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POTENTIATION BY 2′-DEOXYCOFORMYCIN OF THE INHIBITORY EFFECT OF XYLOSYLADENINE ON NUCLEAR RNA SYNTHESIS IN L1210 CELLS *IN VITRO*

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Abstract—The effect of the adenosine deaminase inhibitor, 2′-deoxycoformycin (dCF), on the inhibitory effect of 9-β-D-xylofuranosyladenine (XA) on nuclear RNA synthesis was examined in L1210 cells *in vitro*. Pretreatment of cells for 15 min with a 100 per cent inhibitory dose (1×10^{-6} M) of dCF resulted in approximately a 3- to 8-fold reduction in the 50 per cent inhibitory dose (ID_{50}) of XA for [^{3}H]uridine and [^{3}H]thymidine incorporation into RNA and DNA respectively. The ID_{50} for XA for RNA synthesis vs DNA synthesis was 5-fold lower in the absence of dCF and 20-fold lower in the presence of dCF, indicating the greater sensitivity of RNA synthesis to this inhibitor. Fractionation of nuclear RNA into rRNA, non-poly-(A)heterogeneous RNA and poly(A)heterogeneous RNA revealed the latter species of RNA to be less sensitive to XA in the absence of dCF; however, in the presence of dCF, all three species of nuclear RNA showed similar sensitivities. Nuclear polyadenylic acid synthesis was among the most sensitive RNA fractions to XA, and was also inhibited to a greater degree by pretreatment of cells with dCF. These results indicate that XA is potentiated markedly by inhibition of adenosine deaminase, and that deamination serves as a major catabolic route for this drug.

Several adenosine analogs have been shown recently to serve as potent anti-cancer drugs when administered in combination with the adenosine deaminase inhibitor, dCF* [1–3]. The effectiveness of this chemotherapeutic approach is based on the ability of dCF to prevent the catabolism of those drugs which serve as substrates for adenosine deaminase [2, 4, 5]. One drug which is particularly sensitive to potentiation by dCF is XA [2]. The median survival time of tumor-bearing animals was increased 2-fold by a combination of XA and dCF, while, *in vitro*, the growth inhibitory effect was potentiated approximately 10-fold [2]. The mechanism for this effect is unknown; however XA has been found to be converted rapidly to the 5′-triphosphate in ascites tumor cells [6] and to inhibit *de novo* purine synthesis at millimolar concentrations [6]. Similar effects were shown to pertain *in vivo* after closely scheduled multiple doses of XA [7]. Since there are structural similarities between XA and another inhibitor of RNA synthesis, cordycepin (3′-deoxyadenosine) [8], it appeared likely that XA might also effectively inhibit nuclear RNA synthesis in an analogous fashion. Moreover, cordycepin is similarly deaminated by adenosine deaminase and potentiated by dCF [1]. Since no experimental evidence existed on the mechanism of action of XA on nuclear RNA biosynthesis, the present study was initiated to explore this question and to determine the relative potentiation by dCF of the effect of XA on different species of nuclear RNA.

*Abbreviations used are: dCF 2′-deoxycoformycin [(R)-3-2-deoxy-β-D-*erythro*-pentofuranosyl)-3,6,7,8-tetrahydroimidazo[5,4-*d*] [1,3]diazepin-8-ol]; hnRNA, heterogenous nuclear RNA; poly(A), polyadenylic acid; XA, 9-β-D-xylofuranosyladenine; formycin A, 7-amino-3-(β-D-ribofuranosyl)-pyrazolo[4,5-*d*]pyrimidine; and nRNA, nuclear RNA.

MATERIALS AND METHODS

Materials. dCF, formycin A and XA were kindly supplied by Dr. Harry B. Wood, Jr., Drug Synthesis and Chemistry Branch, National Cancer Institute, Bethesda, MD. [5,6-^{3}H]uridine (47.9 Ci/m-mole), [methyl-^{3}H]thymidine (64.7 Ci/m-mole) and [2,8-^{3}H] adenosine (31 Ci/m-mole) were obtained from New England Nuclear Corp., Boston, MA.

