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INHIBITION OF HIV-REPLICATION BY 3'-FLUORO-MODIFIED NUCLEOSIDES WITH LOW CYTOTOXICITY

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From a series of newly synthesized 3'-fluoro-modified nucleosides the C-5-chloro-substituted derivative of 2',3'-dideoxy-3'-fluoro-uridine (FddUrd) and the 4-thio analogue of 2',3'-dideoxy-3'-fluorothymidine (FddThd) emerged as the most efficient and selective anti-HIV agents. Their antiviral doses (ED_{50}) proved to be 700-and 480-fold below their toxic doses (CD_{50}) in MT-4 cells. The 50% inhibitory dose of cell proliferation of the 5-chloro-substituted FddUrd and its parent agent FddUrd was found to be in the millimolar range for various other human cell-lines and for mouse CFU-GM. The 5'-triphosphate of FddUrd as well as of its 5-chloro derivative are demonstrated to be two of the most active and selective inhibitors of the HIV-reverse transcriptase (IC_{50} = 0.07 ± 0.01 and 0.04 ± 0.006 μM).

2',3'-Dideoxy-3'-fluorothymidine (FddThd) has been described as the most effective inhibitor of HIV-1 replication in various cellular systems, being at least 5 times more effective than 3'-azido-2',3'-dideoxythymidine (AZT) (1-5). However, both compounds have also considerable cytotoxic effects (1-4). We therefore looked for compounds with sufficient anti-HIV activity and much less cytotoxicity than that of FddThd or AZT. Such compounds may be found among 3'-fluoro-modified pyrimidine 2'-deoxyribonucleosides as shown by the corresponding properties of 2',3'-dideoxy-3'-fluorouridine (FddUrd) (1-4). In the work presented here we examined to what extent the C-5-halogeno substitution of FddUrd changes the compounds' properties on HIV-replication, on cell proliferation, and (as 5'-triphosphates) on HIV-1 reverse transcriptase (HIV-RT) and the cellular DNA polymerases α and β. The

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same studies were done with the C-4-thio analogue of FddThd. Our results indicate that the 5-chloro derivative of FddUrd and the 4-thio analogue of FddThd are promising antiviral agents with low cytotoxicity. At the level of polymerases, the 5'-triphosphate of FddUrd and of its 5-chloro derivative proved to be two of the most active and selective inhibitors of HIV-RT.

MATERIAL AND METHODS

Test compounds: The 4-thio analogue of FddThd was newly synthesized, on which we will report elsewhere, whereas the other 3'-fluoro-modified deoxynucleosides and the nucleoside triphosphates were prepared according to published procedures (6).

Polymerase assays: The DNA polymerase α was prepared from calf thymus as described (7); the DNA polymerase β obtained from rat liver (spec.act. 3 units/ug) was kindly provided by Dr.A. Krayevsky, Moscow. Both enzymes were assayed exactly as described (7). The recombinant HIV-1 reverse transcriptase (HIV-RT) was generously provided by Dr.K. Moelling, Berlin-West (8) and tested in comparison to lysed HTLV-III_B as described (9),omitting Triton X-100, however. No differences between the two enzymes could be detected concerning their inhibition by more than 10 nucleoside triphosphate analogues (Matthes et al., unpublished). All required reagents were of the quality used previously (9).

Inhibition assay for the cytopathic effect of HIV-1: MT-4 cells exposed to HTLV-III_B suspension for 90 min at a multiplicity of infection of 0.04 (10,11) were adjusted to 2×10^4 per 0.2 ml RPMI 1640 supplemented medium (1), as were the uninfected cells, and incubated either alone or in the presence of the desired drug concentrations for 6 days at 37°C, and the viable cells were counted (1). The effective doses required to protect 50% of the cells against the cytopathic effect of HTLV-III_B (ED_{50}) and the cytotoxic doses required to reduce the number of viable uninfected cells to 50% (CD_{50}) were estimated (5).

Cell growth assay: The given human cells were incubated for 48 hr at 37°C alone or in the presence of the desired drug concentrations (1). The cells were counted in a Picoscale particle counter (Hungary) and the doses estimated, which result in 50% inhibition of cell proliferation.

