Antiviral Research, 1 (1981) 383–391
Elsevier Biomedical Press

A NOVEL METABOLIC EFFECT OF THE ADENOSINE DEAMINASE INHIBITOR COFORMYCIN, A POTENTIATOR OF ANTIVIRAL ADENOSINE ANALOGUES

HEINZ C. SCHRÖDER, DAN K. SCHUSTER, RUDOLF K. ZAHN and WERNER E.G. MÜLLER*

Institut für Physiologische Chemie, Universität, Duesbergweg, 6500 Mainz, F.R.G.

(Received 23 June 1981; accepted 4 September 1981)

Coformycin [(*R*)-3-(β-D-erythropentofuranosyl)-3,6,7,8-tetrahydroimidazo [4,5 d]-[1,3] diazepin-8-(*R*)-ol] is an inhibitor of adenosine deaminase and thus increases the antiviral potency of a series of adenosine analogues. The influence of chemically synthesized coformycin triphosphate on RNA-polymerizing enzymes (RNA polymerases from *E. coli* and from calf thymus, poly(A) polymerase) and on DNA-synthesizing enzymes (DNA polymerases and terminal deoxynucleotidyltransferase from calf thymus) was studied. Only the poly(A) polymerase was found to be sensitively inhibited by coformycin triphosphate. The inhibition is non-competitive with respect to ATP (K_i = 2.9 μM). Inhibition was reversed by simple dilution. The inhibitor does not act as chain terminator.

coformycin coformycin triphosphate poly(A) polymerase

INTRODUCTION

The search for a useful antiviral agent has led to the discovery of several adenosine analogues, e.g. 9-β-D-arabinofuranosyladenine [9,25], 3′-deoxyadenosine or cordycepin [23,27] and (*S*)-9-(2,3-dihydroxypropyl)adenine [11] which have been shown to be agents for some infections caused by DNA viruses. Most of these compounds were found to be inactivated by adenosine deaminase (reviews in ref. 19). Therefore, it was one of the most exciting developments in the enhancement of the therapeutic efficacy of the adenine nucleoside analogues to discover the naturally occurring nucleoside inhibitors of adenosine deaminase coformycin [24,29] and 2′-deoxycoformycin [32]. The two inhibitors gained additional importance in the control of the adenosine deaminase activity in precursor lymphocytes of patients with a distinct, severe immunodeficiency [14]. Since the precise additional metabolic effects of the adenosine deaminase inhibitors are not known only a judicious use is recommended [9,30]. Experimental data show that coformycin inhibits phytohemagglutinin-induced proliferative response of precursor T cells by an unknown metabolic effect [2]. In addition, it is now known that coformycin is not degraded by L5178y cells, but is converted to its phosphorylated derivatives [24].

* To whom correspondence should be addressed.

0166-3542/81/0000–0000/$02.75

In the present contribution the influence of the triphosphate derivative of coformycin [(*R*)-3-(β-D-erythropentofuranosyl)-3,6,7,8-tetrahydroimidazo [4,5 d]-[1,3] diazepin-8-(*R*)-ol] on ribonucleic acid-synthesizing enzyme systems, in vitro, was studied. These experiments revealed a strong inhibition of poly(A) polymerase by coformycin triphosphate (CFTP). The chemical synthesis of CFTP was described for the first time in this paper.

MATERIALS AND METHODS

Compounds

The following materials were obtained: [^{3}H]ATP (specific activity, 11 Ci/mmol) from The Radiochemical Centre (Amersham, England); DNA-dependent RNA polymerase (*Escherichia coli*) isolated according to the method of Burgess [5] and unlabeled deoxyribonucleoside and ribonucleoside triphosphates from Boehringer Mannheim (Tutzing, F.R.G.); [^{14}C]dATP (specific activity 52.8 mCi/mmol) from New England Nuclear (Boston, MA, U.S.A.).

