Biochemical Pharmacology, Vol. 38, No. 1, pp. 109–119, 1989.
Printed in Great Britain.

0006-2952/89 $3.00 + 0.00

AN ANALYSIS OF THE INHIBITION OF REPLICATION OF HIV AND MULV BY SOME 3′-BLOCKED PYRIMIDINE ANALOGS

HERVE BAZIN,* JYOTI CHATTOPADHYAYA,* ROELF DATEMA,†‡ ANN-CHRISTIN ERICSON,† GUSTAV GILLJAM,§ NILS GUNNAR JOHANSSON,† JUTTA HANSEN,‖ RIE KOSHIDA,* KARIN MOELLING,‖ BO ÖBERG,† GERALD REMAUD,* GÖRAN STENING,† LOTTA VRANG,† BRITTA WAHREN§ and JIN CHENG WU*

*Department of Bioorganic Chemistry, University of Uppsala, Biomedical Center, S-751 25 Uppsala; †Department of Antiviral Therapy, Astra Alab AB, S-151 85 Södertälje; §National Bacteriological Laboratory, S-105 21 Stockholm, Sweden; ‖Max-Planck-Institut für Molekulare Genetik, Ihnestrasse 63-73, D-1000 Berlin 33, Federal Republic of Germany

(*Received* 21 *March* 1988; *accepted* 28 *July* 1988)

Abstract—Some 3′-blocked pyrimidine analogs were synthesized and tested as inhibitors of replication of human immunodeficiency virus (HIV) and Moloney-murine leukemia virus (MuLV). The analogs were of 3 kinds: (1) analogs of 3′-azido-3′-deoxythymidine (AZT) in which the C-5 CH_3 of the base was exchanged for H (AZU) or C_2H_5 (AZEU); (2) 3′-fluoro-3′-deoxythymidine (FLT) and analogs thereof, in which the C-5 CH_3 of the base was exchanged for H (FLU), C_2H_5 (FLEU) or nC_3H_7 (FLPU); (3) the *threo* analogs of AZT (AZT↑) and AZU (AZU↑).

All analogs were less active inhibitors of HIV replication than AZT, except FLT, which was as active as AZT. The 3′-fluoro analogs and AZEU did not inhibit MuLV replication at non-cytotoxic concentrations. Oral administration of FLT to MuLV-infected mice result in antiviral effects only at toxic drug levels. AZU and FLU were less potent inhibitors of HIV replication than AZT or FLT, but the 2′-deoxy uridine analogs were also less cytotoxic to human embryonic fibroblasts than the thymidine analogs.

The 5′-triphosphates of AZU, AZT, AZEU, FLT and FLEU were tested as inhibitors of the HIV- and MuLV-reverse transcriptases. Ranking of the K_i/K_m values for HIV-RT resulted in the following order of potency of the 5′-triphosphates AZT = FLT > AZU > AZEU > FLEU. The 5′-triphosphates of AZEU, FLT and FLEU did not inhibit the MuLV-RT, which explains, in part, the lack of effect of these analogs against MuLV replication.

The *threo* forms (azido "up") of AZU and AZT were less active inhibitors of HIV replication than the erythro forms (azido "down"). A ^{15}N-NMR and ^{1}H-NMR study showed that the furanose moieties of analogs with the azido function "up" assume a conformation distinct from that of the analogs with azido "down". This is due to intramolecular stabilisation of the "N" conformer in the *threo* ("up") diastereomer, due to interaction of the azido functions with the nucleobase and possibly the OH group of C-5′ of the furanose. As discussed, this conformation might explain the decreased biological activity of *threo* forms compared with the *erythro* forms.

The viral nucleic acid polymerases are preferred targets for antiviral agents [1–4]. Also for the treatment of HIV-induced disease, analogs of nucleosides, nucleotides and pyrophosphate, targeted towards HIV-reverse transcriptase (RT),¶ are currently receiving considerable attention [5, 6]. The thymidine analog, 3′-azido-3′-deoxythymidine (AZT) is, for example, a potent inhibitor of HIV reverse transcription [7], and has shown clinical efficacy [8]. The antiviral effect of AZT is probably caused by the nucleoside analog-triphosphate (AZT-TP) inhibiting the viral reverse transcriptase [9, 10].

Here we present a study on some analogs of AZT. Specifically, we have investigated antiviral (HIV and MuLV) and cytotoxic effects of (1) analogs of AZT modified in C-5, (2) analogs of 3′-fluoro-3′-deoxy thymidine (FLT), another anti-retroviral agent [11], modified in C-5, and (3) the *threo* and *erythro* forms of AZT and AZU. See Fig. 1 for structures. In addition, to obtain a chemical basis for structure–activity relationships, we studied inhibition of HIV- and MuLV-RT by triphosphates of AZT- and FLT-analogs and the conformations of the *erythro* and *threo* forms of AZT and AZU.

MATERIALS AND METHODS

Chemicals. Commercially available deoxynucleoside triphosphates were from Sigma Chemical Co.

‡ Corresponding author.

¶ Abbreviations used: RT, reverse transcriptase; HIV, human immunodeficiency virus; Mo-Mulv, Moloney murine leukemia virus; Ra-MuLV, Rauscher murine leukemia virus; TP, 5′-*O*-triphosphate; AZEU, 1-(3′-azido-2′,3′-dideoxy-β-D-*erythro*-pentofuranosyl)-5-ethyluracil; AZU, 1-(3′-azido-2′,3′-dideoxy-β-D-*erythro*-pentofuranosyl)uracil; AZU↑, 1-(3′-azido-2′,3′-dideoxy-β-D-*threo*-pentofuranosyl)uracil; FLEU, 1-(3′-fluoro-2′,3′-dideoxy-β-D-*erythro*-pentofuranosyl)-5-ethyluracil; FLU, 1-(3′-fluoro-2′,3′-dideoxy-β-D-*erythro*-pentofuranosyl)uracil; FLPU, 1-(3′-fluoro-2′,3′-dideoxy-β-D-*erythro*-pento-furanosyl)-5-propyluracil; FLT, 1-(3′-fluoro-2′,3′-dideoxy-β-D-*erythro*-pentofuranosyl)thymine; AZT, 1-(3′-azido-2′,3′-dideoxy-β-D-*erythro*-pentofuranosyl)thymine.

(St. Louis, MO). [^{3}H]dTTP was purchased from New England Nuclear Corp. (Boston, MA). (Riboadenylic acid)$_n$ (deoxythymidylic acid)$_{12-18}$ ((rA)$_n$(dT)$_{12-18}$) was from Pharmacia, Uppsala, Sweden.

The Moloney-MuLV reverse transcriptase was obtained from Pharmacia. The HIV-reverse transcriptase was a recombinant product purified from cultures of *E. coli* expressing the cloned *pol*-gene [12].

Reverse transcriptase assays. For HIV-reverse transcriptase, the 100-μl reaction mixture contained 50 mM Tris–HCl (pH 8.0), 50 mM KCl, 4 mM dithithreitol, 6 mM $MgCl_2$, 50 μg of bovine serum albumin Fraction V, 1 μg of (rA)$_n$(dT)$_{12-18}$, the indicated concentrations of [^{3}H]dTTP (0.5–10 μM; specific activity 5500–1100 cpm/pmol) and 0.03 U enzyme preparation.

After incubation at 37° for 40 min, 40 μl of the reaction mixtures (in duplicate) were spotted on paper disks (Munktell No. 5; 24 mm) and washed four times in ice-cold 5% trichloroacetic acid/0.02 M pyrophosphate and three times in ethanol. The dried paper disks were counted in 3 ml of Econofluor scintillation solution. At saturation with respect to (rA)$_n$(dT)$_{12-18}$ and dTTP, 20 μl of enzyme preparation incorporated 45 pmol of dTMP per 40 min at 37°. The enzyme reactions were linear with time in the experiments described.

The Moloney-MuLV reverse transcriptase assay was done in 100 μl reaction mixture containing 50 μM Tris–HCl (pH 8.3), 10 mM dithiothreitol, 8 mM $MgCl_2$ and 16 μg bovine serum albumin Fraction V, 0.16 μg (rA)$_n$(dT)$_{12-18}$, the indicated concentrations of [^{3}H]dTTP and 0.25 U enzyme (as defined by the producer). The mixtures were incubated for 30 min at 37°, and radioactivity incorporated into acid-precipitable material was measured as described above.

Viruses. Human immunodeficiency virus (HIV) was obtained from the culture supernatant of a persistently HTLV-III$_B$-infected H$_9$ cell line [13]. Moloney murine leukemia virus (Mo-MuLV, referred to below as MuLV unless indicated otherwise) was obtained from Dr. E. M. Fenyö (Karolinska Institute, Dept. of Virology, Stockholm). Virus stocks were prepared from the supernatant of infected 3T3 cells at day 3 p.i. Rauscher-MuLV (RVB-3) was obtained from Dr. M. Strand (Johns Hopkins University, Baltimore). Virus stocks were prepared from spleens of infected Balb/c mice (see below), taken 18 days p.i., according to the protocol described below.