Animals. L1210 cells were inoculated into CDF_1 mice with an inoculum of 10^5 cells/0.1 ml of Hanks' balanced salt solution. Cells were harvested 6 days after inoculation, washed once in RPMI 1630 medium and diluted with the same medium to 10^7 cells/ml.

Incubations. Incubations were carried out at 37° in a shaking water bath at 100 rev/min and consisted of: 5 ml of RPMI 1630 medium, either 5 μCi [^{3}H] uridine (50 mCi/m-mole) or 5 μCi [^{3}H]thymidine (50 mCi/m-mole), XA and/or 1×10^{-6} M dCF as indicated, and 1×10^7 cells. When RNA was extracted and fractionated, each assay was increased 5-fold with respect to number of cells, volume of medium and amount of isotope.

Isotope incorporation. Incorporation of [^{3}H]uridine and [^{3}H]thymidine into total RNA and DNA, respectively, was measured by cooling the incubation flasks on ice for 15 min and adding ice-cold trichloroacetic acid to a final concentration of 10% (w/v). Precipitates were collected on glass fiber filter discs, washed three times with 5% trichloroacetic acid and twice with 95% ethanol. Discs were dried and counted in 10 ml Aquasol in a Searle Mark III liquid scintillation system.

RNA extraction. After incubation, cells were centrifuged at 200 *g* for 15 min at 4° and washed once with RPMI 1630 medium. Nuclei were prepared according to the procedure of Daskal *et al.* [9] using Triton X-100. Nuclear rRNA, non-poly(A)hnRNA and poly(A)hnRNA were extracted by the sodium dodecyl sulfate–phenol extraction method previously described [8]. With this procedure, rRNA is extracted with equal parts of 0.1% sodium dodecyl sulfate–0.1 mM 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 hnRNA 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 [10]. Poly(A)hnRNA was isolated on poly(U) Sepharose as described [8].

Poly(A) isolation. Poly(A)hnRNA was digested with 20 μg RNase A and 20 units RNase T_1, according to the method of Eiden and Nichols [11]. Incubations were similar to those with [^{3}H]uridine except that 250 μCi [^{3}H]adenosine (31 Ci/m-mole) was used as labeled precursor. The radioactivity coinciding with 4S poly(A) was used to calculate poly(A) synthesis.

Electrophoresis. Disc gel electrophoresis of either the three species of nRNA or poly(A) was carried out by a modification of the method of Rosen *et al.* [12] using 1.75 or 2.5% agarose, respectively, in 6 M urea–0.6 mM citric acid–25 mM Na_2HPO_4 (pH 8.0) with 0.6 mM citric acid–25 mM Na_2HPO_4 (pH 8.0) as the running buffer. Electrophoresis was performed at 4° at 2 mA/gel. Gels were dissolved in 0.3 ml of 60% perchloric acid and mixed with 10 ml Aquasol. Radioactivity was determined in a Searle Mark III liquid scintillation system.

Adenosine deaminase assay. L1210 cells were incubated with dCF for 30 min in Dulbecco's medium fortified with 0.25% glucose. After incubation, the cells were washed three times with ice-cold Dulbecco's medium and centrifuged at 2000 *g*. Adenosine deaminase was assayed in the supernatant fluid prepared from washed L1210 cells homogenized using a glass–Teflon homogenizer in 0.1 M potassium phosphate buffer (pH 7.4) and centrifuged at 100,000 *g* for 1 hr at 4°. Assays contained in a volume of 1.0 ml: 0.3 mM formycin A as substrate, 70 mM potassium phosphate buffer (pH 7.4) and approximately 0.5 mg of supernatant protein. Activity was assayed at 37° at 305 nm in a Gilford recording spectrophotometer. Specific activity was calcuated using an extinction coefficient of 7.0 mM cm^{-1} [13]. The use of formycin A as substrate served to enhance the sensitivity of the assay since deaminase activity could be measured at a wavelength devoid of interference by proteins and nucleic acids in the 100,000 *g* supernatant fluid.

