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Inhibition of the Phosphorylation of Nonhistone Chromosomal Proteins by Cordycepin and Xylosyladenine in L1210 Cells *in Vitro*

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SUMMARY

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Cordycepin and xylosyladenine were tested for their inhibitory effect upon the phosphorylation of nuclear proteins in L1210 cells *in vitro*. As indicated by isoelectric focusing of urea-soluble nuclear proteins, the phosphorylation of acidic nonhistone chromosomal proteins was strongly inhibited by both nucleosides, particularly in the presence of the adenosine deaminase inhibitor, 2′-deoxycoformycin. These observations may explain, in part, the inhibitory effect of both drugs upon transcription.

INTRODUCTION

Cordycepin (3′-deoxyadenosine) and xylosyladenine (9-β-D-xylofuranosyladenine) inhibit the growth of tumor cells in culture and *in vivo* (1–4). Cordycepin is an inhibitor of rRNA and mRNA synthesis (5–12) and xylosyladenine inhibits *de novo* purine synthesis (2), as well as nuclear RNA synthesis (13). However, recent observations have indicated that cordycepin is an effective competitive inhibitor *in vitro* of cyclic nucleotide independent protein kinases associated with the nucleus of normal and neoplastic liver (14, 15). Cordycepin was also found to inhibit the phosphorylation of NHCP[2] in isolated rat liver nuclei (16). These observations have led to a reconsideration of the mechanism of action of cordycepin as an antitumor drug, particularly in view of the increasingly important role that phosphorylation of NHCP may play in transcription (17–19). As an extension of our previous studies (16), cordycepin and its stereochemically similar congener, xylosyladenine, (Fig. 1) were tested in L1210 cells *in vitro* to ascertain if they affected the phosphorylation of NHCP in intact cells. In addition, this system permitted us to assess the effect of the adenosine deaminase inhibitor, dCF, upon the action of the two nucleoside inhibitors, since several previous investigations have demonstrated that dCF potentiates the inhibitory effects of cordycepin and xylosyladenine upon RNA synthesis (6, 13, 20) and cell growth (20–23).

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[2] The abbreviations used are: dCF, 2′-deoxycoformycin ([R]-3-[2-deoxy-β-D-*erythro*-pentofuranosyl]-3,6,7,8-tetrahydroimidazo [4,5-*d*]diazepin-8-ol); HEPES, N-2-hydroxy-ethylpiperazine-N′-2-ethanesulfonic acid; NHCP, nonhistone chromosomal proteins; SDS, sodium dodecyl sulfate; TCA, trichloroacetic acid.

MATERIALS AND METHODS

Materials. dCF, cordycepin, and xylosyladenine were kindly supplied by Dr. Harry Wood, Jr., National Cancer Institute. ^{32}P as orthophosphoric acid in HCl-free water was obtained from New England Nuclear Corp., Boston, MA. Ampholine am-

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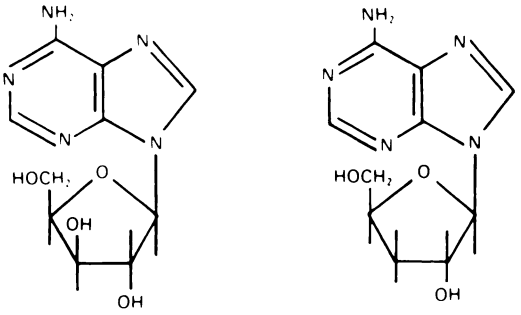

FIG. 1. *Structures of compounds studied*

pholytes (pH 3.5–10 and pH 4–6) were purchased from LKB Instruments, Rockville, MD. Calf thymus mixed histones, DNase I, RNase T_1 and RNase A were obtained from Sigma Chemical Co., St. Louis, MO.

Animals. L1210 cells were inoculated into CDF_1 mice at an inoculum of 10^5 cells/0.1 ml Hank's balanced salt solution. Cells were harvested 6 days after inoculation, washed once in phosphate-free RPMI 1630 medium supplemented with 20 mM HEPES, pH 7.4, and diluted with the same medium to 10^7 cells/ml.

Incubations. Incubations were carried out for 2 hr at 37° in a shaking water bath at 100 rpm and consisted of 5×10^7 cells in 25 ml of RPMI 1630 (Na_2HPO_4-free) medium containing 20 mM HEPES (pH 7.4), 150 μCi of ^{32}P, and either cordycepin or xylosyladenine as indicated. When dCF was used, cells were first preincubated for 10 min before addition of ^{32}P and drugs.

