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INHIBITION OF POLY(2′-FLUORO-2′-DEOXYADENYLIC ACID)-DIRECTED-REVERSE TRANSCRIPTASE ACTIVITY

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Some intercalating and nonintercalating drugs have been tested as inhibitorrs on the DNA synthesis reaction catalyzed by avian myeloblastosis virus (AMV) reverse transcriptase, in the presence of polyriboadenylic acid (poly(rA)) and poly(2′-fluoro-2′-deoxyadenylic acid) (poly(dAfl)) as templates. In both cases, the inhibition was higher with the intercalating drug ethidium bromide than with the nonintercalating analog tetramethyl ethidium bromide. Ethidium bromide inhibited more efficiently the poly(rA)- than the poly(dAfl)-directed reverse transcriptase reaction; in the latter case, the inhibition was non-competitive in relation to TTP. On the other hand, the reaction catalyzed in the presence of the 2′-fluorinated polynucleotide as template was inhibited to a higher extent by other nonintercalating drugs, berenil, netropsin, and distamycin. The inhibitions of both reactions by dideoxy TTP, novobiocin and HPA-23 are also discussed.

KEY WORDS: Reverse transcriptase, poly(dAfl), inhibition

INTRODUCTION

Reverse transcriptase [E.C 2.7.7.7] can utilize polyribonucleotides and polydeoxyribonucleotides as templates, with complementary deoxyribo-oligomers as primers.[1–3] Synthetic polynucleotide analogs have been tested in the reactions catalyzed by reverse transcriptase resulting in different effects. In the presence of Mn^{2+} as divalent cation, poly(2′-O-methylcytidylic acid) (poly(dAfl)) can be recognized as a template by retroviral DNA polymerases and it use as a specific template for RNA-dependent DNA polymerase has been suggested.[4] Poly(dAfl) was also an efficient template in the reactions catalyzed by AMV reverse transcriptase.[5,6] On the other hand, 2′O-alkyl derivatives of polyadenylic acid, and poly(2′-O-ethylcytidylic acid) are inhibitors of murine leukemia virus[7] and AMV[8] reverse transcriptases, respectively.

Substitution by a fluorine atom in the 2′-position of ribose confers greater stability to the polynucleotide duplexes, as observed through physicochemical properties.[9] This fact was supported by the findings that poly(dAfl)- and not polyl(rA)- nor

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polydeoxyadenylic acid-directed reverse transcriptase reaction was less affected in the presence of polycytidylic acid derivatives.[6]

In the present paper, we describe the effect of intercalating and nonintercalating drugs such as ethidium bromide, tetramethyl ethidium bromide, berenil, netropsin and distamycin on the activities of reverse transcriptase with poly(rA) or poly(dAfl) as templates. The effects of dideoxy TTP, novobiocin and HPA–23 have also been studied.

MATERIALS AND METHODS

Chemicals

AMV reverse transcriptase was purchased from Boehringer Mannheim. Poly(rA), TTP, dideoxy TTP, dithiothreitol, bovine serum albumin, oligo(dT)$_{12-18}$, novobiocin, ethidium bromide, and berenil were obtained from Sigma Chemical Company (St. Louis, MO). Poly(dAfl), distamycin A and netropsin were gifts from Dr. W. Guschlbauer (Service de Biochimie, Saclay, France), and tetramethyl ethidium bromide and HPA–23 from Dr. S. Litvak (Institute de Biochimie Cellulaire, Bordeaux, France). Tritium-labeled TTP was obtained from New England Nuclear (Du Pont). All other chemical used in ths work were of the highest purity available.

Preparation of template-primers

The annealing of the template-primer duplexes was performed as previously described.[10] The template : primer ratio was kept at 5:1 in order to avoid multi-strand formation or aggregates.[11]

Reverse transcriptase assay

The incubation mixture in a final volume of 0.05 ml contained: 50 mM Tris-HCl (pH 7.9), 2 mM dithiothreitol, 200 μg/ml bovine serum albumin, 40 mM KCl, 6 mM magnesium acetate or 0.2 mM $MnCl_2$, 0.24 A_{260} units/ml template-primer, 10 μM [^{3}H] TTP (1000 cpm/pmol) and 2 units of reverse transcriptase. Incubation was carried out at 37°C for 30 min. The reaction was stopped by the addition of 1 ml of ice-cold 10% trichloroacetic acid solution. The precipitates were filtered through Millipore nitrocellulose membranes, washed with ice-cold 2% trichloroacetic acid solution, containing 0.1 M sodium pyrophosphate, dried and counted in a PPO/POPOP/toluene scintillation mixture.