Cells. H$_9$ cells (H$_9$ is a human CD$_4$-positive lymphoid cell line) were grown in suspension in RPMI-1640 medium supplemented with 10% fetal calf serum. Flow 5000 is a human embryo fibroblast cell line used between passages 15–25; 3T3 (CCL 92) is a Swiss mouse embryo fibroblast cell line. Flow 5000 and 3T3 cells were grown in minimal essential medium with Earle's salts, supplemented with non-essential amino acids, 10 mM Hepes buffer (pH 7.2) and 10% fetal calf serum.

Determination of antiviral effects in cell cultures. The inhibitory effect of the compounds on expression of HIV antigens was determined as follows. Uninfected H$_9$ cells (50,000 in 0.5 ml) were plated in 24-well microplates together with tenfold dilutions of each drug (1.0 ml). Immediately following this plating, 0.5 ml of HIV in two concentrations (4-fold apart) was added to each well, giving a total volume of 2 ml and final drug concentrations ranging from 0.001 μM to 100 μM. After incubation in 5% CO_2 in air at 37° for 6 days without medium change, HIV antigen content was measured in cells by immunofluorescence.

For immunofluorescence, cells from each well were washed, spread on 8-well slides and stained with human anti-HIV anti-serum (containing IgG to all major HIV components) following by fluorescein-isothiocyanate labelled sheep anti-human IgG (all reagents from SBL, Stockholm, Sweden). The stained cells were counted and the concentration of drug giving 50% of control (without drug) was the estimate of the IC_{50}.

To determine the inhibition of Moloney- and Rauscher-MuLV replication the XC-plaque test was used [14]. Cultures of growing 3T3 cells (in minimal essential medium and 10% fetal calf serum) were infected with 0.2 ml of a virus dilution in presence of polybrene (4 μg/ml). The virus dilution of every preparation was determined so as to obtain *ca.* 100 syncytia per microscope field. Inhibitors were added immediately after infection. Confluent cultures of infected cells, after approx. 3 days, were UV-irradiated and XC cells (10^5 cells/ml) were added. Two days after this addition, syncytia were counted after staining with crystal-violet, according to standard procedures. The effects of the compounds of the replication of Moloney- and Rauscher-MuLV were identical, and only the values for the Moloney virus are reported.

Determination of drug toxicity in proliferating cells. Effects on cell proliferation were studied as described previously [15], test compound being added to actively growing cells, and the effects were determined after 48 hr. Cell numbers were determined by a cell counter (Analysinstrument AB, Stockholm, Sweden).

Animals and virus inoculation. Male Balb/c mice weighing 16–18 g and five weeks of age were inoculated intravenously (tail vein) with approximately 10^6 SFU (syncytium forming units) of Ra-MuLV per mouse [16]. This results in disease as described below. Treatment was by drugs dissolved in tap water given orally via the drinking water, and was started immediately after infection, and contained for 15 days.

To determine viral titers of spleens of infected mice, spleens were homogenized in minimal essential medium containing 10% fetal calf serum and serial dilutions of the homogenate were added to growing 3T3 cells. White blood cell counts were determined in 20 μl blood according to standard procedures using a cell counter. Student's *t*-test was used for statistical evaluation of mean numbers and animal weights. The viral titers were compared by the Mann-Whitney U-test. Differences of $P < 0.05$ for two-tailed tests were considered significant.

NMR-spectroscopy. ^{15}N NMR spectra have been recorded at 27.4 MHz on a Jeol JNM GX 270 spectrometer using a probe-head of 10 mm. The ^{15}N chemical shifts were determined from

gated-proton decoupled spectra (without NOE) and were referenced against an external solution of $CH_3{}^{15}NO_2$ in CD_3NO_2. Typical parameters used were: about 35° pulse angle, pulse delay approx. 35 sec, 12 hr for accumulation time. The ^{15}N resonances of the azido group of AZT and AZT↑ were studied as function of temperature. Due to difficulties in recording ^{15}N-NMR spectra at natural abundance [17], the ^{15}N resonances of AZU and AZU↑ were only recorded at 35°.

^1H NMR spectroscopy for conformational studies has been carried out at 270 MHz on the same spectrometer using a probe with tubes of 5 mm o.d. TMS was used as internal reference in acetone-d_6.

Synthesis. FLT was obtained from Calbiochem. The other 3'-fluoro analogs were prepared by trimethylsilyl trifluoromethanesulfonate catalyzed transglycosylation from silylated FLT. In addition, FLEU was also synthesized by a method used for synthesis of FLT [18, cf. also 11]. The two different methods used for synthesis of FLEU gave identical compounds. 5-Ethyl-2'-deoxyuridine was obtained from Robugen GmbH, Erlangen (F.R.G.).

AZU, AZEU and AZU↑ were prepared analogous to the syntheses described for AZT and AZT↑, respectively [10]. They were converted to their respective 5'-triphosphates, by reaction with phosphorous oxychloride followed by bis-(tributylammonium)-pyrophosphate [10, 19].

Miscellaneous. Melting points were uncorrected. ^1H-NMR spectra at 90 MHz and ^{13}C NMR at 23.7 MHz were recorded with Jeol FX 900 instrument, and at 200 MHz with Jeol FX 200 spectrometer. Tetramethylsilane was used as the internal standard and the chemical shifts are reported in ppm (δ scale). UV absorption spectra were recorded with a Varian-Cary 2200 instrument and Jeol DX 303 instrument was used for recording the mass spectra. IR absorption was recorded with Perkin-Elmer 298 spectrometer. Thin-layer chromatography (TLC) was performed on Merck precoated $60F_{254}$ plates. Merck Kieselgel G was used for short column chromatography. Before collection of spectral data and biological results, the compounds were examined by TLC (see below) for homogeneity, and only of pure material, results are reported.

1-(5'-O-*trityl*-2'-*deoxy*-β-D-*erythro-pentofuranosyl*)-5-*ethyl-uracil* (**1a**). 5-Ethyl-2'-deoxyuridine (350 mg, 1.36 mmol) was dissolved in dry pyridine (5 ml) and trityl chloride (454 mg, 1.63 mmol) was added. The mixture was stirred at 100° for 2.5 hr and then poured into a saturated solution of sodium bicarbonate (30 ml). The suspension was extracted with dichloromethane (2 × 30 ml), the organic phase was dried over $MgSO_4$ and evaporated to give a residue. The residue was purified upon a silica gel column to give **1a**, yield 575 mg (85%). ^1H NMR ($CDCl_3$): 9.12 (broad, s, 1H) NH; 7.32 (m, 16H) arom., H-6; 6.42 (t, $J_{1',2'}$ = 7.8 Hz, 1H) H-1'; 4.55 (m, 1H) H-3'; 4.00 (m, 1H) H-4'; 3.41 (m, 2H) H-5',5''; 2.85 (br., 1H) OH; 2.34 (m, 2H) H-2',2''; 1.87 (m, 2H) CH_2; 0.84 (t, $J_{CH_2-CH_3}$ = 7.6 HZ, 3H) CH_3.

1-(5'-O-*trityl*-3'-O-*methylsulfonyl*-2'-*deoxy*-β-D-*erythro-pentofuranosyl*)-5-*ethyl-uracil*) (**2a**). To a cold solution of compound **1a** (555 mg, 1.11 mmol) in dry pyridine (6 ml) was added methylsulfonyl chloride (381 mg, 3.33 mmol) and the mixture was kept at 0° overnight. Cold water (1 ml) was added and the reaction was kept at 0° for another hour. The solution was poured into crushed ice–water (200 ml) slowly with vigorous stirring. The precipitate was filtered and washed generously with cold water till free of pyridine. The solid was dried, yield 510 mg (80%). ^1H NMR ($CDCl_3$): 8.48 (br., 1H) NH; 7.34 (m, 16H) arom., H-6; 6.42 (m, 1H) H-1'; 5.41 (m, 1H) H-3'; 4.32 (m, 1H) H-4'; 3.49 (m, 2H) H-5',5''; 3.03 (s, 3H) CH_3SO_2; 2.25 (m, 2H) H-2',2''; 1.93 (m, 2H) CH_2CH_3; 0.85 (t, $J_{CH_2CH_3}$ = 7.6 HZ) CH_2CH_3.

1-(5'-O-*trityl*-2,3'-*anhydro*-2'-*deoxy*-β-D-*erythro-pentofuranosyl*)-5-*ethyl-uracil* (**3a**). To a solution of compound **2a** (497 mg, 0.81 mmol) in dry dichloromethane (5 ml) was added diazobicycloundecane (DBU) (164 mg, 0.9 mmol) and the mixture was stirred at room temperature overnight. One more portion of DBU (164 mg, 0.9 mmol) was added and stirring was continued till starting material disappeared. Solvent was removed and the residue was purified on a silica gel column to give **3a**, yield 369 mg (95%). ^1H-NMR ($CDCl_3$): 7.32 (m, 15H) arom.; 6.85 (s, 1H) H-6; 5.49 (d, $J_{1',2'}$ = 3.4 Hz) H-1'; 5.11 (m, 1H) H-3'; 4.25 (m, 1H) H-4'; 3.36 (d, $J_{4',5'}$ = 6.4 Hz) H-5',5''; 2.56–2.24 (m, 4H) H-2',2'',CH_2CH_3; 1.13 (t, $J_{CH_2CH_3}$ = 7.2 HZ, 3H) CH_2CH_3.

