ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, May 1986, p. 867–872
0066-4804/86/050867-06$02.00/0
Copyright © 1986, American Society for Microbiology
Vol. 29, No. 5

PP_i Analogs as Inhibitors of Human T-Lymphotropic Virus Type III Reverse Transcriptase

LOTTA VRANG[1]† AND BO ÖBERG[2]*

Department of Virology, Karolinska Institute, SBL, S-105 21 Stockholm,[1] and Department of Antiviral Chemotherapy, Research and Development Laboratories, Astra Läkemedel AB, S-151 85 Södertälje,[2] Sweden

Received 16 December 1985/Accepted 7 February 1986

Twenty-six PP_i analogs were tested for inhibitory effects on human T-lymphotropic virus type III reverse transcriptase. The structural requirements for inhibition and mechanism of action of the most active inhibitors have been investigated. Foscarnet (phosphonoformic acid) was the most potent inhibitor of human T-lymphotropic virus type III reverse transcriptase with 50% inhibition at 0.5 μM. The mechanism was a noncompetitive type of inhibition of a (riboadenylic acid)$_n$ · (deoxythymidylic acid)$_{12\text{-}18}$ [(rA)$_n$(dT)$_{12\text{-}18}$]-directed transcription at varied dTTP concentration. At constant substrate (dTTP) concentration and varied amounts of template, (rA)$_n$(dT)$_{12\text{-}18}$, the inhibitory action of foscarnet was of an uncompetitive type. The same pattern of inhibition was seen when the less active inhibitor carbonyldiphosphonate was studied under identical conditions. The structural requirements for inhibition of human T-lymphotropic virus type III reverse transcriptase by PP_i analogs were similar to those shown by other reverse transcriptases.

The importance of finding inhibitors of reverse transcriptase has dramatically increased since the discovery of human T-lymphotropic virus type III (HTLV-III) or lymphadenopathy-associated virus as the etiological agent causing acquired immune deficiency syndrome (8, 10). Some inhibitors of HTLV-III reverse transcriptase have been reported such as ammonium-21-tungsto-9-antimonate (2), suramin (7), foscarnet (11, 12) and 3′-azidothymidine (M. H. St. Clair, K. Weinhold, C. A. Richards, D. W. Barry, and P. A. Furman, Program Abstr. 25th Intersci. Conf. Antimicrob. Agents Chemother., abstr. no. 439, 1985). In the case of suramin the inhibition seems to be unspecific and due to a general protein binding (1).

The inhibitors of HTLV-III reverse transcriptase foscarnet, ammonium-21-tungsto-9-antimonate, and 3′-azidothymidine also have been inhibitory to HTLV-III multiplication in cell cultures. The clinical evaluation of these inhibitors is ongoing, but no conclusive results are available yet.

The severity of the acquired immune deficiency syndrome situation makes it necessary to search for and evaluate inhibitors of HTLV-III and potentially useful inhibitors are those acting on the reverse transcriptase. In this report we describe the structure-activity relationship for PP_i analogs tested for activity against HTLV-III reverse transcriptase. We have also investigated the mechanism of action for two of the most active inhibitors.

MATERIALS AND METHODS

Chemicals. The deoxynucleotide triphosphates were from Sigma Chemical Co., St. Louis, Mo. [^{3}H]dTTP and [^{3}H]GTP were purchased from New England Nuclear Corp., Boston, Mass. (Riboadenylic acid)$_n$ · (deoxythymidylic acid)$_{12\text{-}18}$ [(rA)$_n$(dT)$_{12\text{-}18}$], (ribocytidylic acid)$_n$ · (deoxyguanylic acid)$_{12\text{-}18}$ [(rC)$_n$(dG)$_{12\text{-}18}$], and (deoxycytidylic acid)$_n$ · (deoxyguanylic acid)$_{12\text{-}18}$ [(dC)$_n$(dG)$_{12\text{-}18}$] were from Collaborative Research, Inc., Waltham, Mass.

