μCi [^{14}C]uridine. The −2, −3, −4 and −5 markers represent the net charge of mono-, di-, tri- and tetranucleotides, respectively, eluted with the NaCl gradient.

structure. dCF, when used as a single agent at 1×10^{-6} M (or at concentrations as high as 1×10^{4} M), did not affect either the methylation or synthesis of nRNA.

Since base methylation was preferentially reduced by tubercidin, dCF plus 8-azaadenosine, and dCF plus formycin, nRNA was fractionated by electrophoresis in denaturing polyacrylamide gels to ascertain if this effect pertained specifically to 4S nRNA which contains several methylated bases (Fig. 3). At 4×10^{-6} M tubercidin (Fig. 3, panel B) or dCF plus 1×10^{-5} M 8-azaadenosine (Fig. 3, panel C), methylation of >18S nRNA (at the top of the gel) was inhibited to a greater extent than its synthesis, while no differential effect was noted for dCF plus 1×10^{-4} M formycin (Fig. 3, panel D). A different pattern of inhibition of 4S nRNA was noted for the three adenosine analogs; 4×10^{-6} M tubercidin, and dCF plus 1×10^{-4} M formycin, preferentially reduced methylation vs [^{14}C]uridine incorporation, while no differential effect was noted for dCF plus 1×10^{-5} M 8-azaadenosine.

The preferential inhibition of methylation vs synthesis shown at the lowest concentration of drug tested was largely eliminated at a 10-fold higher concentration of each drug with the exception of the effect of the combination of dCF plus 8-azaadenosine on >18S nRNA (Table 2).

Analysis of the methylated nucleosides in 4S nRNA by two-dimensional thin-layer chromatography revealed that the degree of methylation in m^1A was relatively insensitive to the effects of the three drugs, while inhibition of methylation in m^2G, m_2^2G, m^7G and m^1G accounted for most of the effects of the agents on this species of nRNA (Table 3). Methylation in m^5C was reduced to a greater