Herring sperm DNA, isolated according to Zahn et al. [35], was activated according to Aposhian and Kornberg [1] with the characteristics described earlier [20].

Synthesis of coformycin triphosphate

The monophosphate derivative was synthesized from the unprotected coformycin according to a described procedure [34] which was slightly modified. The dried coformycin (9 mg), dissolved in 150 μl triethylphosphate, reacted with 9 μl phosphoryl chloride at 0°C for 3 h. The reaction product was obtained by ethyl ether precipitation and subsequently added to 40 μl triethylamine (in 4 ml water); yield of the reaction being 52%. The 5′-monophosphate was converted to the 5′-triphosphate [16] as follows: 12.5 mg of coformycin monophosphate was activated with 22 mg carbonyldiimidazole in 750 μl dimethylformamide (4 h at 20°C). Subsequently 140 mg tributylammonium pyrophosphate (in 400 μl dimethylformamide) were added and kept at 20°C for 14 h. After removal of the sediment, the solution was treated with methanol and the reaction products were obtained by ethyl ether precipitation. The triphosphate was purified by DEAE-cellulose chromatography [16]. The resulting triethylammonium salt was converted into the lithium form using Dowex 50 X 4 (Li-form). Yield of this reaction: 45%. Coformycin triphosphate was determined to be chromatographically pure as proven in an ascending system on cellulose plate with the system isobutyric acid : ammonia : H_2O (60 : 1 : 40); the R_f values are: coformycin, 0.71; coformycin monophosphate, 0.37; coformycin triphosphate, 0.18. Coformycin triphosphate had the same UV spectrum as coformycin (λ_{max} 282 nm and λ_{min} 255 nm in water, pH 7). In addition, the triphosphate was hydrolyzed to coformycin (as determined by thin-layer chromatography) by *E. coli* alkaline phosphatase [4].

Enzyme preparations

The DNA-dependent RNA polymerases I, II and III were isolated from mouse liver, and solubilized and fractionated according to Roeder and Rutter [28] as described [26]; the specific activities were as follows: type I, 0.21 nmol AMP incorporated into RNA/10 min per mg protein; type II, 1.39 nmol/10 min per mg; type III, 0.12 nmol/10 min per mg. The DNA-dependent DNA polymerases α and β were prepared from calf thymus and separated by sucrose gradient centrifugation [10,26]; the specific activities achieved were: type α, 11 nmol dAMP incorporated into DNA/60 min per mg protein; type β, 0.8 nmol/60 min per mg. Terminal deoxynucleotidyltransferase was isolated from calf thymus [7]; step VII with a specific activity of 940 nmol dAMP incorporated/60 min per mg. Poly(A) polymerase (Mn^{2+}-dependent) was prepared from calf thymus as described previously [31]. Fraction V, with a specific activity of 1840 nmol AMP incorporated/30 min per mg protein (7 mg/ml), was used for the studies. With the exception of poly(A) polymerase, all the other enzymes used were only partially purified.

Enzyme assays

DNA-dependent RNA polymerase activities of types I, II and III were measured by incorporation of [^{3}H]ATP (specific activity 850 dpm/pmol) into acid-insoluble material using activated DNA as template [26]. Acid-insoluble material from the reactions was spotted on Whatman GF/C glass fiber disks and processed as has been described [8]. DNA-dependent RNA polymerase activity of the *E. coli* enzyme was determined as described [33]. DNA-dependent DNA polymerases α and β assays were carried out using [^{3}H]dATP (specific activity 120 dpm/pmol), activated DNA as template/initiator and the components described earlier in detail [26]. Terminal deoxynucleotidyltransferase reactions were carried out with [^{14}C]dATP (specific activity 130 dpm/nmol) and $d(pA)_3$ as initiator as described [7,22]. Poly(A) polymerase reactions were routinely carried out at 37°C for 30 min in the presence of 200 mM Tris-HCl (pH 8.3), 4 mM 2-mercaptoethanol, 20 μg/ml bovine serum albumin, 0.5 mM $MnCl_2$, 5 μg oligo$(pA)_{10}$, varying amounts of [^{3}H]ATP (14 dpm/pmol) and 20 μl enzyme in a final volume of 100 μl. Products of the reaction were detected as acid-insoluble radioactivity as previously described [8].

