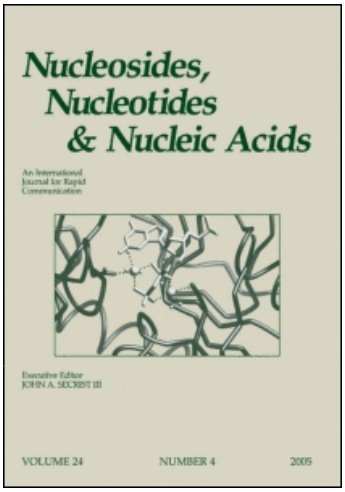

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Some 6-Aza-5-substituted-2'-deoxyuridines Show Potent and Selective Inhibition of Herpes Simplex Virus Type 1 Thymidine Kinase

I. Basnak[a]; M. Sun[a]; T. A. Hamor[a]; F. Focher[b]; A. Verri[b]; S. Spadari[b]; B. Wroblowski[c]; P. Herdewijn[c]; R. T. Walker[a]
[a] School of Chemistry, University of Birmingham, Birmingham, UK [b] CNR, IGBE, Pavia, Italia [c] Rega Institute, Katholieke, Universiteit Leuven, Leuven, Belgium

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SOME 6-AZA-5-SUBSTITUTED-2′-DEOXYURIDINES SHOW POTENT AND SELECTIVE INHIBITION OF HERPES SIMPLEX VIRUS TYPE 1 THYMIDINE KINASE

I. Basnak,[§] M. Sun,[§] T.A. Hamor,[§] F. Focher,[†] A. Verri,[†] S. Spadari,[†] B. Wroblowski,[‡] P. Herdewijn[‡] and R. T. Walker[§]*

School of Chemistry, University of Birmingham, Birmingham B15 2TT, UK.[§]

IGBE, CNR, Via Abbiategrasso, 207, 27100 Pavia, Italia;[†] Rega Institute, Katholieke, Universiteit Leuven, Minderbroedersstraat 10, B-3000 Leuven, Belgium.[‡]

ABSTRACT: The synthesis and X-ray crystal structures of a series of 5-substituted-6-aza-2′-deoxyuridines is reported. These nucleoside analogues inhibit the phosphorylation of thymidine by HSV-1 TK but have no effect on the corresponding human enzyme. Detailed examination of one analogue proves it to be a competitive inhibitor of thymidine with a Ki of 0.34 μM and is a very poor substrate. The analogues are not substrates for the enzyme and also do not inhibit the degradation of thymidine by thymidine phosphorylase. Molecular modelling showed that the inhibitors fit well in the active site of HSV-1 TK, provided the conformation of the sugar moiety is the same for thymidine in the complex.

INTRODUCTION

For many years, chemists involved in the synthesis of nucleoside analogues as antiherpesvirus agents have focused on the viral thymidine kinase (TK) as a legitimate target.[1,2] Certainly the well established antiviral agents, 5-iodo-2′-deoxyuridine (IDU), acyclovir (ACV) and *E*-5-(2-bromovinyl)-2′-deoxyuridine (BVDU) are all substrates for herpesvirus TK. For ACV and BVDU, the phosphorylation is quite specific but for IDU which is also a substrate for cellular kinases, associated toxicity is seen.[3]

This paper is dedicated to the memory of Professor Tsujiaki Hata

Phone: 44-(0)-121-414-4454
Fax: 44-(0)-121-414-4403

Even for the non-chain-terminating antiviral nucleosides such as IDU and BVDU, it is assumed that the 5′-triphosphate is the active form of the drug, even though a precise explanation of how viral replication is inhibited is a matter of conjecture. Thus, BVDU is not active against HSV-2 because the HSV-2 thymidylate kinase does not process efficiently BVDUMP to BVDUDP.[4] More recently, further examples of 5-substituted pyrimidine nucleosides have been synthesized[5,6] and shown to have potent activity against HSV-1. Attempts have been made by modelling studies to predict and explain the TK affinity for such compounds[7] now that the X-ray structure for HSV-1 TK is known.[8]

The family of 6-azapyrimidine-2′-deoxynucleosides, first synthesized in the 1960's, rarely shows any significant antiviral activity against herpesvirus[9,10] and it has long been assumed that these analogues are normally not substrates for HSV-1 TK. We present here the synthesis and X-ray crystal structures of a series of 6-aza-2′-deoxyuridine analogues (SCHEME 1) members of which are competitive inhibitors of HSV-1 TK, are thymidine phosphorylase resistant and which might have a clinical use in the prevention of viral pathogenicity.

RESULTS AND DISCUSSION

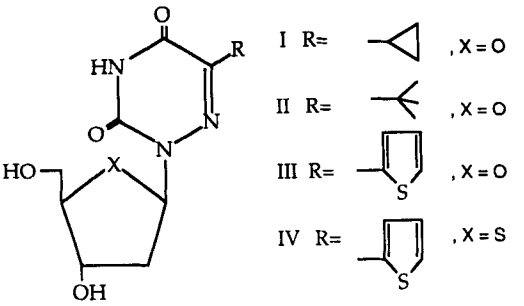
Chemical synthesis

The syntheses of two of the 6-aza-2′-deoxyuridines [I and III, SCHEME 1] in this communication have previously been published.[9] Compounds II and IV were made in an analogous manner by condensation of the appropriate bases and sugar moiety. As expected, compound II was obtained in high yield as one anomer (β)[11] whereas the 4′-thio-2′-deoxy-analogue (IV) gave a much lower (57%) yield of nucleoside which was an α/β, 1:1 mixture, from which the required β-anomer (IV) could be purified by crystallization following deprotection of the mixture with BCl_3. The loss of control of the stereochemistry during the production of 4′-thio-2′-deoxynucleosides is well known; this loss automatically decreases the yield and makes isolation of the final product often very difficult.[12]

Crystal structures

Selected geometrical parameters for the four 6-aza-2′-deoxyuridines (I-IV) whose crystal structures have been determined are listed in TABLE 1. 5-(1-Thienyl)-6-aza-4′-thio-2′-deoxyuridine (IV) crystallises with two independent molecules in the unit cell, labelled A and B in the Table. Stereoscopic views[13] of the molecules are presented in FIGURE 1; since molecules A and B of IV have, apart from a 22° difference in the glycosidic torsion angle, similar conformations, only molecule A is shown.

All four nucleosides have glycosidic torsion angles (χ) within the narrow range -100.0 to -121.8°, *anti* conformation.[14] However the relative flexibility of this conformational



SCHEME I

parameter is demonstrated by the observation that the two chemically identical molecules of structure IV are at extreme opposites of the range.