NHCP and histone extraction. After 2 hours of incubation, cells were centrifuged at $200 \times g$ for 10 min at 4°. Nuclei were isolated according to the procedure of Daskal *et al.* (24) using Triton X-100, and washed in a gradient of 0.34 M sucrose-5 mM $MgCl_2$/0.88 M sucrose-5 mM $MgCl_2$ (25). NHCP were then extracted twice with 1 ml of 8 M urea-50 mM phosphate buffer, pH 7.6 (26). The proteins were precipitated with cold 10% TCA and the radioactivity filtered on glass fiber filter discs as previously described (27). Histones were extracted from the residual pellet following urea-extraction with 0.25 N HCl (26).

RNA extraction and digestion. RNA was extracted from the ^{32}P-labeled urea-soluble extract by adding SDS to 0.1% (wt/vol) and mixing with an equal volume of phenol mixture (phenol:m-cresol:H_2O, 7:2:1, vol/vol). RNA was precipitated overnight at −20° with 2% potassium acetate in 95% ethanol. RNA was dissolved in 0.1 ml of 10 mM Tris-HCl (pH 7.2)-10 mM $MgCl_2$-500 mM NaCl containing either 40 μg of DNase I or 40 μg each of RNase A and RNase T_1. Incubations were carried out for 1 hr at 37° and radioactivity was measured after precipitation of RNA on glass fiber filter discs with 10% TCA.

Isoelectric focusing. Extracts of NHCP were precipitated overnight at −20° with 2% (wt/vol) potassium acetate in 95% ethanol and proteins were redissolved in 8 M urea. Nuclear proteins were focused according to the procedure of Gronow and Griffiths (26) as modified by Lea *et al.* (28). Gels contained 10% sucrose to increase the mechanical stability of the gel and 2% (wt/vol) ampholytes of either pH 3.5–10 or pH 4–6. The anode solution was 5% orthophosphoric acid and the cathode solution consisted of 5% 1,2-diaminoethane. Gels containing 100–200 μg of nuclear proteins were focused at 150 V constant voltage for 15 hours. The pH gradient was measured in a duplicate gel run in parallel for every experiment by soaking 2 mm slices in 2 ml of water. Gels were stained with Coomassie blue as described (26) and scanned at 650 nm in a Gilford spectrophotometer equipped with a linear transport. Radioactivity was measured by sectioning the gels into 2 mm slices, adding 10 ml of Aquasol (New England Nuclear, Boston, MA) and counting in a Searle Mark III liquid scintillation system.

RESULTS

Initial studies were performed to determine the distribution of radioactivity in nuclear fractions obtained from L1210 cells incubated for 2 hr with ^{32}P (Table 1). The extraction procedure of Gronow and Griffiths was used (26). Nuclei were extracted with 8 M urea buffer and the urea-insoluble residue was extracted with 0.25 N HCl to yield a soluble fraction containing histones (as corroborated by disc gel electrophoresis) and an insoluble residue containing pri-

TABLE 1

Distribution of ^{32}P in fractions of isolated nuclei following urea extraction

L1210 cells were incubated with ^{32}P and nuclei were isolated and extracted with 8 M urea-50 mM phosphate buffer as described in MATERIALS AND METHODS. The pellet obtained after centrifugation was extracted with 0.25 N HCl and the material obtained after centrifugation is termed "insoluble residue."

Nuclei Fraction	Radioactivity incorporated (dpm)[a]	%
8 M urea extract	3,103,500	73
0.25 N HCl extract	310,890	7
Insoluble residue	863,720	20

[a] Disintegrations per min.

marily DNA. Approximately 73% of the incorporated ^{32}P was present in the urea-soluble fraction while phosphorylated histones accounted for only 7% of the radioactivity, a result in agreement with previous studies (26, 28). Extraction of the urea-soluble fraction by the SDS-phenol procedure yielded RNA which accounted for 35% of the radioactivity in this fraction (results not shown). Greater than 98% of the RNA was hydrolyzed by RNase A and RNase T_1.

Dose-response experiments of the effect of cordycepin and xylosyladenine on the phosphorylation of the urea-soluble fraction from nuclei are presented in Table 2. Cordycepin and xylosyladenine alone inhibited the incorporation of ^{32}P, with the latter inhibitor being 4-fold more potent than cordycepin. Preincubation of L1210 cells with the adenosine deaminase inhibitor, dCF, produced a moderate potentiation of the effect of cordycepin at 0.04 mM, and markedly synergized with all concentrations of xylosyladenine. Similar quantitative effects were produced by cordycepin and xylosyladenine on the phosphorylation of the HCl-soluble histone fraction (results not shown). Varying the incubation time of cells with nucleoside inhibitors from 30 min to 2 hr did not quantitatively affect the extent of inhibition.

