Vol. 148, No. 1, 1987 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
October 14, 1987 Pages 78-85

INHIBITION OF HIV-ASSOCIATED REVERSE TRANSCRIPTASE BY SUGAR-MODIFIED DERIVATIVES OF THYMIDINE 5'-TRIPHOSPHATE IN COMPARISON TO CELLULAR DNA POLYMERASES α AND β [1]

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Received July 23, 1987

The sugar-modified dTTP analogues 2',3'-didehydro-2',3'-dideoxythymidine 5'-triphosphate (ddeTTP), 2',3'-dideoxythymidine 5'-triphosphate (ddTTP), 3'-fluorothymidine 5'-triphosphate (FdTTP), and 3'-azidothymidine 5'-triphosphate (N_3dTTP) are demonstrated to be very effective and selective inhibitors of the HIV-associated reverse transcriptase (HIV-RT). This conclusion is based on a comparison of the ID_{50} values of the compounds for the HIV-RT (ranging from 0.03 µM for ddeTTP to 0.1 µM for ddTTP) and the cellular DNA polymerase α (> 200 µM). DNA polymerase β is partially affected by N_3dTTP (ID_{50}=31 µM) and by the other analogues (ID_{50}= 1-2.2 µM). FdTTP has proved as effective as N_3dTTP (ID_{50}= 0.05 µM) in suppressing the HIV-RT activity. Kinetic analysis revealed for both dTTP analogues a competitive type of inhibition and the same K_i values (about 0.05 µM).

Particular attention has been focused recently on 3'-azidothymidine (N_3ddThd) which has been described not only as a strong inhibitor of cellular infection by the human immunodeficiency

Abbreviations: HIV: human immunodeficiency virus, HIV-RT : HIV-associated reverse transcriptase; dTTP: thymidine 5'-triphosphate; ddeTTP : 2',3'-didehydro-2',3'-dideoxythymidine 5'-triphosphate; ddTTP : 2',3'-dideoxythymidine 5'-triphosphate; FdTTP : 2',3'-dideoxy-3'-fluorothymidine 5'-triphosphate; CldTTP : 3'-chloro-2',3'-dideoxythymidine 5'-triphosphate; N_3dTTP : 3'-azido-2',3'-dideoxythymidine 5'-triphosphate; ddeThd : 2',3'-didehydro-2',3'-dideoxythymidine; ddTdR : 2',3'-dideoxythymidine; FddThd : 2',3'-dideoxy-3'-fluorothymidine; ClddThd : 3'-chloro-2',3'-dideoxythymidine; N_3ddThd : 3'-azido-2',3'-dideoxythymidine.

[1] Presented in part at FEBS advanced course: "Antiviral Drug Development: A Multidisciplinary Approach", Il Chiocco, Italy, May 10-23, 1987.

0006-291X/87 $1.50

virus (HIV) but also as the first agent to give some promising clinical improvements and even life prolonging effects in the treatment of the acquired immunodeficiency syndrome (AIDS) (1,2,3). A strong inhibition of the HIV-associated reverse transcriptase (HIV-RT) can be considered to be the basis for its activity (4).

In a search for new potential inhibitors of HIV-RT we have investigated the sugar-modified dTTP analogues 2',3'-didehydro-2',3'-dideoxythymidine 5'-triphosphate (ddeTTP), 2',3'-dideoxythymidine 5'-triphosphate (ddTTP), 2',3'-dideoxy-3'-fluorothymidine 5'-triphosphate (FdTTP), 3'-chloro-2',3'-dideoxythymidine 5'-triphosphate (CldTTP), and 3'-azido-2',3'-dideoxythymidine 5'triphosphate (N_3dTTP) (Fig.1). One essential demand on such compounds is their ability to discriminate effectively between cellular DNA polymerases and the reverse transcriptase (RT), thus avoiding an interference with normal cell growth. Therefore we have examined additionally the dTTP analogues in respect to their effects on DNA polymerases α and β. Our results indicate that, with exception of the 3'-chloro derivative, all tested dTTP analogues are very strong inhibitors of HIV-RT, which only partially affected the DNA polymerase β, but hardly the DNA polymerase α. The findings of the present studies qualify both nucleosides, FTdR and ddeTdR, for further antiviral testing at cellular level.