The 2′-deoxyribose moieties of the three molecules I-III have closely similar conformations (TABLE 1). Compound I can best be described[14] as 3′-endo (^{3}E) with only a very small 2′-exo displacement, whereas II and III, although predominantly 3′-endo, exhibit significant 2′-exo displacements ($^3T_2$ conformation). However both independent molecules of the thiosugar (IV) have a quite different, 3′-exo, 4′-endo ($_3T^4$) sugar pucker. IV differs from I-III also with respect to the orientation about the C4′-C5′ bond (γ), *gauche-trans* (g-) in both independent molecules of IV, and *trans* (t) in I-III.

In all four structures the pyrimidine ring is essentially planar, with root mean square atomic deviations ranging from 0.005 A in I and molecule A of IV, to 0.029 A in II. The 5-thienyl substituents (structures III and IV) are oriented such that the sulfur atom is *trans* to C4 and nearly coplanar with the pyrimidine ring. This orientation may be favoured since it brings the hydrogen atom linked to C8 into a suitable position for possible hydrogen bond to O4; distance H8...O4, *ca.* 2.4 A, angle C8-H8...O4 *ca.* 115°.

Crystallographic structural data appear to be available for only two other 6-aza-nucleosides. In the closely analogous 6-azathymidine,[15] the glycosidic torsion angle is -86.6° (high *anti*) and the sugar has an almost regular C2′-exo, C3′-endo (^3_2T) pucker (pseudorotation angle *P*, 359.1°), similar to our structures I-III. However the orientation about the C4′-C5′ bond is *gauche-gauche* (g+). The second 6-azanucleoside, 6-azauridine,[16] (which is of course a ribonucleoside and is of less relevance), crystallises with two independent molecules, both of which have similar conformations to our structures I-III, with χ -93.1 and -94.8°, sugar pucker $^3T_4$ and $^3T_2$, C4′-C5′ orientation, both *trans*. Thus all the 6-azanucleosides whose structures have been determined, have glycosidic torsion angles in the range -87 to -122°.

TABLE 1. Selected geometrical parameters for structures I - IV. X4' represents O4' in I, II and III, and S4' in structure IV.

	I	II	III	IV molecule A	IV molecule B
	Bond lengths(Å)				
C1'-C2'	1.512(3)	1.508(4)	1.502(5)	1.528(10)	1.509(9)
C2'-C3'	1.514(3)	1.515(4)	1.530(6)	1.515(10)	1.530(9)
C3'-C4'	1.521(2)	1.511(3)	1.517(5)	1.512(10)	1.519(10)
C1'-X4'	1.437(2)	1.430(3)	1.438(4)	1.809(8)	1.821(7)
C4'-X4'	1.443(2)	1.450(3)	1.450(4)	1.818(7)	1.823(7)
C1'-N1	1.460(2)	1.467(3)	1.475(4)	1.489(8)	1.480(8)
	Bond angles(°)				
C1'C2'C3'	104.5(2)	104.1(2)	103.6(3)	110.4(6)	110.1(6)
C2'C3'C4'	102.6(2)	102.9(2)	102.6(3)	106.6(5)	107.5(5)
C3'C4'X4'	105.4(1)	106.3(2)	105.1(3)	104.8(4)	105.2(4)
C4'X4'C1'	110.4(1)	110.0(2)	110.7(3)	94.2(3)	93.6(3)
X4'C1'C2'	106.3(2)	106.7(2)	106.3(3)	107.2(5)	108.6(5)
X4'C1'N1	107.7(2)	108.7(2)	109.1(3)	113.2(5)	112.3(4)
	Torsion angles(°)				
C4'X4'C1'C2' (υ_0)	2.3(2)	7.9(2)	6.8(4)	7.4(5)	7.9(5)
X4'C1'C2'C3' (υ_1)	-21.8(2)	-24.9(2)	-25.6(3)	16.6(7)	14.7(7)
C1'C2'C3'C4' (υ_2)	31.8(2)	31.4(2)	34.0(3)	-38.7(8)	-36.1(7)
C2'C3'C4'X4' (υ_3)	-30.8(2)	-27.2(2)	-30.2(3)	42.3(6)	40.4(7)
C3'C4'X4'C1' (υ_4)	18.1(2)	12.4(2)	15.1(3)	-29.0(5)	-27.9(5)
O5'C5'C4'C3'(γ)	-167.8(2)	-174.5(2)	-173.2(3)	-64.1(8)	-65.2(7)
C5'C4'C3'O3' (δ)	87.1(2)	91.1(3)	89.1(4)	160.2(6)	160.4(5)
X4'C1'N1C2 (χ)	-104.7(2)	-109.2(2)	-109.6(4)	-121.8(6)	-100.0(6)
X4'C1'N1N6	70.3(2)	64.7(3)	70.0(4)	59.8(6)	73.2(6)
C8C7C5C4	109.2(3)	65.1(3)	-11.1(6)	-5.1(9)	-3.6(9)
S11C7C5C4	-	-	172.1(3)	175.5(5)	-179.8(5)
	Pseudorotation parameters(°)				
Phase angle(P)	14.2	4.0	7.0	207.5	209.0
Degree of pucker (ψ_m)	32.8	31.5	34.3	43.6	41.3
Sugar conformation	^3E	$^3T_2$	$^3T_2$	$_3T^4$	$_3T^4$
Glycosidic conformation	anti	anti	anti	anti	anti
C4'-C5' conformation	t	t	t	g-	g-