1-(5'-O-*trityl*-3'-*azido*-2',3'-*dideoxy*-β-D-*erythro-pentofuranosyl*)-5-*ethyl-uracil* (**4a**). The mixture of compound **3a** (360 mg, 0.75 mmol), lithium azide (110 mg, 2.25 mmol) and ammonium chloride (80 mg, 1.5 mmol) in dry dimethylformamide (8 ml) was stirred at 110° overnight. Solvent was removed by coevaporations with toluene. The residue was partitioned between ethyl acetate (100 ml) and water (20 ml). The organic phase was washed with water (20 ml), evaporated to dryness and subsequently purified by a silica gel column to give **4a** yield 219 mg (56%). ^1H-NMR ($CDCl_3$): 8.55 (br., 1H) NH; 7.34 (m, 16H) arom. H-6; 6.26 (t, $J_{1',2'}$ = 6.8 Hz, 1H) H-1'; 4.30 (m, 1H) H-3'; 3.99 (m, 1H) H-4'; 3.43 (m, 2H) H-5',5''; 2.40 (m, 2H) $H_{2',2''}$; 1.98 (m, 2H) CH_2CH_3; 0.86 (t, $J_{CH_2CH_3}$ = 7.4 Hz, 3H) CH_2CH_3; IR (KBr): ν_{max} 2100 cm^{-1} (azido). $C_{30}H_{29}N_5O_4$: calc. 523.2220, found 523.2371.

1-(3'-*Azido*-2',3'-*dideoxy*-β-D-*erythro-pentofuranosyl*)-5-*ethyluracil* (*AZEU*) (**5a**). Compound **4a** (296 mg, 0.57 mmol) was dissolved in 80% acetic acid and the solution was heated under reflux for 10 min. The reaction mixture was cooled and the volatile matters were evaporated. Water (20 ml) was added and the suspension was filtered. The solid was washed with water (10 ml) and the filtrate was extracted with hexane (2 × 10 ml). The aqueous phase was concentrated and freeze-dried to give AZEU yield 125 mg (90%). ^1H-NMR (DMSO-d_6): 9.1 (br., 1H) NH; 7.71 (s, 1H) H-6; 6.15 (t, $J_{1',2'}$ = 6.5 Hz, 1H) H-1'; 4.40 (m, 1H) H-3'; 3.92 (m, 1H) H-4'; 3.66 (m, 2H) H-5',5''; 2.31 (m, 4H) H-2',2'', CH_2CH_3; 1.10 (t, $J_{CH_2CH_3}$ = 6.4 Hz, 3H) CH_2CH_3. IR (KBr): ν_{max} 2100 cm^{-1} (azido). ^{13}C NMR ($CDCl_3$): 86.9 (C-4'); 84.5 (C-1'); 61.8 (C-5'); 59.9 (C-3'); 37.2 (C-2'); 20.0 (CH_2CH_3); 12.4 (CH_2CH_3).

$C_{11}H_{15}N_5O_4$: calc. 282.1202 $(M + H)^+$, found 282.1234.

1-(3'-*Azido*-2',3'-*dideoxy*-5'-*tetra*-(*triethylammonium*)*triphospho*-β-D-*erythro*-*pentofuranosyl*)-5-*ethyl*-*uracil* (*AZEU-TP*) (**6a**). The tetra (triethylammonium) salt of AZEU-PPP was prepared from 14 mg of AZEU, analogous to what has been described for the preparation of AZT-TP [10]. Yield 28 mg (70%). ^{1}H-NMR (D_2O): 7.70 (s, 1H) H-6; 6.28 (t, $J_{1',2'}$ = 6.6 Hz, 1H) H-1'; 4.47 (m, 1H) H-3'; 4.22 (d, 3H) H-4',5',5''; 3.18 (q, 24H) NCH_2CH_3; 2.41 (m, 4H) H-2',2'', CH_2CH_3; 1.27 (t, 36H) NCH_2CH_3, 1.08 (t, $J_{CH_2CH_3}$ = 7.4 Hz, 3H) CH_2CH_3, ^{31}P NMR (D_2O): −10.1 (d, J = 19.9 Hz) γ-P; −11.4 (d, J = 19.3 Hz). α-P; −22.9 (t) β-P. IR (KBr): ν_{max} 2100 cm^{-1} (azido). UV (water): λ_{max} 267 (ε = 8900).

1-(5'-O-*trityl*-2'-*deoxy*-β-D-*erythro*-*pentofuranosyl*)*uracil* (**1b**). Yield = 92%. ^{1}H-NMR ($CDCl_2$): 9.10 (br., 1H) NH; 7.75 (d, $J_{5,6}$ = 8.4 Hz, 1H) H-6; 7.36 (m, 15H) arom., 6.28 (t, $J_{1',2'}$ = 6.0 Hz, 1H) H-1'; 5.38 (d, 1H) H-5; 4.46 (m, 1H) H-3'; 4.01 (m, 1H) H-4'; 3.44 (m, 2H) H-5',5''; 2.38 (m, 2H) H-2',2''.

1-(5'-O-*trityl*-3'-O-*methylsulfonyl*-2'-*deoxy*-β-D-*erythro*-*pentofuranosyl*)*uracil* (**2b**). Yield = 90%. ^{1}H-NMR ($CDCl_3$): 9.15 (br., 1H) NH; 7.68 (d, $J_{5,6}$ = 7.68 Hz, 1H) H-6; 7.36 (m, 15H) arom.; 6.35 (dd, $J_{1',2'}$ = 6.1 Hz, $J_{1',2''}$ = 5.9 Hz, 1H) H-1'; 5.40 (m, 2H) H-5, H-3'; 4.32 (m, 1H) H-4'; 3.50 (m, 2H) H-5',5''; 3.01 (s, 3H)-CH_3; 2.55 (m, 2H) H-2',2''.

1-(5'-O-*trityl*-2',3'-*anhydro*-2'-*deoxy*-β-D-*erythro*-*pentofuranosyl*) *uracil* (**3b**). Compound (**2b**) (2.19 g, 4 mmol) was dissolved in ethanol (80 ml) and sodium hydroxide (1 M, 4 ml) was added. The mixture was refluxed 7 min. Solvent was evaporated to dryness and the residue was separated with silica gel column to give **3b**. Yield 1.25 g (68%). ^{1}H-NMR ($CDCl_3$): 7.30 (m, 16H) arom., H-6; 5.85 (d, $J_{5,6}$ = 7.2 Hz, 1H) H-5; 5.54 (d, $J_{1',2'}$ = 3.5 Hz, 1H) H-1'; 5.09 (m, 1H) H-3'; 4.23 (m, 1H) H-4'; 3.32 (m, 2H) H-5',5''; 2.50 (m, 2H) H-2',2''.

1-(5'-O-*trityl*-3'-*azido*-2',3'-*dideoxy*-β-D-*erythro*-*pentofuranosyl*) *uracil* (**4b**). Compound **3b** (232 mg, 0.5 mmol), NaN_3 (98 mg, 1.5 mmol) was suspended in dimethylformamide (4 ml) and water (0.5 ml). The mixture was heated at 110° overnight. The insoluble matters were removed by filtration and the filtrate was coevaporated with toluene to dryness. The residue was purified with silica gel column to give **4b**. Yield 150 mg (60%). ^{1}H-NMR ($CDCl_3$): 9.41 (br., 1H) NH; 7.84 (d, $J_{5,6}$ = 8.1 Hz, 1H) H-6; 7.34 (m, 15H) arom., 6.20 (t, $J_{1',2'}$ = 5.9 Hz, 1H) H-1'; 5.41 (d, 1H) H-5; 4.34 (m, 1H) H-3'; 3.94 (m, 1H) H-4'; 3.47 (m, 2H) H-5',5''; 2.44 (m, 2H) H-2',2''. IR (KBr) = 2100 cm^{-1} (azido). $C_{28}H_{25}N_5O_4$: calc. 496.1985 $(M + H)^+$, found 496.1874.

