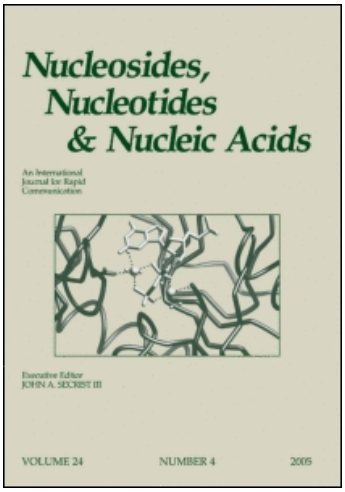

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Relationship Between Conformation and Antiviral Activity-III. 3'-Azidothymidine (AZT) and 3'-Azido-2', 3'-dideoxy-5-hydroxymethyluridine

Sagar V. Gupta[a]; Sashi V. P. Kumar[a]; Allan L. Stuart[a]; Ruili Shi[a]; Keith C. Brown[b]; Wajdi M. Zoghaib[b]; Jung Li[b]; Louis T. J. Delbaere[c]
[a] Department of Veterinary Physiological Sciences, University of Saskatchewan, Saskatoon, Saskatchewan, Canada [b] Department of Chemistry, University of Saskatchewan, Saskatoon, Saskatchewan, Canada [c] Department of Biochemistry, University of Saskatchewan, Saskatoon, Saskatchewan, Canada

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RELATIONSHIP BETWEEN CONFORMATION AND ANTIVIRAL ACTIVITY-III. 3′-AZIDOTHYMIDINE (AZT) AND 3′-AZIDO-2′,3′-DIDEOXY-5-HYDROXYMETHYLURIDINE+

Sagar V. Gupta[3*], Sashi V.P. Kumar[3], Allan L. Stuart[3], Ruili Shi[3], Keith C. Brown[2], Wajdi M. Zoghaib[2], Jung Li[2] and Louis T.J. Delbaere[1]

[1]Department of Biochemistry, [2]Department of Chemistry and [3]Department of Veterinary Physiological Sciences, University of Saskatchewan, Saskatoon, Saskatchewan, Canada, S7N 5B4

ABSTRACT

3′-Azido-2′,3′-dideoxy-5-hydroxymethyluridine (AZHMddUrd) was synthesized to improve the potency of 5-hydroxymethyl-2′-deoxyuridine (HMdUrd) against human immunodeficiency virus (HIV). AZHMddUrd was a very poor inhibitor of HIV replication ($ED_{50} > 200$ μM) and was also nontoxic up to 400 μM (highest concentration tested) to HT4-6C (HeLa CD_4) cells. AZT was phosphorylated by human cellular thymidine kinase. In contrast, AZHMddUrd and HMdUrd were poor substrates for the kinase. The relationship between molecular conformation and antiretroviral activity for 3′-azidothymidine (AZT), HMdUrd and AZHMddUrd is discussed.

INTRODUCTION

Antiretroviral chemotherapy with azidothymidine (3′-azido-2′,3′-dideoxythymidine, AZT), in patients with acquired immunodeficiency syndrome (AIDS) and AIDS-related disease complex (ARC), has proven to be useful in slowing progression of the disease, delaying mortality and decreasing the frequency of opportunistic infections (1,2). The drug also appears to be of some

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+For Part II see Gupta *et al.* Antiviral Chem. Chemother. 1992, 3, 15.

*To whom all correspondence should be addressed.
Telephone (306) 966-7355; Fax (306) 966-7376.

value in delaying the onset of AIDS in asymptomatic sero positive human immunodeficiency virus (HIV) carriers (3). The potency of AZT against HIV in MT-4 cells is approximately 100 to 200 fold higher than in ATH-8 cells. The ED_{50} (50% antiviral effective dose) for AZT was 4 nM in MT-4 and PBM cells (4-7) and 0.4 μM in ATH-8 cells (8). As a substrate analogue, AZT inhibits viral replication by reversible inhibition of reverse transcriptase; after incorporation into the growing DNA chain it prevents further polymerization (chain termination) due to a lack of a 3′-OH group (9-11).