Colony assay of mouse granulocyte-macrophage precursors (CFU-GM): Bone marrow cells obtained from the femurs of 8-12 week-old DBA/2 mice were washed twice in PBS. 5×10^4 cells were plated into petri dishes containing in 1 ml 0.3 ml agar (Bacto-Agar; Difco Laboratories, Detroit), McCoy 5A medium, supplemented with 20% FCS (FKS Ladeburg, Berlin), 4% lung-conditioned medium as a source of colony-stimulating factor, prepared according to Metcalf (12), and the desired drug concentrations. The plates were incubated for 5 days at 37°C in a humidified 5% CO_2-air atmosphere. The plating efficiency was about 0.5%. The drug effects on the numbers of colonies consisting of 50 or more cells were estimated.

RESULTS AND DISCUSSION

Protection of MT-4 cells against the cytopathic effect of HIV-1.

We compared the in vitro activity against HIV-1 of some new 3'-fluoro-modified nucleosides obtained from the parent compounds

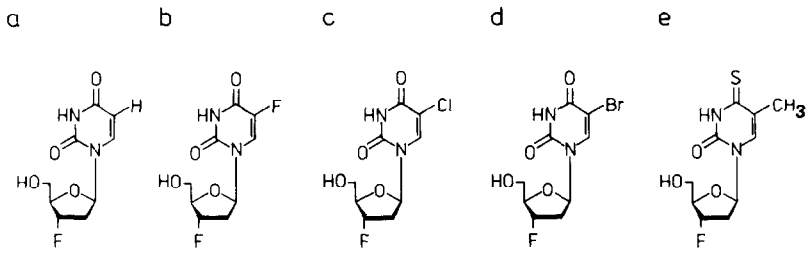

Fig.1. Structural modifications of 3'-fluoro-modified nucleoside analogues tested as inhibitors of HIV-replication. (a): FddUrd, (b): its 5-fluoro-, (c): its 5-chloro-, (d): its 5-bromo derivative; (e): 4-thio analogue of FddThd.

FddUrd and FddThd by the replacement of the 5-hydrogen of FddUrd by fluorine, chlorine or bromine, and the C-4-oxygen of FddThd by the thio group, respectively (Fig.1). As shown in Tab.1, the 5-chloro compound was the most active one among the 5-halogeno-substituted derivatives (13), requiring a dose of 0.7 μM for a 50% (ED_{50}) and of 5.2 μM for a complete protection of MT-4 cells against the cytopathic effect of HIV-1. Though the antiviral activity is about 2.5 times lower than that of the parent compound FddUrd, its cytotoxicity is 6-fold lower on uninfected MT-4 cells. In contrast, the 5-bromo, and 5-fluoro derivatives were found to be less active or less selective. The 4-thio analogue

Table 1. The anti-HIV activity and cytotoxicity of some 3'-fluoro-modified nucleoside analogues in MT-4 cells.

Compound	50% Effective dose ED_{50}; μM	50% Cytotoxic dose CD_{50}; μM
2',3'-Dideoxy-3'-fluoro-thymidine*	0.003	1.1
2',3'-Dideoxy-3'-fluoro-4-thiothymidine	1.0	480
2',3'-Dideoxy-3'-fluoro-uridine	0.275	75
2',3'-Dideoxy-3'-fluoro-5-fluorouridine	50	50
2',3'-Dideoxy-3'-fluoro-5-chlorouridine	0.7	490
2',3'-Dideoxy-3'-fluoro-5-bromouridine	5.0	190

Mean values of at least 3 experiments were given.
*Data from reference (1).

Table 2. The effects of some 3'-fluoro-modified nucleoside triphosphates on the activities of the HIV-RT and of the cellular DNA polymerases α and β. The concentrations required for a 50% inhibition of the enzyme activities (IC_{50}) are given.