PP_i analogs. PP_i (I), glycolic acid (XVI), and oxalic acid (XVII) were from E. Merck, Darmstadt, Federal Republic of Germany. Imidodiphosphate (X) and α-hydroxyl phosphonoacetic acid (XXIII) were from Sigma. Disodium ethane-1-hydroxy-1,1-diphosphonate (V) was a gift from The Procter & Gamble Co., Cincinatii, Ohio. Methanefluorodiphosphonate (VI), methanedifluorodiphosphonate (VII), α-fluoro phosphonoacetic acid (XXIV), and αα-difluoro phosphonoacetic acid (XXV) were kindly given by M. Blackburn, The University, Sheffield, United Kingdom. Methanedibromodiphosphonate (VIII) and methanedichlorodiphosphonate (IX) were kindly given by D. Hutchinson, University of Warwick, Coventry, United Kingdom. 2-Hydroxy-2-phosphonopropionic acid (XXI) was synthesized by the method of H. Blum and K. H. Worms (German patent 2, 310, 450). Methanediphosphonate (II) tetrasodium carbonyldiphosphonate (III), disodium methanehydroxydiphosphonate (IV), phosphonoacetamide (XX), disodium hypophosphate (XI), trisodium phosphonoformate (foscarnet; XII), methyl disodium oxycarbonylphosphonate (XIII), disodium ethoxycarbonylphosphonate (XIV), phosphonoacetic acid (XVIII), 2-phosphonopropionic acid (XIX), and 3-phosphonopropionic acid (XXVI) were synthesized at the Department of Antiviral Chemotherapy at Astra Läkemedel AB as previously described (3). Data on 2-phosphonovaleric acid (XXII) and methylphosphinoformic acid (XV) will be published separately (Johansson et al., manuscript in preparation).

Enzyme. Tissue culture medium from U937p/HTLV-III$_B$ cells, first clarified at low-speed centrifugation, was centrifuged at 132,000 × *g* for 30 min. The sedimented virus was disrupted in 33 mM Tris hydrochloride (pH 8.0), 533 mM KCl, 2.5 mM dithiothreitol, and 0.33% Triton X-100 and stored in working samples at −70°C until use. These prepa-

* Corresponding author.
† Present address: Department of Antiviral Chemotherapy, Research & Development Laboratories, Astra Läkemedel AB, S-151 85 Södertälje, Sweden.

TABLE 1. Effects of diphosphonates on HTLV-III reverse transcriptase activity

	Compound	Conc. (μM) giving 50% inhibition		
		$(rA)_n(dT)_{12-18}$	$(rC)_n(dG)_{12-18}$	$(dC)_n(dG)_{12-18}$
I	HO–P(=O)(OH)–O–P(=O)(OH)–OH	500		
II	HO–P(=O)(OH)–CH_2–P(=O)(OH)–OH	>500		
III	HO–P(=O)(OH)–C(=O)–P(=O)(OH)–OH	4	110	>200
IV	HO–P(=O)(OH)–CH(OH)–P(=O)(OH)–OH	500		
V	HO–P(=O)(OH)–C(OH)(CH_3)–P(=O)(OH)–OH	>500		
VI	HO–P(=O)(OH)–CH(F)–P(=O)(OH)–OH	>500		
VII	HO–P(=O)(OH)–C(F)(F)–P(=O)(OH)–OH	>500		
VIII	HO–P(=O)(OH)–C(Br)(Br)–P(=O)(OH)–OH	>500		
IX	HO–P(=O)(OH)–C(Cl)(Cl)–P(=O)(OH)–OH	>500		
X	HO–P(=O)(OH)–NH–P(=O)(OH)–OH	>500		
XI	HO–P(=O)(OH)–P(=O)(OH)–OH	50	117	100

rations were kindly done by E.-M. Fenyö and B. Åsjö, Karolinska Institute, Stockholm.

Reverse transcriptase assay. The 100-μl reaction mixture contained 50 mM Tris hydrochloride (pH 8.0), 50 mM KCl, 4 mM dithiothreitol, 6 mM $MgCl_2$, 100 μg of bovine serum albumin fraction V, 1 μg of synthetic template-primer, the indicated concentration of [^{3}H]dTTP (0.6 to 40 μM; specific activity, 1,100 to 18,000 cpm/pmol) or [^{3}H]dGTP (0.05 to 4 μM; specific activity, 6,000 cpm/pmol), and 10 μl of enzyme preparation. Standard inhibition assays were performed with 10 μM dTTP.

