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CORDYCEPIN ANALOG OF $(A2'p)_2A$: EVIDENCE THAT IT FUNCTIONS AS A PRODRUG OF CORDYCEPIN

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The effect of the cordycepin trimer analog of $(A2'p)_2A$ on cell growth, cell viability and nucleic acid synthesis was assessed in human colon carcinoma cell line HT-29 in vitro. The cordycepin analog, $(3'dA2'p)_23'dA$ reduced 24 hr cell growth by 50% at 10^{-4}M and decreased cell viability by 98% under these conditions. The cytotoxicity and inhibitory effects of $(3'dA2'p)_23'dA$ on DNA and RNA synthesis were potentiated 5-10-fold by the presence of the adenosine deaminase inhibitor, 2'-deoxycoformycin, and closely resembled those of the parent drug, cordycepin. Chromatographic analyses of the stability of $(3'dA2'p)_2$-3'dA in the tissue culture medium indicated that it was hydrolyzed to the dimer and monomer forms with a half life of approximately 2 hr. No intact $(3'dA2'p)_23'dA$ was detectable intracellularly, but large concentrations of cordycepin nucleotide metabolites were formed, particularly in the presence of 2'-deoxycoformycin.

One means by which interferons are believed to express their antiviral and antiproliferative effects is via the induction of the double-stranded RNA-dependent 2',5'-oligoadenylate synthetase (1-4). The product of this reaction, $ppp(A2'p)_nA$ ($n > 2$), activates an endonuclease, RNase L which in turn degrades cellular and viral RNA (5-9). Although, RNase L, is the only known biological effector of $ppp(A2'p)_nA$, a recent report has described several $ppp(A2'p)_nA$ binding proteins in the nucleus of interferon-treated Ehrlich ascites tumor cells (10) in contrast to a single major acceptor protein (RNase L) in the cytoplasm (11).

Because of the potential importance of the 2',5'-oligoadenylate-RNase L pathway for the expression of the anticellular effects of interferon, several laboratories have synthesized analogs of $ppp(A2'p)_nA$ in an attempt to pharmacologically circumvent the need for interferon induction of the synthetase. One such analog is the cordycepin (3'-deoxyadenosine) congener of $ppp(A2'p)_2A$

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which was first synthesized enzymatically by Doetsch et al. (12,13). The dephosphorylated "core" of the cordycepin analog, i.e. $(3'dA2'p)_2 3'dA$, was effective in inhibiting viral transformation by Epstein-Barr virus but was not cytotoxic to normal lymphocytes (13,14). However, a recent study reported that $(3'dA2'p)_2 3'dA$ was more active than $(A2'p)_2 A$ in inhibiting DNA synthesis in mouse 3T3 fibroblasts (15). In neither case was the stability of $(3'dA2'p)_2 3'dA$ measured intracellularly or in the culture medium to assess whether the cordycepin trimer core was hydrolyzed to the cytotoxic parent nucleoside, cordycepin, or whether the inactivity of the cordycepin analog was due to its rapid hydrolysis and deamination in the medium.

In the present study, we have determined the cytotoxicity, metabolism and inhibitory effects on nucleic acid synthesis of both $(3'dA2'p)_2 3'dA$ and cordycepin in the presence and absence of a potent and highly specific inhibitor of adenosine deaminase, 2'-deoxycoformycin (dCF).

METHODS AND MATERIALS

Materials: [5-^{3}H]Uridine (25 Ci/mmole) and [methyl-^{14}C]thymidine (53 mCi/mmole) were purchased from New England Nuclear (Boston, MA). $(3'dA2'p)_2 3'dA$ and cordycepin were obtained from the Drug Synthesis and Chemistry Branch, NCI. $(3'dA2'p)_2 3'dA$ was also generously provided by Dr. Robert J. Suhadolnik, Temple University School of Medicine.

Cell culture. HT-29 cells were maintained as monolayer cultures under an air atmosphere in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum, 40 mM hepes buffer (pH 7.4) and gentamicin, 50 μg/ml. Cell viability was determined by soft agar cloning and cell growth by cell counting as previously described (4).

DNA and RNA synthesis: Cells were incubated at an initial density of 10^5 cells per 10 ml of medium in 25 cm^2 plastic flasks. Logarithmically (3 day) growing cells were treated for 24 hr with varying concentrations of cordycepin or $(3'dA2'p)_2 3'dA$ in the presence or absence of 1 x 10^{-6}M dCF. Cells were labeled during the last hr of treatment with 0.5 μCi [^{3}H]uridine and 0.5 μCi [^{14}C]thymidine and cold trichloroacetic acid-precipitable radioactivity was measured (4).