5-Hydroxymethyl-2′-deoxyuridine (HMdUrd), a naturally-occurring nucleoside in bacteriophages, has moderate anti-HIV activity (ED_{50} = 2-8 μM) and was effective against Friend leukemia virus in mice (12). In order to try to improve the potency of HMdUrd against HIV, the 3′-azido analogue (3′-azido-2′,3′-dideoxy-5-hydroxymethyluridine, AZHMddUrd) was synthesized, and its molecular conformation was determined (13). In this paper, the relationship between molecular conformation, anti-HIV activity and affinity for cellular thymidine kinase of HMdUrd, AZHMddUrd and AZT is discussed.

RESULTS

Biological Activity

The antiviral activity of HMdUrd, AZHMddUrd and AZT against HIV-1 was determined by a quantitative focal immunoassay using HT4-6C (HeLa CD_4) cells. AZT and HMdUrd were included as positive controls. AZHMddUrd had an ED_{50} of >200 μM compared to HMdUrd (ED_{50} = 8-10 μM) and AZT (ED_{50} = 0.1 μM). The ID_{50} concentrations required to inhibit cell growth by 50% were >400 μM, 30-40 μM and 6-7 μM for AZHMddUrd, HMdUrd and AZT respectively.

The affinity for human cellular thymidine kinase (HTK) was also determined. AZT showed high affinity for the kinase; the concentration required to inhibit 50% of dThd phosphorylation (IC_{50}) for the enzyme was about 11 μM. When assayed under similar conditions, HMdUrd and AZHMddUrd showed significantly less affinity with IC_{50} values of 311 μM and 676 μM respectively.

Molecular conformation

The structural formulae for AZHMddUrd and AZT are shown in Fig. 1. In order to obtain a better understanding of the relationship between anti-HIV activity and molecular structure, the two molecules (AZHMddUrd and AZT) were compared with the natural substrate, dThd, using

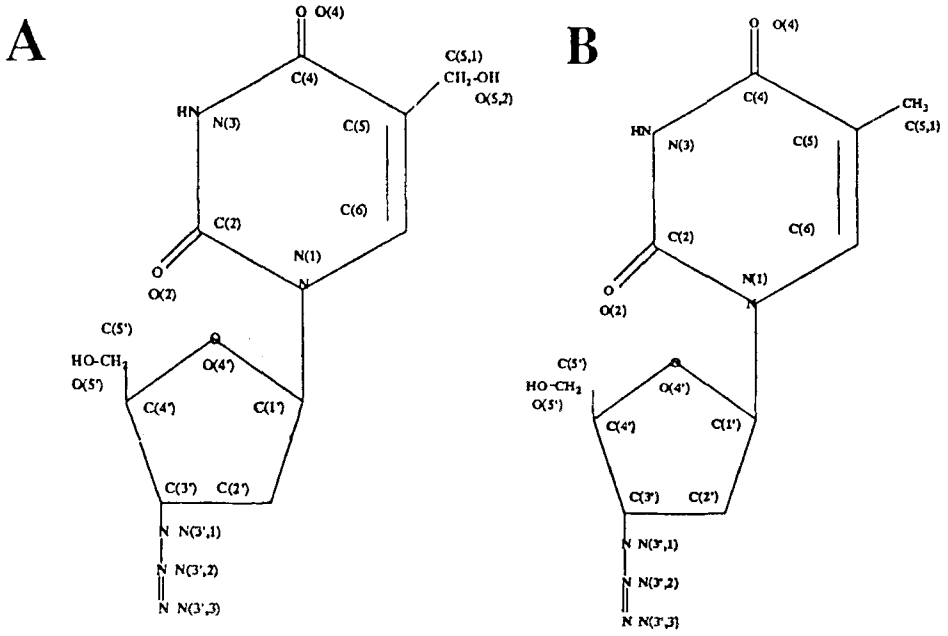

FIG. 1 Structure and atomic numbering of (A) 3′-azido-2′,3′-dideoxy-5-hydroxymethyldeoxyuridine and (B) 3′-azidothymidine.