MATERIALS AND METHODS

Inhibitors. The triphosphates of 2',3'-didehydro-2',3'-dideoxythymidine (ddeThd), 2',3'-dideoxy-3'-fluorothymidine (FddThd), 3'-chloro-2',3'-dideoxythymidine (CldThd), and 3'-azido-2',3'-dideoxythymidine (N_3ddThd) were synthesized and identified as described (5). ddTTP was purchased from Pharmacia (Uppsala).
Substrate and primer templates. dTTP was obtained from Boehringer (Mannheim); [^{3}H]dTTP (spec.act.29.7 Ci/mmole) from Zentralinstitut fuer Kernforschung (Rossendorf). The purity of both compounds was checked periodically by TLC. PolyA·oligo(dT)$_{12}$ was obtained from Boehringer (Mannheim); polydA·oligo(dT)$_{10}$ was a product of Calbiochem (San Diego); oligo(dT)$_{13}$ was from Pharmacia (Uppsala).
DNA polymerase α and β assays. DNA polymerases α and β were prepared as described (6). In some experiments DNA polymerase α from calf thymus (spec.act. 44 U/mg) obtained from Pharmacia (Uppsala) and a DNA polymerase β from rat liver (spec.act. 3 units/µg) kindly provided by Dr. A. Krayevsky, Moscow, were applied with the same results. The activities of both enzymes were assayed essentially as described (6). However, the assay mixtures contained in 20 µl : 0.15 units of DNA polymerase α or 0.25 units of DNA polymerase β, 10 µM dTTP, 1 µCi [^{3}H]dTTP and 0.01 OD polydA·oligo (dT)$_{10}$, the incubation time was 15 min. One unit of enzyme activi-

ty represents 1 nmole of [^{3}H]dTMP incorporated into polydA·oligo $(dT)_{10}$ per hour.

Purification of HIV. HTLV-III$_B$ infected H9 cells were grown to a density of 0.5-1.0 x 10^6 cells/ml as described (7,8); the pooled virus-containing culture supernatants were concentrated about 10-20 fold by the Hollow-Fiber System (Amicon) using cartridges Type H1 MP01-43 and subsequently the virus was sedimented onto a 4 ml cushion of 39% sucrose in TEN (20 mM Tris-HCl, pH 7,4, 1 mM EDTA, 100 mM NaCl) through a 10-15 ml layer of 20% sucrose in TEN in a Beckman SW 27 rotor at 20000 rpm for 1.5 h at 4°C. The virus-containing interphase fraction, monitored at 280 nm, was obtained by a gradient collector, diluted 1:4 with 8.5% sucrose in TEN and sedimented again through 20 ml of 20% sucrose in TEN. The pelleted virus was resuspended in TEN (giving about 1 µg protein/ µl), aliquoted and frozen at -20°C.

Assay for HIV-associated reverse transcriptase (HIV-RT). The optimal conditions for detection of HIV-RT described by Hoffman et al. (9) were used here with small modifications. The assay mixture contained the following components in a total volume of 20 µl : 50 mM Tris-HCl, pH 8.0, 5 mM DTT, 5 mM $MgCl_2$, 150 mM KCl, 0.05% Triton X-100, 0.3 mM GSH, 0.5 mM EGTA, 10 µM dTTP, 0.01 OD polyA-oligo$(dT)_{12}$; 4 µl of the virus suspension was added and the reaction was started with 1 µCi [^{3}H]dTTP. The mixtures were incubated at 37°C for 30min. The amount of virus suspension equivalent to about 30 µg protein gives a max. RT activity of about 1 nmole [^{3}H]dTMP incorporated into polyA·oligo$(dT)_{12}$ per hour. When replacing polyA·oligo$(dT)_{12}$ by polydA·oligo$(dT)_{10}$ in the assay mixture, no detectable incorporation of [^{3}H]dTMP was found,whereas oligo$(dT)_{13}$ produced only about 2.6% of the incorporation reached by polyA·oligo$(dT)_{12}$. After incubation, 2 x 5 µl aliquots of the mixtures were transferred to FN-8 paper disks (Niederschlag, GDR) and processed for radioactivity counting as described (6). The activity of all enzymes tested was proportional to enzyme conc. and time.