HPLC analysis: Cordycepin and $(3'dA2'p)_2 3'dA$ were incubated at 37° in cell-free medium or medium plus serum in a final volume of 0.5 ml. At 2 or 24 hr, 100 μl aliquots were injected into a Brownlee RP-18 column (0.4 x 10 cm) connected in tandem to an RP-18 precolumn (0.4 x 3 cm) and eluted at 3 ml/min with 10% methanol in 0.02 M KH_2PO_4 (pH 3.8).

For analysis of intracellular levels of cordycepin or $(3'dA2'p)_2 3'dA$, cells were grown in 75 cm^2 plastic flasks and incubated with drug for 2 or 24 hr in the presence or absence of 1 x 10^{-6}M dCF. Cells were harvested by trypsinization, washed with cold phosphate-buffered saline and extracted with 100 μl of cold 5% trichloroacetic acid. Extracts were neutralized by shaking with 2 volumes of 0.5 M trioctylamine in trifluorotrichloroethane. Neutralized extracts were assayed directly by HPLC as described above or treated with 4.3

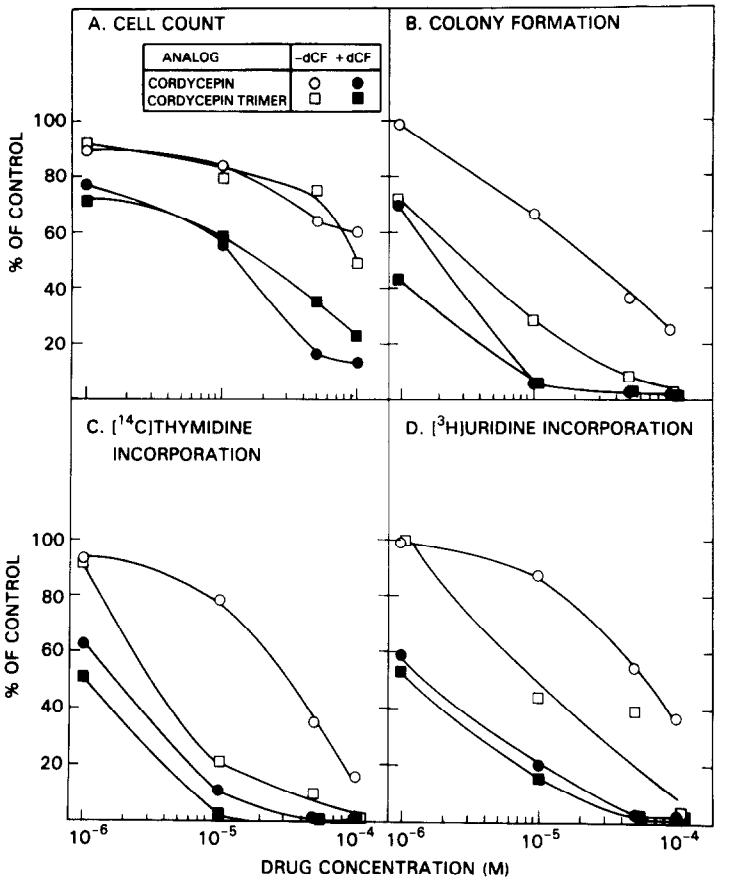

Fig. 1. Cell growth, cell viability and DNA and RNA synthesis following treatment with cordycepin trimer core and cordycepin. Cells were treated for 24 hr with cordycepin trimer core or cordycepin in the presence or absence of 1 x 10^{-6}M dCF. Cell growth was measured by cell count (A), cell viability by colony formation in soft agar (B), and DNA synthesis (C) and RNA synthesis (D) by the incorporation of [^{14}C]thymidine and [^{3}H]uridine, respectively, during the last hr of drug treatment. Results are expressed as a percentage of control values in the absence of all drugs or in the presence of only dCF. Each value is the mean of three determinations.

units of bacterial alkaline phosphatase (43 units/mg) for 1 hr and then assayed by HPLC.

