Anti-(herpes simplex virus) activity of 4′-thio-2′-deoxyuridines: a biochemical investigation for viral and cellular target enzymes[1]

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The antiviral activity of several nucleoside analogues is often limited by their rapid degradation by pyrimidine nucleoside phosphorylases. In an attempt to avoid this degradation, several modified nucleosides have been synthesized. A series of 4′-thio-2′-deoxyuridines exhibits an anti-[herpes simplex virus (HSV)] activity significantly higher (20–600 times) than that shown by the corresponding 4′-oxy counterpart. We investigated the mode of action of these compounds and we found that: (i) several 4′-thio-2′-deoxyuridines are phosphorylated to the mono- and di-phosphates by HSV-1 thymidine kinase (TK) more efficiently than their corresponding 4′-oxy counterpart; (ii) both are inhibitors of cellular thymidylate synthase; (iii) 4′-thio-2′-deoxyuridines are resistant to phosphorolysis by human thymidine phosphorylase; (iv) both 4′-oxy- and 4′-thio-2′-deoxyuridines are phosphorylated to deoxyribonucleotide triphosphate in HSV-1-infected cells and are incorporated into viral DNA; (v) 4′-thio-2′-deoxyuridines are better inhibitors than their 4′-oxy counterparts of [^{3}H]thymidine incorporation in HSV-1-infected cells; (vi) 4′-thio-2′-deoxyuridines are not recognized by HSV-1 and human uracil-DNA glycosylases. Our data suggest that 4′-thio-2′-deoxyuridines, resistant to pyrimidine phosphorylase, can be preferentially or selectively phosphorylated by viral TK in HSV-infected cells, where they are further converted into triphosphate by cellular nucleotide kinases. Once incorporated into viral DNA, they are better inhibitors of viral DNA synthesis than their corresponding 4′-oxy counterpart, either because they are not recognized, and thus not removed, by viral uracil-DNA glycosylase, or because they preferentially interfere with viral DNA polymerase.

Key words: inhibitors, nucleoside analogues, thymidine kinase, thymidine phosphorylase.

INTRODUCTION

The antiviral activity of 5′-bromovinyl-2′-deoxyuridine (BVdU) and similar analogues is often limited by their rapid degradation by pyrimidine nucleoside phosphorylases, which cleave the glycosidic bond between the pyrimidine ring and the sugar moiety. In an attempt to avoid this degradation, modified nucleosides, such as carbocyclic derivatives [1,2], have been synthesized in which the sugar moiety has been replaced by a cyclopentyl or a cyclobutyl ring, or enantiomeric derivatives [3,4], in which the D-deoxyribose moiety has been replaced by the L enantiomer [3–6]. More recently, several 4′-thio-2′-deoxyribonucleosides have been synthesized and studied for their possible antiviral activity [7]. We show here that the anti-herpetic activity of a large series of these derivatives, whose chemical structures are reported in Table 1, is always significantly higher than that of the corresponding 4′-oxy derivatives. In order to investigate the mode of action of these compounds, we have thus studied a number of biochemical parameters such as their resistance to phosphorolysis by intact human blood platelets or by recombinant human thymidine phosphorylase (TP) and their ability to act as inhibitors and substrates for cellular and viral thymidine kinases (TKs). Two reference nucleosides were ^{3}H-labelled, thus allowing us to investigate whether they are phosphorylated to mono- and di-phosphate by herpes simplex virus type-1 (HSV-1) TK or if they are inhibitors of human thymidylate synthase (TS), human uracil-DNA glycosylase (UDG) and HSV-1 UDG and whether they are phosphorylated to 4′-thio-2′-deoxyribose triphosphate in infected cells, any or all of which may interfere with viral DNA polymerase and DNA synthesis.

MATERIALS AND METHODS

Chemicals

Commercial reagents and solvents of analytical grade were used unless otherwise stated. [*methyl*-^{3}H]Thymidine ([*methyl*-^{3}H]Thd; sp. radioactivity 25 Ci/mmol), [γ-^{32}P]ATP (sp. radioactivity 3000 Ci/mmol) and [5-^{3}H]deoxycytidine (sp. radioactivity 12 Ci/mmol) were from Amersham (Arlington Heights, IL, U.S.A.),

Abbreviations used: HSV-1, herpes simplex virus type 1; HSV-2, herpes simplex virus type 2; VZV, varicella zoster virus; CMV, cytomegalovirus; VV, vaccinia virus; VSV, vesicular-stomatitis virus; TK, thymidine kinase; TP, thymidine phosphorylase; TS, thymidylate synthase; UDG, uracil-DNA glycosylase (EC 3.2.2.3; systematic name uridine ribohydrolase); dU, 2′-deoxyuridine; SdU, 4′-thio-2′-deoxyuridine; Thd, thymidine; SThd, 4′-thio-thymidine; EdU, 5-ethyl-2′-deoxyuridine, SEdU, 4′-thio-5-ethyl-2′-deoxyuridine; IPdU, 5-isopropyl-2′-deoxyuridine; SIPdU, 4′-thio-5-isopropyl-2′-deoxyuridine; CPdU, 5-cyclopropyl-2′-deoxyuridine; SCPdU, 4′-thio-5-cyclopropyl-2′-deoxyuridine; t-BdU, 5-t-butyl-2′-deoxyuridine; StBdU, 4′-thio-5-t-butyl-2′-deoxyuridine; CBdU, 5-cyclobutyl-2′-deoxyuridine; SCBdU, 4′-thio-5-cyclobutyl-2′-deoxyuridine; CPedU, 5-cyclopentyl-2′-deoxyuridine; SCPedU, 4′-thio-5-cyclopentyl-2′-deoxyuridine; CHdU, 5-cyclohexyl-2′-deoxyuridine; SCHdU, 4′-thio-5-cyclohexyl-2′-deoxyuridine; BVdU, 5-bromovinyl-2′-deoxyuridine; SBVdU, 4′-thio-5-bromovinyl-2′-deoxyuridine; IdU, 5-iodo-2′-deoxyuridine; SIdU, 4′-thio-5-iodo-2′-deoxyuridine; HEL, human embryonic lung fibroblasts; $CCID_{50}$, 50% cell culture infective dose; DMEM, Dulbecco's modified Eagle's medium; FCS, foetal-calf serum; PFU, plaque-forming units; MOI, multiplicity of infection.