After incubation at 37°C for 30 min, 40 μl of reaction mixture (in duplicate) was spotted on paper disks (Munktell no. 5; 24 mm) and washed four times in ice-cold 5% trichloroacetic acid–0.02 M sodium PP_i and three times in ethanol. The dried paper disks were counted in 3 ml of

TABLE 2. Effects of compounds containing phosphono and carboxylic groups on HTLV-III reverse transcriptase

	Compound	Conc. (μM) giving 50% inhibition		
		$(rA)_n(dT)_{12-18}$	$(rC)_n(dG)_{12-18}$	$(dC)_n(dG)_{12-18}$
XII	HO–P(=O)(OH)–C(=O)OH (Foscarnet)	0.5	4	100
XIII	HO–P(=O)(OCH_3)–C(=O)OH	500		
XIV	HO–P(=O)(OH)–C(=O)OC_2H_5	100		
XV	CH_3–P(=O)(OH)–C(=O)OH	100	475	>600
XVI	HO–CH_2–C(=O)OH	>500		
XVII	HO(O=)C–C(=O)OH	100		
XVIII	HO–P(=O)(OH)–CH_2–C(=O)OH	>500		
XIX	HO–P(=O)(OH)–CH(CH_3)–C(=O)OH	>500		
XX	HO–P(=O)(OH)–CH_2–C(=O)NH_2	>500		
XXI	HO–P(=O)(OH)–C(OH)(CH_3)–C(=O)OH	>500		
XXII	HO–P(=O)(OH)–CH(C_3H_7)–C(=O)OH	>500		
XXIII	HO–P(=O)(OH)–CH(OH)–C(=O)OH	66	325	>600
XXIV	HO–P(=O)(OH)–CH(F)–C(=O)OH	>500		
XXV	HO–P(=O)(OH)–C(F)(F)–C(=O)OH	>500		
XXVI	HO–P(=O)(OH)–CH_2–CH_2–C(=O)OH	>500		

Econofluor scintillation solution. At saturation with respect to $(rA)_n(dT)_{12-18}$ and dTTP, 10 μl of the enzyme preparation incorporated 20 pmol of dTMP in 30 min at 37°C.

RESULTS

Inhibition of HTLV-III reverse transcriptase with PP_i analogs. Table 1 gives the concentrations of various diphosphonates causing a 50% inhibition of HTLV-III reverse transcriptase activity. Compounds inhibiting the $(rA)_n(dT)_{12-18}$-directed transcription were also tested for effect with $(rC)_n(dG)_{12-18}$-and $(dC)_n(dG)_{12-18}$-directed dGTP incorporation.