RESULTS AND DISCUSSION

It has been observed that poly(dAfl) could replace poly(rA) as template for the DNA synthesis reaction catalyzed by reverse transcriptase, to about 50% and 100%, in the presence of Mg^{2+} and Mn^{2+}, respectively.[6] In contrast to poly(rA)- and poly(dA)-, the poly(dAfl)- directed reverse transcriptase activity was not affected by polycytidylic acid analogs.[6]

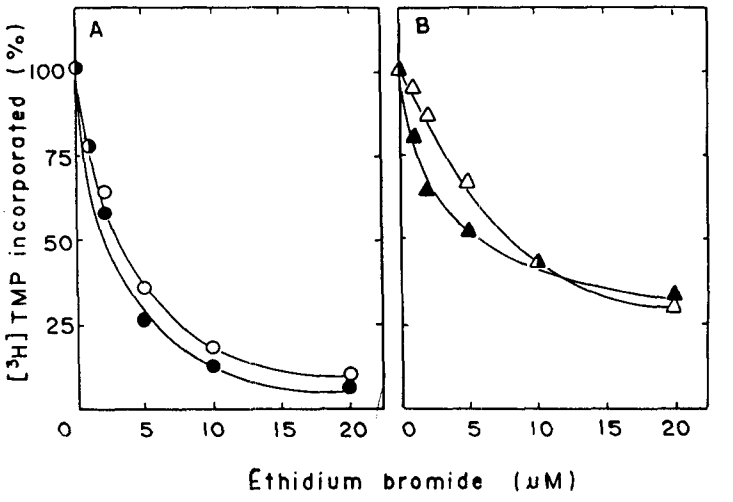

FIGURE 1 Inhibition of reverse transcriptase activity by ethidium bromide. The assay conditions were as described in Materials and Methods, in the presence of poly(rA) (A) or poly(dAfl) (B) as template, and Mg^{2+} (○, △) or Mn^{2+} (●, ▲) as divalent cation.

In this work, we have analyzed the effect of other compounds on the poly(dAfl)-dependent reverse transcriptase reaction.

Effect of intercalating and nonintercalating drugs

The inhibitory effect of ethidium bromide depends on the different synthetic polynucleotides used as template. The inhibition of tumor virus DNA polymerases by ethidium bromide was more pronounced when polymers containing A–T bases were used as template-primers, as compared to G–C containing templates.[12]

As shown in Figure 1, the intercalating drug ethidium bromide inhibited reverse transcriptase activity in the presence of poly(rA) (A) and poly(dAfl) (B) as templates, independently of the divalent cation Mg^{2+} and Mn^{2+}.

In order to define the type of inhibition of the poly(dAfl)-directed reverse transcriptase reaction by ethidium bromide, we have studied the effect of the drugs at different concentrations of TTP. Figure 2 shows a noncompetitive inhibition by ethidium bromide in relation to TTP with a K_i value of 5 μM. Analogous results were observed in the presence of Mn^{2+} as divalent cation (not shown). The inhibition by ethidium bromide was noncompetitive with regard to poly(rA) for AMV reverse transcriptase.[13,14]

The effect of the nonintecalating drug tetramethyl ethidium bromide was studied on the reverse transcriptase activity with poly(rA) and poly(dAfl) as template. As shown in Figure 3, a lower inhibitory effect by tetramethyl ethidium bromide was observed when compared with the intercalating drug ethidium bromide. Tetramethyl ethidium bromide has been reported to be a noncompetitive inhibitor in relation