RESULTS

The effects of cordycepin or $(3'dA2'p)_2 3'dA$ on cell growth, cell viability and DNA and RNA synthesis in the presence and absence of dCF are shown in Fig. 1. In the absence of the adenosine deaminase inhibitor, cordycepin and $(3'dA2'p)_2$-3'dA produced equivalent effects on 24 hr cell growth (Fig. 1A). In the presence of 1 x 10^{-6}M dCF, the growth inhibitory effect of cordycepin was potentiated 15-fold while growth inhibition produced by the trimer analog was increased 5-fold. In contrast, cell lethality produced by $(3'dA2'p)_2 3'dA$ in the absence of

dCF was 10-fold greater than cordycepin alone (Fig. 1B), but a similar degree of potentiation by dCF was obtained as for inhibition of cell growth, i.e. a 5-fold increase for $(3'dA2'p)_2 3'dA$ and a 15-fold increase for cordycepin.

Measurements of [^{14}C]thymidine incorporation following 24 hr with the analogs revealed a pattern which mirrored the cell viability results (Fig. 1C). The cordycepin trimer analog was 10-fold more inhibitory than cordycepin in the absence of dCF but was potentiated to a lesser degree in the presence of the adenosine deaminase inhibitor, viz. 3-fold for $(3'dA2'p)_2 3'dA$ and 18-fold for cordycepin. A similar pattern was obtained for the incorporation of [^{3}H]-uridine in the absence of dCF (Fig. 1D); however, both analogs were potentiated 10-fold in the presence of dCF revealing the selective inhibitory activity of cordycepin on RNA synthesis (16).

In order to see whether the cordycepin trimer analog was entering the cells intact or whether it was serving as a prodrug of cordycepin, cells were treated for 24 hr with cordycepin or $(3'dA2'p)_2 3'dA$ in the presence or absence of dCF, and cell extracts were analyzed by HPLC. No free cordycepin or $(3'dA2'p)_2 3'dA$ was present intracellularly after 2 or 24 hr treatment with the analogs (results not shown). However, if the cell extracts were incubated with alkaline phosphatase and then chromatographed, cordycepin was readily detectable under all treatment conditions (Fig. 2). It was also apparent that greater concentrations of cordycepin existed as nucleotide metabolites (prior to phosphatase treatment) in the presence of dCF, particularly from the cordycepin trimer analog (Fig. 2E *vs.* 2C and 2F *vs.* 2D). Approximately 2-fold higher levels of cordycepin metabolites were formed from $(3'dA2'p)_2 3'dA$ *vs.* cordycepin in either the absence or presence of dCF, although the concentrations of metabolites were elevated about 20-fold by dCF in each case. The concentration of cordycepin metabolites formed from $(3'dA2'p)_2 3'dA$ in the presence of dCF was 7.7 nmoles per 10^6 cells *vs.* 4.7 nmoles per 10^6 cells from cordycepin under similar conditions.

Since no intact $(3'dA2'p)_2 3'dA$ was present in cells as early as 2 hr after its addition to the medium (results not shown), it was probable that the