Inhibitor	IC_{50}: μM Polymerases		
	HIV-RT*	α	β
2',3'-Dideoxy-3'-fluoro-uridine 5'-triphosphate	0.07 ± 0.01	> 200	3.0
2',3'-Dideoxy-3'-fluoro-5-fluorouridine 5'-triphosphate	0.45 ± 0.08	> 200	15.5
2',3'-Dideoxy-3'-fluoro-5-chlorouridine 5'-triphosphate	0.04 ± 0.006	200	5.0
2',3'-Dideoxy-3'-fluoro-5-bromouridine 5'-triphosphate	0.08 ± 0.019	90	5.0
2',3'-Dideoxy-3'-fluoro-4-thiothymidine 5'-triphosphate	0.2 ± 0.045	140	7.0

* Mean values and standard variations of 4 experiments were given.
0.01 OD of the corresponding primer templates and 10 μM of dTTP as substrate were generally used.

of FddThd, however, is almost as effective (ED_{50}=1.0 μM) and selective as the chloro derivative of FddUrd (13).

Inhibition of HIV-RT and the cellular DNA polymerases α and β.

The described nucleoside analogues must be phosphorylated inside of cells to the 5'-triphosphates before they can attack the HIV-RT, their virtual viral target. Besides the viral polymerases also the cell-own polymerases are exposed to these analogues. Therefore, we have examined the susceptibility of both the HIV-RT and the cellular DNA polymerases α and β to the inhibition by the 3'-fluoro nucleoside 5'-triphosphates. Tab.2 summarizes the results obtained from at least 3 different dose response curves as the concentrations of the analogues required for a 50% inhibition of the enzyme activities (IC_{50}). The 5'-triphosphate of FddUrd and of its 5-bromo derivative emerged as very strong inhibitors of the HIV-RT (IC_{50}=0.07 ± 0.01 and 0.08 ± 0.019 μM). A replacement of hydrogen at the C-5 position by chlorine results in the most effective inhibitor (IC_{50}=0.04 ± 0.006 μM), being slightly more active than the 5'-triphosphates of FddThd and AZT (ID_{50}=0.05 μM) (9,14). The 5-fluoro substitution reduced the activity of the 5'-

Table 3. Inhibition of the proliferation of human cell-lines by 3'-fluoro-modified nucleosides. Doses reducing the cell number to 50% (ID_{50}) are given.

2',3'-Dideoxy-3'-fluoro-	Cell-lines* ID_{50};μM				
	K-562	REH	K-37	H9	MOLT-4
-thymidine+	45	160	260	1000	n.d.
-thiothymidine	575	500	700	800	1000
-uridine	>1000	9000	>1000	>2500	910
-5-chlorouridine	>2000	>1000	>1000	>2000	4200
-5-bromouridine	830	1000	>5000	500	2000

*Deviation: K-562: acute myeloic leukemia; REH: acute lymphatic leukemia; K-37, H9, and MOLT-4: immortalized T-cells.
+Data from reference (1).
Mean values of 3 experiments were given.

triphosphate of FddUrd about 10-fold. The corresponding 4-thio analogue of FddThd was 4-fold less efficient than the parent compound. The data show furthermore an almost complete insensitivity of polymerase α and a partial inhibition of polymerase β, as described earlier for the 5'-triphosphates of sugar-modified nucleosides such as 2',3'-didehydro-2',3'-dideoxythymidine, 2',3'-dideoxythymidine, and FddThd or AZT (9). These results suggest that mechanisms other than inhibition of DNA polymerases α or β must be responsible for the extreme differences in cytotoxicity, e.g., of FddThd and the 5-chloro derivative of FddUrd (see Table 3 and Fig.3).

Kinetics of inhibition of the HIV-RT. Increasing concentrations of the substrate dTTP were used to characterize the nature of HIV-RT inhibition by the 5'-triphosphates of FddUrd and its 5-chloro derivative. As reflected by the double reciprocal plots of the experimental data in Fig.2, the inhibition by the 5'-triphosphate of FddUrd was competitive with dTTP. The K_m value of HIV-RT for dTTP was estimated to be 11.4 ± 1.9 μM, the inhibitor constant K_i was 0.08 ± 0.018 μM) (n=4). Unexpectedly, the Lineweaver-Burk plot suggests a noncompetitive type of inhibition for the 5'-triphosphate of the 5-chloro analogue of FddUrd (Fig.2, right).