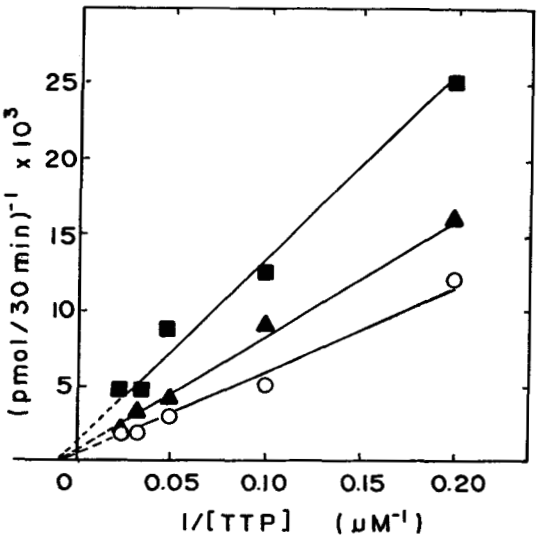

FIGURE 2 Lineweaver-Burk plots for kinetics of inhibition of reverse transcriptase with ethidium bromide. The assay conditions were as described in Materials and Methods, with varying concentration of TTP incubated in the absence (○) and in the presence of 5 μM (▲) and 10 μM (■) of ethidium bromide.

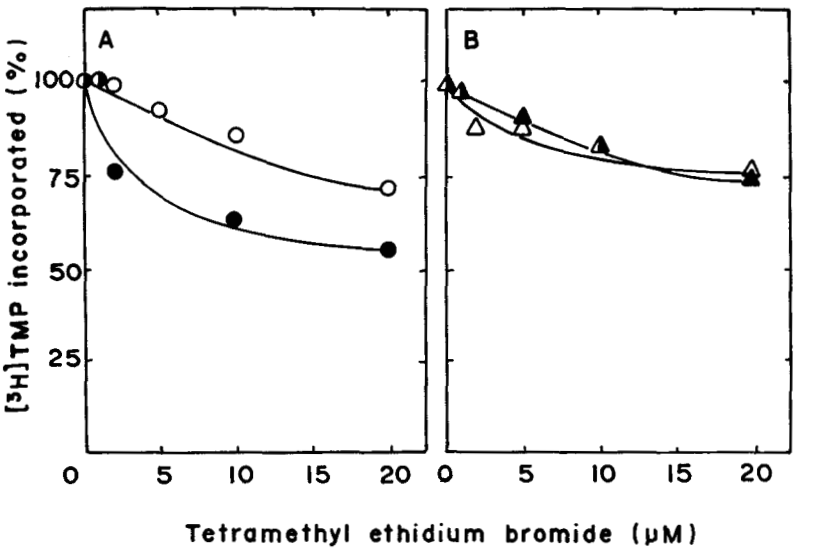

FIGURE 3 Inhibition of reverse transcriptase activity by tetramethyl ethidium bromide. The assay conditions were as described in Materials and Methods, in the presence of poly(rA) (A) or poly(dAfl) (B) as template, and Mg^{2+} or (○, △) or Mn^{2+} (●, ▲) as divalent cation.

TABLE 1
Effect of nonintercalating drugs on the reverse transcriptase activity

Addition	Conc. (M)	DNA synthesis(%)	
		Poly(rA)-oligo(dT)	Poly(dAfl)-oligo(dT)
None		100 (147)	100 (60)
Berenil	2.0×10^{-6}	95	68
Berenil	10.0×10^{-6}	82	30
Netropsin	1.7×10^{-3}	46	18
Netropsin	3.4×10^{-3}	17	9
Distamycin	0.7×10^{-3}	49	6
Distamycin	1.4×10^{-3}	15	4

Picomoles of radioactive precursor, [^{3}H] TMP, incorporated are given in brackets.

to TTP in the poly(rA)-dependent reverse transcriptase reaction.[13] Ethidium bromide and tetramethyl ethidium bromide could be affecting a site other than the TTP and the template-primer sites in the reverse transcriptase.