RESULTS AND DISCUSSION

Inhibition of the HIV-RT by ddeTTP,ddTTP,FdTTP,CldTTP, and N_3dTTP. The effects of increasing conc. of sugar-modified dTTP analogues (Fig.1) on the activity of HIV-RT using polyA·oligodT as template primer and 10 µM dTTP as substrate are summarized in Fig.2. The highest inhibitory activity was found for ddeTTP, with a nearly complete inhibition of the enzyme at a conc. of about 0.8 µM and

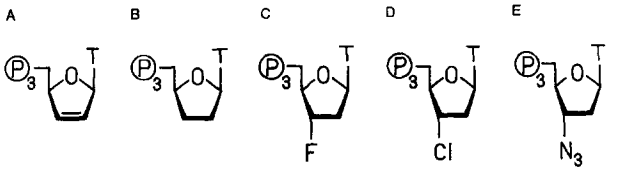

Fig.1. Structural modifications of the deoxyribose moiety of dTTP analogues tested as inhibitors of HIV-RT and the cellular DNA polymerases α and β . (A) ddeTTP; (B) ddTTP; (C) FdTTP; (D) CldTTP; (E) N_3dTTP; Ⓟ$_3$-represents the triphosphate group.

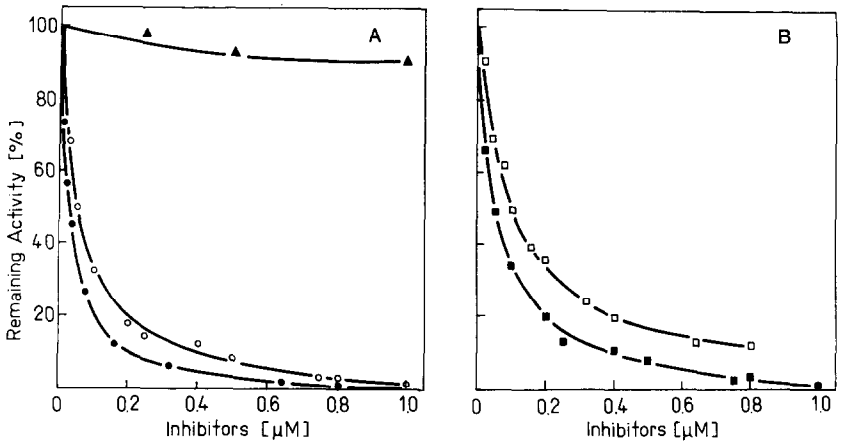

Fig.2. Inhibition of the HIV-RT by sugar-modified dTTP analogues. The activity of the enzyme was estimated as described in the presence of the indicated conc. of (A) : ddeTTP (●); FdTTP (○); CldTTP (▲) or of (B) : N_3dTTP (■); ddTTP (□). Mean values of at least 4 experiments were given. One hundred per cent of enzyme activity ranged from 39-42 pmoles [^{3}H]dTMP incorporation per 30 min.

a 50% inhibition at 0.03 μM. FdTTP was found to be slightly less effective, and 1.0 μM has to be applied for maximum inhibition. An apparently identical inhibitory effectivity was obtained with N_3dTTP assayed simultaneously with FdTTP. Therefore, we have calculated the conc. required for a 50% inhibition of the enzyme (ID_{50}) from the dose response curves by means of non linear regression analysis and have obtained an ID_{50} value of 0.058 ± 0.011 μM for FdTTP (n=6) and of 0.052 ± 0.005 μM for N_3dTTP (n=5). From this we conclude that there does not seem to exist a significant difference between the two compounds in their effectiveness. For ddTTP an ID_{50} of 0.1 μM was determined; thus, the order of effectiveness of the dTTP analogues can be written as follows: ddeTTP > FdTTP = N_3dTTP > ddTTP. A uniform IC_{50} value for the last three of these compounds of about 0.04 μM was published by Cheng et al. (10) during preparation of our manuscript. The chloro derivative failed to cause any significant inhibition of the HIV-RT activity (Fig.2a).