the computer program PROFIT (14). The stereoscopic representations of the molecules are shown in Fig. 2 and 3. The planar pyrimidine ring of AZHMddUrd was superimposed on the two crystallographic independent conformational states A and B of AZT and dThd and distances between equivalent atoms were calculated (Tables 1 and 2). The conformational parameters of the three molecules are compared in Table 3.

The bond lengths and angles of AZHMddUrd (13) are similar to those reported for dThd (15) and AZT (16). The conformation about the glycosidic bond χ [C(2)-N(1)-C(1′)-O(4′)] for all three molecules is within the normal range for pyrimidine-2′-deoxyribonucleosides with the **anti** conformation. The χ value of AZHMddUrd (219.3°) is higher than for molecule B (188.0°) of AZT but lower than that for molecule A (234.1°) of AZT. The furanose rings of AZHMddUrd and dThd have an envelope conformation: AZHMddUrd has C(2′)-**endo** puckering and dThd

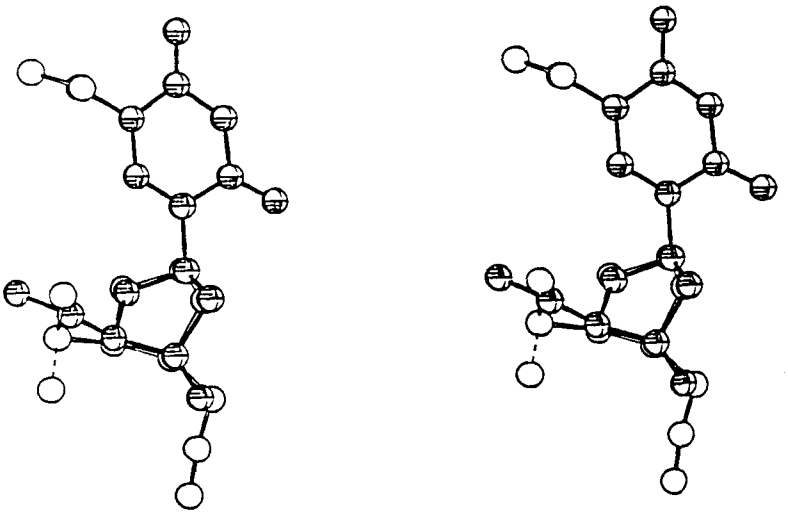

FIG. 2 Stereo view of the superimposed molecules. Hydrogen atoms are omitted for clarity. AZHMddUrd (open spheres and open bonds) and dThd (shaded spheres and solid bonds).

has C(3′)-**exo** puckering. In contrast, the two crystalline forms of AZT exhibit a twist conformation: molecule A is C(2′)-**endo**-C(3′)-**exo** and molecule B is C(3′)-**exo**-C(4′)-**endo**. The exocyclic (C5′) side chain in AZHMddUrd adopts two disordered conformations g^+ and g^- with $\gamma = 58.6°$ and - 58.2°, which correspond to occupancies of 0.74 and 0.26, respectively. Pictorial representation of the three possible C(5′) exocyclic side chain conformers is shown in Fig. 4. The C(5′)-hydroxyl group in the thymidine molecule has the t conformation ($\gamma = 172.8°$) and the conformation for molecules A and B of AZT is g^+ ($\gamma = 49.7°$) and t ($\gamma = 173.7°$) respectively. Comparison of the bond distances between equivalent atoms indicate that the pyrimidine ring and the furanose ring in AZHMddUrd and dThd are well aligned (Fig. 2, Table 1). The puckering of the furanose ring in AZHMddUrd is only slightly different from that seen in dThd. However, the torsion angle γ, C(3′)-C(4′)-C(5′)-O(5′), is completely different; g^+ and g^- for AZHMddUrd and t for dThd (Table 3). For the azido analogues, AZT (molecule A)