Since the urea-soluble extract of nuclei was a heterogeneous fraction consisting of RNA, NHCP, as well as residual histones (26, 28), isoelectric focusing of the urea-soluble fraction was carried out to separate these three components. Densitometric scans of focused urea-soluble extracts using two pH gradients revealed marked differences in the resolution of proteins stained with Coomassie blue (Fig. 2). The major protein band at the extreme alkaline end of the gradient of pH 3.5–10 (Fig. 2A) represented histones, as confirmed with calf thymus mixed histones in a parallel gel (results not shown), while the NHCP represented predominantly those proteins with pI of 4–6. The use of ampholytes of pH 4–6 markedly compressed the distribution of proteins migrating toward the cathode (Fig. 2B).

Xylosyladenine was next tested for its effect on the phosphorylation of the ^{32}P-labeled urea-soluble fraction of nuclei isolated from L1210 cells incubated with the inhibitor. With a gradient of pH 3.5–10, ^{32}P-labeled histones focused at pH 9.5 (fractions 1–3), labeled NHCP at pH 4–6.5 (fractions 10–20) and RNA at pH 2.5 (fractions 30–34) (Fig. 3). Precipitation of the urea-soluble fraction with ethanol followed by incubation with RNase A and RNase T_1 completely eliminated the radioactive peak focusing at pH 2.5 (results not shown). Xy-

TABLE 2

Inhibition by cordycepin and xylosyladenine of the phosphorylation of the urea-soluble fraction of nuclei

L1210 cells were incubated with ^{32}P and either cordycepin or xylosyladenine in the presence or absence of dCF, and nuclei were isolated and extracted as described in MATERIALS AND METHODS. Each value is the mean of 2–4 experiments. Numbers in parentheses represent the range of values.

Treatment	% Inhibition[a]	
	−dCF	+1 × 10^{-6} M dCF
Urea-soluble fraction:		
Cordycepin:		
1.6 mM	56 (48–63)	55 (50–60)
0.4 mM	36 (32–43)	47 (36–50)
0.04 mM	27 (19–38)	46 (45–48)
Xylosyladenine:		
0.4 mM	44 (43–45)	82 (80–84)
0.1 mM	33 (30–36)	60 (56–64)
0.01 mM	18 (16–20)	40 (38–41)

[a] Percent inhibition was determined with respect to appropriate controls without drug treatment or with dCF treatment alone. dCF was without significant effect by itself (0–6% inhibition).

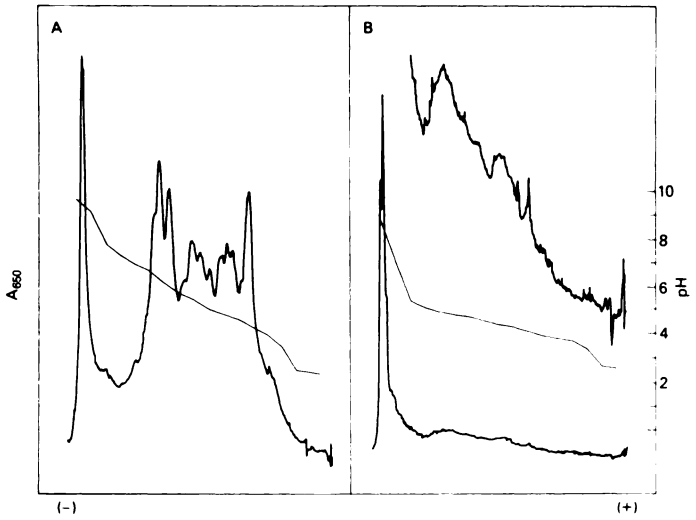

FIG. 2. *Densitometric scans of polyacrylamide gels focused with two pH ranges*

Urea-soluble nuclear proteins were focused in polyacrylamide gels using ampholytes of pH 3.5–10 (A) and pH 4–6 (B). Gels were stained and scanned as described in MATERIALS AND METHODS. The upper tracing in (B) represents a 10-fold expansion in A_{650} for proteins focusing between pH 2.5–6. The *bold* line represents A_{650} and the *fine* line represents the pH gradient.