1-(5'-O-*trityl*-3'-*azido*-2',3'-*dideoxy*-β-D-*threo*-*pentofuranosyl*)*uracil* (**7**). Compound **2b** (1.1 g, 2 mmol) was dissolved in dry tetrahydrofuran (20 ml) and a dry crystalline complex of potassium azide and 18-crown-6 (1.1 g, 2.56 mmol) was added and refluxed under nitrogen for 24 hr. The reaction mixture was cooled and diethyl ether (10 ml) was added. The precipitate was filtered off and the filtrate was evaporated to give a gum which was dissolved in dichloromethane (50 ml) and washed with saturated potassium chloride (20 ml), water (20 ml). The organic phase was evaporated and purified with silica gel column to give **5**. Yield 0.68 g (69%). ^{1}H-NMR ($CDCl_3$): 9.20 (br., 1H) NH; 7.32 (m, 16H) arom., H-6; 6.15 (dd, $J_{1',2'}$ = 2.3 Hz, $J_{1',2''}$ = 7.2 Hz, 1H) H-1'; 5.65 (d, $J_{5,6}$ = 7.8 Hz, 1H) H-5; 4.23 (m, 2H) H-3', H-4'; 3.52 (m, 2H) H-5',5''; 2.64–2.28 (m, 2H) H-2',2''. IR (KBr) = 2100 cm^{-1} (azido). $C_{28}H_{25}N_5O_4$: calc. 496.1985 $(M + H)^+$, found 496.2103.

1-(3'-*Azido*-2',3'-*dideoxy*-β-D-*erythro*-*pentofuranosyl*)*uracil* (*AZU*) (**5b**). Yield 87%. ^{1}H-NMR (DMSO-d_6): 8.25 (d, $J_{5,6}$ = 8.1 Hz, 1H) H-6; 6.48 (t, $J_{1',2'}$ = 6.4 Hz, 1H) H-1'; 6.05 (d, 1H) H-5; 5.03 (br., 1H) OH; 4.80 (m, 1H) H-3'; 4.25 (m, 1H) H-4'; 4.02 (m, 2H) H-5',5''; 2.74 (m, 2H) H-2',2''; ^{13}C NMR (D_2O + CD_3COCD_3): 85.0 (C-4'); 84.2 (C-1'); 60.8 (C-5') 59.8 (C-3'); 36.2 (C-2'). IR (KBr): 2100 cm^{-1} (azido). $C_9H_{11}N_5O_4$: calc. 254.0889 $(M + H)^+$, found 254.1011.

1-(3'-*Azido*-2',3'-*dideoxy*-β-D-*threo*-*pentofuranosyl*)*uracil* (*AZU*↑) (**8**). Yield 76%. ^{1}H-NMR (DMSO-d_6): 7.64 (d, $J_{5,6}$ = 7.8 Hz, 1H) H-6; 6.02 (dd, $J_{1',2'}$ = 3.0 Hz, $J_{1',2''}$ = 6.6 Hz, 1H) H-1'; 5.68 (d, 1H) H-5; 5.07 (br., 1H) OH; 4.47 (m, 1H) H-3'; 4.04 (m, 1H) H-4'; 3.70 (m, 2H) H-5',5''; 2.87–1.96 (m, 2H) H-2',2''; ^{13}C NMR (D_2O): 84.8 (C-1'); 83.0 (C-4'); 60.3 (C-3'); 59.6 (C-5'); 37.5 (C-2'). IR (KBr): 2100 cm^{-1} (azido). $C_9H_{11}N_5O_4$: calc. 254.0889 $(M + H)^+$, found 254.0997.

1-(3'-*Azido*-2',3'-*dideoxy*-5'-*tetra*-(*triethylammonium*)*triphospho*-β-D-*erythro*-*pentofuranosyl*)*uracil* (*AZU-TP*) (**6b**). Yield 53%. ^{1}H-NMR (D_2O): 7.83 (d, $J_{5,6}$ = 8.4 Hz, 1H) H-6; 6.13 (t, $J_{1',2'}$ = 6.8 Hz, 1H) H-1'; 5.83 (d, 1H) H-5; 4.46 (m, 1H) H-3'; 4.09 (d, 3H) H-4', H-5',5''; 2.37 (q, 2H) H-2',2''; 3.18 (m, 24H) NCH_2CH_3; 1.14 (t, 36H) NCH_2CH_3, ^{31}P-NMR (D_2O): −9.1 (d, J = 17.7 Hz) δ-P; −11.2 (d, J = 21.3 Hz) α-P; −22.3 (t, J = 19.5 Hz) β-P. UV (H_2O) λ_{max} 260 nm (ε = 8200). IR (KBr): 2100 cm^{-1} (azido).

1-(3'-*Fluoro*-2',3'-*dideoxy*-β-D-*erythro*-*pentofuranosyl*)-5-*ethyluracil* (*FLEU*) (**10**). 5-Ethyluracil (51 mg) and 3'-F-3'-deoxythymidine (48 mg) were suspended in acetonitrile (1.2 ml) and N,O-bis(trimethylsilyl)-acetamide (0.35 ml) was added. The mixture was stirred at room temperature for 1.5 hr. Trimethylsilyl trifluoromethane sulfonate (0.05 ml) was added. After stirring at room temperature for about 160 hr, the solvent was evaporated in vacuum, water (0.5 ml) was added, the mixture was filtered and washed with water (0.5 ml). The combined water phase was applied to a C_{18}-column (HPLC) and eluted with methanol–water (1:3) at a rate of 8.0 ml/min. The desired β-anomer, FLEU, eluted after 12.3 min and the α-anomer after 16.4 min. Yield 7.3 mg (14%). UV λ_{max} (H_2O): 266 nm. MS: M^+ 258 (7%), 140 (100%), 119 (64%).

1-(3'-*Fluoro*-2',3'-*dideoxy*-β-D-*erythro*-*pentofuranosyl*)*uracil* (*FLU*) (**11**). FLU was prepared analogous to FLEU. The crude product was separated on a C_{18}-HPLC column, using methanol–water (1:9) at a rate of 9.5 ml/min to give after 8.0 min the α-anomer and after 9.75 min the desired β-anomer, FLU. Yield 1.6 mg (3%). ^{1}H-NMR (Jeol

FX 200, DMSO-d_6) δ: 4.26 (dt, 1H, $J_{3'F,4'}$ = 29.4 Hz, $J_{4'5'}$ = 1.5 Hz, H-4'); 5.40 (dd, 1H, $J_{3'F,3'}$ = 53.7 Hz, $J_{2',3'}$ = 4.9 Hz, H-3'); 5.66 (d, 1H, $J_{5,6}$ = 8.3 Hz, H-5); 6.31 (dd, 1H, J = 5.7, J = 8.8 Hz, H-1'); 7.81 (d, 1H, $J_{5,6}$ = 8.3 Hz, H-6).

1-(3'-*Fluoro*-2',3'-*dideoxy*-β-D-*erythro*-*pentofuranosyl*)-5-*propyluracil* (*FLPU*) (**12**). FLPU was prepared analogous to FLEU. The crude product was separated on a C_{18}-HPLC column, using methanol–water (35:65) at a rate of 7.0 ml/min to give after 12.9 min the desired β-anomer FLPU and after 18.0 min the α-anomer. Yield 3.6 mg (7%). UV λ_{max} (H_2O): 267 nm. MS: M^+ 276 (6%), 154 (100%), 119 (76%).

Preparation of FLEU by the method of Kowollik and Langen [18]:

1,(3',5'-*Di*-O-*methylsulfonyl*-2'-*deoxy*-β-D-*erythro*-*pentofuranosyl*)-5-*ethyluracil* [20]. 5-Ethyl-2'-deoxyuridine (15.9 g) in dry pyridine (180 ml) was cooled on an ice-bath. Methanesulfonyl chloride (18.0 ml) was added slowly and the solution was stirred at about 0° overnight, after which ice water was added (12 ml). After another 1 hr the mixture was poured onto ice water (3.4 L) with vigorous stirring and was left at 0° for 5 hr. The mixture was filtered and washed with water (1.5 L), 90% aq. ethanol (200 ml), ethanol (200 ml) and diethyl ether (2 × 200 ml). The residue was dried *in vacuo* to give 23.7 g of a crude product (93%, mp 138–148°). Recrystallization from about 500 ml of 90% aq. ethanol gave 11.1 g (43.4%) of a product with mp 140.5–142° ($C_{13}H_{20}N_2O_9S_2$; % calc. C 37.9, H 4.9, N 6.8; % found C 37.4, H 4.8, N 6.7). The solution was concentrated to give another 9.2 g (36%) of a product with mp 147–148.5 g ($C_{13}H_{20}N_2O_9S_2$; % calc. C 37.9, H 4.9, N 6.8, % found C 37.6, H 4.8, N 6.8). Thin layer chromatography (TLC; silica, chloroform–methanol (9:1)) was identical for the two crystalline crops (R_f = 0.33) as well as the ^{1}H NMR spectra.

2,3'-*Anhydro*-1-(2'-*deoxy*-5'-O-*methylsulfonyl*-β-D-*threo*-*pentofuranosyl*)-5-*ethyluracil* (cf. Ref. 21). The 3',5'-dimesylate of 2'-deoxy-5-ethyluridine (6.19 g) in absolute ethanol (750 ml) was heated at reflux. NaOH dissolved in ethanol (15.5 ml, 0.967 M) was added and the heating was continued for 1 hr. The solution was left at room temperature overnight, after which the precipitate (CH_3SO_3Na) was filtered off. The solution was evaporated to dryness and the residue was recrystallized from ethanol (100 ml) to give 2.7 g of the 2,3'-anhydro compound, mp 138–140°. The solution was concentrated to a 50 ml volume and another 1.2 g of the product was collected. Total yield 82%. TLC (silica, chloroform–methanol (9:1)) R_f = 0.10.