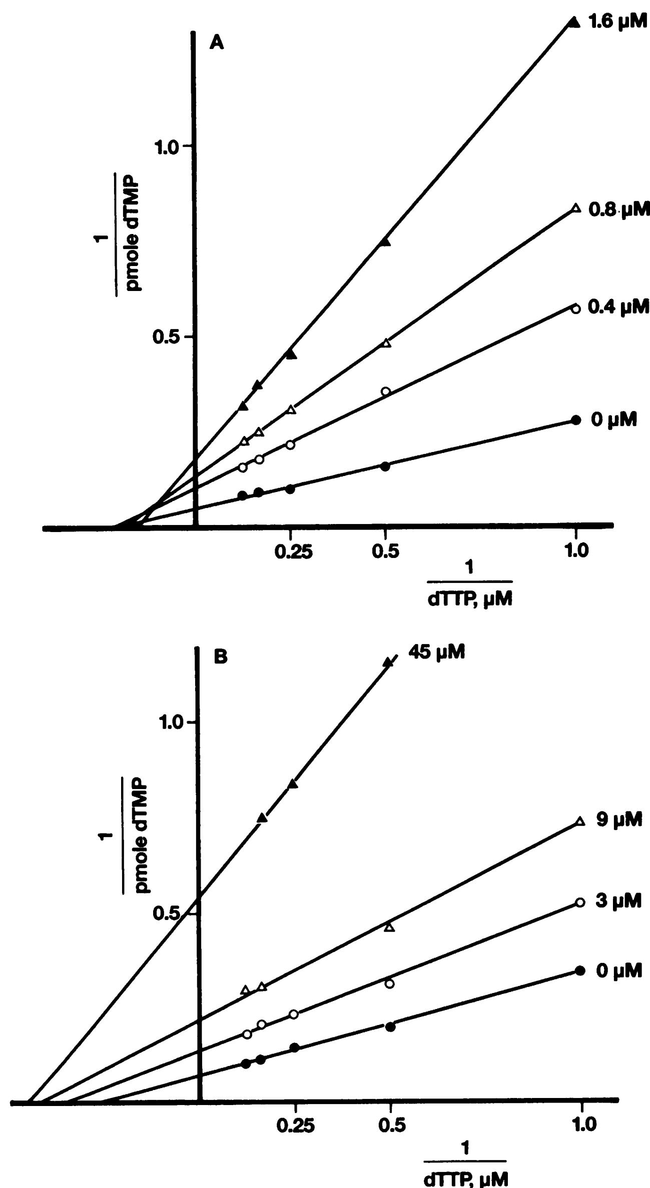

FIG. 1. Inhibition of HTLV-III reverse transcriptase by foscarnet and carbonyldiphosphonate with $(rA)_n(dT)_{12-18}$ as the template-primer and dTTP as the variable substrate. (A) Foscarnet: (●) 0 μM, (○) 0.4 μM, (△) 0.8 μM, and (▲) 1.6 μM. (B) Carbonyldiphosphonate: (●) 0 μM, (○) 3 μM, (△) 9 μM, and (▲) 45 μM. Conditions and assay procedure were as described in Materials and Methods.

TABLE 3. Comparison of inhibitory PP_i analogs tested on reverse transcriptases from HTLV-III and avian myeloblastosis virus (AMV)

	Compound	Conc. (μM) giving 50% inhibition	
		HTLV-III	AMV[a]
I	HO–P(=O)(OH)–O–P(=O)(OH)–OH	500	500
III	HO–P(=O)(OH)–C(=O)–P(=O)(OH)–OH	4	200
X	HO–P(=O)(OH)–NH–P(=O)(OH)–OH	>500	100
XI	HO–P(=O)(OH)–P(=O)(OH)–OH	50	25
XII	HO–P(=O)(OH)–C(=O)–OH	0.5	8
XVII	HO–C(=O)–C(=O)–OH	100	>500
XXIII	HO–P(=O)(OH)–CH(OH)–C(=O)–OH	66	>500

[a] Data are taken from Eriksson et al. (5).

PP_i (I) showed 50% inhibition at 500 μM; placing a carbonyl group, but not a methylene group, between the two phosphate groups raised the inhibitory potential dramatically. Carbonyldiphosphonate (III) inhibited the reverse transcriptase activity by 50% at 4 μM. The other methanediphosphonates (II, V through X) were not inhibitory at 500 μM with the exception of one compound, methanehydroxydiphosphonate (IV), which had an inhibitory capacity at the same level as PP_i (I). Hypophosphate (XI) was also found to inhibit reverse transcriptase at a rather low concentration.

The reaction with $(rA)_n(dT)_{12-18}$ was more sensitive to inhibition by carbonyldiphosphonate than the reactions utilizing $(rC)_n(dG)_{12-12}$ or $(dC)_n(dG)_{12-12}$ as template-primers.

Table 2 gives the results with compounds containing both phosphono and carboxylic groups as inhibitors of HTLV-III reverse transcriptase activity. Foscarnet (XII) was the most potent inhibitor of transcription for all template-primers tested, although the $(dC)_n(dG)_{12-18}$-directed synthesis required a 200 times higher concentration for 50% inhibition than did the $(rA)_n(dT)_{12-18}$-directed reaction. Esterification of phosphono and carbonyl groups, respectively, (XIII) and (XIV), resulted in a reduction of inhibiting activity for both compounds. The slight activity remaining for (XIV) could be explained by trace amounts of foscarnet (XII) as impurity in the dissolved compound. Replacing one hydroxyl in the phosphono group of foscarnet with a methyl gave a structure

TABLE 4. Inhibition of reverse transcriptase activity by 100 μM foscarnet

Template/Primer	Per cent inhibition				
	Avian myeloblastosis virus*	Raucher murine leukemia virus*	Bovine leukemia virus*	Visna virus*	Human T-lymphotropic virus type III
$(rA)_n (dT)_{12-18}$	96	96	88	90	>99
$(Cm)_n (dG)_{12-18}$	2	97	82	92	75
$(dC)_n (dG)_{12-18}$	44	84	14	93	47

* Data are taken from Sundquist and Öberg (14) and Sundquist and Larner (13).