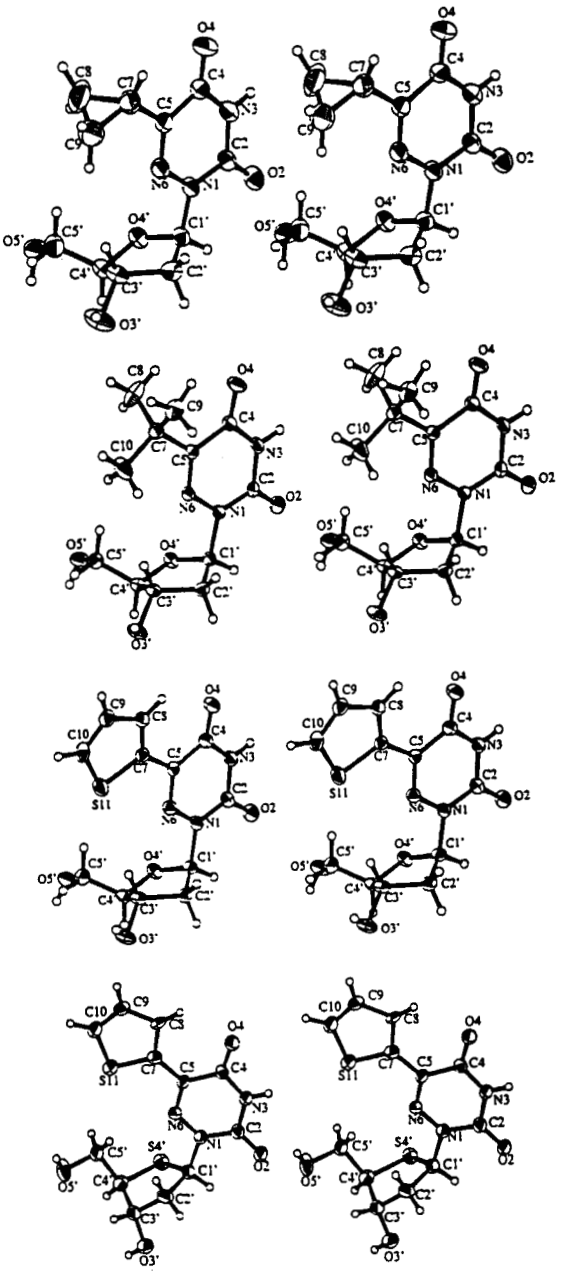


FIG. 1. Stereoscopic views of the molecules I - IV (top I, bottom IV)

The ^{3}E and $^{3}T_{2}$ sugar conformations of our structures I-III are found also in a number of 2′-deoxypyrimidine nucleosides, e.g. 4-*O*-ethylthymidine,[17] 4′-azidothymidine,[18] 5-hydroxymethyl-2′-deoxycytidine[19] and 2′-deoxycytidine hydrochloride.[20] However the $_{3}T^{4}$ conformation of thiosugar IV is rare. Other 4′-thio-2′-deoxynucleosides have nearly regular 2′-endo, 3′-exo puckers ($^{2}_{3}T$, phase angle *P* ca. 180°),[21,22] and a number of 3′-exo, 2′-endo ($_{3}T^{2}$, *P* in range 185-190°) 2′-deoxynucleoside structures are in the literature.[23-25] In terms of the pseudorotational pathway of the sugar ring, these differ from thiosugar (IV) only by some 20-25°.

In all four crystal structures, all the hydrogen atoms linked to O3′, O5′ and N3 take part in the formation of intermolecular hydrogen bonds. Structures I-III are virtually isomorphous and exhibit a similar pattern of hydrogen bonding; O3′ donates a proton to O4′, O5′ donates a proton to O3′ and N3 donates a proton to O5′. A more complicated hydrogen bonding scheme in IV involves two O3′-H...O4 and two N3-H...O5′ hydrogen bonds, together with O5′A-H...O3′B and O5′B-H...S4′A hydrogen bonds. The participation of O4′ in the hydrogen bonding is noteworthy.

The orientation of the 2-thienyl group in both compound III and IV is such that the sulfur atom is *trans*-to-C4. This is opposite to the *cis*-to-C4 orientation of the same sulfur atom in all 5-(2-thienyl)-substituted 2′-deoxyuridines, which possess high antiherpetic activity.[6,7]

The cyclopropyl group is known to be very sensitive towards conjugation and/or inductive properties of the rest of the molecule. This is reflected in its changing conformational behaviour and is related to the unique bonding properties of the cyclopropane ring.[26] It is therefore interesting that the *gauche-cis* conformation of the cyclopropyl group in compound I, with C9-C7-C5-N6 bonds exactly *syn*-periplanar, corresponds to the most populated conformation of the C9-C7-C5-C6 bonds in 5-cyclopropyl-2′-deoxyuridine.[27]

Antiviral assays

Compounds I-IV were submitted for antiviral testing but none showed any activity or even toxicity against HSV-1, HSV-2 or VZV.

Enzyme kinetics

(i) *Effect of compounds I-IV on thymidine phosphorylation.*

Compounds I-IV were assessed for their ability to inhibit the phosphorylation of [^{3}H]-d Thd catalysed by human and HSV-1 TKs.[29] All the compounds exert an inhibitory effect with the viral enzyme but are inactive against the human TK. The results are shown in TABLE 2.

TABLE 2. Effect of compounds I-IV on thymidine phosphorylation by HSV-1 and human TK

	IC_{50} (μM)	
Compound	HSV-1 TK*	Human TK*
I	5.46	>100
II	1.15	>100
III	2.52	>100
IV	0.40	>100

* Thymidine in the TK assays was present at the respective K_m concentrations: 0.8 μM (HSV-1) and 2 μM (human).

(ii) *Mechanism of inhibition.*

In order to determine the mechanism of inhibition of the HSV-1 TK, one of the best inhibitors (compound II) was selected for kinetic analysis. The Lineweaver-Burk plot derived from experiments at variable concentrations of inhibitor and substrate is shown in FIGURE 2A. The kinetics of inhibition is consistent with a competitive mechanism. Inhibition is clearly prevented by sufficient substrate and the Ki value determined by plotting the slope values of the lines reported in panel A *versus* the concentration of inhibitor, was 0.32 μM [FIGURE 2B].

(iii) *Compounds II and III are poor substrates of HSV-1 TK.*

To understand whether these 6-aza-derivatives are *bona fide* substrates subject to phosphorylation, compounds II and III were incubated with HSV-1 TK in the presence of [γ-^{32}P]-ATP as phosphate donor. Conditions promoting the formation of TMP from thymidine were used as a control and at an enzyme concentration which allowed complete phosphorylation of thymidine, only 11% and 15% of compounds II and III respectively, were phosphorylated.

(iv) *Compounds I-IV are resistant to phosphorolysis by human thymidine phosphorylase.*

Human blood platelets contain thymidine phosphorylase (TP) as the sole pyrimidine nucleoside phosphorylase and have been used to monitor the intracellular degradation of thymidine and of various 5-substituted-2′-deoxyuridines.[30] This system has also previously been used to show that BVDU, IDU and other 5-substituted -2′-deoxyuridines are effective substrates of thymidine phosphorylase, which cleaves the uracil ring from the sugar moiety,[30] thus limiting their potential therapeutic activity. The susceptibility of compounds I-IV to thymidine phosphorylase was evaluated by incubating different concentrations of each compound with TP in the presence of 80 μM thymidine as