[1] This paper is dedicated to the late Professor R. T. Walker.

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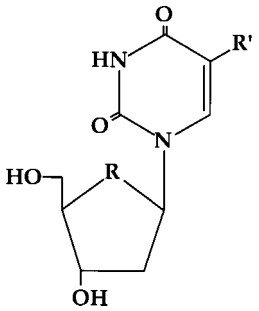
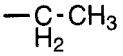
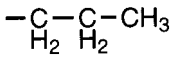
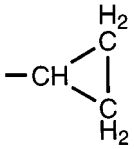
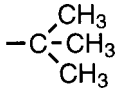
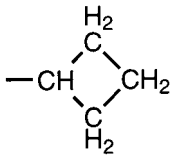
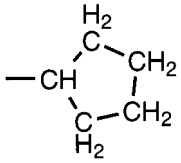
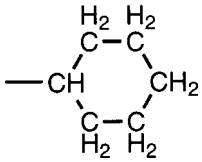
Table 1 4′-Thio-2′-deoxyuridines: chemical structure of the 5-substituents (R′)

R is sulphur (S) or oxygen (O) in 4′-thio and 4′-oxy-derivatives respectively.



R′ substituent	Chemical structure
Hydrogen	
Methyl	
Ethyl (E)	
Isopropyl (IP)	
Cyclopropyl (CP)	
t-Butyl (t-B)	
Cyclobutyl (CB)	
Cyclopentyl (CPe)	
Cyclohexyl (CH)	

whereas [^{3}H]isopropyldeoxyuridine (IPdU) (sp. radioactivity 20 Ci/mmol) and [^{3}H]isopropylthiodeoxyuridine (SIPdU) (sp. radioactivity 2.8 Ci/mmol) were prepared by CEO SibTech, Inc. (Newington, CT, U.S.A.).

Synthesis of nucleosides

The series of 5-substituted 4′-thio-2′-deoxyuridines was synthesized as previously described [7].

Cells and virus assays

HeLa TK$^-$ cells (human epithelial cell line derived from a cervix carcinoma, TK-deficient) and HeLa TK$^-$/HSV-1 TK$^+$ (HeLa TK$^-$ transformed to the TK$^+$ phenotype with a functional copy of the HSV-1 TK gene) were those previously used [3]. Cells were tested for mycoplasma contamination with the Hoechst 33258 staining method and were found to be negative. The cells were grown in Dulbecco's modified Eagle's medium (DMEM) with 10% (v/v) foetal-calf serum (FCS). Media were invariably supplemented with 2 mM L-glutamine and penicillin/streptomycin. HSV-1 (strain LV)-infected HeLa TK$^-$ cells, kindly supplied by Professor R. Manservigi (Institute of Microbiology and Interdepartmental Centre for Biotechnology, University of Ferrara, Ferrara, Italy) were the source of HSV-1 TK enzyme purified as previously described [8].

Confluent human embryonic E_6SM fibroblasts and human embryonic lung (HEL) cells were grown in 96-well microtitre plates and inoculated with the KOS, McIntyre or F strains of HSV-1 (E_6SM), the G, 196 or Lyons strains of HSV-2, VV (E_6SM) and VSV (E_6SM) at 100 $CCID_{50}$ (50% cell culture infective dose) per well, with the OKA and YS strains of VZV (HEL) at an input of 20 plaque-forming units (PFU)/well, or with the AD169 and Davis strains of cytomegalovirus (CMV) (HEL) at 100 PFU/well. After a 1–2 h incubation period, residual virus was removed and the infected cells were further incubated with minimal essential medium (supplemented with 2% inactivated FCS, 2 mM L-glutamine and 0.3% $NaHCO_3$) containing various concentrations (2000, 1000, 500, 200, 50, 20, 5, 2, 0.5, 0.2, 0.05, 0.02 μM) of the compounds until the virus-induced cytopathicity was measured. Antiviral activity was expressed as EC_{50} (50% effective concentration) or compound concentration required to reduce viral plaque formation after 5 days (varicella zoster virus, VZV) or virus-induced cytopathicity [CMV after 7 days and HSV, vaccinia virus (VV) and vesicular-stomatitis (VSV) after 3 days] by 50% compared with the untreated control. Average values were obtained for the results independently obtained for the three HSV-1, three HSV-2, two VZV and two CMV strains included in the present study. The different strains of HSV-1, HSV-2, VZV and CMV are known not to differ significantly in their susceptibility for nucleoside analogues. The cytotoxicity measurements were based on a microscopically visible alteration of normal E_6SM cell morphology (expressed as the minimal cytotoxic concentration) or compound concentration that causes alteration of the morphology of the cell cultures. The cytostatic measurements were based on an inhibition of normal HEL cell growth (expressed as the 50% cytostatic concentration or compound concentration required to inhibit HEL cell proliferation by 50%).