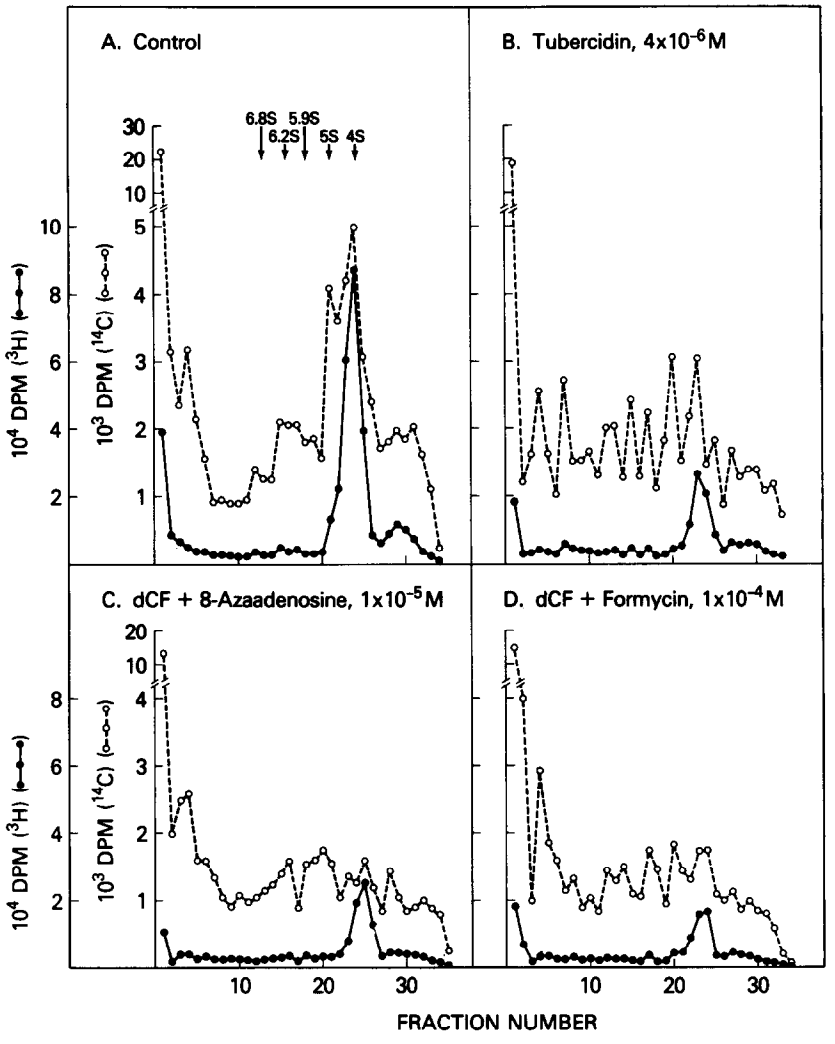

Fig. 3. Polyacrylamide gel electrophoresis of nRNA. L1210 cells (5×10^7 cells/flask) were treated as described in Fig. 2, and electrophoresed in denaturing 8% polyacrylamide-urea gels as described under Materials and Methods.

Table 3. Effects of tubercidin, 8-azaadenosine and formycin on the composition of methylated nucleosides in 4S nRNA from L1210 cells*

	Methylated nucleoside (d.p.m.)							
Treatment	m^7G	m^5C	m^3C	m^2G	m_2^2G	m^1G	m^5U	m^1A
Control	3230	2220	630	4590	4290	2520	3430	300
			% of control					
Tubercidin 4×10^{-6} M	20	54	27	32	36	28	41	88
dCF + 8-azaadenosine 1×10^{-5} M	47	42	27	55	50	42	53	93
dCF + formycin 1×10^{-4} M	25	9	40	30	17	19	31	83

* Each value is the mean of two determinations.

extent vs the other methylated nucleosides by dCF plus formycin, but not by tubercidin or dCF plus 8-azaadenosine (Table 3).

DISCUSSION

One of the unique activities recently found to be associated with some adenosine analogs is their ability to interfere with the methylation of nRNA. Cordycepin (3′-deoxyadenosine) and xylosyladenine are examples of such drugs, and preferentially inhibit 2′-*O*-methylation of nRNA vs RNA synthesis in L1210 cells [14]. For the latter drug, this effect is associated with interference with the synthesis of *S*-adenosyl-L-methionine, and is potentiated by the adenosine deaminase inhibitor, dCF [15]. In the present study, two adenosine analogs which are potentiated by dCF, 8-azaadenosine and formycin, as well as tubercidin which is not a substrate for adenosine deaminase, also were shown to preferentially inhibit the methylation of nRNA vs RNA synthesis. In these instances, however, the impairment in methylation of nRNA was associated primarily with base methylation and not with 2′-*O*-methylation as in the case of cordycepin and xylosyladenine [14]. The actions of tubercidin, 8-azaadenosine and formycin were associated with both high molecular weight (>18S) and 4S nRNA, and in the latter species of RNA, impaired methylation occurred in seven of the eight methylated bases analyzed. Therefore, these investigations demonstrate that three adenosine analogs, which differ structurally in the imidazole moiety of adenine, are potent inhibitors of base methylation in nRNA. Whether this effect is associated with impaired processing of 45S precursor rRNA is not known; it is conceivable, however, that drastically reduced 2′-*O*-methylation at higher concentrations of these drugs could inhibit the processing of 45S precursor rRNA to 28S and 18S RNA as shown for tubercidin and formycin [9, 10].

The mechanism of action of adenosine analogs with regard to their inhibitory effects on the methylation of nRNA is not known. Recently, several analogs of SAH, including 8-azaadenosyl-SAH, were found to inhibit (guanine-N^2)-tRNA methyltransferase [16]. Similarly, SAH analogs of tubercidin inhibited (guanine-7-)-methyltransferase of Newcastle disease virions [17]. However, the present study is the first to show that tubercidin, 8-azaadenosine and formycin can affect the methylation of nRNA. Whether these analogs can act directly or must be anabolized to nucleotide or SAH metabolites remains to be determined.

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