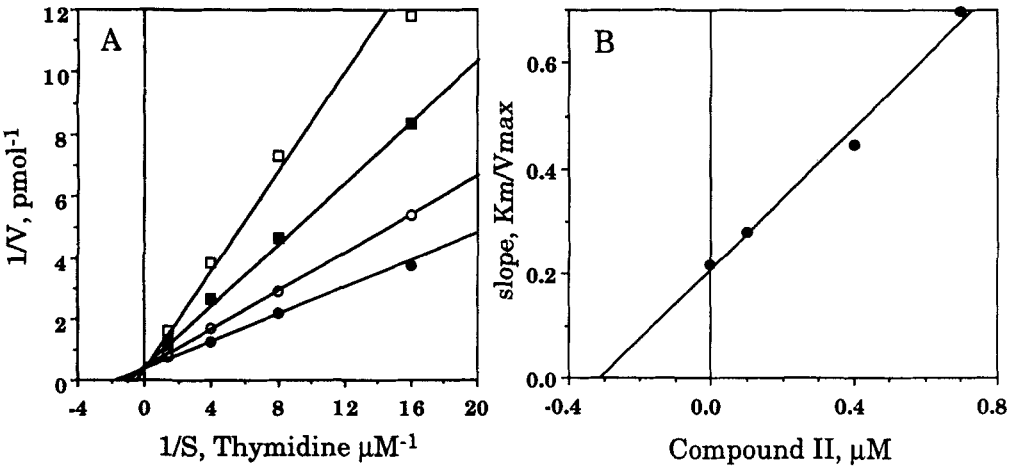


FIG. 2. Panel A: Lineweaver-Burk plot of the effect of compound II on the activity of HSV-1 TK in the presence of increasing concentrations of the substrate [^{3}H]-dThd. Compound II : 0 µM (●), 0.1 µM (○), 0.4 µM (■) and 0.7 µM (□). Panel B: Ki determination of compound II by plotting the slope value of the lines reported in the Lineweaver-Burk plot *vs* concentrations of inhibitor.

described in the experimental. Under these conditions, no compound up to a concentration of 2 mM inhibited the degradation of thymidine. When tested directly for their susceptibility to phosphorolysis, compounds II and III showed no degradation at all (see Experimental) under conditions where thymidine is fully degraded to thymine.

Molecular Modelling

The structures of the inhibitors after docking into the active site of HSV-1 TK followed by energy minimization are shown in FIGURE 3. Upon binding, substrates I-IV adopt a different conformation compared both to their X-ray structures and to thymidine as bound to HSV-1 TK. The azauracil ring is rotated by 30-60° around the χ torsion and also the relative orientation of R is changed to achieve maximal complementarity with the binding pocket (TABLE 3).

The interaction energy between the substrates and the protein, i.e. the sum of electrostatic, van der Waals and non-bonded energies, can be analysed separately for the -R-azauracil- and sugar moieties. In the complexes with R=thienyl, the interaction energy appears to be the same for the azauracil compounds as for the corresponding uracil compound,[7] although their sulfur conformations are opposite. The interaction energies of the t-butyl and cyclopropyl moieties are reduced, because of their smaller size and steric

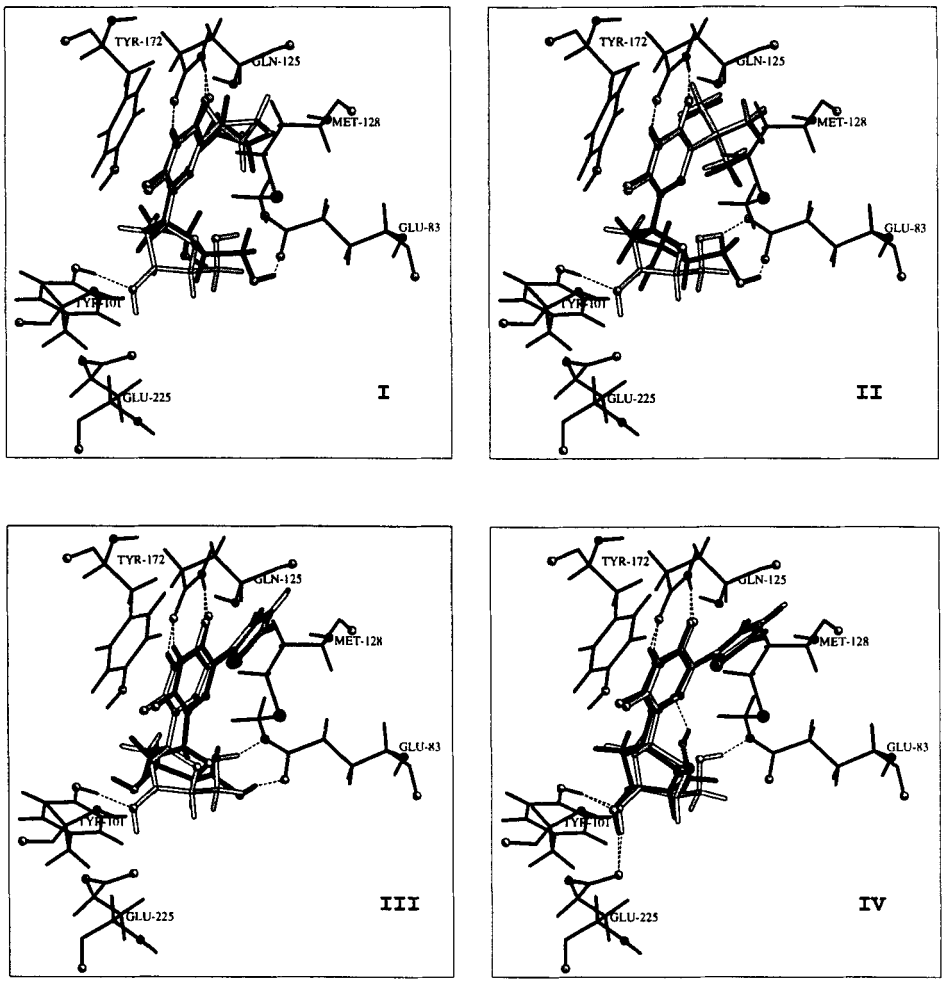


FIG. 3: Compounds I–IV in the binding pocket of HSV1-TK after energy minimization. Compounds with sugar moiety in x-ray conformation are shown in black and in thymidine conformation are shown in grey. Hydrogen bonds are shown by dashed lines, oxygens atoms by light grey spheres, nitrogen atoms by black spheres and sulphur atoms by larger grey spheres.

TABLE 3: Energetic and geometric parameters for compounds I–IV as bound to HSV-1 TK[8]. The compounds which were modelled with the sugar in the same conformation as thymidine in HSV-1 TK are denoted (-alt-s).