Cloning, expression and purification of human TP

The complete cloning of human TP has been extensively described by Focher et al. [9]. Briefly, TP cDNA, contained in plasmid pPL5 [10] (kindly provided by Professor R. Bicknell, Institute of Molecular Medicine, University of Oxford, Oxford, U.K.), was subcloned in pTrcHisC (Invitrogen, Leek, The Netherlands) to give the recombinant bacterial expression vector pHisTP containing the His-tagged TP sequence which encodes for a 509-amino-acid protein. His-tagged TP expression and purification were carried out as described by the manufacturer of the Ni-

NTA (Ni^{2+}-nitrilotriacetate) Superflow resin (Qiagen, Hilden, Germany).

TK assay

HSV-1 TK was purified and assayed as previously described [8] with some modifications. Briefly, the enzyme was incubated at 37 °C for 30 min in a mixture (25 μl) containing 30 mM potassium Hepes, pH 7.5, 6 mM $MgCl_2$, 6 mM ATP, 0.5 mM dithiothreitol and 0.15 μM [*methyl*-^{3}H]deoxythymidine ([*methyl*-^{3}H]dThd) (2200 c.p.m./pmol) or different concentrations of [^{3}H]IPdU (sp. radioactivity 1743 c.p.m./pmol) or [^{3}H]SIPdU (sp. radioactivity 246 c.p.m./pmol). Human cytosolic TK, purified from HeLa cells [8], was assayed under the same reaction conditions, but using [*methyl*-^{3}H]dThd (sp. radioactivity 2200 c.p.m./pmol) at 1.12 μM in place of 0.15 μM. The reaction was terminated by spotting 20 μl of the incubation mixture on a 25 mm-diameter DEAE-paper disk (DE-81 paper; Whatman). The disks were processed and the radioactivity was counted as described in [8].

Nucleoside degradation by recombinant human TP and by intact human blood platelets and HPLC analysis of the reaction products

A mixture (25 μl) containing 0.1 mM thymidine or 0.1 mM of the compound to be tested, 50 mM sodium arsenate, pH 6.2, and an amount of enzyme to give linear reaction rate was incubated at 37 °C for 30 min and then heated 5 min at 90 °C. The tube was then centrifuged at 9000 ***g*** for 5 min, the supernatant (20 μl) was then injected into the HPLC apparatus and analysed by the procedure described by Focher et al. [9]. The relative peak areas of thymine and thymidine were used to determine the enzymic activity of TP.

To determine the phosphorolysis of 4′-thio- and 4′-oxy-2′-deoxyuridines by intact human blood platelets, prepared as described by Desgranges et al. [11], a sample of 3×10^8 platelets/ml was incubated at 37 °C in a mixture (350 μl) containing 10 mM Tris/HCl (pH 7.5), 1 mM sodium phosphate (pH 7.5), 0.15 M NaCl, 1 mM EDTA and 0.1 mM of the compound to be tested. At different times, 100 μl aliquots were removed, rapidly cooled on ice and centrifuged for 10 min at 3000 ***g*** at 4 °C. Sample of the supernatant (20 μl) was analysed by HPLC as described above.

TS assay: determination of ^{3}H release from [^{3}H]deoxycytidine

The activity of TS in HeLa and HeLa TK^-/HSV-1 TK^+ cells was measured by estimation of ^{3}H released from [^{3}H]deoxyuridylate (formed in the cells from [^{3}H]deoxycytidine) in the reaction catalysed by TS [12]. A modified version of this method was followed [4].

UDG assay

UDG activity was assayed as previously described [13]. Recombinant HSV-1 UDG was expressed and purified as previously described [14], and human UDG was purified from HeLa cells as reported by Focher et al. [13].

Effect of IPdU and SIPdU on viral DNA synthesis

HeLa TK^- cells (1.2×10^6 at a concentration of 4.3×10^5/ml) were seeded in a 30 mm-diameter Petri disk in DMEM and incubated at 37 °C. After 16 h the medium was replaced with fresh medium and the virus [1.2×10^7 PFU/Petri dish; multiplicity of infection (MOI) 10] was added. After 1 h, cells were washed with PBS and resuspended in 2 ml of fresh medium containing 5 μM [^{3}H]Thd (sp. radioactivity 430 c.p.m./pmol) and 7.6 μM IPdU (10-fold IC_{50} value) or 3.5 μM SIPdU (10-fold IC_{50} value). Cells were further incubated 30 h. To each sample was added 2 ml of cold 10 % (w/v) trichloroacetic acid, and acid-precipitable radioactivity was bound on GF/C filters by filtering under vacuum. Filters were washed with 10 ml of cold 10 % TCA, then with ethanol and finally dried. Radioactivity was counted in a β-radiation counter after the addition of 1 ml of Betamax scintillation fluid (ICN, Costa Mesa, CA, U.S.A.) to each filter.

