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CORDYCEPIN AND XYLOSYLADENINE: INHIBITORS OF METHYLATION OF NUCLEAR RNA

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SUMMARY

Cordycepin and xylosyladenine inhibited methylation of nuclear RNA to a greater extent than RNA synthesis in L1210 cells in vitro. Inhibition of base methylation, 2'-O-methylation and 5'cap methylation was equal to, 2 to 5-fold greater and 1.5-fold greater, respectively than inhibition of RNA synthesis. Cordycepin was more potent than xylosyladenine in inhibiting 2'-O-methylation of cytidine and adenosine, but not guanosine. These results suggest that impirment in the 2'-O-methylation of nuclear RNA may be one of the major effects that limits the biological activity of rRNA and mRNA by these drugs.

INTRODUCTION

The processing of nuclear rRNA and mRNA via specific methylation processes is believed to be a prerequisite for the maturation of functional cytoplasmic rRNA and mRNA. In particular, 2'-O-methylation of nuclear precursor 45S rRNA determines the specificity of endonucleolytic cleavage to form 28S and 18S rRNA (1-5). Furthermore, methylation of the 5' terminus of nuclear precursors to mRNA to form the 5' "cap" is now believed to be necessary for the formation of a stable initiation complex for translation (6-10).

Cordycepin (3'-deoxyadenosine) is an effective inhibitor of the synthesis of diverse species of nuclear RNA (11-13). It's congener, xylosyladenine (9-β-D-xylofuranosyladenine) has a similar spectrum of activity (A.L. Peale and R.I. Glazer, submitted for publication) although it is 5-fold more potent. In this study, we wish to present evidence indicating that both nucleosides are more effective inhibitors of the methylation of total nuclear RNA than of its synthesis, and that these findings may be contributory to the impairment of the appearance of cytoplasmic rRNA and mRNA after treatment with these drugs.

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MATERIALS AND METHODS

Cordycepin and xylosyladenine were kindly provided by Dr. Harry B. Wood, Jr., National Cancer Institute. [^{3}H-methyl]methionine (63 Ci/mmole) and [^{14}C(U)]uridine (462 mCi/mmole) were purchased from New England Nuclear. E. coli alkaline phosphatase and snake venom phosphodiesterase were obtained from Sigma Chemical Co., and 2'-O-methyl nucleosides and m^{7}guanosine were purchased from P-L Biochemicals.

Animals. L1210 cells were inoculated into CDF_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 washed once in Dulbecco's medium and further diluted with the same medium to 2 x 10^7 cells/ml.

Incubations. Incubations of L1210 cells were carried out for 60 min at 37° in a shaking water bath at 100 rpm and consisted of: 5 ml of Dulbecco's medium, 5 x 10^7 cells, 0.25% glucose, either 50 uCi [^{3}H]methionine (80 mCi/mmole), 5 µCi [^{14}C]uridine (50 mCi/mmole), and either 2.5 x 10^{-4} M cordycepin or 5 x 10^{-5} M xylosyladenine as indicated. Cells were preincubated for 30 min with either drug in Dulbecco's medium with 0.25% glucose before the 60 min labeling period.

RNA extraction: After incubation, cells were centrifuged at 200 x g for 15 min at 4°, and washed once with Dulbecco's medium. Nuclei were prepared according to the procedure of Daskal et al. (14) using Triton X-100 as the detergent. Total nuclear RNA was extracted by the sodium dodecyl sulfate-phenol extraction method previously described (11). With this procedure, rRNA is extracted with equal parts of 0.1% sodium dodecyl sulfate-0.1 M sodium acetate (pH 5.1)-0.005 M EDTA and phenol mixture (phenol:m-cresol:H_2O, 7:2:1 (v/v) containing 0.1% 8-hydroxyquinoline) followed by extraction of heterogeneous RNA from the phenol precipitated interface with an equal volume of 0.1% sodium dodecyl sulfate:0.1 M Tris-HCl (pH 9.0):7 M urea (15). Extracts were combined and precipitated at -20° with 2 volumes of 2% potassium acetate in 95% ethanol. RNA precipitates were washed once with 95% ethanol at 4° and hydrolyzed overnight at 37° with 0.3N KOH.

DEAE Sephadex chromatography. Alkaline hydrolysates of nuclear RNA were neutralized with 0.3 N $HClO_4$ using 0.1% phenol red as internal indicator. Samples were centrifuged at 2,000 x g at 4° after remaining at 4° for 20 min. Neutralized hydrolysates were diluted with 10 ml of 7 M urea-20 mM Tris-HCl (pH 7.6) and adsorbed to DEAE Sephadex equilibrated with the same buffer. Mono-, di- and oligonucleotides were eluted with a linear gradient of 0.1 - 0.7 M NaCl in 7 M urea-20 mM Tris-HCl (pH 7.6). Columns were standardized separately with RNase A hydrolysates of ^{3}H-labeled L1210 nuclear RNA.