Compound	Torsion angle (°)	Interaction energy with HSV-1 TK after energy minimization (kJ/mol)				ΔG_{solv} (kJ/mol)	IC_{50}(pred) (μM)
	χ	sugar-	-aza-U-	-R	total		
I	-97.7	-107.5	-108.6	-20.9	-237.1	4.0	4200
I-alt-s	-113.4	-153.2	-108.2	-21.6	-283.0	4.0	1.2
II	-124.0	-99.8	-104.8	-23.3	-227.8	6.4	9400
II-alt-s	-126.3	-152.2	-103.6	-18.1	-274.0	6.4	2.3
III	-158.6	-87.6	-105.8	-47.6	-241.1	-4.2	56000
III-alt-s	-124.4	-155.3	-105.6	-49.7	-310.7	-4.2	0.2
IV	-117.8	-126.3	-117.3	-49.3	-292.9	-4.2	5.6
IV-alt-s	-113.9	-152.4	-116.5	-49.0	-317.9	-4.2	0.1

hindrance with Tyr-172. The interaction energies between the protein and the azauracil rings are slightly higher than for their uracil counterparts,[7] with the highest value for compound IV. A reason for the latter could be longer C-S bond in IV, which pushes the azauracil ring closer to Tyr-172. In all compounds the azauracil ring is found to be hydrogen bonded via N3 and O4 with Gln-125 in the same manner as the pyrimidine ring in thymidine.[7] A large decrease of interaction energy was seen in the sugar moieties of the inhibitors due to a loss of hydrogen bonding to O3′. This led to poor predictions of the IC_{50} values for all the compounds. However, the sugar moiety exhibits high flexibility and is assumed to be the part with the strongest interaction with the enzyme. Also, earlier studies suggested that the sugar ring binds in the same manner as does thymidine.[7] This led to modelling the sugar in the same orientation as in thymidine, with a regain of hydrogen bonds and interaction energy (FIGURE 3). The predicted IC_{50} values are listed in TABLE 3 and show good agreement with experimental values (FIGURE 4).

CONCLUSIONS

6-Azapyrimidine-2′-deoxynucleosides have received scant attention because they were thought to possess little or no significant biological activity. The data presented here refutes that theory. In particular, the 6-aza analogue of the recently reported antiherpesvirus agent 5-(2-thienyl)-2′-deoxyuridine[31] is shown here to be a specific inhibitor of herpesvirus TK and its 4′-thio congener is even more effective. This property of 6-aza-2′-deoxyuridines has not previously been detected because in antiviral assays in

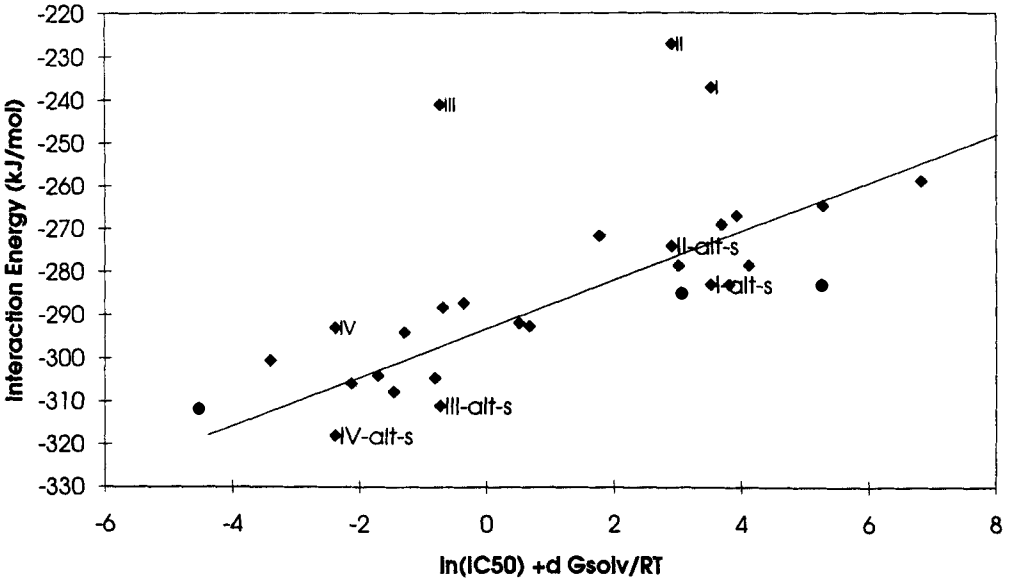


FIG. 4: A plot of $\ln(IC_{50}) + \Delta G_{solv}/RT$ *vs* interaction energy for compounds I–IV and for a series of 5-substituted 2'-deoxyuridine substrates[7]. The compounds which were modelled with the sugar in the same conformation as thymidine in HSV-1 TK are denoted (-alt-s).

vitro, the expression of herpesvirus TK is not necessary for viral replication although it is an important enzyme for the pathogenicity of the virus.[32,33] Not only are the compounds described here inhibitors of herpesvirus TK, the analogues checked in detail (and most probably the others) are very poor substrates. The analogues are also stable to phosphorolysis and should be considered as potential candidates for combatting herpesvirus infections in non-replicating cells such as neurons (herpetic encephalitis) which shortly after birth become TK^- and thymidylate-negative and where virus proliferation depends upon its own TK, or in suppressing reactivation of such infections in the clinic.

EXPERIMENTAL

NMR spectra were recorded on Bruker AC300 and AMX400 spectrometers and the chemical shift values are in ppm. Mass spectra were recorded on a Kratos MS580 spectrometer. Chemical ionization (CI) or fast atom bombardment (FAB) were used as necessary. Precoated Merck silica gel 60 F_{254} plates were used for TLC and compounds were detected under UV light (254 nm). Column chromatography was performed using

Kieselgel 60, 230-400 mesh, type 9385. Glass columns were slurry-packed under gravity. Chloroform was dried by heating under reflux over phosphorus pentoxide and distilled, acetonitrile was dried by heating under reflex over calcium hydride and distilled and methanol was dried by heating under reflux over magnesium methoxide and distilled. All these dried solvents were stored over type-4A molecular sieves. Diethyl ether was dried with sodium wire.

***tert*-Butylglyoxylic acid [V].** *tert*-Butylmethylketone (Aldrich, 98%; 10 g, 0.1 mol), sodium hydroxide (12 g, 0.3 mol) and potassium permanganate (48 g, 0.3 mol) in distilled water (1 λ) were stirred in a stoppered flask at room temperature for 3 hr. Methanol was then added portionwise to decompose the excess of oxidant and the reaction mixture was left overnight at room temperature. The mixture was then filtered with suction through a layer of Celite to remove MnO_2 and the colourless filtrate was concentrated under reduced pressure to a small volume (*ca.* 20 ml), which was then acidified with dilute sulfuric acid and extracted with ether (6 x 60 ml). The combined ethereal extracts were washed with brine (25 ml), dried ($MgSO_4$) and the solvent removed to provide the *title compound* (5.76 g, 44.3%) in crude form which was used directly in the following reaction.