RESULTS

Incubation *in vitro* of L1210 for 15 min with dCF resulted in a dose-dependent inhibition of adenosine deaminase activity assayed in the 100,000 *g* supernatant fluid [14]. The ID_{50} for dCF was 4×10^{-8} M, but 1×10^{-6} M dCF was used in all further experiments to ensure complete inhibition of enzyme. Inhibition was complete within 5 min, but a 15-min pretreatment time was chosen to optimize inhibition. Prior studies have shown that preincubation of adenosine deaminse with dCF is necessary for maximal inhibition [1]. In addition, the ID_{50} for dCF was independent of whether adenosine, formycin A or XA served as substrate.

To obtain a measure of RNA and DNA syntheses, L1210 cells were incubated with [^{3}H]uridine and [^{3}H]thymidine respectively (Fig. 1). The ID_{50} for XA for RNA synthesis was 1×10^{-5} M in the absence of dCF pretreatment and 3×10^{-6} M in its presence

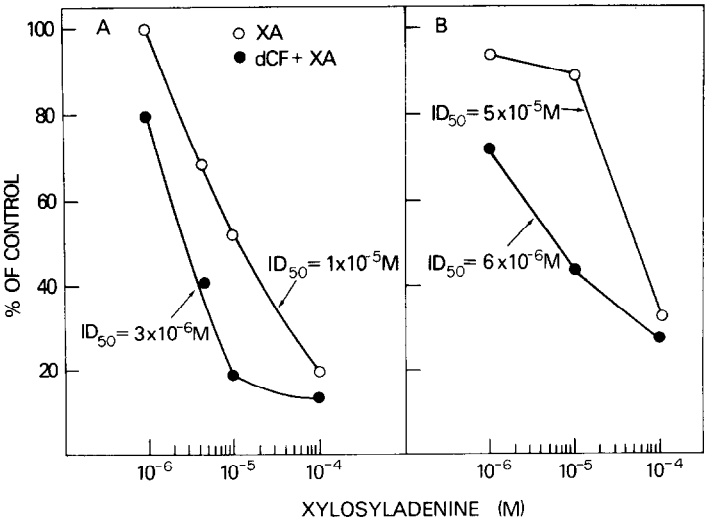

Fig. 1. Dose-response effects of XA on RNA and DNA syntheses. L1210 cells (10^7 cells/flask) were preincubated for 15 min with (●) or without (○) 1×10^{-6} M dCF, and incubated for 30 min with XA. Cells were then labeled for an additional 30 min with [^{3}H]uridine (A) or [^{3}H]thymidine (B). Each result is the mean of duplicate assays.