Extraction of ^{3}H-labelled nucleosides, nucleotides and DNA from HeLa TK^- cells infected with HSV-1

HeLa TK^- cells (1.2×10^6 at a concentration of 5.14×10^5/ml) were seeded in 30 mm-diameter Petri dish in DMEM and incubated at 37 °C. After 16 h the medium was replaced with fresh medium and the virus (6×10^6 PFU/Petri dish; MOI 5) was added. After 1 h cells were washed with PBS and re-suspended in 2 ml of fresh medium containing either 5 μM [^{3}H]Thd (sp. radioactivity 430 c.p.m./pmol) or 5 μM [^{3}H]IPdU (246 c.p.m./pmol) or 5 μM [^{3}H]SIPdU (1743 c.p.m./pmol). Cells were further incubated for 6 h and then extensively washed in PBS. Drained cells were re-suspended in 300 μl of cold 0.5 M $HClO_4$ and kept at room temperature for 30 min. Cell lysate was transferred in an Eppendorf tube and centrifuged for 10 min at 13000 ***g***. The pellet was stored at −20 °C and the supernatant was neutralized with 35 μl of 4 M KOH and 40 μl of 1 M potassium phosphate buffer, pH 7.4, centrifuged for 10 min at 12000 rev./min and freeze-dried overnight. To each sample 500 μl of 20 mM KH_2PO_4 (pH 3)/20 % (v/v) methanol was added. A 200 μl portion of this solution, containing labelled nucleosides and nucleotides, was injected into the HPLC apparatus (see below). The acid-precipitable radioactivity (total DNA) present in the pellet, previously stored at −20 °C, was resuspended in 900 μl of cold 0.5 M $HClO_4$ and heated 15 min at 80 °C. To an aliquot the liquid-scintillation fluid EcoLume (ICN, Costa Mesa, CA, U.S.A.) was added and the mixture counted for radioactivity in a β-radiation counter to determine the total radioactivity incorporated into DNA. An ion-exchange- chromatography method employing an HPLC system was used in order to separate nucleosides from nucleotides. A 4.6 mm × 25 cm TSKgel DEAE-2SW column (TosoHaas, Montgomeryville, PA, U.S.A.) was used at room temperature with the following conditions: injection volume, 200 μl; detection, UV 260 nm; eluents, buffer A [0.02 M KH_2PO_4 (pH 3)/20 % (v/v) methanol] and buffer B [0.6 M KH_2PO_4 (pH 3)/20 % methanol]. Gradient conditions: 50 min linear gradient from buffer A to B. The flow rate was 1 ml/min; 50 fractions (fraction volume 1 ml) were collected and counted for radioactivity in a β-radiation counter after the addition of 4 ml of EcoLume to each sample.

RESULTS

Comparative analysis of antiviral and cytotoxic activity of 4′-thio- and 4′-oxy-2′-deoxyuridines

Table 2 reports the comparison of the antiviral activity of several 4′-thio- and 4′-oxy-2′-deoxyuridine analogues against HSV-1, HSV-2, VV and VSV, and Table 3 reports the comparison of the antiviral activity of the same compounds against VZV and CMV. Both 4′-thio- and 4′-oxy-2′-deoxyuridines are almost inactive against VSV (except SIPdU) and CMV [except SThd (4′-thiothymidine)], but most of the compounds of both classes are active against HSV, VZV and VV with IC_{50} values in the

Table 2 Anti-HSV-1, -HSV-2, -VV and -VSV activity and cytotoxic activity of deoxyuridine analogues in human E_6SM cell cultures

Any abbreviations not already defined in the text are defined in the abbreviations footnote.

Compound	R′	IC_{50}*(μM) HSV-1	HSV-2	VV	VSV	MCC† (μM)
dU	H	> 2000	> 200	> 2000	> 2000	> 2000
SdU	H	16	96	1.6	> 2000	> 2000
Thd	Methyl	> 2000	> 2000	> 2000	> 2000	> 2000
SThd	Methyl	0.013	0.036	0.004	> 2000	> 2000
EdU	Ethyl	12	8.3	7.5	> 2000	> 2000
SEdU	Ethyl	0.048	0.23	0.45	> 2000	> 2000
IPdU	Isopropyl	5.4	770	890	> 1500	> 1500
SIPdU	Isopropyl	0.06	0.151	170	> 50	⩾ 60
CPdU	Cyclopropyl	59	91	35	> 1500	> 1500
SCPdU	Cyclopropyl	0.17	2.6	1.3	> 1500	> 1500
TertBdU	t-Butyl	253	> 1500	850	> 1500	> 1500
STertBdU	t-Butyl	0.73	497	> 1500	> 1500	> 1500
CBdU	Cyclobutyl	34	850	850	> 1500	> 1500
SCBdU	Cyclobutyl	2.8	53	802	> 1500	> 1500
CPedU	Cyclopentyl	115	> 1200	810	> 1200	> 1200
SCPedU	Cyclopentyl	13	676	760	> 1200	> 1200
CHdU	Cyclohexyl	> 1200	> 1200	> 1200	> 1200	> 1200
SCHdU	Cyclohexyl	7.0	800	720	> 1200	> 1200
BVDU	Bromovinyl	0.18	1040	5.8	> 200	⩾ 240
Acyclovir	–	0.330	0.779	> 70	> 70	⩾ 70

* Data represent the average of the IC_{50} values obtained for three HSV-1 strains (KOS, F and McIntyre) and three HSV-2 strains (G, 196 and Lyons); each antiviral assay was performed in two separate experiments.

† Minimal cytotoxic concentration or compound concentration required to cause a microscopically detectable alteration of normal cell morphology.

Table 3 Anti-VZV and anti-CMV and cytostatic activity of deoxyuridine analogues in HEL cell cultures

Abbreviations used: DHPG, 9-(1,3-dihydroxy-2-propoxymethyl)guanine; HPMPC, (*S*)-1-(3-hydroxy-2-phosphonylmethoxypropyl)cytosine; any other abbreviations not already defined in the text will be found in the abbreviations footnote.