For the kinetic experiments to determine the Michaelis constants (K_m), concentrations of the labeled ATP or dATP in the range between 2 and 105 μM were added to the assays. The K_m value and the inhibitor constant (K_i) were calculated according to the method of Lineweaver and Burk [18].

RESULTS

Effect of coformycin triphosphate on the activity of DNA- and RNA-polymerizing enzymes

The data summarized in Table 1 show that RNA polymerase, isolated both from bacteria and from mouse liver, as well as DNA polymerases and terminal deoxynucleotidyltransferase are not affected by coformycin triphosphate. Coformycin triphosphate was added at a concentration of 20 μM to the enzyme assays, consisting of 3 μM radioactively labeled ATP (in the RNA-polymerizing enzyme reaction mixtures) or of 0.8 μM labeled dATP (in the DNA polymerizing enzyme assays). However, the Mn^{2+}-dependent poly(A) polymerase was found to be potently inhibited by coformycin triphosphate.

Inhibition analyses of poly(A) polymerase

To determine the mechanism of inhibition of poly(A) polymerase by coformycin triphosphate, inhibition analyses involving variation of the substrate ATP were conducted (Fig. 1). The inhibition of the enzyme activity was non-competitive with respect to ATP (Michaelis constant K_m = 22.4 μM). The calculated inhibition constant (K_i = 2.9 μM) of the unisubstrate enzyme, poly(A) polymerase, represents the true inhibition constant. This means that an inhibitor concentration of 2.9 μM reduces the maximum velocity (V_{max}) of the reaction to 50% [12].

Published experiments led to the assumption [3] that polyadenylation can be divided into the following two stages: initiation and elongation of the poly(A) chain. To test if only one step was inhibited, time course experiments with coformycin triphosphate were performed (Fig. 2). The results revealed that, firstly, the time course of the poly(A) polymerase reaction is linear also in the presence of coformycin triphosphate and, secondly, the extent of the inhibition was identical irrespective of the addition of coformycin triphosphate to a starting reaction or to a reaction in progress.

To determine approximately the degree of binding of coformycin triphosphate to the enzyme dilution studies were performed (Table 2). The data show that inhibition of poly(A) polymerase activity by coformycin triphosphate is reversed by simple dilution. While in the undiluted experiments the enzyme was inhibited by 63%, the inhibition in the diluted assays was found to be only 15%. This result implies that coformycin triphosphate is a reversible inhibitor for the enzyme. This conclusion is supported by the finding that increasing the amount of poly(A) polymerase from 140 μg to 280 μg has no effect on inhibition by coformycin triphosphate (data not presented).

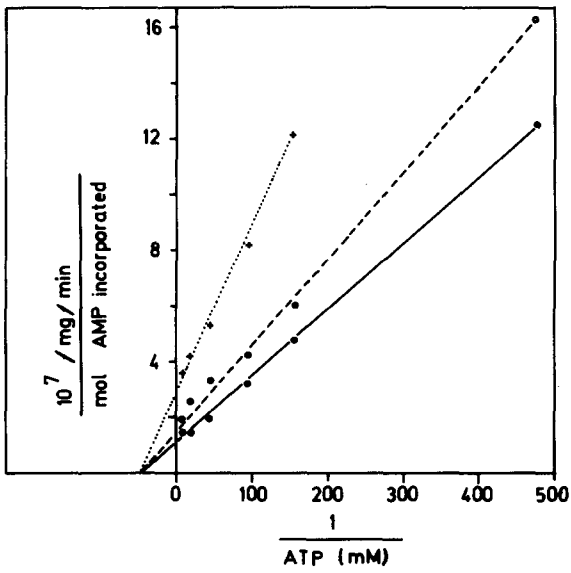
One approach to demonstrate that coformycin monophosphate is not incorporated at the 3′-terminus of the initiator where it may act as a chain terminator is the sequential addition of the inhibitor and the substrate to the reaction. Such an experiment shows (Table 3) that the degree of inhibition of AMP incorporation, caused by coformycin triphosphate, was the same in the case of a preceding incubation of the enzyme with the inhibitor alone, or if the inhibitor was added together with ATP.