***tert*-Butylglyoxylic acid thiosemicarbazone [VI].** A hot aqueous solution of thiosemicarbazide (2.9 g, 31.8 mmol) in water (31 ml) was immediately added to compound V (4.13 g, 31.8 mmol) with stirring and left to cool at room temperature. The product was then collected by suction filtration, washed with a small amount of cold water and dried *in vacuo* to give a crude product (5.3 g, 82.1%) which was used directly in the following reaction. CIMS *m/z* 204 $(M+1)^+$, 186 $(M-17)^+$, 170 $(M-33)^+$, 158 $(M-45)^+$.

5-*tert*-Butyl-3-thioxo-2,3,4,5-tetrahydro-1,2,4-triazin-5-one [VII]. The thiosemicarbazone [VI] (5.2 g, 25.6 mmol) was boiled with sodium hydroxide solution (1 M, 55 ml) under reflux for 20 min. The resulting reaction mixture was then cooled in ice, acidified with HCl and the white solid collected by suction filtration, washed with a small amount of cold water and dried *in vacuo* to provide the title compound VII which was used directly in the following reaction. CIMS *m/z* 186 $(M+1)^+$. ^{1}H-NMR δ (DMSO-d_6) 13.37 and 13.03 (2H, 2bs, 2NH), 1.23 (9H, s, *t*-butyl).

5-*tert*-Butyl-6-azauracil [VIII]. A suspension of compound VII (4.6 g, 24.9 mmol) in water (75 ml) containing chloroacetic acid (12 g, 125 mmol) was stirred under reflux for 60 h. The resulting suspension was cooled to room temperature, the crude product collected by suction filtration, washed with cold water and dried to provide the *title compound* [VIII], (3.28 g, 77%). CIMS *m/z* 170 $(M+1)^+$. ^{1}H-NMR δ (DMSO-d_6) 12.07 and 11.81 (2H, 2bs, 2NH), 1.24 (9H, s, *t*-butyl). Elemental analysis $C_7H_{11}N_3O_2$ (169.16) calculated C, 49.70; H, 6.55; N, 24.83; found C, 49.62; H, 6.66; N, 24.57%.

5-*tert*-Butyl-1-(2-deoxy-3,5-di-*O*-*p*-toluoyl-β-D-*erythro*-pentofuranosyl)-6-azauracil [X]. A suspension of compound VIII (1.35 g, 8 mmol) in hexamethyldisilazane (36 ml) and trimethylsilychloride (5 ml) was heated under reflux until complete solution had been achieved (3 h). The excess of reagents was removed by distillation *in vacuo* and to the resulting crude silylated base [IX] was added a solution of 2-deoxy-3,5-di-*O*-*p*-toluoyl-α-D-erythro-pentofuranosyl chloride (3.2 g, 8.1 mmol) in dry chloroform (70 ml) and the mixture stirred at room temperature with exclusion of moisture for 2.5 h. The reaction was then judged to be complete (TLC, ethyl acetate/n-hexane, 1:1) and the reaction mixture was taken to dryness and the residue purified on a silica gel column (ethyl acetate/n-hexane, 1:1). Appropriate fractions were combined and taken to dryness to give the *title compound* [X] (3.88 g, 93%) as a white stable foam. FABMS *m/z* 522 $(M+1)^+$, 386 $(M-135)^+$, 353 $(M-168)^+$. ^{1}H-NMR δ ($CDCl_3$) 8.25 (1H, bs, NH), 7.93 (4H, m, *p*-Tol), 7.23 (4H, m, *p*-Tol), 6.69 (1H, t, H-1', *J*=5Hz), 5.72 (1H, m, H-3'), 4.52 (3H, m, H-4', H-5', H-5"), 2.99 (1H, m, H-2'), 2.48 (1H, m, H-2"), 2.43 and 2.39 (2 x 3H, 2s, 2xCH_3, *p*-Tol), 1.38 (9H, s, *t*-butyl). Anal. $C_{28}H_{31}N_3O_7$ (521.53) C, 64.48; H, 5.99; N, 8.06. Found C, 64.75; H, 6.01; N, 8.05.

5-*tert*-Butyl-6-aza-2'-deoxyuridine [II]. Compound X (500 mg, 0.96 mmol) was added to a solution of sodium methoxide (25 ml, 5 mM solution prepared by dissolving 56 mg of sodium in 25 ml of dry methanol) and stirred at room temperature overnight. The reaction mixture was then diluted with methanol (50 ml), neutralized with Dowex-50 ion exchange resin, the resin removed by filtration and the organic solution worked up in the usual way to provide a crude yield of the *title compound* [II] (200 mg, 72.8%) which could be recrystallized from methanol, M.p. 121-122 °C FABMS *m/z* 286 $(M+1)^+$. ^{1}H-NMR δ (DMSO-d_6) 12.05 (1H, bs, NH), 6.34 (1H, dd, H-1', $J_{1'2'}$=5 Hz, $J_{1'2''}$=7.5 Hz), 5.18 (1H, d, OH-3', *J*=4.5 Hz, 4.66 (1H, t, OH-5', *J*=5.0 Hz), 4.32 (1H, m, H-3'), 3.68 (1H, m, H-4'), 3.41 (2H, m, H-5', H-5"), 2.41 (1H, m, H-2'), 2.07 (1H, m, H-2"), 1.27 (9H, s, *t*-butyl). Elemental analysis $C_{12}H_{19}N_3O_5$ (285.27) calculated C, 50.52; H, 6.71; N, 14.73; found C, 50.44; H, 6.80; N, 14.64%.

5-(2-Thienyl)-1-(2-deoxy-4-thio-3,5-di-*O*-benzyl-α/β-D-*erythro*-pentofuranosyl)-6-azauracil [XI]. 5-(2-Thienyl)-6-azauracil (5.3 g, 27.2 mmol) and *bis*-trimethylsilylacetamide (11 g, 54 mmol) was stirred in dry acetonitrile (150 ml) for 20 min to give a clear solution. Dried molecular sieves (6 g) were added and the reaction flask was flushed with dry nitrogen and kept under N_2 throughout the following reaction. Benzyl 3,5-di-*O*-benzyl-1,4-dithio-2-deoxy-D-ribofuranoside (12 g, 28 mmol) in acetonitrile (160 ml) was added followed by *N*-iodosuccinimide (3.2 g, 14 mmol) in acetonitrile (70 ml). The reaction mixture immediately turned a dark red colour and was

stirred at room temperature for 7.5 h. The reaction was then judged to be complete (TLC, absence of any sugar starting material), the molecular sieves were filtered off and the organic layer taken to dryness to yield a brown amorphous solid which was collected by suction filtration and treated with chloroform (100 ml) in an ultrasound bath. The resulting fine suspension was removed by filtration to provide 2.12 g (40%) of unreacted base starting material. The filtrate was taken to dryness, the residue dissolved in boiling methanol, decolourized with charcoal and allowed to stand overnight. The resulting solid was isolated by suction filtration to give the *title compound* XI as an inseparable α/β-anomeric mixture (4.03 g, 56.8%). The ^{1}H-NMR spectrum of the protected mixture is complex but clearly identifiable are the two H-1′ protons at δ 6.20 and 6.27 (dd) in the ratio 1:1. FABMS *m/z* 510 $(M+1)^+$. The material so obtained was used directly in the next experiment.