Compound	R′	IC_{50}* (μM) VZV	CMV	CC_{50}† (μM)
dU	H	> 250	> 2000	> 200
SdU	H	35	> 2000	> 200
Thd	Methyl	> 250	> 2000	> 200
SThd	Methyl	0.09	1	0.8
EdU	Ethyl	43	> 2000	⩾ 200
SEdU	Ethyl	0.087	> 2000	⩾ 200
IPdU	Isopropyl	> 185	> 1500	> 200
SIPdU	Isopropyl	1.8	> 1500	> 200
CPdU	Cyclopropyl	166	> 1500	> 200
SCPdU	Cyclopropyl	0.09	> 1500	> 200
TertBdU	t-Butyl	> 170	> 1500	> 200
STertBdU	t-Butyl	> 170	> 1500	> 150
CBdU	Cyclobutyl	> 170	> 1500	> 150
SCBdU	Cyclobutyl	0.38	> 1500	> 150
CPedU	Cyclopentyl	> 170	> 1200	> 150
SCPedU	Cyclopentyl	0.48	> 1200	> 150
CHdU	Cyclohexyl	> 160	> 1200	> 150
SCHdU	Cyclohexyl	⩾ 125	> 1200	> 150
BVdU	Bromovinyl	0.004	–	⩾ 600
Acyclovir	–	2.1	–	⩾ 880
DHPG	–	–	0.95	–
HPMPC	–	–	0.23	–

* Data represent the average of the IC_{50} values obtained for two VZV strains (OKA and YS) and two CMV strains (Davis and AD-169); each antiviral assay was performed in two separate experiments.

† CC_{50} or 50% cytostatic concentration required to inhibit HEL cell proliferation by 50%.

micromolar or nanomolar range. In particular, against HSVs the activity of 4′-thio-2′-deoxyuridines is significantly higher than that of their respective 4′-oxy counterparts. This is the case for SThd, SIPdU, 4′-thio-5-cyclopropyl-2′-deoxyuridine (SCPdU), whose IC_{50} values are, on average, 100-fold lower than their 4′-oxy counterparts with low or no cytostatic or cytotoxic activity (with the exception of SThd) against the cell lines used for these antiviral studies. This interesting behaviour shown by 4′-thio-2′-deoxyuridines against HSVs led us to study *in vitro* the effect of some selected compounds on a number of HSV-1 and cellular enzymic activities with the aim of understanding the reasons for the higher antiviral activity of 4′-thio-2′-deoxyuridines compared with their 4′-oxy-counterparts.

4′-Thio-2′-deoxyuridines selectively inhibit thymidine phosphorylation catalysed by HSV-1 TK

We first studied the effect of several 4′-thio-2′-deoxyuridines (reported in Table 2) in comparison with their 4′-oxy counterparts, on the phosphorylation of [^{3}H]Thd catalysed by HSV-1 and human TKs. Interestingly, all these compounds exert an inhibitory activity against the viral enzyme, while being inactive or much less potent against human TK (Table 4). Each IC_{50} value presented in Table 4 is calculated from the curve of [I] (inhibitor concentration) plotted against $1/v$ (the reciprocal of the enzyme velocity) and represents the average for two experiments in which five different concentrations of the tested compound were used. S.D. never exceeded $\pm 20\%$ of each reported value.

Since 4′-thio-analogues, except SThd and, to a minor extent, SCPdU, do not inhibit [^{3}H]Thd phosphorylation by human TK,

Table 4 Effect of deoxyuridine analogues on the [^{3}H]dThd phosphorylation by HSV-1 and HeLa TKs

Abbreviations not already defined in the text will be found in the abbreviations footnote.

Compound	R	R′	IC_{50} (μM) HSV-1 TK	Human TK
dU	O	H	32	107
SdU	S	H	36	> 100
SThd	S	Methyl	0.09	6.26
EdU	O	Ethyl	0.43	150
SEdU	S	Ethyl	0.12	> 200
IPdU	O	Isopropyl	0.76	N.I.*
SIPdU	S	Isopropyl	0.35	N.I.
CPdU	O	Cyclopropyl	0.87	40
SCPdU	S	Cyclopropyl	0.53	60
TertBdU	O	t-Butyl	0.21	> 200
STertBdU	S	t-Butyl	0.23	N. I.
CBdU	O	Cyclobutyl	1.75	> 100
SCBdU	S	Cyclobutyl	1.5	> 100
CPdU	O	Cyclopentyl	25	N. I.
SCPdU	S	Cyclopentyl	3.6	N. I.
CHdU	O	Cyclohexyl	> 250	N. I.
SCHdU	S	Cyclohexyl	23.4	N. I.
BVdU	O	Bromovinyl	0.07†	> 600†
SBVdU	S	Bromovinyl	0.17	> 600
IdU	O	Iodo	0.12†	6.05†
SIdU	S	Iodo	0.14	21

* N.I., no inhibition up to 200 μM.

† Spadari et al. [4].

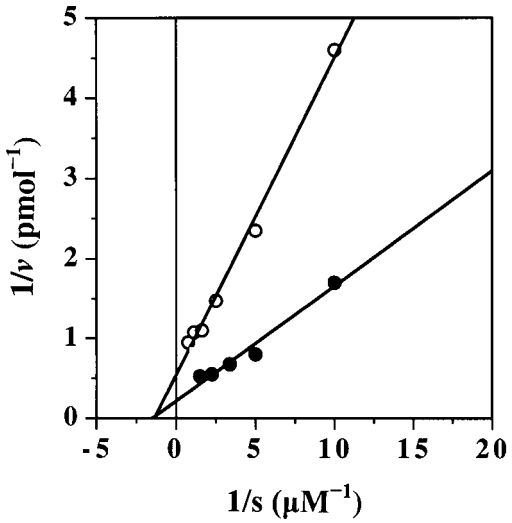


Figure 1 Determination of K_m values for IPdU and SIPdU

Lineweaver–Burk plots showing the kinetics of phosphorylation of [^{3}H]IPdU (○) and [^{3}H]SIPdU (●) by HSV-1 TK at different substrate concentrations.