TABLE 1

Influence of coformycin triphosphate on DNA- and RNA-synthesizing enzymes

Enzyme	Type of enzyme	Source of the enzyme	K_m (μM)		K_i (μM)
			ATP	dATP	
DNA-dependent RNA polymerase	I	Mouse liver	16.8 ± 2.9		No inhibition
	II	Mouse liver	23.9 ± 3.4		No inhibition
	III	Mouse liver	27.1 ± 3.7		No inhibition
DNA-dependent RNA polymerase		*E. coli*	7.4 ± 0.8		No inhibition
Poly(A) polymerase		Calf thymus	22.4 ± 2.8		2.9 ± 0.2
DNA-dependent DNA polymerase	α	Calf thymus		3.9 ± 0.4	No inhibition
	β	Calf thymus		2.4 ± 0.3	No inhibition
Terminal deoxynucleotidyl-transferase		Calf thymus		3.2 ± 0.3	No inhibition

Incorporation of [^{3}H] dATP or [^{3}H] ATP into DNA or RNA was determined, and kinetic constants were evaluated as described in Methods.



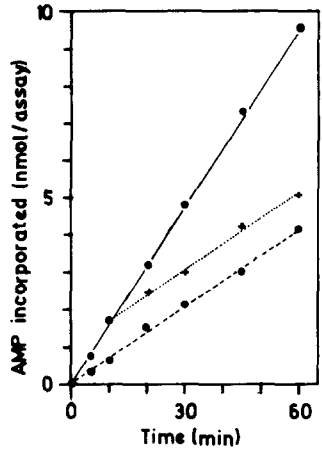


Fig. 1. Non-competitive inhibition of poly(A) polymerase (from calf thymus) by coformycin triphosphate. Lineweaver–Burk [18] plot. K_i of coformycin triphosphate at varying (ATP) concentrations with 0 μM (●), 1.2 μM (○), and 4.5 μM (+) coformycin triphosphate.

Fig. 2. Time course of inhibition of poly(A) polymerase of calf thymus by coformycin triphosphate. The standard reaction mixture, supplemented with 6 μM [^{3}H]ATP, was assayed as described in Materials and Methods (●). Coformycin triphosphate (4.5 μM) was added immediately (○) or 10 min after the start of the reaction (+).

TABLE 2

Effect of dilution on the inhibition of poly(A) polymerase by coformycin triphosphate

Conditions	AMP incorporated (pmol/assay)	
	Undiluted	Diluted
Control (6 μM ATP)	5230	510
+ 4.5 μM CFTP	1940	430
Control (12 μM ATP)	9420	935
+ 4.5 μM CFTP	3660	810

Standard reaction mixtures containing 6 or 12 μM [^{3}H]ATP were assayed as described in Methods. Duplicate standard reaction mixtures (controls) and duplicate mixtures, containing 4.5 μM coformycin triphosphate were incubated for 2 min. Then one of the experimental and one of the control mixtures were diluted 10 times with complete reaction mixtures lacking enzyme and inhibitor. After 30 min (at 37°C), the incorporation rate was determined. CFTP = coformycin triphosphate.