1-(2',3'-*Dideoxy*-3'-*fluoro*-5'-O-*methylsulfonyl*-β-D-*erythro*-*pentofuranosyl*)-5-*ethyluracil* (cf. Ref. 18). The 2'-deoxy-2,3'-anhydro-5'-mesyl-5-ethyluridine compound (1.3 g, dried over P_2O_5) was dissolved under anhydrous conditions (glove-box) in dry, peroxide free dioxane (250 ml) containing 0.05% of hydrogen fluoride (water content <0.005% by Fischer analysis). AlF_3 (0.5 g, dried at 100° over P_2O_5) was added and the mixture was heated in a stainless-steel container (dried in a dessicator over P_2O_5 before use) at 170° for 70 min. The reaction product was isolated as described by Kowollik and Langen for the analogous thymidine compound [18], to give 130 mg. TLC (silica, chloroform–methanol (9:1)) R_f = 0.50. MS: M^+ 336 (25%), 197 (30%) 140 (100%). ^{1}H NMR (Jeol FX 200, DMSO-d_6) δ: 1.17 (t, CH_3), 2.37 (q, CH_2), 2.2–2.6 (m, H-2'), 3.22 (s, CH_3SO_3), 4.38 ("d", H-4', J3'F,4' ≈30 Hz), 4.39 (H-5'), 5-50 ("d", H-3', J3'F,3' ≈50 Hz), 6.39 (dd, H-1'), 7.58 (s, H-6).

1-(5'-O-*acetyl*-2',3'-*dideoxy*-3'-*fluoro*-β-D-*erythro*-*pentofuranosyl*)-5-*ethyluracil*. The 5'-mesyl-3'-fluoro-2'-deoxy-5-ethyluridine compound (110 mg, dried *in vacuo* over P_2O_5) and CH_3CO_2K (110 mg, dried *in vacuo* at 100°) were dissolved in acetic anhydride (12 ml) and stirred at 135° for 3 hr. The product (30 mg) was isolated as described for the thymidine analogue [18]. TLC (silica, chloroform–methanol (19:1) R_f = 0.24. MS: M^+ 300 (12%), 161 (35%), 140 (100%).

1-(2',3'-*Dideoxy*-3'-*fluoro*-β-D-*erythro*-*pentofuranosyl*)-5-*ethyluracil* (*FLEU*). 5'-Acetyl-3'-fluoro-2'-deoxy-5-ethyluridine (30 mg) was dissolved in about 10 ml of methanol saturated with ammonia and kept at 0° overnight. The solution was evaporated and the residue was crystallized from ethylacetate to give 14 mg of the pure product, identical with the sample prepared by transglycosylation, mp 177–179 (corrected). TLC (silica, chloroform-methanol (9:1) R_f = 0.42. ^{1}H NMR (Jeol FX 200, DMSO-d_6) δ: 1.17 (t, CH_3); 2.37 (q, CH_2); 2.2–2.6 (m, H-2'); 3.76 (q, J4',5' = 3.9 Hz, H-5'); 4.21, 4.35 (dt, J3'F,4' = 27.3 Hz, J4',5' = 3.9 Hz, H-4'); 5.28, 5.55 (dd, J3'F,3' = 54.5 Hz, J2',3' = 4.9 Hz, H3'); 6.32, 6.36 (dd, J = 7.8 Hz, J = 5.7 Hz, respectively, H-1'); 7.75 (s, H-6).

RESULTS AND DISCUSSION

Inhibition of HIV- and MuLV-replication in cell culture by AZT-analogs

In HTLV-III_B-infected H_9 cells, AZT and FLT emerged as the most potent antiviral agents. As shown in Table 1, the order of activity was FLT > AZT > AZT↑ > AZU = FLU > FLEU > FLPU > AZEU > AZU↑. Thus, of the *erythro*- and *threo*-forms of AZT and AZU, the *erythro* forms were more potent and selective than the *threo*-forms. Of further interest is the observation that AZEU is inactive towards HIV in H_9 cells, whereas the corresponding 3'-fluoro analog, FLEU, is active, and selective (see also below). However, towards human fibroblasts (Flow 5000 cells) the 3'-azido-analogs were less cytotoxic than the 3'-fluoro-analogs. FLU is a less active anti-HIV agent than FLT in H_9 cells. FLU is, however, also less toxic than FLT towards human embryonic fibroblasts. The results indicate that FLT and FLU deserve further attention with respect to their therapeutic potential.

In the MuLV-3T3 cell system only AZT had an antiviral effect comparable to that in the HTLV-III_B-H_9 cell system. FLT only inhibited MuLV replication at concentrations inhibiting cell growth (Table 1). In fact, the 3'-fluoro-analogs were poor or non-selective inhibitors of MuLV replication, whereas AZT and AZU were selective inhibitors of this virus.

Table 1. Inhibition of replication of HIV and MuLV, and inhibition of cell growth by thymidine analogs

	IC_{50} (μM)*		TC_{50} (μM)†			
			H_9			
	HIV	MuLV	I	II	3T3	F 5000
AZU	0.5	35	—	370	>500	>200 (5%)
AZU↑	>100	—	—	>100	—	>200 (0%)
AZT	0.01	0.01	5	4	50	500
AZT↑	0.26	—	—	—	—	250
AZEU	100	>100 (0%)	80	75	—	500
FLU	0.5	>50 (16%)	—	—	—	>100 (10%)
FLT	0.005	0.2	1	50	0.25	5
FLEU	5	>50 (0)	170	—	—	150
FLPU	>10	>100 (0)	>250 (15%)	—	—	>250 (6%)

Figures in parentheses: inhibition at the concentrations after the > sign.

* The values are the concentrations inhibiting replication of HIV (H_9 cells) or MuLV (3T3 cells) by 50%, and are average values obtained from at least four dose–response curves.

† The values are the concentrations inhibiting the growth of each cell-line by 50%, and are obtained from dose–response curves of eight different concentrations of each drug. Values listed under II are the concentrations giving 50% cell-lysis under conditions of the antiviral test (omitting virus).

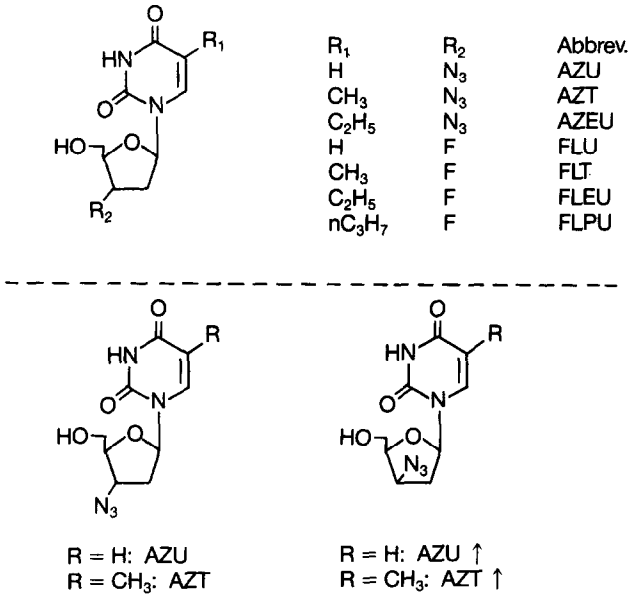


Fig. 1. Structural formulas and abbreviations used for the compounds investigated.

Inhibition of viral reverse transcriptases by triphosphates of AZT-analogs

The activity of HIV-RT, using polyrA·oligo dT as template and dTTP (7 μM) as substrate, was inhibited by the triphosphates of AZU, AZT, AZEU, FLT and FLEU (Fig. 1, Table 2). AZT- and FLT-TP emerged as the most potent inhibitors, followed by AZU, AZEU- and FLEU-TP in this order. This order of activity is reflected in the K_i/K_m values (Table 2). The result that the triphosphates of FLT and AZT are excellent inhibitors of HIV-RT has been described earlier [9, 10, 22–24]. Judging from RT-inhibition data only, AZEU should have been more active than FLEU, but AZEU might not be phosphorylated extensively in H_9 cells. Others also observed a poor anti-HIV effect of AZEU [11].