5-(2-Thienyl)-6-aza-4′-thio-2′-deoxyuridine [IV]. The compounds [XI] (2.54 g, 5 mmol; α/β 1:1) in dry dichloromethane (100 ml), were added with stirring to a solution of BCl_3 in dichloromethane (50 ml, 1 M) and the temperature kept at -90 °C in a cryostat under a stream of dry nitrogen overnight. The reaction mixture was then quenched by the addition of dry MeOH and dichloromethane (100 ml, 1:1) while the temperature was maintained below -70 °C. The reaction mixture was then allowed to warm to room temperature, the solvents removed and the residue recrystallized from methanol to give two crops: first crop 592 mg (86% β, 14% α) and the second crop 618 mg (22% β, 78% α) to give an overall recovery of 74%. Further recrystallization of the first crop from methanol provided the pure *title compound* (83 mg, 5%, M.p. 213-214 °C), which could undoubtedly be improved if required. FABMS *m/z* 328 $(M+1)^+$. ^{1}H-NMR δ (DMSO-d_6) 12.45 (1H, s, NH), 8.00 (1H, dd, H-5"thienyl, $J_{5",4"}$=4Hz, $J_{5",3"}$=1Hz), 7.75 (1H, dd, H-3"thienyl, $J_{3",4"}$=5Hz, $J_{3",5"}$=1Hz), 7.19 (1H, dd, H-4"thienyl, $J_{4",5"}$=4Hz, $J_{4",3"}$=5Hz), 6.27 (1H, dd, H-1′, $J_{1',2'}$=5Hz, $J_{1',2"}$=6Hz), 5.26 (1H, d, OH-3′, *J*=5Hz), 5.00 (1H, t, OH-5′, *J*=5Hz), 4.58 (1H, m, H-3′), 3.80 (1H, m, H-4′), 3.48 and 3.2 (2H, 2m, 2xH-5′), 2.50 (1H, m, H-2′), 2.24 (1H, m, H-2"), Anal. $C_{12}H_{13}N_3O_4S_2$ (327.34) C, 44.02; H, 4.00; N, 12.84; S, 19.59. Found C, 44.31; H, 4.29; N, 12.56; S, 19.32%. The crude α-anomer was not further purified δ 6.18 (1H, dd, H-1′)

X-ray Crystallography. Data for all four structures were measured on Rigaku R-axis II area detector diffractometer with MoKα radiation at 293(2)K. The structures were determined by direct methods[34] and refined[35] by least squares using anisotropic displacement parameters for the non-hydrogen atoms. Hydrogen atoms linked to carbon and nitrogen were placed in calculated positions. Those linked to the oxygen atoms, O3′ and O5′ were located from difference maps in structures I, II and III, and were included in

the refinement with isotropic displacement parameters. In structure IV, these hydrogen atoms were placed in idealised positions, C-O-H angle tetrahedral and torsion angle chosen to maximise the electron density at the hydrogen positions.

Crystal and refinement data are presented in TABLE 4. The final atomic parameters have been deposited.

Viral plaque reduction assays and cytotoxicity assay were performed as previously described.[34]

Enzymes. Both the host- and virus-specific thymidine kinases (TKs) were purified by affinity chromatography by using a CH Sepharose 4B column coupled with thymidine 3′-(*p*-aminophenyl phosphate) as previously described.[29] Thymidine phosphorylase was partially purified from human platelets by ultracentrifugation and ion-exchange chromatography using a HiTrapQ column (Pharmacia).

Cells. HeLa TK^-/HSV-1 TK^+ (HeLa TK^- transformed to the TK^+ phenotype with a functional copy of the HSV-1 TK gene) were obtained from Professor G. Della Valle, Department of Genetics and Microbiology, "A. Buzzati Traverso", University of Pavia, Italy. The cell lines were tested for mycoplasma contamination with Hoechst 33258 stain and found to be negative. The cells were grown in DMEM with 10% Fetal Calf Serum (NBL Laboratories). Media were invariably supplemented with **L**-glutamine (2 mM) and penicillin-streptomycin (GIBCO).

Thymidine Kinase Assays. These assays were performed as previously described.[28] Briefly, enzyme was incubated at 37 °C for 30 min in a mixture (25 µl) containing HEPES K^+ (30 mM, pH 7.5), $MgCl_2$ (6 mM), ATP (6 mM), dithiothreitol (0.5 mM) and [^{3}H]-dThd (0.8 µM, 2200 cpm/pmol). Human cytosolic TK was assayed under the same reaction conditions apart from the concentration of [^{3}H]-dThd (2 µM, 2200 cpm/pmol). The reaction was terminated by spotting 20 µl of the incubation mixture onto a 25 mm DEAE paper disc (DE-81 paper, Whatman). The disc was washed twice in an excess of ammonium formate (1 mM, pH 5.6) in order to remove unconverted nucleoside and then twice in ethanol and then counted in a β-counter.

When [γ-^{32}P]-ATP was used in the TK assay, the enzyme was incubated in the mixture described above but containing [γ-^{32}P]-ATP (1mM, 125 cpm/pmol), $MgCl_2$ (2 mM) and compound II, III or IV (40 µM). After 1 h at 37 °C, to each sample was added EDTA (1.5 µl, 0.5 M) to stop the reaction, followed by a solution (2 µl) containing TMP (10 mM), dThd (10 mM) as markers at 260 nm. Samples were heated for 5 min at 80 °C, centrifuged at 10,000 rpm for 10 min and aliquots (20 µl) were taken for analysis by HPLC.