Table 5 Phosphorylation of [^{3}H]IPdU and [^{3}H]SIPdU by HSV-1 TK

Values represent the percentages of nucleosides (dNs), deoxynucleoside monophosphates (dNMPs) and nucleoside diphosphates (dNDPs) present in the reaction mixture after incubation of the nucleoside with HSV-1 TK. See the Materials and methods section for experimental details.

Compound	dN	dNMP	dNDP
IPdU	5	43	43
SIPdU	14	64	11

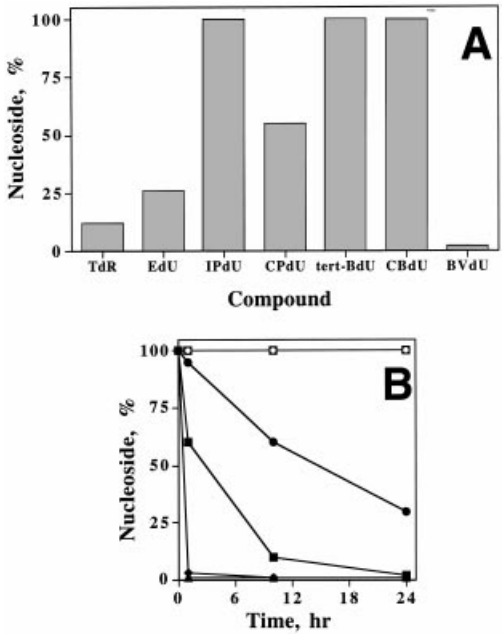


Figure 2 Activity of thymidine phosphorylase on 4′-oxy- and 4′-thio-2′-deoxyuridines

(**A**) Degradation of Thd ('TdR'), BVdU and some 4′-oxy-2′-deoxyuridines by recombinant human TP. Bars represent the amount of remaining nucleoside after 30 min of incubation with 20 ng of recombinant human TP. Abbreviations (not already defined in the text): EdU, 5-ethyl-2′-deoxyuridine; t-BdU, t-butyl-2′-deoxyuridine; CBdU, 5-cyclobutyl-2′-deoxyuridine. (**B**) Resistance of 4′-thio-2′-deoxyuridines to degradation by human thymidine phosphorylase present in intact human blood platelets. IPdU (●), SIPdU (○), CPdU(□), SCIPdU (■), BVdU (▲), SBVdU (△), Thd (◆) and SThd (◇).

we can argue that most of the 4′-thio analogues do not interact with the active site of the enzyme and are not phosphorylated to any significant extent by cellular TK. Moreover, when the 5-substituent remains fairly small (up to t-butyl) the 4′-thio analogues are powerful inhibitors of the phosphorylation of thymidine by HSV-1 TK and are likely to be acting as substrates.

Both 4′-oxy- and 4′-thio-2′-deoxyuridines competitively inhibit viral TK, suggesting a specific interaction of these compounds with the active site of the viral enzyme. In particular, IPdU and SIPdU show K_i values of 0.3 and 0.09 μM respectively.

IPdU and SIPdU are phosphorylated by HSV-1 TK to mono- and di-phosphate

To determine whether IPdU and SIPdU are substrate inhibitors of the viral enzyme or pure non-substrate inhibitors, we incubated HSV-1 TK with [^{3}H]IPdU and [^{3}H]SIPdU as described in the Materials and methods section. Figure 1 shows that, under the assay conditions reported in the Materials and methods section, which are optimal for the phosphorylation of the natural substrate, Thd (K_m 0.25 μM), both IPdU and SIPdU are phosphorylated by the HSV-1 TK. The enzyme shows similar K_m values for both compounds (0.6 μM), but different V_{max} values (1.9 and 5 pmol/30 min for IPdU and SIPdU respectively); this indicates that, under our *in vitro* assay conditions, SIPdU is 2.6-fold more phosphorylated than IPdU. In order to determine whether the enzyme is also able to phosphorylate both compounds to the diphosphate form, as is the case for the natural substrate, Thd, the products of the reaction were resolved by HPLC as described in the Materials and methods section. Table 5 shows a comparison of the enzymic products of the reaction in which [^{3}H]IPdU or [^{3}H]SIPdU were used as substrates. In both cases HSV-1 TK is able to phosphorylate these uridine derivatives up to the diphosphate form, with a preference for the 4′-oxy derivative.

4′-Thio-2′-deoxyuridines are resistant to phosphorolysis by recombinant human thymidine phosphorylase and by intact human blood platelets

We then studied whether 4′-oxy- and 4′-thio-2′-deoxyuridines, like Thd, are *bona fide* substrates of human TP and we found that, under conditions where an excess of purified recombinant TP completely transforms 2 μmol of Thd to thymine in 15 min, all the 4′-thio-2′-deoxyuridines described in the present study were completely resistant to degradation (results not shown). Among the 4′-oxy-2′-deoxyuridines, only the compounds with small 5-substituents (such as methyl, ethyl, bromovinyl) were sensitive to the enzyme (Figure 2A).