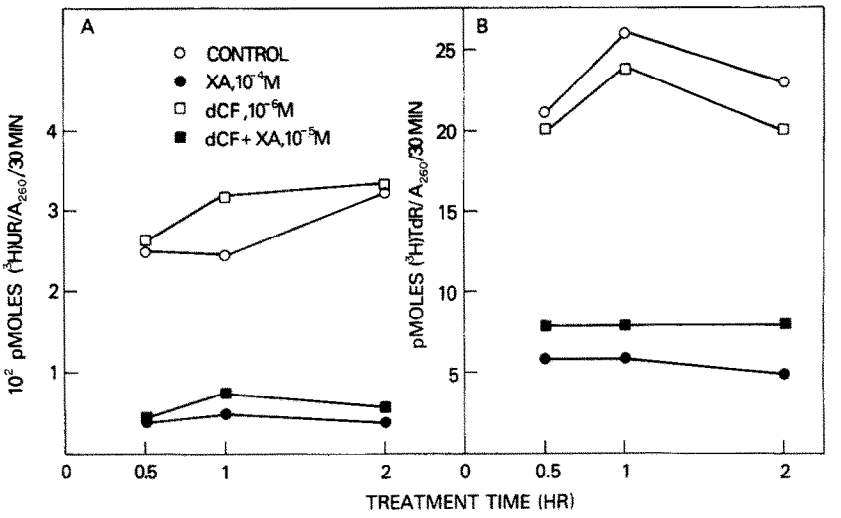

Fig. 2. Time course of inhibition of RNA and DNA syntheses by XA. L1210 cells (10^7 cells/flask) were pre-incubated for 15 min with (□, ■) or without (○, ●) 1×10^{-6} M dCF and then incubated for the indicated times with (●, ■) or without (○, □) XA. Cells were then incubated an additional 30 min with either [^{3}H] uridine (A) or [^{3}H]thymidine (B). Each value is the mean of duplicate determinations.

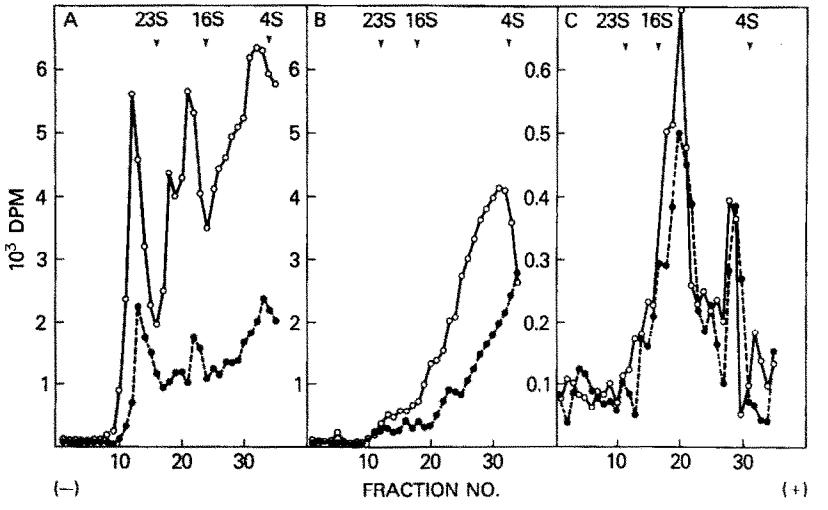

Fig. 3. Agarose-urea gel electrophoresis of nuclear RNA. L1210 cells (5×10^7 cells/flask) were incubated for 30 min in the absence (○) or presence (●) of 5×10^{-5} M XA. RNA was labeled for an additional 30 min with [^{3}H]uridine, and fractionated as described in 'Materials and Methods'. Key: (A) rRNA, (B) non-poly(A)hnRNA, and (C) poly(A)hnRNA.

(Fig. 1A). DNA synthesis was inhibited to a lesser extent than RNA, but the ID_{50} for XA was reduced from 5×10^{-5} to 6×10^{-6} M by pretreatment with dCF (Fig. 1B).

Experiments designed to determine the time course of inhibition by XA of either RNA synthesis (Fig. 2A) or DNA synthesis (Fig. 2B) indicated that inhibition was rapid and essentially complete within 30 min, regardless of whether or not dCF was present.

To test the specificity of action of XA on RNA synthesis, nuclear RNA was fractionated into rRNA, non-poly(A)hnRNA and poly(A)hnRNA. Electrophoresis in agarose-urea gels indicated three distinct species of RNA (Fig. 3). The nuclear RNA extracted at pH 5.1 without urea was ribosomal in character, with 28S and 18S being the predominant species after 30 min of labeling with [^{3}H]uridine (Fig. 3A). XA affected 28S and 18S rRNA to the same extent. Non-poly(A)hnRNA gave a heterodisperse pattern (Fig. 3B), but unlike rat liver hnRNA [10] was of low molecular weight. XA affected the latter species of RNA mainly in the 4S–16S region. In contrast, poly(A)hnRNA was not significantly inhibited except in the region of the prominent 11S species where the labeling was reduced 30 per cent by XA (Fig. 3C).