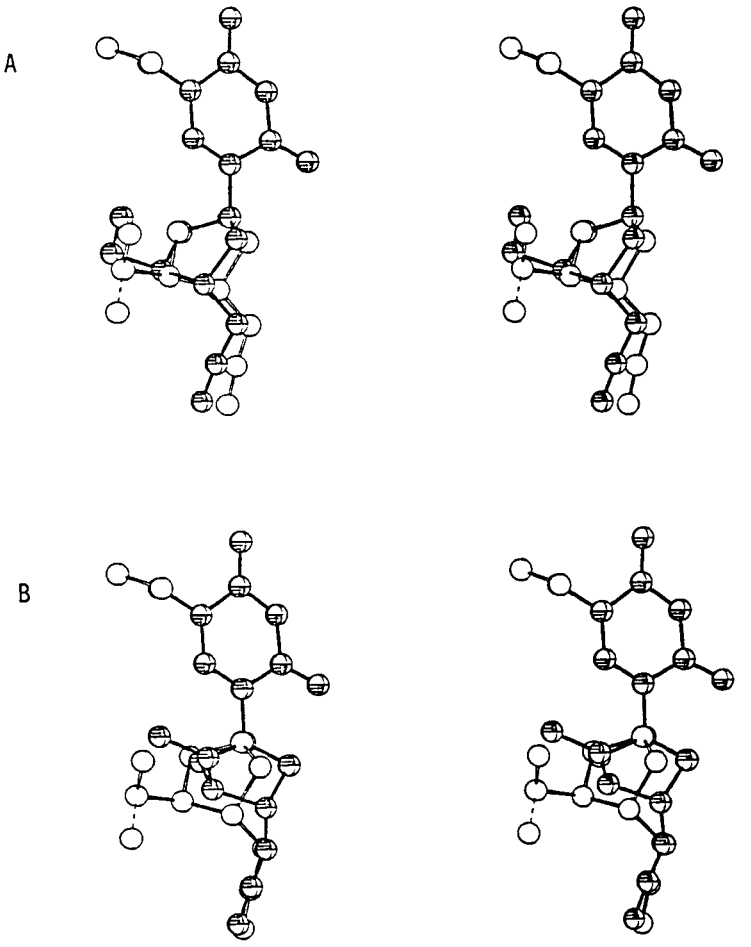

FIG. 3 Stereo view of the superimposed molecules. Hydrogen atoms are omitted for clarity.

A. AZHMddUrd (open spheres and open bonds) and molecule A of AZT (shaded spheres and solid bonds);

B. AZHMddUrd (open spheres and open bonds) and molecule B of AZT (shaded spheres and solid bonds).

TABLE 1

Comparison of AZHMddUrd and dThd distances between equivalent atoms when the pyrimidine ring of AZHMddUrd is superimposed on the pyrimidine ring of dThd.

Atomic numbering		Distance (Å)
Thymidine	3′-AZHMddUrd	
N(1)	N(1)*	0.059
C(2)	C(2)*	0.029
O(2)	O(2)*	0.144
N(3)	N(3)*	0.037
C(4)	C(4)*	0.053
O(4)	O(4)*	0.099
C(5)	C(5)*	0.020
C(5,1)	C(5,1)*	0.112
C(6)	C(6)*	0.039
C(1′)	C(1′)*	0.101
C(2′)	C(2′)	0.132
C(3′)	C(3′)	0.223
C(4′)	C(4′)	0.437
O(4′)	O(4′)	0.186
C(5′)	C(5′)	0.757
O(5′)	O(5′)	1.476

* Atoms used in the least-square refinement in PROFIT for superimposing.

and AZHMddUrd, the alignment of the azido group, the puckering at the C(3′) position and the orientation of the side chain at the 5-position of the pyrimidine ring are different (Fig. 3A, Table 2). Comparison of AZHMddUrd and molecule B of AZT also indicates substantial differences in the sugar-puckering mode and in the orientation of the O(5′) atom (Fig. 3B and Table 2). However, the azido group of these two molecules are well aligned.