A different structure–activity relationship emerged when MuLV-RT was tested, using the same template and dTTP (6 μM). Thus, AZT- and AZU-TP inhibited this enzyme (Fig. 2, Table 2), whereas the triphosphates of the 3′-fluoro-analogs and AZEU were not, or only poor, inhibitors. The lack of specific anti-MuLV effect of FLT, FLEU and AZEU observed in cell culture (Table 1) is in agreement with these findings. As the K_i of FLT-TP for MuLV-RT is >100 μM, the antiviral effect of FLT observed

Table 2. Inhibition of HIV- and MuLV-RT by triphosphates of some thymidine analogs

	RT*			
	HIV		MuLV	
	K_i	K_i/K_m	K_i	K_i/K_m
AZU-TP	0.016	0.003	10	0.08
AZT-TP	0.006	0.001	4	0.003
AZEU-TP	0.12	0.02	>200	>1.67
FLT-TP	0.007	0.001	>100	>0.8
FLEU-TP	2.3	0.38	200	1.67

* K_i values were determined using a competitive inhibitor equation with the K_m value of dTTP being 6 μM (HIV-RT) or 120 μM (MuLV-RT). Average values from three determinations.

uninfected animals, a sign of toxicity, and at higher doses (0.5 mg/ml drinking water) FLT also decreased the spleen weight of uninfected animals.

MuLV-infected cells appear not suitable for screening of 3'-blocked thymidine analogs with a potential to inhibit HIV. FLT, which is not a selective inhibitor of MuLV replication in cell-culture (of both the Rauscher- and Moloney-MuLV), can exert an antiviral effect *in vivo*, but at doses resulting in toxicity, and the antiviral effect observed *in vivo* is probably not caused by inhibition of the viral reverse transcriptase. In fact, several nucleoside analogs inhibiting HIV do not inhibit MuLV replication *in vivo* and *in vitro* (our unpublished results), and alternative strategies need, therefore, to be worked out to determine the antiviral potential of such compounds in small laboratory animals.

Conformation analysis of the erythro and threo forms of AZT and AZU

The *erythro* forms of AZT and AZU inhibited

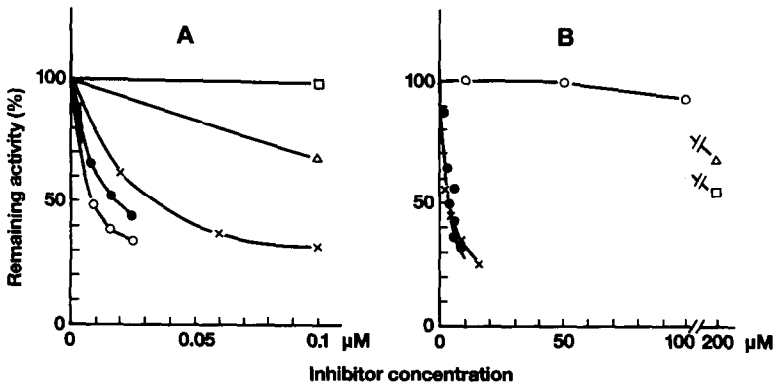


Fig. 2. Inhibition of HIV-RT (A) and MuLV-RT (B) by thymidine analogs. The activity of the enzymes was measured at the indicated concentrations. One hundred per cent activity was the incorporation of 48 pmol of dTMP per 60 min for HIV-RT, and 18 pmol/30 min for the MuLV-RT. Substrate concentrations used were 7 μM dTTP (HIV-RT) and 6 μM dTTP (MuLV-RT). ●, AZT-TP; ×, AZU-TP; △; AZEU-TP; ○, FLT-TP; □, FLEU-TP.

in the MuLV system is likely to be caused by a cytotoxic mechanism (see also below).

Efficacy of FLT and AZT in vivo

To assess the possibility of oral administration of FLT and AZT the drugs were given to mice infected with Ra-MuLV and to uninfected mice.

Rauscher MuLV-infected mice develop a disease characterized by, amongst others, splenomegaly, increased white blood cell count and high titers of infectious virus in the spleen [16, 25]. In our experiments, the pattern of disease development is that shown in Fig. 3. In line with earlier published results [25], AZT treatment via the drinking water prevented disease development (i.e. the increased spleen weight and white blood cell numbers), and decreased virus titers in the spleen (Table 3). FLT treatment was, at a dose of 0.05 mg/ml drinking water, as effective in reducing virus titers and splenomegaly as AZT given at a dose of 0.25 mg/ml drinking water. However, in contrast to AZT, FLT caused a decrease in the number of white blood cells in

replication of HIV at lower concentrations than the *threo* forms. Differences in the character of the 3'-azido group and/or differences in the conformation of the pentose moieties of the two diastereomeric forms could be observed by NMR-spectroscopy, possibly explaining differences in biological effects.

The azido group in AZT, AZU, AZT↑ and AZU↑ can be described by mesomeric forms I and II

$$(\mathrm{I})\mathrm{R}-\mathrm{N}_\alpha=\mathrm{N}_\beta^{\delta+}=\mathrm{N}_\gamma^{\delta-}\leftrightarrow(\mathrm{II})\mathrm{R}-\mathrm{N}_\alpha^{\delta-}-\mathrm{N}_\beta^{\delta+}=\mathrm{N}_\gamma.$$

Thus, N_β is most deshielded due to its electron deficient nature. N_α is the most shielded [26, 27], probably because of a stabilisation of its negative charge (in II) by R. Therefore, the assignment of ^{15}N-NMR resonances for these compounds is done according to the shieldings of the constituent nitrogens: $N_\alpha > N_\gamma > N_\beta$. N^1 and N^3 resonance of the pyrimidine part have been attributed in agreement with the literature [17].

From the data in Table 4, it can be seen that the

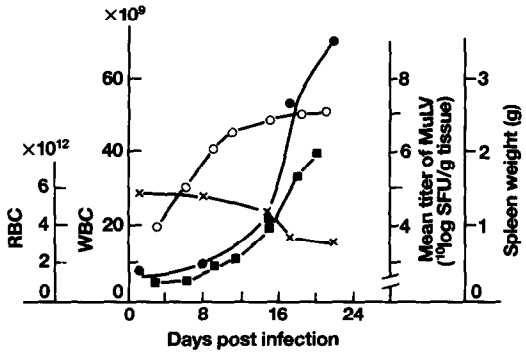


Fig. 3. Development of disease in Ra-MuLV-infected mice. ○, viral titers in spleen; ×, number of red blood cells (RBC); ●, number of white blood cells (WBC); ■, spleen weight. Values shown are mean values from N = 8.

^{15}N shifts showed the azido-nitrogens in AZT↑ to be more shielded than in AZT. This may be due to intramolecular interaction(s) of the azido function with the thymine residue. A decrease of temperature from 30° to −10° resulted in a shielding of 1 ppm for N_γ in AZT↑ while N_γ in AZT was deshielded by *ca.* 3 ppm. These can be rationalized by invoking a loose complex between the C5, C6 double bond and the azido function in AZT↑ as shown in [A] in scheme 1, preventing the flexibility of the azido function. Presumably N_γ is most involved in such a loose complex in AZT↑, shown in [B] in scheme 1, as evident by its characteristic deshielding compared to N_γ of AZT and the shielding of the H6 proton by 0.18 ppm.

Protonation of an sp^2- or sp-hybridized nitrogen usually shifts the protonated-nitrogen to an upfield region due to [17] (i) increase in its π charge density, (ii) variations in the average molecular excitation energy arising from a lack of $n \rightarrow \pi^*$ transition (due to protonation involving the lone-pair), and (iii) difference in its π-bond order. In AZT↑ N_α went upfield by *ca.* 0.7 ppm by raising the temperature from −20° to 30°, which can be due to an interaction between C5′-OH and N_α. This "frozen conformation" at low temperature is favoured when the sugar moiety adopts an N conformation (as shown by ^{1}H-NMR). In AZT, N_α was invariant over the same range of temperature (from +30° to −10°). The noticeable deshielding of N_γ in AZT was presumably due to an increase of the solvation of its lone pair at a low temperature. Examination of a molecular model of AZT does not show any interaction of the azido group and the rest of the molecule. The H6 in AZT underwent a deshielding of *ca.* 0.1 ppm upon a decrease of the temperature from 20° to −20°, presumably due to hydrogen bonding between its 5′-OH and O4′ with H6 while the H6 in AZT↑ moved only by *ca.* 0.03 ppm over a range of temperature of 30° to −40°, showing that the interaction between the azido function and the H5, H6 double bond in AZT↑ is quite pronounced.