Effects on proliferation of human cell-lines. Besides the 5-fluoro analogue, all other nucleosides were evaluated for their effects on proliferation of some human cell-lines. In agreement with previous data (1), the tested cell-lines tolerated much

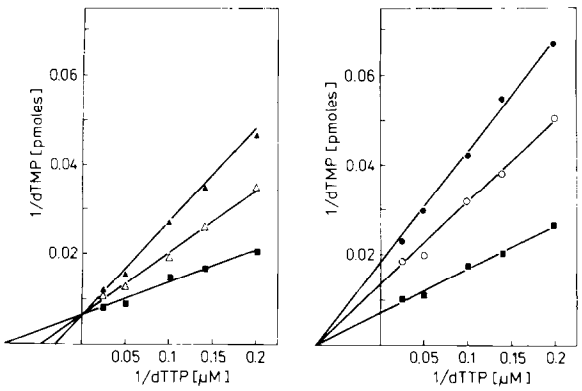

Fig.2. Kinetics of inhibition of HIV-RT by the 5'-triphosphates of FddUrd (left) and its 5-chloro derivative (right). Double reciprocal plots of the reaction velocities in the presence of different concentrations of dTTP with 0.01 OD polyA · oligo(dT)$_{10}$ as template primer. No inhibitor (■); 5'-triphosphate of FddUrd: 0.03 μM (Δ) or 0.1 μM (▲); 5'-triphosphate of the 5-chloro derivative of FddUrd: 0.03 μM (O) or 0.1 μM (●).

higher doses of FddUrd than FddThd (Tab.3). With the exception of the MOLT-4 cells, the doses inhibiting cell proliferation by 50% were noticed for FddUrd at the millimolar range. The 5-chloro analogue of FddUrd hardly influenced the growth of all tested cell-lines at concentrations up to 1 mM. For the 5-bromo analogue of FddUrd and the 4-thio derivative of FddThd the most sensitive cell-lines have an ID_{50} of 500 μM.

Effects on colony formation of mouse myeloid progenitor cells (CFU-GM). In agreement with the effects on human cell-lines, the colony formation of mouse CFU-GM was much less influenced by FddUrd and the 5-chloro, and 5-bromo derivatives and by the 4-thio analogue of FddThd in comparison to FddThd itself (Fig.3). The 50% inhibitory doses (ID_{50}) were 15 μM for FddThd against

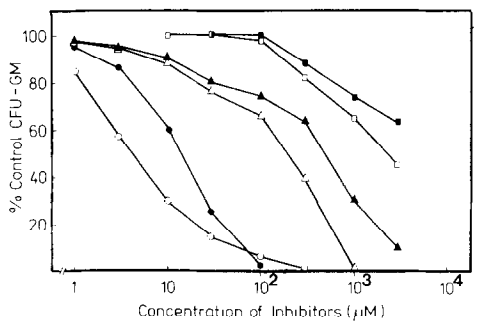

Fig.3. Inhibitory effects of modified nucleosides on colony formation of mouse CFU-GM given as percent of control. FddUrd (□), its 5-chloro- (■), its 5-bromo- (▲) derivative; FddThd (●), its 4-thio analogue (Δ); 2'-deoxy-5-chlorouridine (O).

2200 μM for FddUrd and 9000 μM for its 5-chloro, and 500 μM for its 5-bromo derivative, and 200 μM for the 4-thio analogue of FddThd. AZT gave in our system an ID_{50} of 45 μM, whereas Somadossi et al. (15) have found for human CFU-GM an ID_{50} of 0.9 μM, variations which may be caused by species differences. In any case, the myeloid progenitor cells tolerated much higher doses of the described 3'-fluoro-modified nucleoside analogues than of AZT or FddThd itself. Of special interest is the antiproliferative activity of 2'-deoxy-5-chlorouridine on mouse CFU-GM. The ID_{50} is 4.2 μM but it is, unlike the corresponding 3'-fluorine-containing derivative, without effect against HIV-1 in MT-4 cells up to 5.0 μM (CD_{50}=0.8 μM). These data demonstrate that the replacement of the 3'-OH-group by fluorine generates both its efficient anti-HIV activity and low cytotoxicity.