TABLE 2

Comparison of AZHMddUrd and AZT distances between equivalent atoms when the pyrimidine ring of AZHMddUrd is superimposed on the pyrimidine ring of AZT.

AZT	3′-AZHMddUrd	AZT	
		Molecule A	Molecule B
Atomic numbering		Distance (Å)	
N(1)	N(1)*	0.047	0.063
C(2)	C(2)*	0.030	0.042
O(2)	O(2)*	0.164	0.118
N(3)	N(3)*	0.061	0.056
C(4)	C(4)*	0.054	0.032
O(4)	O(4)*	0.083	0.069
C(5)	C(5)*	0.036	0.029
C(5,1)	C(5,1)*	0.134	0.087
C(6)	C(6)*	0.044	0.036
C(1′)	C(1′)*	0.125	0.051
C(2′)	C(2′)	0.359	0.934
C(3′)	C(3′)	0.477	0.894
N(3′,1)	N(3′,1)	0.618	0.237
N(3′,2)	N(3′,2)	0.715	0.693
N(3′,3)	N(3′,3)	0.858	1.105
C(4′)	C(4′)	0.269	1.500
O(4′)	O(4′)	0.162	0.826
C(5′)	C(5′)	0.544	2.594
O(5′)	O(5′)	0.503	0.999

* Atoms used in the least-square refinement of PROFIT for superimposing.

TABLE 3

Comparison of the conformational parameters of AZHMddUrd, AZT and dThd.

	3´-AZHMddUrd[a]	AZT[b] Molecule A	AZT[b] Molecule B	Thymidine[c]
χ	219.3 *anti*	234.1 *anti*	188.0 *anti*	220.0 *anti*
Puckering mode	^{2}E C2´-*endo*	$^{2}T_{3}$ C2´-*endo*-C3´-*exo*	$^{4}T_{3}$ C3´-*exo*-C4´-*endo*	$_{3}E$ C3´-exo
P	159	171	213	188
τ_m	34	14	11	38
γ	58.6 (*g*+)	49.7 (*g*+)	173.7 (*t*)	172.8 (*t*)
	-58.2 (*g*-)	---	---	---

[a] Ref. 13.
[b] Ref. 16.
[c] Ref. 15.

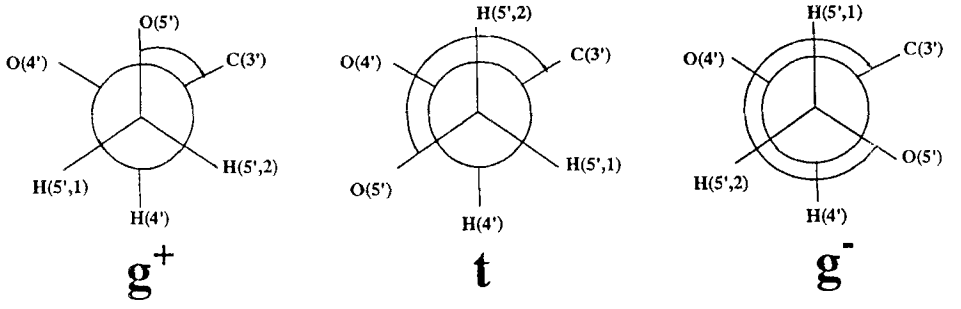

Fig. 4 The three possible C(5´)-exocyclic side chain conformers looking down the C(5´)-C(4´) bond.