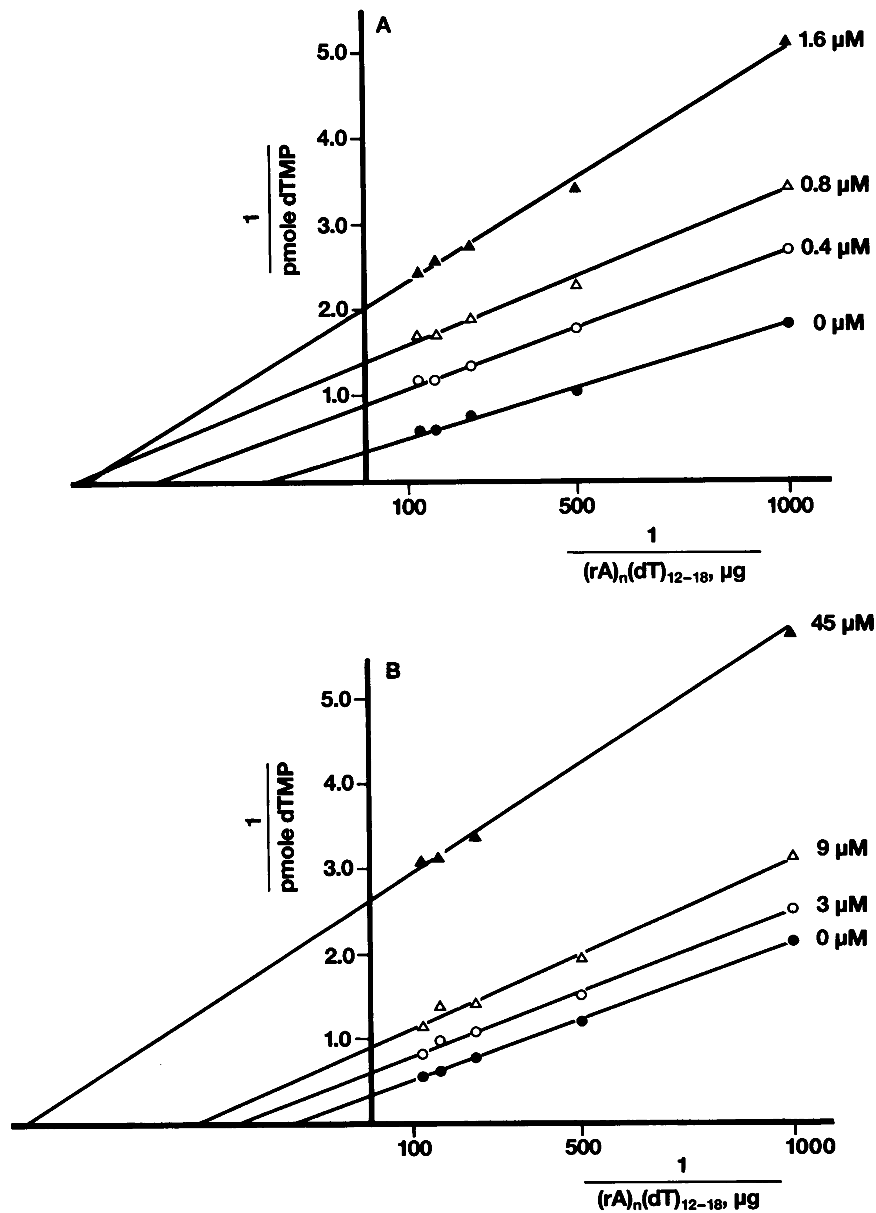

FIG. 2. Inhibition of HTLV-III reverse transcriptase by foscarnet and carbonyldiphosphonate with dTTP as the substrate and $(rA)_n(dT)_{12-18}$ as the variable template-primer. (A) Foscarnet: (●) 0 μM, (○) 0.4 μM, (△) 0.8 μM, and (▲) 1.6 μM. (B) Carbonyldiphosphonate: (●) 0 μM, (○) 3 μM, (△) 9 μM, and (▲) 45 μM. Conditions and assay procedure were as described in Materials and Methods.

that still had some inhibiting activity (50% inhibition at 100 μM). When the entire phosphono group was replaced by a carboxyl as in oxalic acid (XVII) the same inhibitory activity was seen. A longer distance between the phosphono and carboxyl group represented by phosphonoacetic acid (XVIII) and 3-phosphonopropionic acid (XXVI) resulted in complete loss of inhibitory effect up to 500 μM. Several substituted phosphonoacetic compounds (XIX, XXI through XXV) were tested, and only one structure had measurable effect. α-Hydroxy phosphonoacetic acid (XXIII) reduced the $(rA)_n(dT)_{12-18}$-directed transcription by 50% at 66 μM and showed the same template dependence for inhibition as foscarnet (XII).