Kinetics of FdTTP and N_3dTTP inhibition. Increasing substrate conc. were used to characterize the nature of FdTTP and N_3dTTP inhibition of HIV-RT. Double reciprocal plots of the experimental data revealed that the inhibition of FdTTP as well as N_3dTTP proved to be competitive with regard to dTTP (Fig.3). The K_m value of HIV-RT for dTTP was determined to be 22.2 ± 3.1 μM, (n=4) the inhibitor constant K_i for FdTTP was 0.054 ± 0.017 μM (n=5) and for

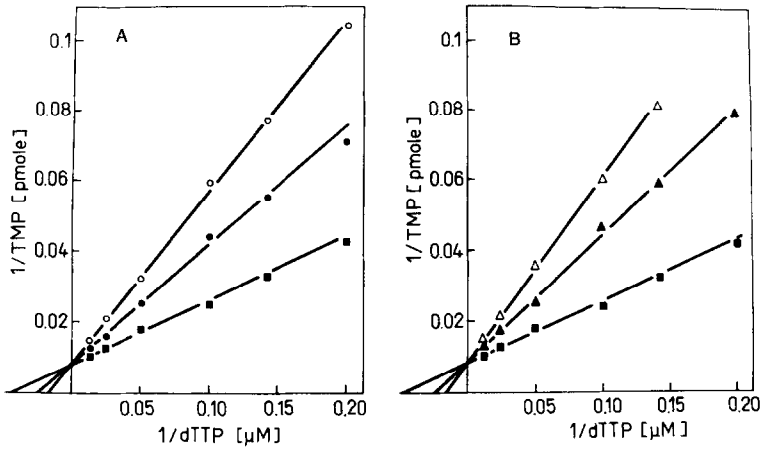

Fig.3. Kinetics of inhibition of HIV-RT by (A) FdTTP and (B) N_3dTTP. Double reciprocal plots of substrate dependent reaction velocities in the presence of no inhibitor (■); 0.04 μM FdTTP (●) or N_3dTTP (▲), and 0.08 μM FdTTP (○) or N_3dTTP (△).

N_3dTTP 0.045 ± 0.010 μM (n=4). All these values were calculated from data obtained by linear regression analysis. Apparently no significant difference between the inhibitor constants exists, indicating the same high affinity of both analogues to the binding site of the normal substrate dTTP (K_m/K_i=440). In agreement with this, Furman et al. (4) described for N_3dTTP an apparent $K_{i_{app}}$ of 0.04 μM.

Preincubation experiments. The DNA-chain terminating incorporation of N_3dTTP is regarded as the major mechanism of its strong inhibitory effects on HIV-RT (1,4). A substantial incorporation of N_3dTTP or FdTTP into the template primer during a preincubation period should therefore markedly reduce the conc. of the active primer ends and, consequently, result in an increased inhibitory efficiency of the compounds. We have preincubated the HIV-RT in the presence of 0.04 μM FdTTP and N_3dTTP, resp., with a reduced template primer conc. as described in Table 1. Following the completion of the assay mixture by the substrate the kinetics of their inhibitory potency were estimated. The data of Table 1 show that a preincubation affected hardly the inhibitory kinetics of the analogues, supporting the idea that the observed effects against HIV-RT are due to their competitive inhibition of the enzyme rather than to their chain terminating incorporation. This suggestion is supported by investigations of N_3dTTP with RT from Rauscher murine leukemia virus (11).