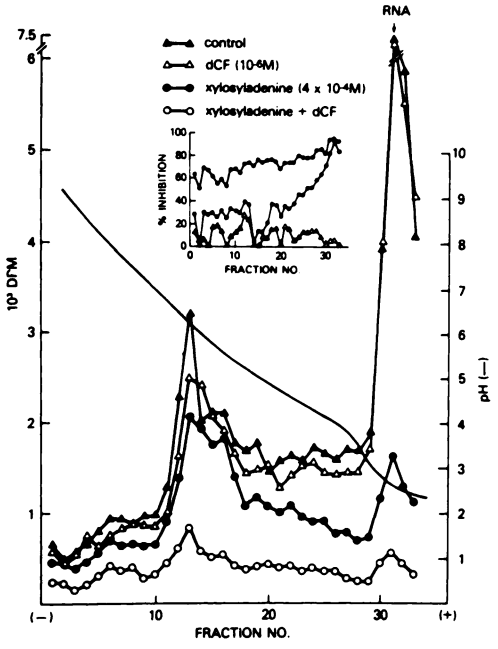

FIG. 3. *Effect of xylosyladenine on ^{32}P-labeled NHCP focused at pH 3.5–10*

^{32}P-labeled urea-soluble nuclear extracts obtained from L1210 cells were incubated with 4×10^{-4} M xylosyladenine in the presence or absence of 1×10^{-6} M dCF and were focused as described in MATERIALS AND METHODS.

losyladenine inhibited the phosphorylation of the NHCP by 20–80% and had a greater effect (50–80% inhibition) on the more acidic NHCP (pH 2.8–4) and RNA synthesis in comparison to NHCP focusing at pH 4.2–7 (inset, Fig. 3). Preincubation of cells with dCF served to accentuate and equalize the inhibitory effect of xylosyladenine across the entire pH gradient.

The use of the gradient of pH 4–6 greatly enhanced the specific radioactivity of NHCP with a pI of 4–6. Increased resolution of the inhibitory effect of xylosyladenine was also observed (inset, Fig. 4) and dCF produced a marked synergistic effect in combination with xylosyladenine on the phosphorylation of the acidic NHCP.

Analogous experiments with cordycepin revealed a major qualitative difference in comparison to xylosyladenine (Fig. 5). While the greatest inhibition of phosphorylation was also found to reside in the more acidic NHCP as was observed with xylosyladenine, dCF did not potentiate the effects of cordycepin to as great an extent (inset, Fig. 5). The enhanced resolution afforded by the use of ampholytes of pH 4–6 (Fig. 6) demonstrated a more consistent potentiation of the inhibitory effects of cor-

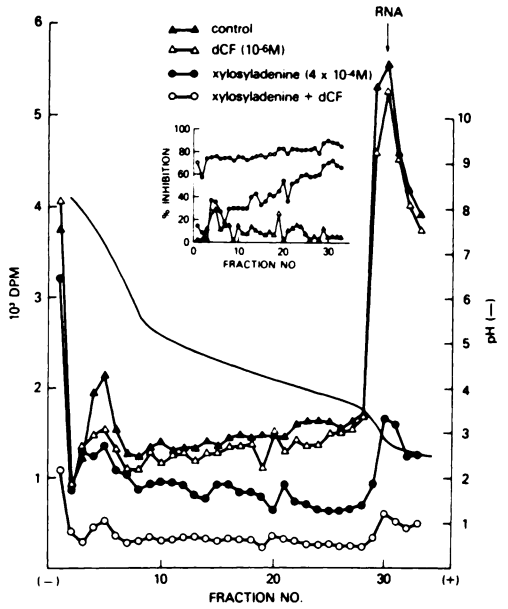

FIG. 4. *Effect of xylosyladenine on ^{32}P-labeled NHCP focused at pH 4-6*

See Fig. 3 for experimental conditions.

