ELSEVIER

Antiviral Research 39 (1998) 129–137

Antiviral Research

Anti-herpesvirus activity profile of 4′-thioarabinofuranosyl purine and uracil nucleosides and activity of 1-β-D-2′-fluoro-4′-thioarabinofuranosyl guanine and 2,6-diaminopurine against clinical isolates of human cytomegalovirus

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Received 13 March 1998; accepted 17 July 1998

Abstract

Newly synthesized 4′-thio- and 2′-fluoro-4′-thioarabinofuranosyl purine and pyrimidine nucleosides were compared with the corresponding 4′-oxo type arabinosyl nucleosides for anti-herpesvirus and anti-cell proliferative potencies. 4′-Thioarabinosyl- and 2′-fluoro-4′-thioarabinofuranosyl 5-substituted uracils had selective antiviral activities, but were not superior to 4′-oxo nucleosides, except for the activity of 5-ethyl-uracil 4′-thio nucleosides against herpes simplex virus. Furthermore, 4′-thio substituted derivatives of sorivudine (BV-araU) and related compounds, and 2′-fluoro-5-methyl-arabinosyluracil exhibited reduced activity against varicella-zoster virus compared with the parent compounds. The 4′-thioarabinosyluracils, except for 5-methyluracil derivatives, were inactive against human cytomegalovirus (HCMV). 4′-Thioarabinofuranosyl guanine and diaminopurine had the most potent anti-HCMV and anti-proliferative activities, whereas arabinosyl guanine and diaminopurine had only marginal antiviral activity. 2′-Fluoro-4′-thioarabinofuranosyl derivatives of guanine (4′-thio-FaraG) and 2,6-diaminopurine (4′-thio-FaraDAP), however, had particularly high activity against all herpesviruses tested with anti-proliferative activity equipotent to that of arabinosyl guanine and diaminopurine. 4′-Thio- and 2′-fluoro-4′-thioarabinofuranosyladenines exhibited biological activities similar to that of arabinosyladenine. Both 4′-thio-FaraG and 4′-thio-FaraDAP had a 6-fold lower ED_{50} than ganciclovir against clinical isolates of HCMV. A ganciclovir-resistant isolate, obtained from a patient who had received long-term ganciclovir-treatment, was susceptible to 4′-thio-FaraG and 4′-thio-FaraDAP.

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0166-3542/98/$ - see front matter
PII S0166-3542(98)00042-4

Dr Imanishi of Kyoto Prefectural University of Medicine, and HAIN-55 cells were used for the anti-cell growth activity test.

2.2. Compounds

4′-Thioarabinofuranosyl purine and uracil nucleosides and their 2′-fluoro substituted derivatives were synthesized as previously reported (Yoshimura et al., 1997a,c). 5-Ethyl, 5-iodo, 5-(*E*-2-bromovinyl), and 5-(*E*-2-chlorovinyl) derivatives of 1-β-D-arabinofuranosyluracil (Et-araU, I-araU, BV-araU, and CV-araU, respectively), 9-β-D-arabinofuranosylguanine (araG), and 5-methyl and 5-(*E*-2-bromvinyl) derivatives of 1-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)uracil (FMAU and FBV-araU, respectively) were synthesized at the Chemistry Laboratory of Yamasa Corporation. 9-β-D-Arabinofuranosyl-2,6-diamnopurine (araDAP) was prepared at Bioproducts Laboratory of Yamasa Corporation. Synthesized analogues were identified by NMR and their mass spectra, and their purity was determined to be 98% or greater by high performance liquid chromatography. AraA and 1-β-D-arabinofuranosylthymine (araT) were commercial products of the Yamasa Corp. 1-(2-Deoxy-2-fluoro-β-D-arabinofuranosyl)-5-ethyluracil (FEAU) and 1-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)-5-iod-ouracil (FIAU) were gifts from Dr J.J. Fox and Dr K.A. Watanabe, Sloan-Kettering Institute, New York. Structures of the arabinofuranosyl 5-substituted uracil nucleosides are shown in Fig. 1. The structures of the sugar moieties of arabinofuranosyl purine nucleosides are the same as those of the 5-substituted uracil nucleosides.

2.3. Antiviral activity tests

Anti-herpesvirus activities were determined by the plaque reduction method as described previously (Machida et al., 1991, 1993). Briefly, confluent monolayers of HEL cells grown in a 12-well plate (Corning Glass Works, Corning, NY) were infected with 50–100 plaque forming units of the HSV-1 VR-3 strain, the HSV-2 MS strain, the cell-associated VZV Oka strain, or the cell-free HCMV AD169 strain. After virus adsorption for 30 min at 37°C, the virus solution was discarded and the HSV-1 or HSV-2-infected cells were overlaid with maintenance medium containing 0.8%