NMR Analysis

The proton coupling constants and conformational populations are summarized in Tables 4 and 5. The conformation of the sugar ring was obtained from the relationship between the proton-proton coupling constants and the pseudorotational properties of the ring using the computer programme PSEUROT (17). The populations of the three rotamers about the exocyclic C(4')-C(5') bond were estimated from the $J_{4'5'}$ and $J_{4'5''}$ coupling constants using the method of Haasnoot *et al.* (18).

DISCUSSION

In an effort to develop better drugs for the treatment of AIDS, a large number of nucleoside analogues have been synthesized. The studies published indicate that **unmodified** nucleosides do not have anti-HIV activity. However, chemical modifications of the sugar moiety convert a normal substrate for nucleic acid synthesis into compounds with potent anti-HIV activity (20,21). HMdUrd, an unmodified nucleoside, inhibits replication of HIV in cell culture and provided significant protection against Friend leukemia virus in mice (12) are interesting findings. It was reasonable to assume that converting HMdUrd to the 3´-azido analogue would result in a more active molecule because the only structural difference between AZHMddUrd and AZT is the presence of a hydroxymethyl side chain instead of a methyl group at the C(5) position of the pyrimidine ring. Unfortunately, the expectation of increased potency was not fulfilled. AZHMddUrd was essentially devoid of activity against HIV.

HMdUrd and AZHMddUrd were weak inhibitors for the human cytoplasmic dThd kinase. Biochemical analysis of the metabolic fate of HMdUrd in mammalian cells demonstrated significant levels of HMdUrd-5'-triphosphate (HMdUTP) and incorporation of hydroxymethyluracil residues into DNA (22-25). HMdUrd-5´-monophosphate is a good inhibitor of thymidylate synthase (26) and HMdUTP is a moderate inhibitor of reverse

TABLE 4

Vicinal coupling constants (Hz) of AZT, AZHMddUrd and HMdUrd.

310 K 37°C	AZT		AZHMddUrd		HMdUrd	
	J exp.[a]	J cal.[b]	J exp.[a]	J cal.[b]	J exp.[a]	J cal.[b]
$J_{1'2'}$	6.3	6.0	6.1	5.9	6.8	7.1
$J_{1'2''}$	6.6	6.8	6.6	6.7	6.5	6.5
$J_{2'2''}$	0.0		0.0		-14.4	
$J_{2'3'}$	6.6	6.8	6.6	6.8	6.7	6.5
$J_{2''3'}$	6.1	5.9	6.1	6.0	4.3	4.5
$J_{3'4'}$	5.0	5.0	5.1	5.0	4.0	4.2
$J_{4'5'}$	4.2		4.8		4.7	
$J_{4'5''}$	3.5		3.5		3.9	
$J_{5'5''}$	-11.8		-12.9		-12.3	

[a] J experimental; precision 0.1-0.2 Hz.
[b] J calculated using PSEUROT with $\tau_m = 36.0°$.

TABLE 5

Conformation populations (%): S- and N-conformers of the furanose ring and the three rotamers of the exocyclic C(5') side chain.

Conformer[a]	Compounds			
	AZT	HMdUrd	dThd[b]	AZHMddUrd
S	50 (10.4°)[c]	63 (152.0°)	60	49 (151.5°)
N	50 (152.7°)	37 (10.6°)	40	51 (7.7°)
g+[d]	60	52	44	57
g-	22	35	21	29
t	18	13	35	14

[a] In the PSEUROT calculations, τ_m was constrained to 36.0°. The rms deviation for each calculation was: AZT - 0.193 E; HMdUrd - 0.138 E; AZHMddUrd - 0.146 E.
[b] DMSO-d_6 at 30°C (19).
[c] Numbers in brackets are the calculated pseudorotational angles - P_S and P_N.
[d] Exocyclic orientation at 37°C (except dThd). The values reported for the side chain of AZT, HMdUrd and AZHMddUrd are averages over all best fit conformational pairs with a deviation of <5%.