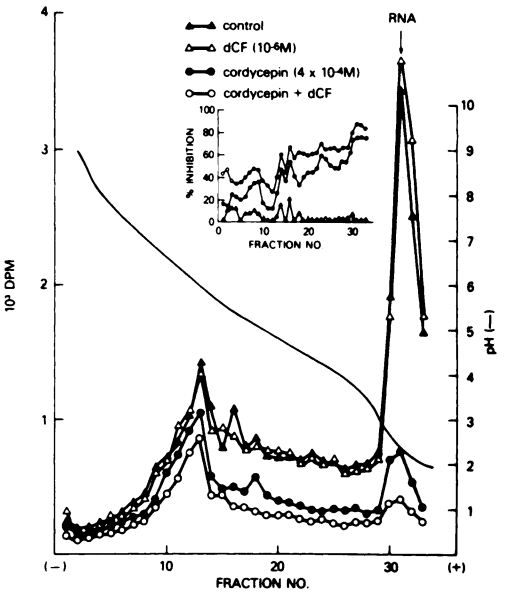

FIG. 5. *Effect of cordycepin on ^{32}P-labeled NHCP focused at pH 3.5-10*

See Fig. 3 for experimental conditions.

dycepin by dCF on the phosphorylation of NHCP (inset, Fig. 6) than that found when the broader pH gradient was used (Fig. 5).

DISCUSSION

Cordycepin (14-16) has previously been characterized as a competitive inhibitor of nuclear protein kinase activities. The diversity of species of nuclear protein kinases in normal and neoplastic tissues (29) has led to the postulation that they may have a regulatory function in gene transcription (17). Indeed, Park *et al.* (30) has demonstrated that the phosphorylation of NHCP can regulate the rate of transcription of histone mRNA *in vitro*. The present study is the first to demonstrate that the nucleoside anticancer drugs, cordycepin and xylosyladenine, inhibit the phosphorylation of NHCP as well as RNA synthesis in intact cells. The differences between the potencies of cordycepin and xylosyladenine may possibly be explained by their relative rates of deamination via adenosine deaminase. Adamson *et al.* (4) has reported that cordycepin and xylosyladenine are deaminated at 52% and 16% the rate of adenosine, respectively. This would imply that lower concentrations of xylosyladenine would be required to produce inhibition of phosphorylation in comparison to cordycepin, by virtue of its limited catabolism. Indeed, this is exactly what is found in L1210 cells for the

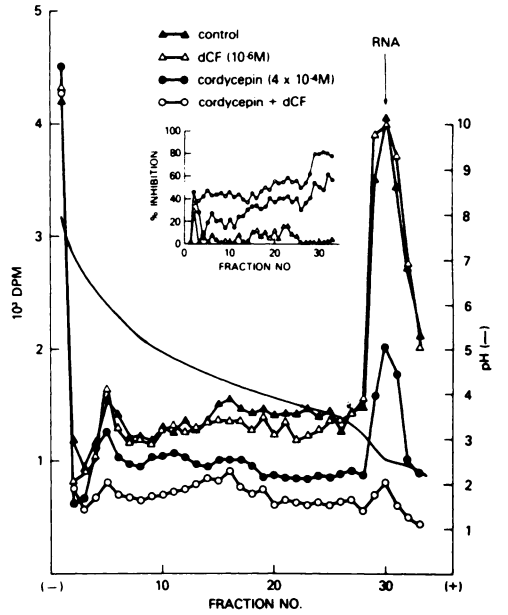

FIG. 6. *Effect of cordycepin on ^{32}P-labeled NHCP focused at pH 4-6*

See FIG. 3 for experimental conditions.

cytotoxic, antitumor, and RNA inhibitory effects of the two drugs (4, 6, 13).

The lesser degree of potentiation of cordycepin as compared with xylosyladenine by dCF may reflect their relative extents of conversion to inhibitory nucleotide metabolites. In ascites tumor cells, 9% of cordycepin is converted to the 5′-triphosphate (31), whereas 18% of xylosyladenine is metabolized to this metabolite (2). Since cordycepin 5′-triphosphate is considerably more potent than cordycepin as an inhibitor of RNA polymerase activities and the phosphorylation of NHCP in isolated nuclei (16), the relative rate of conversion of the nucleoside drugs to inhibitory nucleotides would be expected to reflect their potencies in the presence of an inhibitor of their catabolism such as dCF.

Another important feature of the inhibitory action of cordycepin and xylosyladenine is their greater effect on the NHCP of pI equal to 3–4.5 as compared with NHCP of a higher pI. The activity of the two inhibitors on these acidic species of NHCP correlated closely with their inhibitory action on RNA synthesis. In this regard, acidic NHCP have been shown to exert a greater stimulatory effect on transcription than other NHCP (30). The present evidence that cordycepin and xylosyladenine can affect the phosphorylation of acidic NHCP suggests that this process is closely coupled to transcription and that inhibition of the phosphorylation of NHCP by these drugs is responsible, in part, for their cytotoxic activities.

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