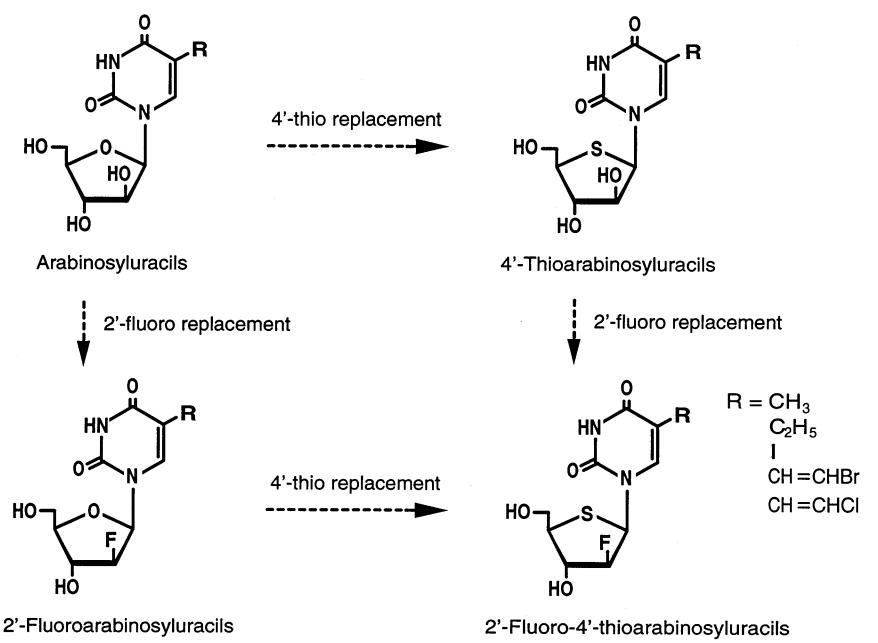

Fig. 1. Structures of arabinofuranosyl, 2′-fluoroarabinofuranosyl, 4′-thioarabinofuranosyl, and 2′-fluoro-4′-thioarabinofuranosyl 5-substituted uracil nucleosides. Arrows show the direction of substitution.

methylcellulose (Nacalai Tesque, Tokyo) and test drugs. For the VZV- and HCMV AD169 strain-infected cultures, drugs were added after virus adsorption for 1 h at 37°C. The HSV-1- or HSV-2-infected cells and the VZV-infected cells were incubated in a 5% CO_2-air incubator at 37°C for 2–3 days and for 4 days, respectively, then stained with a 0.5% crystal violet solution. The HCMV AD169 strain-infected cultures were incubated in the same manner for 4 days, then the cells were overlaid with fresh medium containing 0.4% methyl cellulose and the same concentration of each drug; incubation continued for 3–4 days. For testing antiviral activity against clinical isolates of HCMV, infected cells were treated in the same manner for 4–7 days. The second incubation with methyl cellulose was continued until plaques were observed. Duration of the first and second incubation depended on the rate of viral growth (plaque formation), and total incubation periods were from 15 to 19 days. HCMV-infected cells were strained with May-Gruenwald's and Giemsa solutions.

Dilutions of test drugs were made in serial half-$\log_{10}$ decrements with maximum concentrations of 50 and 100 μg/ml, for VZV- or CMV-infected and HSV-infected cultures, respectively. Four to five drug concentrations (1.5–2 $\log_{10}$ range) were used in duplicate for each drug. Each experiment was repeated two to four times. The number of plaques were counted under a stereoscopic light microscope. Percent inhibition of plaque formation, compared with plaque number in control cultures, was calculated for each drug concentration used. The drug required to reduce plaque number by 50% (ED_{50}) was determined by interpolation from the dose–response curve.

2.4. Anti-proliferative activity test

An inhibitory effect on cell growth was determined with the MTT assay using human T-cell acute lymphoblastoid leukemia cells as described previously (Ashida et al., 1997). Briefly, 90 μl of RPMI 1640 medium supplemented with 10% fetal bovine serum containing 5×10^3 of CCRF-HSB-2 cells was seeded into each well of a 96-well microplate (Nunc, Roskilde, Denmark). Ten μl of drug solution were added simultaneously in triplicate to each well. The plates were incubated at 37°C in a humidified atmosphere of 5% CO_2. After 72 h of incubation, 10 μl of MTT solution (5 mg/ml in phosphate-buffered saline) were added to each well, and the cells were incubated for an additional 4 h at 37°C. Then 100 μl of 50% dimethylformamide (v/v) and 20% SDS (w/v) dissolved in 0.02 N HCI were added to solubilize any MTT-formazan formed. The optical density at 570 nm of each well was measured with an Immuno-Reader NJ-2000 (InterMed Japan, Tokyo, Japan). Growth inhibition test against HAIN-55 cells was also conducted for purine nucleosides according to the methods described previously (Machida et al., 1993). Briefly, HEL cells were plated in 35-mm plastic dishes at 8.5×10^4 per dish. After 4 h, the cells were re-fed with fresh growth medium containing an appropriate amount of the test compound. After 4 days of incubation, cells from duplicate cultures were dispersed by trypsin, and the number of cells was counted under a microscope using a hematocytometer. The 50% inhibitory concentration (IC_{50}) was determined from the dose-response curves.