Mechanism of inhibition of HTLV-III reverse transcriptase by foscarnet and carbonyldiphosphonate. We studied the two most potent inhibitors, foscarnet and carbonyldiphosphonate, kinetically to elucidate their mode of action on HTLV-III reverse transcriptase. Double-reciprocal plots showed a noncompetitive inhibition for both compounds when $(rA)_n(dT)_{12-18}$ was used as the template-primer and dTTP was used as the variable substrate (Fig. 1). This indicates that neither foscarnet nor carbonyldiphosphonate interacts at the binding site for deoxyribotriphosphates on the enzyme. The Michaelis-Menten constant (K_m) and inhibition constant (K_i) were calculated from these double-reciprocal plots. Under the reaction conditions used, K_m for dTTP was 5 μM, and K_is for foscarnet and carbonyldiphosphonate were calculated to be 0.4 and 9 μM, respectively.

When the amount of $(rA)_n(dT)_{12-18}$ was varied and the dTTP concentration was held constant, the type of inhibition for both foscarnet and carbonyldiphosphate was uncompetitive. With a K_m of 0.15 μg/ml the calculated values of K_i were 2 and 36 μM, respectively. The uncompetitive mode of action for the two compounds indicates that the binding of the template-primer to the enzyme is not affected.

DISCUSSION

The structure-activity relationship for activity of PP$_i$ analogs against HTLV-III reverse transcriptase (Tables 1 and 2) shows similarities to the effects on other reverse transcriptases (4, 19). In all cases, foscarnet has been the most active inhibitor. The 50% inhibition observed here at 0.5 μM foscarnet is intermediate between the values reported for HTLV-III by Sandström et al. (11), 0.1 μM, and Sarin et al. (12), 2 μM. It seems likely that minor differences in assay conditions or enzyme preparation could explain these differences. Carbonyldiphosphonate (III) showed a better inhibition of HTLV-III reverse transcriptase than the earlier reported inhibition for avian myeloblastosis virus reverse transcriptase, 4 versus 200 μM for 50% inhibition (5). α-Hydroxy phosphonoacetic acid (XXIII) also had a better inhibitory effect against HTLV-III reverse transcriptase than against the avian myeloblastosis virus enzyme, 66 versus 500 μM (5). A comparison between the inhibition patterns of HTLV-III and avian myeloblastosis virus reverse transcriptases is shown in Table 3.

The influence of template-primer on the inhibition of HTLV-III reverse transcriptase by foscarnet has similarities to the pattern shown by bovine leukemia virus reverse transcriptase, but is less similar to the effect on visnavirus reverse transcriptase (Table 4). This is somewhat unexpected, considering the close relationship between visnavirus and HTLV-III (6). The reason for the template-primer dependence for inhibition by foscarnet (Table 4) is not understood.

The mechanism of action of foscarnet and carbonyldiphosphonate was a noncompetitive inhibition with regard to dTTP and an uncompetitive inhibition with respect to template primer (Fig. 1 and 2). This is in agreement with previous results with reverse transcriptase from avian myleloblastosis virus (5). In contrast to this type of inhibition, the activity of 3′-azidothymidine triphosphate is competitive to dTTP (St. Clair, et al., ICAAC), and the activity of ammonium-21-tungsto-9-antimonate is competitive to template-primer (2). These differences might be of importance, and combinations could be used to prevent resistance development or to obtain synergistic effects.

The use of reverse transcriptase inhibitors against HTLV-III infections are expected to prevent the infection of new cells, when reverse transcriptase is required, but not to eliminate virus from infected cells, where synthesis of viral RNA is carried out by cellular enzymes. To have any permanent clinical use against HTLV-III infections it will thus not be enough to inhibit the reverse transcriptase, but the treatment must also result in the elimination of infected cells. It seems likely that this can only be achieved by the use of combinations of inhibitors of reverse transcriptase and other drugs.