To determine the conformation of the 2′-deoxyribose moieties ^{1}H-NMR data can be used. The

Table 3. Treatment of Rauscher-MuLV-infected mice and uninfected mice with FLT or AZT

Treatment	Spleen weight		Number of white blood cells		Medium virus titer per gram spleen (range) (^{10}log SFU)
	Infected animals	Uninfected animals	Infected animals (×10⁹/L)	Uninfected animals (×10⁹/L)	
Exp. A					
None	1.268 ± 0.31	0.108 ± 0.015	10.9 ± 4.4	10.5 ± 3.8	7.12 (6.76–7.40)
FLT, 0.01 mg/ml	1.557 ± 0.24	0.109 ± 0.017	8.0 ± 2.9	7.3 ± 0.9	6.28 (5.18–7.18)***
FLT, 0.05 mg/ml	0.369 ± 0.14*	0.109 ± 0.010	5.3 ± 1.2**	4.6 ± 1.5**	4.84 (3.95–8.87)***
Exp. B					
None	1.490 ± 0.28	0.116 ± 0.010	21.4 ± 7.6	12.0 ± 1.3	7.98 (7.32–8.29)
FLT, 0.1 mg/ml	0.196 ± 0.019*	0.100 ± 0.032			4.74 (4.45–4.81)***
AZT, 0.25 mg/ml	0.498 ± 0.17*	0.121 ± 0.010	9.9 ± 0.8**	12.0 ± 4.8	5.45 (4.15–6.28)***
Exp. C					
None	1.360 ± 0.33	0.103 ± 0.008			8.20 (8.08–8.36)
FLT, 0.5 mg/ml	0.142 ± 0.059*	0.066 ± 0.087			<3.0
AZT, 0.5 mg/ml	0.291 ± 0.038*	0.116 ± 0.013			<2.5

Determinations were done on material taken 15 days post-infection. Values shown are mean values ± SD, unless indicated otherwise.

Abbrev.: SFU, syncytium forming units.

[a]Drugs were provided, dissolved in the drinking water at the indicated concentrations. Treatment was started immediately after infection. Groups of 8 (infected) or 5 (uninfected) animals were used.

*P < 0.001 (*t*-test) relative to untreated controls; **P < 0.02 (*t*-test) relative to untreated control; ***P < 0.01 (Mann-Whitney U-test) relative to untreated controls.

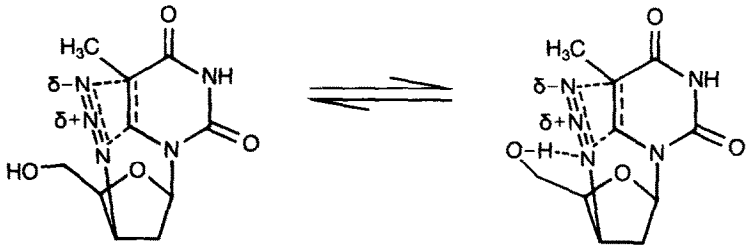
Table 4. ^{15}N-chemical shifts* of the *erythro* and *threo* diastereomers of AZT and AZU

Compound	Temperature 30°	−10°	−20°
AZT↑†	N_β = −135.2 N_α = −305.9 N_γ = −169.3 N^3 = −227.7 N^1 = −237.1	N_β = −135.2 N_α = −306.6 N_γ = −170.4 N^3 = −228.1 N^1 = −237.1	N_β = −136.2 N_α = −306.7 N_γ = −170.6 N^3 = −228.2 N^1 = −237.1
AZT	N_β = −134.6 N_α = −303.3 N_γ = −167.0 N^3 = −227.7 N^1 = −237.8	N_β = −135.5 N_α = −303.5 N_γ = −164.0 N^3 = −228.1 N^1 = −238.0	
AZU↑‡	N_β = −135.9 N_α = −305.7 N_γ = −168.4 N^3 = −224.0 N^1 = −231.9		
AZU‡	N_β = −135.7 N_α = −308.4 N_γ = −165.8 N^3 = −223.9 N^1 = −232.9		

* In ppm, with respect to an external reference: $CH_3\ ^{15}NO_2$ concentration ~0.4 M in acetone, a negative value denotes an upfield shift.

† α, β and γ defined as in $R - N_\alpha = N_\beta^{\delta+} = N_\gamma^{\delta-}$.

‡ Measured at 35°.



Scheme 1.

chemical shifts of AZU, AZT, AZU↑, AZT↑ and those of the two thymidine isomers 2'-deoxy-1-β-D-*erythro*furanosylthymine (dThd) and 2'-deoxy-1-β-D-threofuranosylthymine (dThd↑) are shown in Table 5. Two distinct conformations [28, 29] occur in the 2'-deoxy ribose ring denoted as N-type (population of conformers from C3' *endo* to C2' *exo*) and S-type (population of conformers from C3' *exo* to C2' *endo*).

These conformers can be conveniently described by the phase angle of pseudoration [P], the puckering amplitude [ϕm] [28, 29]. From the five vicinal couplings ($^3J_{1',2'}$ $^3J_{1',2''}$ $^3J_{2',2''}$ $^3J_{2'',3'}$ and $^3J_{3',4'}$), using the program PSEUROT developed by De Leeuw and Altona [30] the geometry of the N and S conformers can be determined together with their respective molar-fractions. The simulation of spin-spin couplings of the J network of the sugar moieties have been carried out only for AZU although accurate values for vicinal couplings are prerequisite to get right values for P and ϕm. An estimation of the preference in the conformational equilibrium (N ↔ S), obtained from spin-spin coupling constants directly extracted from a ^{1}H-NMR spectrum (Table 6) shows that when the azido group is "down" (*erythro*) the %N is *ca.* 45% at room temperature whereas in AZT↑ and AZU↑ the N ↔ S equilibrium is displaced to *ca.* 85% N. It is, therefore, clear that the conformation of the sugar moiety is mainly dictated by the *configuration* of the 3'-substituent and not by the nature of substitution on the nucleobase (i.e. C5-Me in AZT and AZT↑ versus C5-H in

Table 5. ^{1}H-chemical shifts* of thymidine analogs

Compound	H1′	H2′	H2″	H3′	H4′	H5′	H5″
AZT↑†	6.22	2.40	2.52	4.51	3.95	3.86	3.81
AZT†	6.10	2.85	2.22	4.54	4.17	3.91	3.91
AZU↑†	6.31	2.55	2.63	4.61	4.09	3.98	3.93
AZU†	6.25	2.97	2.38	4.66	4.30	4.02	4.02
dThd‡	6.18	2.07	2.09	4.26	3.77	3.60	3.55
dThd↑‡	6.07	1.85	2.55	4.24	3.79	3.72	3.64

* At 270 MHz, referenced against TMS.
† Recorded in acetone-d_6 at 30°.
‡ Recorded in DMSO-d_6 at 20°.

Table 6. Coupling constants* of thymidine analogs

Compound	$J_{1',2'}$	$J_{1',2''}$	$J_{2',3'}$	$J_{2'',3'}$	$J_{3',4'}$	$J_{4',5'}$	$J_{4',5''}$
AZT↑†	6.5	6.6	6.8	4.8	4.6	3.3	3.2
AZT†	3.6	7.7	2.1	6.5	4.0	5.7	5.7
AZU↑†	6.6	6.4	6.9	4.9	4.5	3.5	2.9
AZU†	3.4	7.8	2.0	6.2	4.1	6.0	5.5
dThd‡	2.2	8.2	1.0	5.1	3.0	6.7	4.5
dThd↑‡	6.6	7.4	3.5	5.1	3.3	3.5	3.7

* In Hz, estimated accuracy ±0.4 Hz.
† Recorded in acetone-d_6 at 30°.
‡ Recorded in DMSO-d_6 at 20°.

AZU and AZU↑ does *not* have any influence on the sugar conformation). It has been found [31] in 2′-deoxyadenosine (3′-OH "down") that the sugar is in a slightly preferred S conformation while in the corresponding epimer with 3′-OH "up", the conformation is biased to N conformation. Similar features were also found in thymidine (3′-OH "down") and in its epimer with the 3′-OH "up" (i.e. *threo* analogue) (Table 6), as shown for 5-methoxy-methyl-1-(2′-deoxy-β-D-*lyxo*furanosyl)uracil [32].

Furthermore [31], with a 3′-substituent "down", the conformation around the 4′-5′ bond is usual, i.e. mainly γ^+, while, due to electronic and steric interactions, the conformation around 4′-5′ bond in a compound with the 3′-substitution "up" (i.e. *threo*) is mainly γ^t. This can be seen qualitatively in the 4′-5′ and 4′-5″ coupling constants in the compounds investigated (Table 6). This is in agreement with a recent crystallographic study of AZT [33].

Thus, *threo* conformations (in AZT↑ and AZU↑) are possibly stabilized by interaction between the azido group and the C5, C6 double bond in the pyrimidine part. A hydrogen bond between N_α and 5′-OH is presumably allowed when γ^t is the main conformation across the 4′-5′ bond. It is, however, clear that the latter interaction will be completely absent in triphosphate derivatives.

The difference in the antiviral effect of AZT and AZT↑, and AZU and AZU↑ can be due to this drastic difference in their conformations, which can influence the rate of enzymatic phosphorylation of their 5′-OH functions. In addition, the intramolecular stabilisation of the *threo* conformation may decrease binding of the 3′-azido substituent, as in the *erythro*-diastereomers, with an essential site in an enzyme. It is also likely that in the triphosphate derivatives of AZT, AZT↑, AZU and AZU↑, the Watson-Crick base pairing with the viral RNA template, mediated by viral reverse transcriptase, could be impaired depending upon the glycosidic bond angles in those derivatives. In fact, a K_i value of 0.0022 μM for the *erythro* form of AZT-TP has been observed using HIV RT from disrupted U937/HTLV-III, whereas the *threo* form was less active, with a K_i value of 0.2 μM [10].