The effect of other nonintercalating drugs can be observed in Table 1. Berenil, netropsin and distamycin A more efficiently inhibited the poly(dAfl)- than the poly(rA)-dependent reverse transcriptase reaction. Spectrophotometric and hydrodynamic binding studies of berenil have indicated a selective affinity for dA-dT pairs in DNA, but it can also bind to RNA, synthetic homopolymers and heat-denatured DNA.[15,16] The antibiotics netropsin and distamycin A are well known for their antiviral effects; they form highly ordered complexes with DNA and show a strong dependence on the A-T content.[17–19] Netropsin and distamycin can be used as specific conformational probes due to their exclusive affinity for the B-type double helix in solution.[17,20,21] Circular dichroism binding studies with netropsin and distamycin revealed that the poly(dAfl)-poly(dT) could adopt a B-like conformation contrary to poly(rA)-poly(dT).[22,23] The ability of a fluoropolymer to undergo the A to B transition upon netropsin and distamycin binding appears to be possible because of the smaller size of the fluorine.[22,23] These nonintercalating drugs all bind in the minor groove of the DNA double helix.[18,24] Our present results shown in Table 1 which indicate a higher inhibition of the poly(dAfl)-directed reverse transcriptase reaction strongly support the above mentioned findings.

Effect of dideoxy TTP, novobiocin and HPA–23

Other compounds have been tested as inhibitors on the reactions catalyzed by reverse transcriptase with poly(rA) and poly(dAfl) as template. As shown in Table 2, dideoxy TTP, novobiocin and HPA–23 inhibited the reverse transcriptase activity independently of the synthetic template used, in contrast to the results obtained with nonintercalating drugs where poly(rA)-dependent reaction was less affected

TABLE 2
Effect of dideoxy TTP, novobiocin and HPA-23 on the reverse transcriptase activity

Addition	Conc. (M)	DNA synthesis (%)	
		Poly(rA)-oligo(dT)	Poly(dAfl)-oligo(dT)
None		100 (132)	100 (55)
Dideoxy TTP	1.0×10^{-6}	26	9
Didoexy TTP	5.0×10^{-6}	6	3
Novobiocin	1.0×10^{-3}	75	62
Novobiocin	2.0×10^{-3}	39	30
HPA-23	7.3×10^{-7}	77	88
HPA-23	1.4×10^{-6}	68	75

The conditions were the same as described in Materials and Methods, except for dideoxy TTP where 1 μM [^{3}H]TTP (8,000 cpm/pmol) was used. Picomoles of radioactive precursor incorporated are given in brackets (in the case of dideoxy TTP 10.5 pmoles and 2.1 pmoles were the values corresponding to 100% of incorporation with poly(rA) and po1y(dAfl), respectively).

by the inhibitors when compared with poly(dAfl) (Table 1). Dideoxy TTP and other terminating derivatives modified at the 3'-position have been shown to be competitive inhibitors in relation to TTP for Rauscher murine leukemia virus reverse transcriptase.[25] Dideoxy TTP inhibited also the DNA synthesis reaction catalyzed by HIV reverse transcriptase and by wheat germ DNA polymerase A, a γ-like enzyme.[26] Novobiocin has been previously shown to inhibit the DNA polymerase α and AMV reverse transcriptase activities; however, in the latter case, an irreversible inactivation of the viral enzyme could be occurring.[27] The drug HPA-23 (ammonium-21-tungsto-9-antimoniate) has been used as an inhibitor of RNA-dependent DNA polymerase of AIDS retroviruses and as competitive inhibitor relative to poly(rA)-oligo(dT).[28] We have previously observed that HPA-23 was a more potent inhibitor of cellular DNA polymerases when compared with the inhibition of reverse transcriptase.[10,29]

In this paper, we have shown that the presence of fluorine in the sugar moiety of polyadenylic acid did not confer stability when this compound was used as template in the AMV reverse transcriptase reaction in the presence of intercalating, nonintercalating and other drugs. When working with polynucleotides analogs as inhibitors of reverse transcriptase, we have observed a stabilizing effect of 2'-fluoropolymers as template.[30]