Inhibition of cellular DNA polymerases α and β. The effects of ddeTTP, ddTTP, FdTTP, and N_3dTTP on the activity of cellular DNA

Table 1. Influence of preincubation of HIV-RT with FdTTP or N_3dTTP on the kinetics of inhibition

Addition of the Analogue		% Remain. Activity		
Preincub.	Incub.	10 min	20 min	30 min
-	-	100(3.7)*	100(7.9)	100(14.4)
FdTTP	-	54	58	56
-	FdTTP	62	57	60
N_3dTTP	-	57	52	54
-	N_3dTTP	62	58	59

Lysed virus suspensions were preincubated for 15 min with a reduced polyA·oligodT conc. (0.003 OD/20 µl) in the presence or absence of 0.04 µM FdTTP or N_3dTTP. The reactions were started by adding the substrate (10 µM [^{3}H]dTTP) alone or together with the analogue and terminated at the indicated times. Linearity of the reactions up to 30 min can be seen. The percentage of activity remaining from the control was given.
* The figures in parentheses represent the pmoles [^{3}H]dTMP incorporated into polyA·oligodT of preincubated controls and were found to be about 95% of those obtained from non preincubated probes.

polymerases α and β were investigated with 10 µM dTTP as substrate and polydA·oligodT as template primer. We described recently that in analogy to ddTTP the DNA polymerase α is much less sensitive against FdTTP than DNA polymerase β (6). The same holds true for

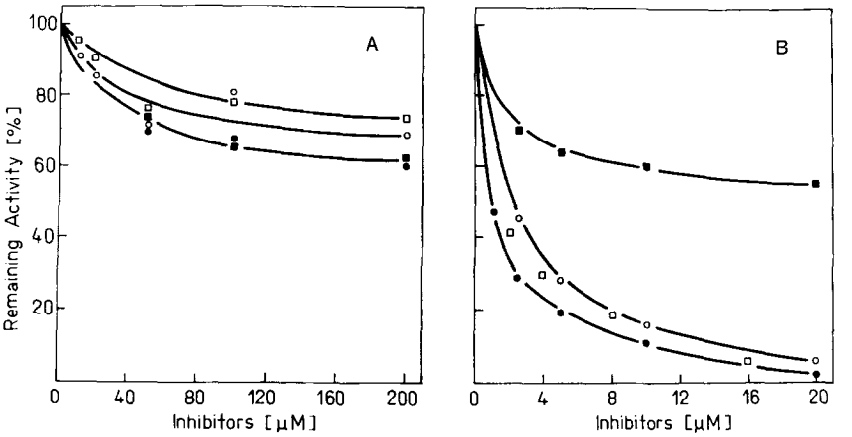

Fig.4. Effects of sugar-modified dTTP analogues on the activity of (A) : DNA polymerase α and (B) : DNA polymerase β. The activity was estimated in the presence of the indicated conc. of FdTTP (o) and ddTTP (□), completed data from (6); ddeTTP (●) and N_3dTTP (■). Mean values of 3 experiments were given. One hundred per cent of activity represents for DNA polymerase α 16-21 pmoles [^{3}H]dTMP incorporation per 15 min and 29-35 pmoles in case of DNA polymerase β.

Table 2. Comparison of conc. of sugar-modified dTTP analogues required for a 50% inhibition (ID_{50}) of HIV-RT and the cellular DNA polymerases α and β

Polymerase	ID_{50}, μM			
	ddeTTP	FdTTP	N_3dTTP	ddTTP
HIV-RT	0.03	0.05	0.05	0.10
α	>200	>200	>200	>200
β	1.0	2.2	31.0	1.4

0.01 OD of the primer templates and 10 μM of dTTP as substrate were generally used.

ddeTTP and N_3dTTP (Fig.4). Even if the substrate-to-inhibitor ratio was 1:20 the activity of DNA polymerase was inhibited only to about 30-40% . In contrast, the results with DNA polymerase β indicated a partial inhibition of its activity. The conc. required for a 50% inhibition of the enzyme activity was estimated to be 1.0 μM for ddeTTP, 1.4 μM for ddTTP, 2.2 μM for FdTTP, and 31.0 μM for N_3dTTP.