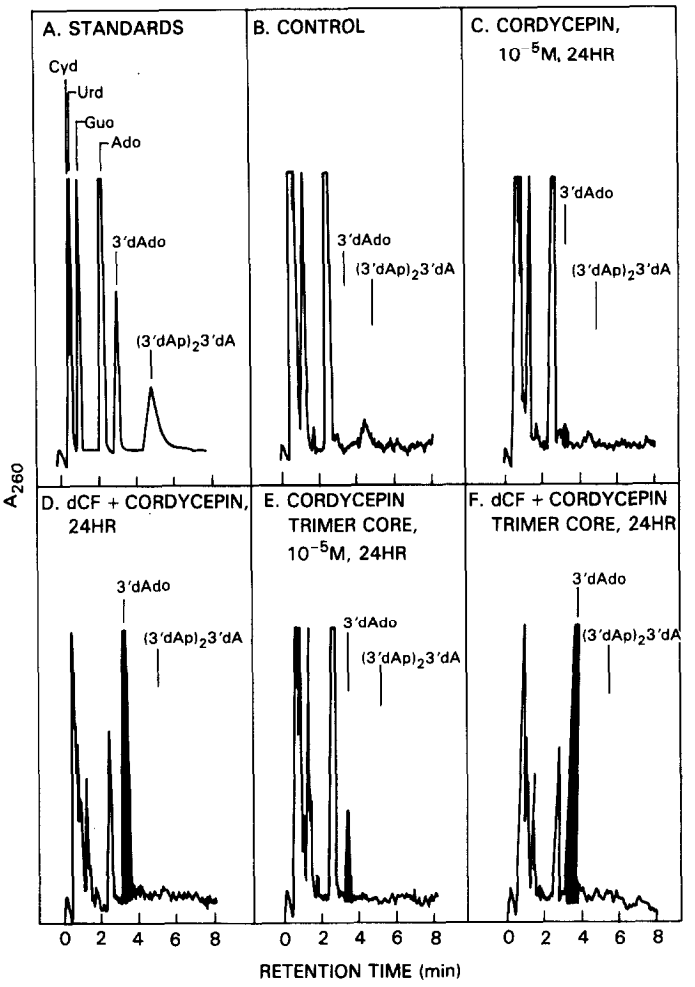

Fig. 2. HPLC analysis of intracellular cordycepin metabolites following treatment of cell extracts with alkaline phosphatase. Cells were incubated as described in Fig. 1. Analyses were conducted as described under "Materials and Methods." Cyd, cytidine; Urd, uridine; Guo, guanosine; Ado, adenosine; 3'dAdo, cordycepin; $(3'dA2'p)_2 3'dA$, cordycepin trimer core.

cordycepin trimer analog was degraded in the tissue culture medium to cordycepin which was then transported into the cell. To ascertain this possibility, $(3'dA2'p)_2 3'dA$ was incubated with dCF in either cell-free medium alone or cell-free medium supplemented with 10% heat-inactivated fetal calf serum, and analyzed by HPLC (Fig. 3). Cordycepin trimer analog was not degraded by incubation for 2 hr (Fig. 3A) or 24 hr (results not shown) at 37° in medium alone. However, in the presence of serum supplemented medium, approximately 50% of $(3'dA2'p)_2 3'dA$ remained after 2 hr, while 35% was present as the dimer analog and 15% as cordycepin (Fig. 3B). Following 24 hr incubation of $(3'dA2'p)_2 3'dA$ in serum

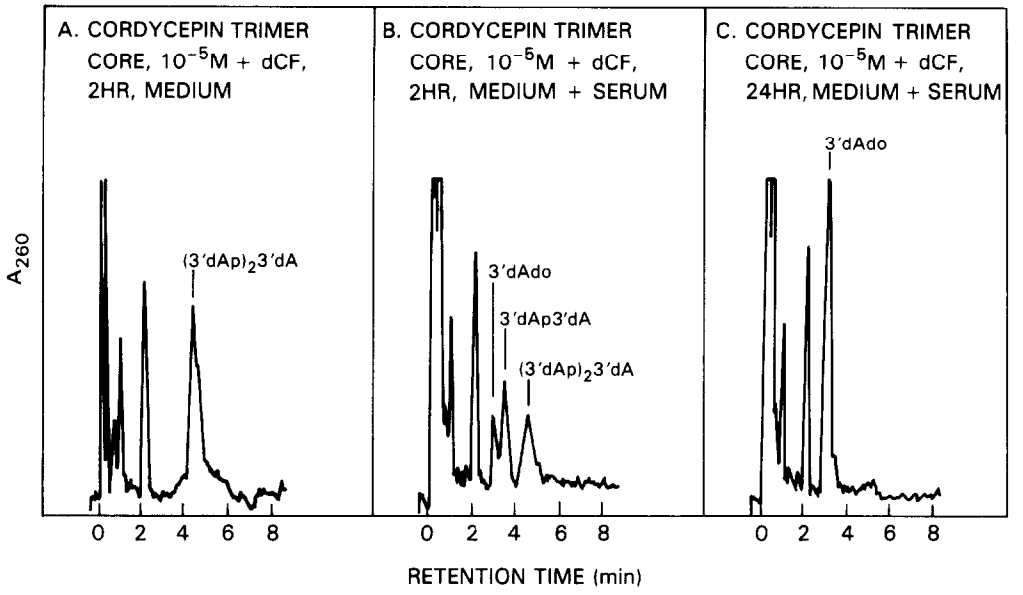

Fig. 3. HPLC analysis of cordycepin trimer core metabolism in cell-free medium and cell-free serum supplemented medium. Cordycepin trimer core was incubated with RPMI 1640 medium in the absence (A) or presence (B,C) of 10% fetal calf serum. HPLC analyses were conducted as described under "Materials and Methods."

supplemented medium, all of the cordycepin trimer analog was present as cordycepin (Fig. 3C).