In order to address the same question by using a natural system, we prepared human intact blood platelets [11], known to contain high levels of TP. In this system it was previously found that several 5-substituted 2′-deoxyuridines are degraded by TP, a fact that limits their potential therapeutic activity [15]. To compare the susceptibility of 4′-oxy- and 4′-thio-2′-deoxyuridines in this system, we incubated intact human blood platelet with

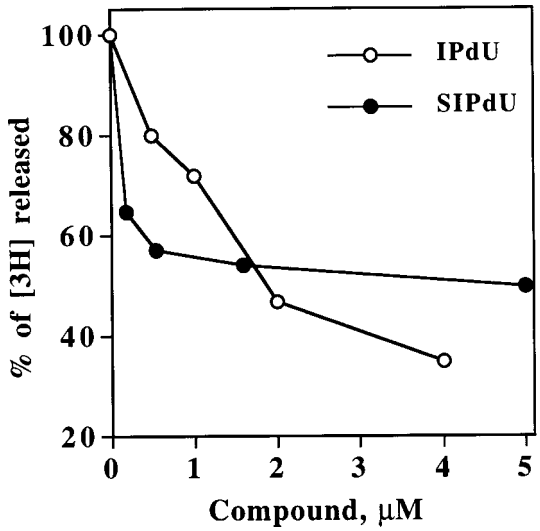


Figure 3 Effect of IPdU (○) and SIPdU (●) on tritium release by TS from deoxy[5-^{3}H]uridylate in cells exposed to deoxy[5-^{3}H]cytidine

Each point is the average of three determinations.

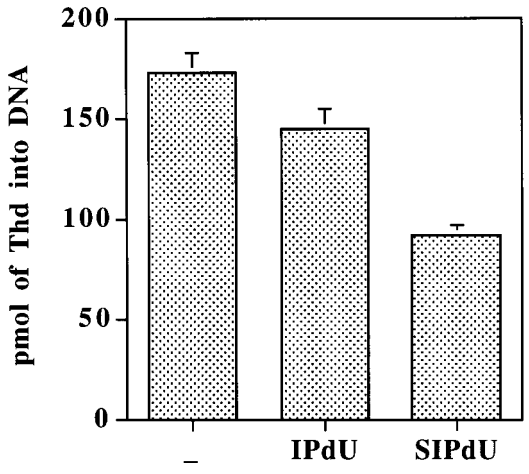


Figure 4 Effect of IPdU and SIPdU on viral DNA synthesis

[^{3}H]dThd incorporation in viral DNA in the presence of IPdU and SIPdU concentrations 10-fold higher than their K_m values, namely 7.6 and 3.5 μM for IPdU and SIPdU respectively.

0.1 mM Thd, IPdU, 5-cyclopropyl-2′-deoxyuridine (CPdU) and BVdU or their corresponding 4′-thio counterparts as described in the Materials and methods section. Aliquots of the incubation mixture were removed at 0, 1, 10 and 24 h and analysed by HPLC. Also under these assay conditions all the 4′-thio-2′-deoxyuridines tested were found to be fully resistant to enzymic degradation (Figure 2B), but, surprisingly, among the corresponding 4′-oxy-2′-deoxyuridines previously found resistant to degradation by recombinant TP, IPdU was partially hydrolysed. Thus its observed resistance to degradation by recombinant TP (even at 10-fold higher enzyme concentrations; results not shown) might suggest that other enzymic activities could be involved in its *in vivo* catabolism.

In vivo effect of IPdU and SIPdU on TS activity

It is known that several 5-substituted dUMP analogues, such as 5-fluoro-dUMP and 5-bromovinyl-dUMP, are potent inhibitors of cellular TS [16]. This inhibition depletes the dTTP pool in infected cells, directly affecting DNA synthesis and contributing to their cytotoxicity. Therefore we investigated and compared the effect of IPdU and SIPdU on TS activity in HeLa TK$^-$/HSV-1 TK$^+$ cells. TS represents the *de novo* pathway of dTMP synthesis, since it catalyses the reductive methylation of dUMP to dTMP, in which the hydrogen atom on C-5 of the uracil ring is replaced by a methyl group. Thus the activity of TS in intact cells can be measured by estimating the ^{3}H release from [5-^{3}H]dUMP formed upon administration of deoxy[5-^{3}H]uridine or deoxy[5-^{3}H]cytidine as described by Balzarini et al. [12]. In fact, both labelled nucleosides are converted into [5-^{3}H]dUMP: deoxy[5-^{3}H]uridine directly by phosphorylation, and deoxy[5-^{3}H]cytidine either via deamination to deoxy[5-^{3}H]uridine and phosphorylation to [5-^{3}H]dUMP or via phosphorylation to [5-^{3}H]dCMP and deamination to [5-^{3}H]dUMP.

The results shown in Figure 3, obtained by administering [5-^{3}H]cytidine to growing HeLa TK$^-$/HSV-1 TK$^+$ cells, where both IPdU and SIPdU are phosphorylated by viral TK, show that they inhibit the ^{3}H release from [5-^{3}H]dUMP with comparable activity. This suggests that both IPdU and SIPdU interfere with TS activity *in vivo*.

Effect of 4′-thio- and 4′-oxy-2′-deoxyuridine analogues on human and HSV-1 UDGs

When some deoxyuridine analogues reported in Table 4 were assayed *in vitro* against HSV-1 recombinant UDG and human UDG, the 4′-thio-2′-deoxyuridines tested were found inactive against both enzymes. On the other hand, the 4′-oxy-2′-deoxyuridines CPdU and IPdU were able to inhibit HSV-1 and human UDGs with a strong preference for the viral enzyme. The corresponding modified bases were inactive (results not shown). This might imply that, in contrast with the 4′-oxy-, the modified 4′-thio-2′-deoxyuridines, once incorporated into viral DNA by viral DNA polymerase, are not recognized by the viral UDG and would not be removed, thus increasing their antiviral potency.