Dose–response studies of the effect of XA on nuclear rRNA, non-poly(A)hnRNA and poly(A)hnRNA revealed the following order of sensitivity to XA

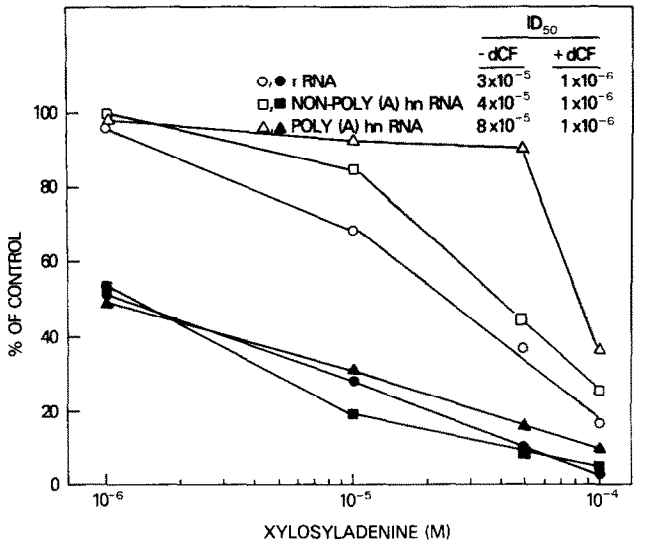

Fig. 4. Dose–response effects of XA on nuclear RNA synthesis L1210 cells (5×10^7 cells/flask) were incubated for 15 min with (●, ■, ▲) or without (○, □, △) 1×10^{-6} M dCF and incubated for 30 min with XA. RNA was labeled for an additional 30 min with [^{3}H]uridine. Each value is the mean of five determinations. Control values (mean ± SE, pmoles [^{3}H]uridine incorporated/A_{260}/30 min) were: rRNA, 660 ± 10; nonpoly(A)-hnRNA, 710 ± 40; and poly(A)hnRNA, 180 ± 20.

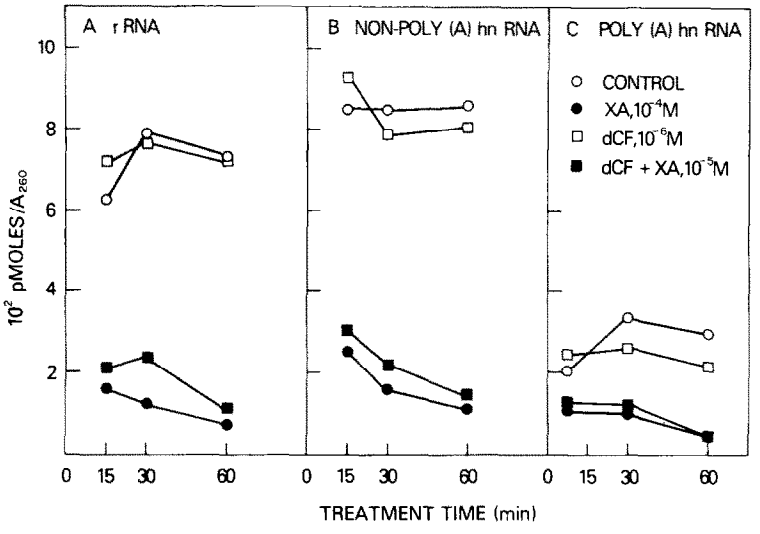

Fig. 5. Time course of inhibition of nuclear RNA synthesis by XA. L1210 cells (5×10^7 cells/flask) were incubated as described in Fig. 2. Refer to Fig. 2 for an explanation of the legend on the picture. Each value represents the mean of three determinations.

inhibition: rRNA > nonpoly(A)hnRNA > poly(A)-hnRNA (Fig. 4). However, in the presence of dCF, equal ID_{50} were obtained.