transcriptase (27). Earlier studies have shown that HMdUrd inhibits proliferation of tumor cells in culture (28-30) and prolongs the life of mice implanted with L1210 cells (24,28). On the basis of these results, it is logical to assume that the active form of the drug is HMdUTP following phosphorylation of HMdUrd by HTK, *albeit*, at a considerably slower rate than dThd. The biological effects elicited by HMdUrd are probably due to the inhibition of DNA polymerization (DNA polymerase/reverse transcriptase) at the substrate level and by incorporation into DNA. The lack of anti-HIV activity of AZHMddUrd is probably due to such a slow rate of phosphorylation by HTK that physiologically significant levels of AZHMddUTP are never reached in infected cells.

The very slow rate of phosphorylation by HTK of thymidine analogues, HMdUrd and AZHMddUrd, appears to be partly due to the altered conformation of the exocyclic 5'-OH group and to a lesser extent, due to the conformation of the furanose ring. The C(5')-hydroxyl group of AZT (molecule B) and deoxythymidine has a *t* conformation which may be the preferred orientation for phosphorylation by HTK. In contrast, for HMdUrd, the exocyclic 5'-OH group has a g^+ rotamer conformation (31, 32). In AZHMddUrd, the side chain on C(5') is partially disordered and exhibits two distinct conformations g^+ (74%) and g^- (26%). The NMR data agrees to a large extent with the solid state data. AZT and AZHMddUrd are both predominantly in the g^+ rotamer (Table 5). Thymidine is also predominantly in the g^+ rotamer but 35% is *t*. In contrast, the population of *t* rotamer is considerably lower for AZT (18%), HMdUrd (13%) and AZHMddUrd (14%). Thus, the geometry of the 5'-OH group of the furanose ring appears to play a role in determining the substrate specificity toward HTK but the hydroxyl group on C(5,1) probably has the predominant role.

In conclusion, results of these investigations indicate that a hydroxymethyl substituent at the C(5) position of the pyrimidine moiety (instead of a methyl group) has an influence on the conformation of the exocyclic C(5') side chain of the furanose ring, which in turn seems to

determine, at least in part, the substrate specificity for phosphorylation and ultimately biological activity.

EXPERIMENTAL SECTION

Thymidine kinase (TK) activity

Fresh peripheral blood lymphocytes were stimulated with phytohemagglutinin. After pelleting, the cells were suspended in 50 mM Tris-HCl (pH 8.0) containing 2.5 mM dithiothreitol, sonicated (2 fifteen seconds bursts at 20 watts each) and the suspension was centrifuged at 100,000 xg for 60 min. The supernatant was divided into aliquots and stored at -70°C.

The standard assay mixture contained 50mM Tris-HCl (pH 8.0), 5 mM ATP, 5 mM $MgCl_2$, 10 mM KF, 2.5 mM dithiothreitol, 1mg/ml bovine serum albumin, 5 μM dThd, tritiated [^{3}H] thymidine 1 μCi (Sp. act. 84 Ci/mmol), varying concentrations of the test compounds (AZT, HMdUrd or AZHMddUrd) and 25 μl enzyme extract. Total reaction volume was 100 μl. The assay mixtures were incubated for 30 min at 37°C and the reaction was terminated by chilling the samples to 0°C in an ice bath. The samples (50 μl) were applied onto DE51 cellulose discs, dried, washed with 1 mM ammonium formate followed by absolute ethanol and dried. Radioactivity was counted using a toluene based scintillation fluid (6 g PPO and 0.075 g POPOP per litre of scintillation grade toluene).