The comprehensive analysis of potential antiviral compounds by spectroscopic, biochemical and virological procedures, as described here, may facilitate design of new antiviral 3′-blocked pyrimidine analogs.

Acknowledgements—We gratefully acknowledge the competent technical assistance of Anna Sundqvist, Siv Nordlund, Barbro Levén, Mats Berg and Gunilla Brännström. Part of the work was supported by the Swedish Board for Technical Development (STU), the Swedish Natural Science Research Council (NFR) and funds from the Wallenbergstiftelsen to Jyoti Chattopadhyaya for 270 MHz NMR facilities. Merit Kropp provided competent secretarial assistance.

REFERENCES

1. Helgstrand E and Öberg B, Enzymatic targets in virus chemotherapy. *Antibiot Chemother* **27**: 22–69, 1980.
2. Öberg B, Inhibitors of virus-specific enzymes. In: *Problems of Antiviral Therapy* (Eds. Stuart-Harris CH and Oxford J), pp. 35–69. Academic Press, New York, 1983.

3. Datema R, On the antiviral efficacy of some drugs interfering with nucleic acid synthesis in virus-infected cells. *Chemica Scripta* **26**: 49–53, 1986.
4. Frank KB, Chiou JF and Cheng YC, Interaction of DNA polymerase and nucleotide analog triphosphates. *Adv Enzyme Regul* **24**: 377–389, 1986.
5. De Clercq E, New selective antiviral agents active against the AIDS virus. *Trends Pharmacol Sciences* **8**: 339–345, 1987.
6. Mitsuya H and Broder S, Strategies for antiviral therapy in AIDS. *Nature (Lond)* **325**: 773–778, 1987.
7. Mitsuya H, Weinhold KJ, Furman PA, St. Clair MH, Nusinoff-Lehrman S, Gallo RC, Bolognesi D, Barry DW and Broder S, 3'-Azido-3'-deoxythymidine (BW A509U): an antiviral agent that inhibits the infectivity and cytopathic effect of human T-lymphotropic virus type III/lymphadenopathy-associated virus *in vitro*. *Proc Natl Acad Sci USA* **82**: 7096–7100, 1985.
8. Fischl MA, Richman DD, Grieco MH, Gottlieb MS, Volberding PA, Laskin OL, Leedom JM, Groopman JE, Mildvan D, Schooley RT, Jackson GG, Durack DT, King D and the AZT Collaborative Working Group. The efficacy of azidothymidine (AZT) in the treatment of patients with AIDS and AIDS-related complex. *N Engl J Med* **317**: 185–191, 1987.
9. St. Clair MH, Richards CA, Spector T, Weinhold KT, Miller WH, Langlois AJ and Furman PA, 3'-Azido-3'-deoxythymidine triphosphate as an inhibitor and substrate of purified human immunodeficiency virus reverse transcriptase. *Antimicrob Agents Chemother* **31**: 1972–1977, 1987.
10. Vrang L, Bazin H, Remaud G, Chattopadhyaya J and Öberg B, Inhibition of the reverse transcriptase from HIV by 3'-azido-3'-deoxythymidine triphosphate and its *threo* analogue. *Antiviral Res* **7**: 139–149, 1987.
11. Herdewyn P, Balzarini J, De Clercq E, Pauwels R, Baba M, Broder S and Vanderhaeghe H, 3'-Substituted 2',3'-dideoxynucleoside analogues as potential anti-HIV (HTLV-III/LAV) agents. *J Med Chem* **30**: 1270–1278, 1987.
12. Hansen J, Schulze T and Moelling K, RNase H activity associated with bacterially expressed reverse transcriptase of human T-cell lymphotropic virus III/lymphadenopathy-associated virus. *J Biol Chem* **262**: 12393–12396, 1987.
13. Gallo RC, Salahuddin SZ, Popovic M, Shearer GM, Kaplan M, Haynes BF, Palker TJ, Redfield R, Oleski J, Safal B, White G, Foster P and Markham PD, Frequent detection and isolation of cytopathic retroviruses (HTLV-III) from patients with AIDS and at risk for AIDS. *Science* **224**: 500–502, 1984.
14. Rowe WP, Pugh WE and Hartley JW, Plaque assay techniques for murine leukemia viruses. *Virology* **42**: 1136–1139, 1970.
15. Stenberg K, Wangenheim J and Tribukait B, Quantitative analysis of antiviral drug toxicity in proliferating cells. *Cell Biol Toxicol* **2**: 441–445, 1986.
16. Steeves RA, Strand M and August JT, Lymphoproliferative disease in Rauscher murine leukemia virus-infected mice. *J Virol* **14**: 187–193, 1974.
17. Martin GJ, Martin ML and Gouesnard JP, ^{15}N-NMR spectroscopy. In: *NMR Basic Prin Prog.* 18 Springer-Verlag, Berlin, 1981.
18. Kowollik G and Langen P, 1-(2,3-Dideoxy-3-fluoro-H$_T$-D-ribofuranosyl)thymine (3'-deoxy-3'-fluorothymidine). In: *Nucleic Acid Chemistry* (Eds. Townsend LB and Tipson RS), pp. 299–302. Wiley, New York, 1978.
19. Yoshikawa M, Kato T and Takenishi T, Studies of phosphorylation. III. Selective phosphorylation of unprotected nucleosides. *Bull Chem Soc Jap* **42**: 3505–3508, 1969.
20. Michelson AM and Todd AR, Deoxyribonucleosides and related compounds. Part V.* Cyclothymidines and other thymidine derivatives. The configuration at the glycosidic centre in thymidine. *J Chem Soc* 816–823, 1955.
21. Horwitz JP, Chua J, Urbanski JA and Noel M, Nucleosides. III. 1-(2'-Deoxy-3',5'-epoxy-β-D-*threo*-pentofuranosyl)thymine. *J Org Chem* **28**: 942, 1963.
22. Furman PA, Fyfe JA, St. Clair MH, Weinhold K, Rideout JL, Freeman GA, Nusinoff-Lehrman S, Bolognesi DP, Broder S, Mitsuya H and Barry DW, Phosphorylation of 3'-azido-3'-deoxythymidine and selective interaction of the 5'-triphosphate with human immune deficiency virus reverse transcriptase. *Proc Natl Acad Sci USA* **83**: 8333–8337, 1986.
23. Cheng YC, Dutschman GE, Bastow KF, Sarngadharan MG and Ting RYC, Human immunodeficiency virus reverse transcriptase. *J Biol Chem* **262**: 2187–2189, 1987.
24. Matthes E, Lehmann CH, Scholz D, von Janta-Lipinski M, Gaertner K, Rosenthal HA and Langen P, Inhibition of HIV-associated reverse transcriptase by sugar-modified derivatives of thymidine 5'-triphosphate in comparison to cellular DNA polymerases α and β *Biochem Biophys Res Commun* **148**: 78–86, 1987.
25. Ruprecht RM, O'Brien LG, Rossoni LD and Nusinoff-Lehrman S, Suppression of mouse viraemia and retroviral disease by 3'-azido-3'-deoxythymidine. *Nature (Lond)* **323**: 467–469, 1986.
26. Müller VJ, ^{15}N NMR spectroscopy of carbon azides. *Z Naturforsche* **346**: 437–441, 1979.
27. Müller VJ and Schröder HF. Preparation and spectroscopic properties of ^{15}N-labelled phosphoru azides. *Z Anorg Allg Chem* **450**: 149–164, 1979.
28. Altona C and Sundaralingam M, Conformational analysis of the sugar ring in nucleosides and nucleotides. A new description using the concept of pseudorotation. *J Am Chem Soc* **94**: 8205–8212, 1972.
29. Altona C and Sundaralingam M, Conformational analysis of the sugar ring in nucleosides and nucleotides. Improved method for the interpretation of proton magnetic resonance coupling constants. *J Am Chem Soc* **95**: 2333–2344, 1973.
30. De Leeuw FAAM and Altona C, Computer-assisted pseudorotation analysis of five-membered rings in means of proton spin-spin coupling constants: program PSEUROT. *J Comp Chem* **4**: 428–437, 1983.
31. Koole LH, Buck HM, Nyilas A and Chattopadhyaya J, Structural properties of modified deoxyadenosine structures in solution. Impact of the gauche and anomeric effects on the furanose conformation. *Can J Chem* **65**: 2089–2094, 1987.
32. Wilson Quail J, Ekiel I, El-Kabbani OAL, Tourigny G, Delbaere LTJ, Stuart AL and Gupta SV, Structure and conformation of 5-methoxymethyl-1-(2'-deoxy-β-D-lyscofuranosyl)uracil. *Can J Chem* **64**: 1355–1359, 1986.
33. Birnbaum GI, Giziewicz J, Gabe EJ, Lin TS and Prusoff NH, Structure and conformation of 3'-azido-3'-deoxythymidine (AZT), an inhibitor of the HIV (AIDS) virus. *Can J Chem* **65**: 2135–2139, 1987.