DISCUSSION

It is evident from the present study that the cordycepin trimer core analog of 2',5'-oligoadenylate serves as a prodrug of cordycepin under tissue culture conditions. HPLC analysis indicated that intact (3'dA2'p)$_2$3'dA was not present intracellularly, but rather that the trimer analog was hydrolyzed to cordycepin in the serum supplemented medium. It is also apparent that (3'dA2'p)$_2$3'dA is progressively hydrolyzed to the dimer and monomer species by the esterases in serum, but we have not as yet assessed whether they are nonspecific or similar to the 2',5'-oligoadenylate phosphodiesterase. These analyses were greatly facilitated by the addition to the medium of the highly specific inhibitor of adenosine deaminase, dCF (17).

Previous results with (3'dA2'p)$_2$3'dA can easily be explained by our findings. The inactivity of the cordycepin trimer analog in lymphocytes (13) may have been the result of low esterase and/or high adenosine deaminase

activities in either the serum used in the medium or in these cells. The cytotoxic activity (as measured by [^{3}H]thymidine incorporation) of (3'dA2'p)$_2$3'dA in 3T3 fibroblasts (15) was likely due to degradation of the trimer analog to cordycepin. Although an equimolar concentration of cordycepin was inactive (15), this result can simply be explained by the 10-fold lesser inhibitory activity of cordycepin vs. (3'dA2'p)$_2$3'dA on DNA synthesis in the absence of dCF (Fig. 1C), a result undoubtedly due to the rapid deamination of the nucleoside in the medium, as well as intracellularly.

Thus, (3'dA2'p)$_2$3'dA is apparently the first oligonucleotide prodrug to be tested for antiproliferative activity. Despite the fact that it is unlikely functioning as an activator of the ppp(A2'p)$_n$A-activated RNase L in situ, it is unique in that it is resistant to deamination by adenosine deaminase and adenylate deaminase (unpublished results) and may serve as a sustained release form of cordycepin to provide a longer maintained antitumor activity in vivo. Further development of this new class of antitumor analog is clearly warranted.

REFERENCES

1. Zilberstein, A., Kimchi, A., Schmidt, A. and Revel, M. (1978) Proc. Natl. Acad. Sci. U.S.A. 75, 4734-4738.
2. Kerr, I.M. and Brown, R.E. (1978) Proc. Natl. Acad. Sci. U.S.A. 75, 256-260.
3. Verhaegen-Lewalle, M., Kuwata, T., Zhang, Z.X., DeClerq, E., Cantell, K. and Content, J. (1982) Virology 117, 425-434.
4. Chapekar, M.S. and Glazer, R.I. (1983) Cancer Res. 43, 2683-2687.
5. Eppstein, D.A. and Samuel, C.E. (1978) Virology 89, 240-251.
6. Wreschner, D.H., James, T.C., Silverman, R.H. and Kerr, I.M. (1981) Nucleic Acids Res. 9, 1571-1581.
7. Hovanessian, A.G., Wood, J., Meurs, E. and Montagnier, L. (1979) Proc. Natl. Acad. Sci. U.S.A. 76, 3261-3265.
8. Slattery, E., Ghosh, N., Samanta, H. and Lengyel, P. (1979) Proc. Natl. Acad. Sci. U.S.A. 76, 4778-4782.
9. Baglioni, C., Minks, M.A. and Maroney, P.A. (1978) Nature 273, 684-686.
10. St. Laurent, G., Yoshie, O., Floyd-Smith, G., Samanta, H., Sehgel, P.B. and Lengyel,P. (1983) Cell 33, 95-102.
11. Floyd-Smith, G., Yoshie, O. and Lengyel, P. (1982) J. Biol. Chem. 257, 8584-8587.
12. Doetsch, P., Wu, J.M., Sawada, Y. and Suhadolnik, R.J. (1981) Nature 291, 355-358.
13. Doetsch, P., Suhadolnik, R.J., Sawada, Y., Mosca, J.D., Flick, M.B., Reichenbach, N.L., Dang, A.Q., Wu, J.M., Charubala, R., Pfleiderer, W. and Henderson, E.E. (1981) Proc. Natl. Acad. Sci. U.S.A. 78, 6699-6703.
14. Henderson, E.E., Doetsch, P.W., Charubala, R., Pfleiderer, W. and Suhadolnik, R.J. (1982) Virology 122, 198-201.
15. Eppstein, D.A., Marsh, Y.V., Schryver, B.B., Larsen, M.A., Barnett, J.W., Verheyden, J.P.H. and Prisbe, E.J. (1982) J. Biol. Chem. 257, 13390-13397.
16. Glazer, R.I., Lott, T.J. and Peale, A.L. (1978) Cancer Res. 38, 2233-2238.
17. Glazer, R.I. (1980) Cancer Chemother. Pharmacol. 4, 227-235.