CONCLUSIONS

The criterion of a high degree of sensitivity and a sufficient selectivity for HIV-RT in comparison to cellular DNA polymerases α and β appears to be met by the inhibitors examined (Table 2). The potency for the corresponding nucleosides to become effective anti-HIV agents is entirely dependent on their intracellular phosphorylation to the 5'-triphosphates. While an insufficient phosphorylation might explain the only slight activity of ddThd in this respect (12), ddeThd has been applied successfully in preventing HIV infection in a cellular system (13). Results from studies under way have shown that FddThd, originally synthesized as a cytostatic agent in our department (14), can be readily phosphorylated in T-cells and seems to be able to protect MT-4 cells against the cytopathic effect of HIV as efficiently as N_3ddThd (15,16).

ACKNOWLEDGMENT: The skillful technical assistance of H. Sciborski is gratefully acknowledged.

REFERENCES

1. Mitsuya, H., Weinhold, K.J., Furman, P.A., St.Clair, M.H., Nusinoff-Lehrman, S., Gallo, R.C., Bolognesi, D., Barry, D.W., and Broder, S. (1985) Proc.Natl.Acad.Sci. USA 82, 7096-7100.
2. Yarchoan, R., Klecker, R.W., Weinhold, K.J., Markham, P.D., Lyerly, H.K., Durack, D.T., Gelmann, E., Nusinoff-Lehrman, S., Blum, R.M., Barry, D.W., Shearer, G.M., Fischl, M.A., Mitsuya, H., Gallo, R.C., Collins, J.M., Bolognesi, D.P., Myers, C.E., and Broder, S. (1986) Lancet i, 575-580.
3. Mitsuya, H., Broder, S. (1987) Nature 325, 773-778.
4. Furman, P.A., Fyfe, J.A., St.Clair, M.H., Weinhold, K., Rideout, J.L., Freeman, G.A., Nusinoff-Lehrman, S., Bolognesi, D.P., Broder, S., Mitsuya, H., and Barry, D.W. (1986) Proc. Natl.Acad.Sci. USA 83, 8333-8337.
5. Scheit, K.H., and Faerber, P. (1978) In Nucleic Acid Chemistry (L.B. Townsend and R.S. Tipson, Eds.), pp. 793-799. Wiley-Interscience, New York.
6. Matthes, E., Lehmann, Ch., Drescher, B., Büttner, W., and Langen, P. (1985) Biomed.Biochim.Acta 44, K63-K73.
7. Popovic, M., Sarngadharan, M.G., Read, E., and Gallo, R.C. (1984) Science 224, 497-500.
8. Ladhoff, A.-M., Scholz, D., Rosenthal, S., and Rosenthal, H.A. (1986) Z.Klin.Med. 41, 2209-2214.
9. Hoffman, A.D., Banapour, B., and Levy, J.A. (1985) Virology 147, 326-335.
10. Cheng, Y., Dutschman, G.E., Bastow, K.F., Sarngadharan, M.G., and Ting, R.Y.C. (1987) J. Biol. Chem. 262, 2187-2189.
11. Ono, K., Ogasawara, M., Iwata, Y., Nakane, H., Fujii, T., Sawai, K., and Saneyoshi, M. (1986) Biochem.Biophys.Res.Commun. 140, 498-507.
12. Mitsuya, H., and Broder, S. (1986) Proc.Natl.Acad.Sci. USA 83, 1911-1915.
13. Baba, M., Pauwels, R., Herdewijn, P., De Clercq, E., Desmyter, J., Vandeputte, M. (1987) Biochem. Biophys. Res. Commun. 142, 128-134.
14. Langen, P., Etzold, G., Hintsche, R., and Kowollik, G. (1969) Acta biol. med. germ. 23, 759-766.
15. WP A61K/292 826 3, 24.7.86.
16. Matthes, E., Lehmann, Ch., Scholz, D., von Janta-Lipinski, M., Rosenthal, H.A., and Langen, P. (1987) Submitted.