ACKNOWLEDGMENTS

We thank E.-M. Fenyö and B. Åsjö for kindly preparing HTLV-III reverse transcriptase, M. Blackburn and D. Hutchinson for the gift of PP$_i$ analogs, and M. Kropp for typing the manuscript.

LITERATURE CITED

1. **Basu, A., and M. J. Modak.** 1985. Observations on the suramin-mediated inhibition of cellular and viral DNA polymerases. Biochem. Biophys. Res. Commun. **128**:1395–1402.
2. **Dormont, D., B. Spire, F. Barré-Sinoussi, L. Montagnier, and J. C. Chermann.** 1985. Inhibition of RNA-dependent DNA polymerases of AIDS and SAIDS retroviruses by HPA-23 (ammonium-21-tungsto-9-antimonate). Ann. Inst. Pasteur **136E**:75–83.
3. **Eriksson, B., A. Larsson, E. Helgstrand, N.-G. Johansson, and B. Öberg.** 1980. Pyrophosphate analogues as inhibitors of herpes simplex virus type I DNA polymerase. Biochim. Biophys. Acta **607**:53–64.
4. **Eriksson, B., and B. Öberg.** 1984. Phosphonoformate and phosphonoacetate, p. 127–142. *In* Y. Becker (ed.), Antiviral drugs and interferon: the molecular basis of their activity. Martinus Nijhoff, Boston.
5. **Eriksson, B., G. Stening, and B. Öberg.** 1982. Inhibition of reverse transcriptase activity of avian myeloblastosis virus by pyrophosphate analogues. Antiviral Res. **2**:81–95.
6. **Gonda, M. A., F. Wong-Staal, R. C. Gallo, J. E. Clements, O. Narayan, and R. V. Gilden.** 1985. Sequence homology and morphologic similarity of HTLV-III and visna virus, a pathogenic lentivirus. Science **227**:173–177.
7. **Mitsuya, H., M. Popovic, R. Yarchoan, H. Matsushita, R. C. Gallo, and S. Broder.** 1984. Suramin protection of T-cells in vitro against infectivity and cytopathic effect of HTLV-III. Science **226**:172–174.
8. **Montagnier, L., J. C. Cherman, F. Barré-Sinoussi, S. Chamaret, J. Gruest, M. T. Nugeyre, F. Rey, C. Danquet, C. Axler-Blin, F. Vezinct-Brun, C. Rouzioux, A. G. Saimot, W. Rozenbaum, J. C. Gluckman, D. Klatzmann, E. Vilmer, C. Griscelli, C. Gazengel, and J. B. Brunet.** 1984. A new human T-lymphotropic retrovirus: characterization and possible role in lymphadenopathy and acquired immune deficiency syndromes, p. 363–370. *In* R. C. Gallo, M. Essex, and L. Gross (ed.), Human t-cell leukemia/lymphoma virus. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

9. **Öberg, B.** 1983. Antiviral effects of phosphonoformate (PFA, foscarnet sodium). Pharmacol. Ther. **19:**387–415.
10. **Popovic, M., M. G. Sarngadharan, E. Read, and R. C. Gallo.** 1984. Detection, isolation, and continuous production of cytopathic retroviruses (HTLV-III) from patients with AIDS and pre-AIDS. Science **224:**497–500.
11. **Sandström, E. G., R. E. Bryington, J. C. Kaplan, and M. S. Hirsch.** 1985. Inhibition of human T-cell lymphotropic virus type III in vitro by phosphonoformate. Lancet **i:**1480–1482.
12. **Sarin, P. S., Y. Taguchi, D. Sun, A. Thornton, R. C. Gallo, and B. Öberg.** 1985. Inhibition of HTLV-III/LAV replication by foscarnet. Biochem. Pharmacol. **34:**4075–4078.
13. **Sundqvist, B., and E. Larner.** 1979. Phosphonoformate inhibition of visna virus replication. J. Virol. **30:**847–851.
14. **Sundqvist, B., and B. Öberg.** 1979. Phosphonoformate inhibits reverse transcriptase. J. Gen. Virol. **45:**273–281.