TABLE 3

Effect of a sequential addition of coformycin triphosphate (4.5 μM) and ATP (6 μM) on the inhibition of poly(A) polymerase

Conditions	AMP incorporated (nmol/assay)
Control	5.2
Time 0 min: [^{3}H]ATP + CFTP	1.9
Time 0 min: CFTP Time 30 min: [^{3}H] ATP	2.0

The incubation in the presence of [^{3}H] ATP was performed for 30 min; in the last experiment the enzyme was preincubated (at 37°C) for 30 min in the presence of CFTP and subsequently incubated (at 37°C) for 30 min with [^{3}H] ATP. CFTP = coformycin triphosphate.

DISCUSSION

The detailed analysis of the mode of action of coformycin triphosphate, which is obviously formed in some mammalian cell systems [24], revealed a highly selective inhibition of poly(A) polymerase of calf thymus. Consequently, this antibiotic affects cell metabolism at two different sites: a) in the nucleoside form on the level of adenosine deaminase, inhibiting the deamination of adenosine (or its antiviral analogues 9-β-D-arabinofuranosyladenine, 3′-deoxyadenosine and (*S*)-9-(2,3-dihydroxypropyl)adenine to inosine (or its hypoxanthine analogues) (reviews in ref. 24); and b) in the triphosphate form at the level of poly(A) polymerase, i.e. during the process of mRNA biosynthesis. Coformycin itself has no influence on the activity of DNA and RNA polymerases studied in this paper. The inhibition of poly(A) polymerase was determined to be of the non-competitive type with respect to AMP. In contrast to the binding property of coformycin to adenosine deaminase, which is not reversible [6], coformycin triphosphate associates in a relatively reversible manner with the poly(A) polymerase.

The consequences of the data in this paper for antiviral chemotherapy are two-fold. 1. The inhibitory influence of coformycin is not restricted to a single enzymic reaction (adenosine deamination) which is of benefit for the application of some antiviral adenosine analogues, but must now be extended to an additional metabolic process (polyadenylation). It remains to be determined whether the latter effect, observed in in vitro assays, limits the in vivo application of the antiviral adenosine analogues. 2. Adenosine deaminase inhibitors have been shown to increase the inhibitory effect of adenosine analogues either in a synergistic [15,29] or in an additive fashion [9,13], depending on the biological system used. One key for the understanding of the differences may be found at the level of ribonucleotide reductase. It has been established that this enzyme, isolated from uninfected mammalian cells, is inhibited by dATP and requires ATP for full

activity, while the herpes virus-induced reductase is resistant to inhibition by dATP and is inhibited by ATP [17]. Therefore it could well be that the triphosphates of coformycin and deoxycoformycin affect the host cell- and the virus-induced reductase differentially and in an opposite way.

An interesting task for future studies is the elucidation of the mode of action of coformycin in combination with cordycepin on cell metabolism, especially on poly(A) metabolism. Both compounds are inhibitors of poly(A) polymerase after their intracellular phosphorylation to the triphosphate state. However, while coformycin triphosphate is a non-competitive inhibitor of this enzyme (this study), cordycepin triphosphate is a potent competitive inhibitor of poly(A) polymerase as described earlier by us [21, 23].

ACKNOWLEDGEMENTS

Appreciation is expressed to Professor Hamao Umezawa, Institute of Microbiol Chemistry, Tokyo, for the coformycin provided for this study. The authors express their gratitude to Ms. Renate Steffen for excellent technical assistance. This work was supported by a grant from Fonds der Chemischen Industrie (W.E.G.M.; No. 1191).