TABLE 4. Crystallographic and experimental data

	I	II	III	IV
formula	$C_{11}H_{15}N_3O_5$	$C_{12}H_{19}N_3O_5$	$C_{12}H_{13}N_3O_5S$	$C_{12}H_{13}N_3O_4S_2$
f w	269.3	285.3	311.3	327.4
cryst. sys.	orthorhombic	orthorhombic	orthorhombic	monoclinic
space grp.	$P2_12_12_1$	$P2_12_12_1$	$P2_12_12_1$	$P2_1$
a, Å	13.070(6)	13.558(8)	13.160(12)	10.453(10)
b, Å	17.193(9)	17.896(8)	18.33(2)	19.182(11)
c, Å	5.780(2)	5.850(3)	5.649(4)	6.789(4)
β,deg	-	-	-	91.49(2)
V, Å^3	1299(1)	1419(1)	1363(2)	1361(2)
Z	4	4	4	4
D_c, g cm^{-3}	1.377	1.335	1.517	1.598
μ(MoKα), mm^{-1}	0.110	0.105	0.264	0.411
crystal size, mm	0.4 x 0.2 x 0.2	0.4 x 0.3 x 0.2	0.3 x 0.2 x 0.2	0.3 x 0.2 x 0.2
θ range,deg	2.4 - 25.2	1.9 - 25.2	1.9 - 25.2	2.0 - 25.2
unique rflns[I>σ(I)]	2263	2500	2342	3977
Δρ, maxm. +ve, eÅ^{-3}	0.10	0.14	0.16	0.34
Δρ, maxm. -ve, eÅ^{-3}	0.12	0.17	0.25	0.32
R	0.0415	0.0554	0.0606	0.0749,
wR2[a]	0.0987	0.1133	0.1185	0.1897
w(a,b)[b]	0.051, 0.19	0.058, 0.37	0.048, 0.73	0.107, 0.0

[a] $wR2 = [\Sigma w(Fo^2-Fc^2)^2/\Sigma w(Fo^2)^2]^{1/2}$

[b] $w = 1/[\sigma^2(Fo^2) + (aP)^2 + bP]$ where $P = (Fo^2 + 2Fc^2)/3$

Nucleoside and Nucleotide Separation by Reverse-phase HPLC. The reverse-phase method employing the Bio-Rad 100 MAPS preparative system was used in order to separate nucleosides from nucleotides. An 0.4 x 15 cm Reverse Phase C_{18} BioSil ODS-5S column was used at room temperature under the following conditions: injection volume, 20 µl; detection, UV 260 nm; eluents, buffer A (20 mM KH_2PO_4; pH 5.6), buffer B (20 mM KH_2PO_4, pH 5.6, 60% methanol). Gradient conditions: 0-5 min, 0% buffer B; 15 min, 50% buffer B; 16-28 min, 100% buffer B. Flow rate: 0.5 ml/min, 56 fractions (250 µl) collected and counted in a β-counter.

Thymidine Phosphorylase Assays. Human thymidine phosphorylase was assayed as follows: enzyme, partially purified as described from human platelets,[28] was incubated at 37 °C for 30 min in a mixture (25 µl) containing Na-maleate (40 mM, pH 5.7), NaH_2PO_4 (10 mM, pH 5.7), and [^{3}H]-Thd (80 µM, 270 cpm/pmol). The reaction was stopped by the addition of trichloroacetic acid (5 µl, 100% w/v), reaction tubes were then centrifuged (10,000 rpm, 5 min) and the thymine produced assayed as follows: aliquots (2 µl) were loaded on to a silica gel plate (Merck) which was developed in ethyl acetate/water/formic acid, 60:35:5. Lanes were then cut out and radioactivity from single compounds counted in a β-counter.

Nucleoside Degradation by Intact Human Blood Platelets. The phosphorolysis of compounds II and III and thymidine by intact human blood platelets was measured by an HPLC method. The nucleoside degradation assay and HPLC analysis were formed as described by Desgranges *et al.*[30] Nucleosides were separated from their corresponding bases by HPLC on a Reverse-Phase C_{18} BioSil ODS-55 column eluted with the following linear gradient and conditions: injection volume, 20 µl; detection, UV 330 nm for compounds II and III and 260 nm for thymidine; eluents, buffer A (10 mM KH_2PO_4, pH 4.6), buffer B (10 mM KH_2PO_4, pH 4.6, 60% methanol). Gradient conditions: 0-5 min, 0% buffer B; 35 min, 100% buffer B. Flow rate: 0.5 ml/min.

Molecular Modelling. The modelling followed closely the procedure described for a series of 5-substituted-2′-deoxyuridine substrates.[7] As a starting model, the 2.8 Å resolution crystal structure of HSV-1 TK complexed with thymidine[8] was used. Molecule 2 of the asymmetric unit and all crystallographic water were removed. Hydrogen atoms were added using MacroModel[37] and HBPlus.[38] All histidine residues were assumed to be neutral and protonated at Nε (residues 58, 142 and 213) or Nδ (residues 105, 283, 323 and 351). Atomic partial charges for the four compounds were calculated by use of a standard procedure for fitting the electrostatic potential at the STO-3G level of theory.[39] The *ab initio* calculations were done using GAMESS.[40] The substrates were docked into the active site of HSV-1 TK by fitting the azauracil rings onto the pyrimidine ring of

thymidine in the complex. Alternatively the sugar moieties of the substrates were placed in the same orientation and conformation as for thymidine. After elimination of thymidine, the whole complex was energy minimized until the energy gradient dropped below 0.01 kJ/mol Å. For the energy calculations the BatchMin module of MacroModel with its AMBER 3 force field[41] was used. A distance-dependent dielectric constant was applied and a non bonded cutoff distance of 10 Å. Residues having no atoms within 3 Å of the substrates were restrained to their crystallographic positions with a harmonic force constant of 1000 kJ/mol $Å^2$.

The total solvation free energies of the compounds were calculated through its decomposition into electrostatic and non-polar components. The electrostatic part was determined by solving the Poisson-Boltzmann equation as implemented by the DelPhi program.[42] The solvent accessible surface area was calculated with the DMS program of MidasPlus.[43] Probe radii and dielectric constants were 1.4 Å and 80 for the solvent and 0 Å and 2 for the vacuum calculations. The atomic partial charges were obtained by the CHELPG procedure of GAMESS after 6-31G** *ab initio* calculation.[40] The non-polar component of the free solvation energy was estimated by extrapolation from a least-squares fit of the relationship of the surface area of alkanes with experimental transfer energies.[44] The least squares equation used for the prediction of IC_{50} values is:[7] $\ln(IC_{50}) = 0.177\ IE - \Delta G_{solv}/RT + 51.9$, where IE as interaction energy in kJ/mol, ΔG_{solv} is the solvation energy of the substrate in kJ/mol, and the units RT and IC_{50} are kJ/mol and μM respectively.

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