Thin layer chromatography. Mononucleotide, dinucleotide and oligonucleotide fractions corresponding to a net charge of -2, -3, and -5.5, respectively, were desalted of urea by adsorption of samples diluted 10-fold with water to columns (1.5 x 10) of DEAE cellulose. Each fraction was eluted with 50 ml of 1.0 M triethylammonium carbonate and lyophilized. Lyophilized samples were redissolved in 2 ml of water and transferred to 13 x 100 mm test tubes and lyophilized again. Samples were dissolved in 0.1 ml of 50 mM Tris-HCl (pH 8.5)-0.005 M $MgCl_2$ containing 10 µg of alkaline phosphatase and 20 µg of phosphodiesterase, and incubated for 2 hr at 37° (17). After incubation, samples were spotted on cellulose-coated thin layer sheets (Eastman Kodak). The solvent systems were: A) ethyl acetate:2-propanol:7.5 M NH_4OH:1-butanol (3:2:2:1, v/v) and B) 2-propanol:concentrated HCl:H_2O (68:17.6:14.3, v/v) (17).

RESULTS

Alkaline hydrolysates of control and drug-treated nuclear RNA double-labeled with [^{3}H]methionine and [^{14}C]uridine showed three distinct peaks of ^{3}H radioactivity

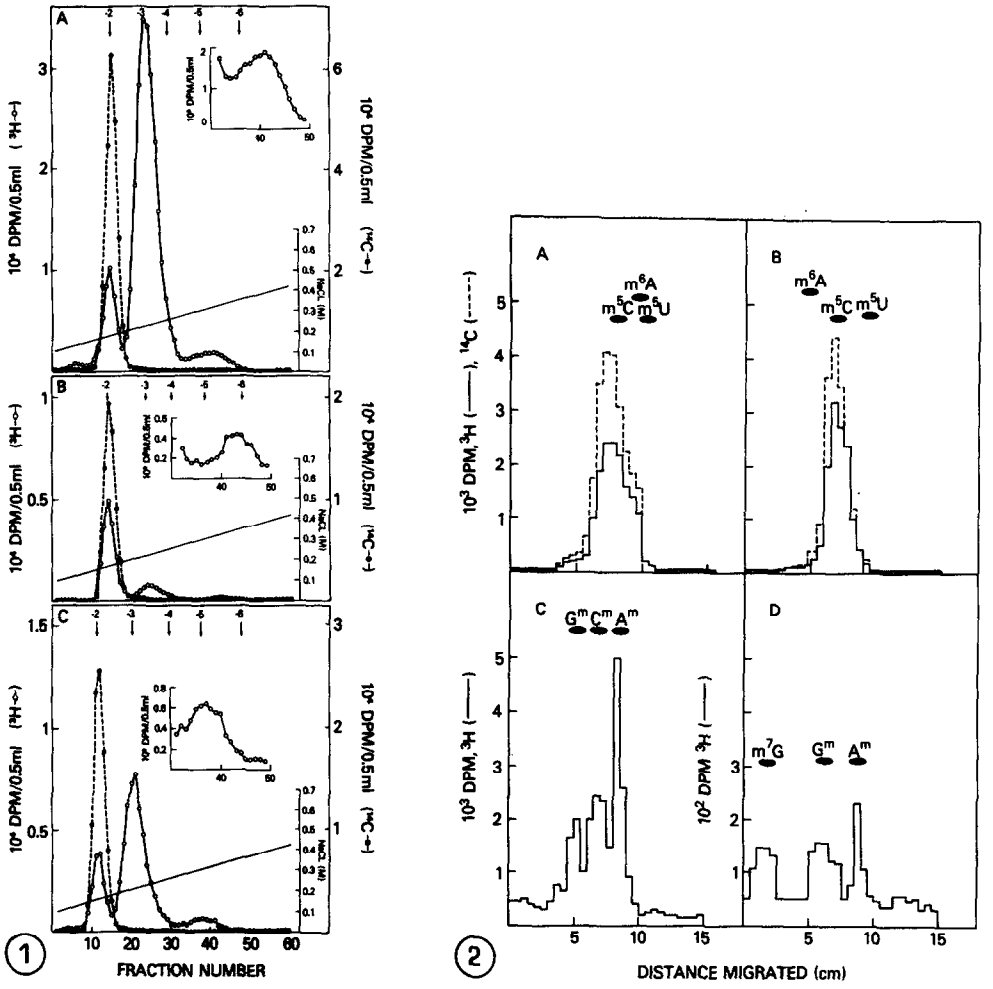

Figure 1. DEAE Sephadex chromatography of alkaline hydrolysates of nuclear RNA labeled with [H]methionine and [C]uridine. L1210 cells were incubated in the absence (A) or presence of 2.5 x 10^{-4} M cordycepin (B) or 5 x 10^{-5} M xylosyladenine (C). Total nuclear RNA was extracted, hydrolyzed and chromatographed as described in MATERIALS AND METHODS. A total of 100 fractions were collected but only the first 60 fractions are shown since no radioactivity was eluted in the last 40 fractions. The insets represent the -5.5 oligonucleotide region with the scale on the ordinate decreased 10-fold.