CONCLUSIONS

The 5'-triphosphate of FddUrd and of its 5-chloro, and 5-bromo derivatives (IC_{50}=0.04-0.08 μM) proved to be some of the most active inhibitors of HIV-1 reverse transcriptase, whereas their efficiency as nucleosides to block the viral cytopathic effect on MT-4 cells varies widely (ED_{50}=0.275-5.0 μM). This probably reflects great differences in their intracellular metabolism, especially the phosphorylation (16). Preliminary results show that the 5-chloro derivative of FddUrd has a more than 10-fold higher affinity to the thymidine kinase than the parent compound. The 5'-monophosphate of FddUrd seems to be both methylated and further phosphorylated, resulting in small amounts of the 5'-triphosphate of FddThd inside of cells.

The virtual absence of an antiproliferative activity of FddUrd and its 5'-chloro derivative and the low cytotoxicity of the 4-thio analogue of FddThd together with their high anti-HIV activity recommend these nucleoside analogues for further evaluation as selective antiretroviral drugs, especially for the treatment of AIDS.

During the preparation of this manuscript a paper by Balzarini et al. (16) came to our knowledge describing a similar anti-HIV-1 activity of the 5-chloro derivative of FddUrd but showing different data concerning the cytotoxicity of the parent compound.

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REFERENCES

1. Matthes, E., Lehmann, Ch., Scholz, D., Rosenthal, H.A. and Langen, P. (1988) Biochem.Biophys.Res.Commun. 153, 825-831.
2. Hartmann, H., Vogt, M.W., Durno, A.G., Hirsch, M.S., Hunsmann, G., and Eckstein, F. (1988) AIDS Res. and Human Retroviruses 4, 457-466.
3. Bazin, H., Chattopadhyaya, J., Datema, R., Ericson, A.-C., Gilljam, G., Johansson, N.G., Hansen, J., Koshida, R., Moelling, K., Öberg, B., Remaud, G., Stening, G., Vrang, L., Wahren, B., and Wu, J.C. (1989) Biochem.Pharmacol. 38, 109-119.
4. Balzarini, J., Baba, M., Pauwels, R., Herdewijn, P., and De Clercq, E. (1988) Biochem.Pharmacol. 37, 2847-2856.
5. 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.
6. Matthes, E., Lehmann, Ch., Scholz,D., von Janta-Lipinski,M., Gaertner, K., Langen, P., and Rosenthal, H.A. (1987) EP 0 254 268 A2.
7. Matthes, E., Lehmann, Ch., Drescher, B., Büttner, W., and Langen, P. (1985) Biomed.Biochim.Acta 44, K63-K73.
8. Hansen, J., Schulze, T., and Moelling, K. (1987) J.Biol.Chem. 262, 12393-12396.
9. Matthes, E., Lehmann,Ch., Scholz, D., von Janta-Lipinski,M., Gaertner, K., Rosenthal, H.A., and Langen, P. (1987) Biochem. Biophys.Res.Commun. 148, 78-85.
10. Popovic, M., Sarngadharan, M.G., Read, E., and Gallo, R.C. (1984) Science 224, 497-500.
11. Harada, S., Koyanagi, Y., and Yamamoto, N. (1985) Science 229, 563-566.
12. Metcalf, D. (1984) In Clonal Culture of Hemopoietic Cells pp. 125-126. Elsevier, Amsterdam.
13. Matthes, E., Scholz, D., Sydow, G., von Janta-Lipinski, M., Rosenthal, H.A., and Langen, P. (1989) Z.Klin.Med., in press.
14. Cheng, Y., Dutschman, G.E., Bastow, K.F., Sarngadharan, M.G., and Ting, R.Y.C. (1987) J.Biol.Chem. 262, 2187-2189.
15. Sommadossi, J.-P., and Carlisle, R. (1987) Antimicrob.Agents Chemother. 31, 452-454.
16. Balzarini, J., Van Aerschot, A., Herdewijn, P., and De Clercq,E., (1989) Biochem.Pharmacol. 38, 869-874.