REFERENCES

1 Aposhian, H.W. and Kornberg, A. (1962) J. Biol. Chem. 237, 519.
2 Ballet, J.J., Insel, R., Merler, E. and Rosen, F.S. (1976) J. Exp. Med. 143, 1271.
3 Brawerman, G. (1976) Prog. Nucleic Acid Res. Mol. Biol. 17, 117.
4 Breter, H.J., Maidhof, A. and Zahn, R.K. (1978) Biochim. Biophys. Acta 518, 205.
5 Burgess, R.R. (1969) J. Biol. Chem. 244, 6160.
6 Cha, S., Agarwal, R.P. and Parks, R.E. (1975) Biochem. Pharmacol. 24, 84.
7 Chang, L.M.S. and Bollum, F.J. (1971) J. Biol. Chem. 246, 909.
8 Chang, L.M.S. and Bollum, F.J. (1971) J. Biol. Chem. 246, 5835.
9 Cohen, S.S. (1979) In: Nucleoside Analogues, eds. R.T. Walker, E. De Clercq and F. Eckstein (Plenum, New York) p. 225.
10 Coleman, M.S., Hutton, J.J. and Bollum, F.J. (1974) Blood 44, 19.
11 De Clercq, E., Descamps, J., De Somer, P. and Holy, A. (1978) Science 200, 563.
12 Dixon, M. and Webb, E.C. (1979) Enzymes (Longman Ltd., London).
13 Drach, J.C. and Shipman, C. (1977) Ann. N.Y. Acad. Sci. 284, 396.
14 Giblett, E.R., Anderson, J., Cohen, F., Pollara, B. and Meuwissen, H.J. (1972) Lancet 2, 1067.
15 Glazer, R.I., Lott, T.J. and Peale, A.L. (1978) Cancer Res. 38, 2233.
16 Hoard, D.E. and Ott, D.G. (1965) J. Am. Chem. Soc. 87, 1785.
17 Huszar, D. and Bacchetti, S. (1981) J. Virol. 37, 580.
18 Lineweaver, H. and Burk, D. (1934) J. Am. Chem. Soc. 56, 408.
19 Müller, W.E.G. (1979) In: Antiviral Agents and Viral Diseases of Man, eds. G.J. Galasso, T.C. Merigan and R.A. Buchanan (Raven, New York) p. 77.
20 Müller, W.E.G., Maidhof, A., Zahn, R.K. and Shannon, W.M. (1977) Cancer Res. 37, 2282.
21 Müller, W.E.G., Seibert, G., Beyer, R., Breter, H.J., Maidhof, A. and Zahn, R.K. (1977) Cancer Res. 37, 3824.

22 Müller, W.E.G., Zahn, R.K. and Arendes, J. (1978) FEBS Lett. 94, 47.
23 Müller, W.E.G., Zahn, R.K., Arendes, J. and Falke, D. (1979) Virology 98, 200.
24 Müller, W.E.G., Zahn, R.K., Arendes, J., Maidhof, A. and Umezawa, H. (1978) Hoppe-Seylers Z. Physiol. Chem. 359, 1287.
25 Müller, W.E.G., Zahn, R.K., Bittlingmaier, K. and Falke, D. (1977) Ann. N.Y. Acad. Sci. 284, 34.
26 Müller, W.E.G., Zahn, R.K. and Falke, D. (1978) Virology 84, 320.
27 North, T.W. and Cohen, S.S. (1978) Proc. Natl. Acad. Sci. U.S.A. 4684.
28 Roeder, R.G. and Rutter, W.J. (1970) Proc. Natl. Acad. Sci. U.S.A. 65, 675.
29 Sawa, T., Fukagawa, Y., Homma, I., Takeuchi, T. and Umezawa, H. (1967) J. Antibiot. 22A, 227.
30 Suhadolnik, R.J. (1979) Nucleosides as Biological Probes (Wiley, New York).
31 Tsiapalis, C.M., Dorson, J.W. and Bollum, F.J. (1975) J. Biol. Chem. 250, 4486.
32 Woo, P.W.K., Dion, H.W., Lange, S.M., Dahl, L.F. and Durham, L.J. (1974) J. Heterocyc. Chem. 11, 641.
33 Yamazaki, Z.I., Müller, W.E.G. and Zahn, R.K. (1973) Biochim. Biophys. Acta 308, 412.
34 Yoshikawa, M., Kato, T. and Takenishi, T. (1967) Tetrahedron Lett. 50, 5065.
35 Zahn, R.K., Tiesler, E., Kleinschmidt, A.R. and Lang, D. (1962) Biochem. Z. 336, 281.