Fate of 4′-thio- and 4′-oxy-2′-deoxyuridines in HSV-1 infected cells

When HeLa TK$^-$ cells were infected by HSV-1 in the presence of labelled [^{3}H]Thd and concentrations of unlabelled IPdU or SIPdU 10-fold higher than their respective IC_{50} values, we observed a decrease in [^{3}H]Thd incorporation into DNA (Figure 4). This could be due either to inhibition of the synthesis of pyrimidine nucleotides through their interference with viral TK and human TS or, most probably, to a direct incorporation of their triphosphate forms into DNA. In order to address this second point we determined the fate of IPdU and SIPdU in virus-infected cells to verify if both compounds can reach the triphosphate form and can be incorporated into viral DNA. To this purpose we have infected HeLa TK$^-$ cells with HSV-1 in presence of 5 μM of either [^{3}H]Thd or [^{3}H]IPdU or [^{3}H]SIPdU. Cells were further incubated 6 h, when viral TK is maximally expressed, and then extensively washed in PBS (90% of the labelled material is removed in this step). From the cell pellets the remaining radioactivity was extracted as described in the

Table 6 Fate of [^{3}H]dThd, [^{3}H]IPdU and [^{3}H]SIPdU in HSV-1 infected cells after 6 h from the infection

The values reported represent the percentage of ^{3}H recovered as nucleosides (dNs), nucleotides (dNMPs, dNDPs and dNTPs) and DNA. See the Materials and methods section for experimental details.

Compound	dN	dNMP	dNDP	dNTP	DNA
dThd	2.0	65.0	10.0	3.8	19.0
IPdU	0.6	56.0	22.0	15.0	5.3
SIPdU	0.8	82.0	9.2	5.0	3.1

Materials and methods section. As reported in Table 6, very low levels of labelled nucleosides were present in the cell extracts; this indicates both a lack of contamination of extracellular labelled material and the complete phosphorylation of the nucleosides once incorporated into the cells. The data reported in Table 6 also demonstrate that both [^{3}H]IPdU and [^{3}H]SIPdU are phosphorylated *in vivo* up to the triphosphate form and that viral DNA polymerase can incorporate both compounds into DNA.

DISCUSSION

In order to improve the biological activity of 2′-deoxynucleoside analogues as anticancer and antiviral agents it has been thought important to decrease their susceptibility to degradation by nucleoside phosphorylase. To this purpose, chemical modifications of the sugar ring, such as replacement of the 4′-oxygen by a methylene group [1,2] or replacement of the D-2′-deoxyribose or the carbocyclic D-2′-deoxyribose by their respective L enantiomers [3–5] have indeed given rise to nucleosides resistant to such degradation and which remain active as anticancer and antiviral agents [17]. Replacement of the 4′-oxygen by sulphur was also studied, but only in the case of ribonucleosides [18]. On the basis of the finding that the 4′-thio modification introduced in ribonucleosides conferred resistance to nucleoside cleavage as well, only a few deoxyribonucleosides, such as 2′-deoxy-5-fluoro-4′-thiouridine [19], SThd [20,21] and SBVdU [20–22], have been synthesized and tested against HSV infections. These compounds showed potent antiviral activity against HSV-1 and CMV, but still possess severe cytotoxicity [19–22]. Thus a novel series of 5-substituted 4′-thio-2′-deoxypyrimidines were synthesized and evaluated for their potential anti-HSV activity [7]. In the present work we have compared the antiviral activity of these compounds with their 4′-oxy counterparts and have interestingly found that all 4′-thio-2′-deoxyuridines are not only more active against HSV-1, HSV-2 and VZV than their 4′-oxy counterparts but also poorly or not cytotoxic against mammalian cells.

In order to define their chemotherapeutic potential, in the present study we also evaluated their effect on a number of viral and cellular enzymic activities. We thus demonstrated that the higher efficacy of the 4′-thio-2′-deoxyuridines as anti-herpetic agents compared with their 4′-oxy counterparts could be based not only their higher affinity for HSV-1 TK, which selectively phosphorylates them to the mono- and di-phosphate form (the triphosphate is then made by cellular enzymes), but also upon their resistance to phosphorolysis by human TP. 4′-Thio-2′-deoxyuridines were indeed resistant to phosphorolysis by recombinant TP or by intact blood platelets, whereas the most active 4′-oxy counterparts, being those which possess small substituents in position 5 (methyl, ethyl, cyclopropyl and bromovinyl), were all substrates for human TP. Those with more bulky 5-substituents become progressively more resistant to phosphorolysis, but show lower antiviral activity because they are poorer substrates for viral TK.

Furthermore, the higher efficacy of some 4′-thio-2′-deoxyuridines as anti-herpetic agents compared with their 4′-oxy counterparts could be also explained by the fact that the 4′-thio-2′-deoxyuridines, in contrast with their corresponding 4′-oxy-2′-deoxyuridines, are not recognized by HSV-1 and human UDGs. This suggests that the 4′-thio-2′-deoxyuridines do not affect post-replicative base excision repair in replication foci [23] in non-infected cells. However, it is also possible that, once incorporated into viral genome by viral DNA polymerase, 4′-thio-2′-deoxyuridines might not be recognized nor removed by cellular or viral UDGs, thus interfering with viral DNA replication and transcription.

We also found that both 4′-oxy- and 4′-thio-2′-deoxyuridines, in the phosphorylated form, are inhibitors of cellular thymidylate synthase. This indicates that they affect the *de novo* synthesis of TMP only in HSV-1-infected cells, but do not interfere with cellular TS activity in non-infected cells, where they are not present in the monophosphate form because of the absence of viral TK.

The results of the present study support our hypothesis that 4′-thio-2′-deoxyuridines have a potential chemotherapeutic use as antiviral agents and may help to rationalize their target specificity.

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