Figure 2. Thin layer chromatography of DEAE Sephadex fractions of alkaline hydrolysates of nuclear RNA. Nucleotide fractions were separated by DEAE Sephadex chromatography, desalted, freeze-dried and digested with alkaline phosphatase and phosphodiesterase as described in MATERIALS AND METHODS. The abbreviations used for the nucleoside markers are: m^5C, 5-methylcytidine; m^6A, 6-methyladenosine; m^5U, 5-methyluridine; m^7G, 7-methylguanosine; G^m, 2'-O-methylguanosine; C^m, 2'-O-methylcytidine; A^m, 2'-O-methyladenosine.
A) -2 fraction with solvent system A B) -2 fraction with solvent system B
C) -3 fraction with solvent system A D) -5.5 fraction with solvent system A.

at -2, -3 and -5.5 net charge and ^{14}C radioactivity coinciding with only the mononucleotide peak (Fig. 1). Equipotent (with respect to inhibition of [^{14}C]uridine incorporation into nuclear RNA) concentrations of cordycepin and xylosyladenine markedly inhibited RNA synthesis, but even more significantly, reduced methylation of nuclear RNA in the mononucleotide (-2 charge), dinucleotide (-3 charge) and oligonucleotide (-5.5 charge) fractions (Figs. 1B and 1C) compared to control RNA (Fig. 1A). Methylation of the dinucleotide fraction containing 2'-O-methylated dinucleotides was inhibited by 73-92% in contrast to inhibition of base methylation of 45-58% in the mononucleotide fraction and 71-74% inhibition of methylation of the oligonucleotide fraction (TABLE 1).

Methylated nucleosides in each of the three fractions were separated by thin layer chromatography. The mononucleotide fraction consisted predominantly of 5-methylcytidine as determined in solvent systems A (Fig. 2A) and B (Fig. 2B). The dinucleotide fraction contained 2'-O-methyladenosine with lesser quantities of 2'-O-methylguanosine and 2'-O-methylcytidine (Fig. 2C) while the oligonucleotide fraction consisted of 7-methylguanosine, 2'-O-methylguanosine and 2'-O-methyladenosine (Fig. 2D). Cordycepin and xylosyladenine inhibited each of the methylated nucleosides to the same extent as found for their respective fractions after DEAE Sephadex chromatography (TABLE 1).

DISCUSSION

The present study has demonstrated that the nucleoside anticancer drugs, cordycepin and xylosyladenine, are effective inhibitors of base and sugar methylation in nuclear RNA of L1210 cells *in vitro*. 2'-O-Methylation of adenosine, guanosine and cytidine were particularly sensitive to these agents and inhibition of sugar methylation was far more sensitive than impairment of RNA synthesis as assessed by uridine incorporation. Inhibition of the methylation in the oligonucleotide fraction which represents the 5' "cap" of nuclear RNA (16-18) was intermediate in sensitivity. Although the mononucleotide, dinucleotide and oligonucleotide fractions from total RNA obtained by DEAE Sephadex chromatography represent a mixture of RNA species, it

TABLE 1

Inhibition of methylation of L1210 nuclear RNA hydrolysates

by cordycepin and xylosyladenine

Treatment	RNA fraction							
	-2				-3		-5.5	
	^{3}H		^{14}C		^{3}H		^{3}H	
	Total dpm (x 10^{-3})							
		%		%		%		%
Control	130.1 ± 16.7	100	670.9 ± 130.7	100	684.8 ± 86.6	100	53.1 ± 5.0	100
Cordycepin, 2.5 x 10^{-4}M	54.4 ± 4.1	42	287.1 ± 49.1	43	186.9 ± 18.1	27	15.2 ± 1.5	29
Xylosyladenine, 5 x 10^{-5}M	71.4 ± 1.4	55	278.8 ± 59.4	42	53.7 ± 4.6	8	14.0 ± 1.2	26

Incubations and isolation of L1210 nuclear RNA were carried out as described in MATERIALS AND METHODS. The total radioactivity represents that coinciding with the peaks of -2, -3, and -5.5 charge in Fig. 1. Each value is the mean ± S.E. of 3 separate experiments.

is believed that the dinucleotide fraction is mainly associated with rRNA (16-18) and the 5' "cap" with poly(A)-containing heterogeneous nuclear RNA (18). The potent inhibition by cordycepin and xylosyladenine of these methylation processes may explain, in part, their inhibitory effects on nuclear RNA synthesis, and in particular, the activity of cordycepin in impairing the processing of precursor rRNA (19).

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