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References

1. Sarngadharan, M.G., Robert-Guroff, M. and Gallo, R.C. (1978) *Biochim. Biophys. Acta.*, **516**, 419–487.
2. Verma, M. (1981) *The Enzymes* (Boyer, P.D., ed), vol.14 A, pp. 87–103. New York; Academic Press.
3. Gerard, G.F. (1983) *Enzymes of Nucleic Acid Synthetic and Modification* (Jacob, S.T., ed.), vol. I, pp. 1–38. Boca Raton, Florida; CRC Press.
4. Gerard, G.F., Rottman, F. and Green, M. (1974) *Biochemistry*,**13**, 1632–1641.
5. De Clercq, E., Fukui, T., Kakiuchi, N. and Ikehara, M. (1979) *Cancer Lett.*, **7**, 23–37.
6. Aoyama, H., Sarih-Cottin, L., Tarrago-Litvak, L., Kakiuchi, N., Litvak, S. and Guschlbauer, W. (1985) *Biochim. Biophys. Acta.*, **824**, 225–232.
7. Arya, S.K. Carter, W.A., Alderfer, J.L. and Ts'o, P.O.P. (1974) *Biochem. Biophys. Res. Commun.*, **59**, 608–615.
8. Mikke, R., Kielanowska, M., Shugar, D. and Zmudzka, B. (1976) *Nucleic Acids Res.*, **3**, 1603–1611.
9. Kakiuchi, M., Marck, C., Rosseau, N., Leng, M., De Clerqc, E. and Guschlbauer, W. (1982) *J. Biol. Chem.*, **257**, 1924–1928.
10. Aoyama, H., Jucá, M.B., Castroviejo, M. and Litvak, S. (1993) *Plant Mol. Biol.*, **23**, 1055–1060.
11. Tambly, T.M. and Wells, R.D. (1975) *Biochemistry*, **14**, 1412–1425.
12. Fridlender, B. and Weissbach, A. (1971) *Proc. Nat. Acad Sci. USA*, **68**, 3116–3119.
13. Sarih, L., Hevia-Campos, E., Tharaud, D. and Litvak, S. (1980) *FEBS Lett.*, **122**, 100–104.
14. Benzair, A.B., Rhodes-Feuillete, A., Emanoil–Ravier, R. and Periez, J. (1985) *Ann. Inst. Pasteur/ Virology*, **136E**, 91–96.
15. Newton, B.A. (1967) *Biochem. J.*, **105**, 50P–51P.
16. Zimmer, C. and Wahnert, U. (1986) *Progr. Biophys. Molec. Biol.*, **47**, 31–112.
17. Wartell, R.M., Larson, J.E. and Wells, R.D. (1974) *J. Biol. Chem.*, **249**, 6719–6731.
18. Braithwaite, A.W. and Baguley, B.C. (1980) *Biochemistry*, **19**, 329–348.
19. Waring, M.J. (1981) *Ann. Rev. Biochem.*, **50**, 159–192.
20. Zimmer, C., Reinert, K.E., Luck, G., Wahnnert, U., Lober, G. and Thrum, H. (1971) *J. Mol. Biol.*, **72**, 329–348.
21. Zimmer, C. (1975) *Progr. Nucleic. Acids. Res. Mol. Biol.*, **15**, 285–318.
22. Zimmer, C., Kakiuchi, N. and Guschlbauer, W. (1982) *Nucleic Acids Res.*, **10**, 1721–1732.
23. Marck, C., Kakiuchi, N. and Guschlbauer, W. (1982) *Nucleic Acids Res.*, **10**, 6149–6161.
24. Berman, H.M., Neidle, S., Zimmer, C. and Thrum, H. (1979) *Biochim. Biophys. Acta.*, **561**, 124–131.
25. Ono, K., Ogasawara, M., Iwata, Y., Nakane, H., Fujii, K. and Saneyoshi, M. (1986) *Biochem. Biophys. Res. Commun.*, **140**, 498–507.
26. Laquel, P., Sallafranque-Andreola, M., Tarrago-Litvak, L., Castroviejo, M. and Litvak, S. (1990) *Biochim. Biophys. Acta.*, **1048**, 139–148.
27. Sarih, L., Garret, M., Aoyama, H., Graves, P.V., Tarrago-Litvak, L. and Litvak, S. (1983) *Biochem. Int.*, **7**, 79–88.
28. Dormont, D., Spire, B., Barré-Sinoussi, F., Montagnier, L. and Chermann, J.C. (1985) *Ann. Inst. Pasteur/Virology*, **136E**, 75–83.
29. Aoyama, H., Jucá, M.B., Zourgui, L. and Litvak, S. (1991) *Arq. Biol. Tecnol.*, **34**, 459–466.
30. Jucá, M.B. and Aoyama, H. (1994) *J. Enz. Inhib.*, in press.