Compounds

3'Azido-2',3'-dideoxy-5-hydroxymethyluridine (AZHMddUrd): 5'-Acetyl-3'-azido-2',3'-dideoxythymidine (1.08 g, 3.4 mmole) was dissolved in anhydrous CCl_4 (250 ml) under reflux using two 300 W lamps. Argon was bubbled through the solution at a moderate rate. Bromine (0.189 ml, 3.65 mmoles) in dry CCl_4 (100 ml) was added slowly over 4 h. The residual Br_2 and HBr was removed by bubbling argon in the reaction mixture at a rapid rate for

1 h. The solvent was removed under reduced pressure at 30°C, the residue was dissolved in dioxane (20 ml), $NaHCO_3$ (5%, 20 ml) was added and the reaction mixture was stirred at 25°C for 1 h and then at 60°C for 10 min. The solvent was evaporated *in vacuo* and the residue was extracted with dichloromethane (300 ml) in a soxhlet for 4 h. The dichloromethane was evaporated and the crude 5'-acetyl-3'-azido-2',3'-dideoxy-5-hydroxymethyluridine was isolated as an amorphous powder. The residue was dissolved in methanol, saturated with ammonia at 0°C and left overnite at 25°C to remove the blocking groups. The solvent was evaporated, and the residue was dissolved in water and purifed by HPLC using a reverse phase C 18 column (1 x 25 cm Supelcosil LC-18, 5μ). The mobile phase was methanol (20%) with a flow rate of 5 ml/min. The eluate was evaporated. AZHMddUrd crystallized as fine needles from MeOH-EtOAc (208 mg; yield 21%); m.p. 133-136°; UV, λ_{max} 264 nm (ϵ 9,650) and λ_{min} 234 nm (ϵ 2,350). Anal: Calc. for $C_{10}H_{13}N_5O_5$: C, 42.38; H, 4.63; N, 24.73; Found: C, 42.40; H, 4.68; N, 24.60.

Azidothymidine (AZT) was purchased from Raylo Chemicals, Edmonton, Alberta. HMdUrd was synthesized in our laboratory. Spectra were recorded in phosphate buffer, 0.1 M, pH 7.0. For antiviral assays, stock solutions were made in phosphate buffered saline and stored at 4°C.

Focal immunoassay using HT4-6C (HeLa CD_4) cells

HIV-1 strain MO was isolated from an AIDS patient and was obtained from the Centre for Disease Control, Ottawa, Ontario, Canada. HUT-78 cells and MT-2 cells were obtained from American type culture collection. HT4-6C (HeLa CD_4) cell line was kindly made available by Dr. B. Chesebro, Rocky Mountain Laboratories, Hamilton, Montana, U.S.A. The virus was grown in HUT-78 cells until about 90% HIV cytopathic effect. The virus titre was determined using MT-2 cells as previously described (33).

Antiviral activity was determined by a quantitative focal immunoassay according to the recommendation of Chesebro and Wehrly (34). Briefly, confluent monolayers of Hela CD_4 cells (2.5×10^4 cells/well) were infected with 50-200 plaque forming units of HIV-1 per well in a microtitre tray (24 wells) and immediately thereafter exposed to increasing concentrations of the test compound. After incubation at 37°C for 3 days in a humidified CO_2 (5%) atmosphere, the monolayers were fixed, immunochemically stained and enumerated using a microscope. From dose response curves, the concentration required to reduce the number of plaques by 50% (ED_{50}) was determined. In each experiment, toxicity controls (containing test compound and medium only), cell controls (containing media only) and virus controls (containing virus and media) were run simultaneously.

NMR Analysis

The NMR experiments were carried out using a Brucker 300 spectrometer. Solutions were made to a concentration of 0.1 M in D_2O. Spectra were recorded in the Fourier transform mode at 5°, 25°, 37° and 50°C. ^1H NMR spectra were simulated with the aid of the Brucker routine PANIC and final coupling constants have a precision of 0.1-0.2 Hz. Calculations of coupling constants and pseudorotational parameters were performed using PSEUROT (17) assuming a maximum puckering amplitude $\tau_m = 36.0°$. Exocyclic side chain populations were calculated using PANIC experimental coupling constants.

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