The kinetics of inhibition of the three species of nuclear RNA revealed maximum inhibition within 15–30 min of XA treatment, regardless of whether cells were pretreated with dCF (Fig. 5).

Analysis of nuclear poly(A) synthesis in cells pulsed for 30 min with [^{3}H]adenosine indicated that this species of RNA was less sensitive than nuclear rRNA, but equally as sensitive as non-poly(A)hnRNA and poly(A)hnRNA (Table 1). Pretreatment of cells with dCF resulted in approximately a 10-fold reduction in the XA concentration required for an equipotent effect.

DISCUSSION

Since XA serves almost as efficiently as does adenosine as a substrate for adenosine deaminase [2, 4, 5], one would expect its activity to be compromised in cells with high levels of this enzyme. Indeed, inhibition of adenosine deaminase by dCF resulted in a high degree of potentiation of inhibition of

Table 1. Effect of xylosyladenine on nuclear poly(A) synthesis*

Treatment	Poly(A) synthesis (pmoles/A_{260}/30 min)	%
Control	0.30 ± 0.02	100
XA, 1×10^{-5} M	0.30	100
1×10^{-4} M	0.20	65
dCF, 1×10^{-6} M	0.27 ± 0.02	100
dCF + XA, $\times 10^{-5}$ M	0.13	48
dCF + XA, 1×10^{-4} M	0.05	19

*Values for control and dCF-treated cells represent the mean ± S. E. of three determinations. Other results represent the mean of two determinations

RNA synthesis by XA. Moreover, the increase in potency of XA produced by pretreatment with dCF was of the same magnitude as that seen for its growth inhibitory and antitumor properties [2]. From the results of precursor incorporation, it appears that RNA synthesis is preferentially affected by XA (Fig. 1). However, in the presence of dCF, this difference becomes less distinct since inhibition of DNA synthesis becomes markedly enhanced, although it is still inhibited to a lesser degree than RNA synthesis.

XA, like its congener, cordycepin, is a potent inhibitor of nuclear RNA synthesis. Its specificity is such that rRNA, as well as nonpoly(A)hnRNA poly(A)hnRNA and poly(A) are inhibited by approximately the same order of magnitude. Unlike cordycepin [15], XA appears to be more inhibitory to non-poly(A)hnRNA.

In consideration of the mechanism of action of XA, inhibition of *de novo* purine synthesis *in vivo* [7] and *in vitro* [6] has been reported. In the latter study, 90 per cent inhibition of incorporation of adenine into total RNA of TA3 cells or of the synthesis of 5-phosphoribosyl-1-pyrophosphate occurred at 1 μmole XA/10^7 cells. The concentration of XA required to inhibit total RNA synthesis by 80–90 per cent in our studies was 2–20 nmoles XA/10^7 cells (Fig. 1). Therefore, it appears that L1210 cells are more sensitive to the nucleoside inhibitor than TA3 cells, although the relationship between inhibition of nRNA synthesis and *de novo* purine synthesis is unknown.

One possible mechanism of action of XA apart from inhibition of *de novo* purine synthesis may be its conversion to the 5′-triphosphate metabolite which, in turn, would inhibit transcription and polyadenylylation. That such a conversion takes place has been established by Ellis and LePage [6]. They found that approximately 17 per cent of the XA was converted to the 5′-triphosphate. This is twice the conversion rate found for cordycepin [16]. Since cordycepin 5′-triphosphate, but not cordycepin itself is a potent inhibitor of RNA polymerases and poly(A) polymerase [17, 18], it is possible that the effects of XA are mediated in a similar manner.

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