3. Results

3.1. Selective anti-herpesvirus activity of 4′-thioarabinofuranosyl uracil nucleosides

5-Substituted arabinofuranosyluracil derivatives of 4′-thio- and 2′-fluoro-4′-thio nucleosides had selective antiviral activity against HSV-1 and HSV-2, but the potencies were not superior to, and were sometimes lower than, the corresponding 4′-oxo nucleosides with the exception of 4′-thio-Et-araU which exhibited higher anti-HSV-1 and HSV-2 activities than did Et-araU (Table 1). Furthermore, potent activities of BV-araU and CV-araU against VZV and HSV-1 and of FMAU and FBV-araU against VZV were reduced by the 4′-thio replacement. The 4′-thio- and 2′-fluoro-4′-thio arabinosyluracil analogues were inactive against HCMV, except that 4′-thio-FMAU exhibited activity equipotent to that of FMAU. It was also noted that 4′-thio-FIAU was much less in-

Table 1
Anti-herpesvirus and anti-proliferative activities of arabinofuranosyl uracil nucleosides

Compound	Anti-herpesvirus activities (ED_{50}, μM)[a]				IC_{50} for cell growth (μM)[b]
	HSV-1	HSV-2	VSV	CMV	
Arabinofuranosyluracils					
araT	1.78	1.74	1.43	112	232
Et-araU	7.46	242	>184	>184	>367
I-araU	23.5	30.8	70	>135	>270
BV-araU	0.072	178	0.0037	>143	>286
CV-araU	0.085	>382	0.0056	>164	>328
4′-Thioarabinofuranosyluracils					
4′-Thio-araT	2.8	16.8	24.1	160	>365
4′-Thio-Et-araU	1.49	22.5	>173	>173	>347
4′-Thio-I-araU	9.1	35	70	>129	>259
4′-Thio-BV-araU	2.2	159	0.55	>137	>274
4′-Thio-CV-araU	3.0	220	>156	>156	>312
2′Fluoroarabinofuranosyluracils					
FMAU	0.085	0.11	0.11	0.35	1.73
FEAU	0.020	0.62	1.09	230	>366
FIAU	0.073	0.16	0.21	0.059	4.0
FBV-araU	0.14	24	0.023	>142	>285
2′-Fluoro-4′-thioarabinofuranosyluracils					
4′-Thio-FMAU	1.56	3.6	89	0.32	2.5
4′-Thio-FEAU	0.052	0.25	101	>172	>344
4′-Thio-FIAU	0.046	0.47	17.5	>129	144
4′-Thio-BV-araU	0.54	16.3	0.98	>136	272

[a] Determined by the plaque reduction method; each value is the average of the two or three experiments.
[b] Determined by the MTT method; data from a single experiment for many test compounds except some key compounds for which two experiments were conducted.

hibitory to cell growth than FIAU, but approximately equally or slightly less potent against HSV-1 and HSV-2, resulting in a greater therapeutic index. The 4′-thio substitution, however, resulted in loss of anti-HCMV activity and marked reduction of anti-VZV activity.

3.2. Anti-herpesvirus and anti-proliferative activities of 4′-thioarabinofuranosyl purine nucleosides

As shown in Table 2, the 4′-thio-substituted araG and araDAP had two to three orders of magnitude more potent anti-herpes virus activities than those of the 4′-oxo nucleosides, araG and araDAP, although anti-proliferative activity against CCRF-HSB-2 was also enhanced approximately 10-fold by the 4′-thio replacement. As the purine nucleosides showed inhibitory effect on the growth of CCRF-HSB-2 cells, the growth inhibition test was also conducted for these compounds using HAIN-55 cells. Similar results were obtained for the 4′-thio-substituted and 2′-fluoro-4′-thio-substituted purine nucleosides, but araG and araDAP were much less inhibitory to HAIN-55 cells than to CCRF-HSB-2, whereas araA was more inhibitory to HAIN-55 cells (Table 2). The 4′-thio analogues showed the most potent activity against the HCMV AD169 strain and were approximately 10-fold more potent than ganciclovir. On the other hand, 4′-thio-FaraG and 4′-thio-FaraDAP were the most potent against HSV-1 and HSV-2 among the nucleoside tested and 8- to 90-fold more potent than the corresponding 4′-thio nucleoside, while their anti-proliferative activities of 4′-thio-FaraG and 4′-thio-FaraDAP

against CCRF-HSB-2 was 10-fold lower than that of 4′-thio-araG and 4′-thio-araDAP and comparable to the activity of araG and araDAP. However, the anti-proliferative activity of 4′-thio-FaraG and 4′-thio-FaraDAP against HAIN-55 cells was only 2-fold lower than that of the corresponding 4′-thio nucleosides, and 10- to 20-fold higher than that of the corresponding arabinosyl nucleosides. The anti-HCMV activity of 4′-thio-FaraG and 4′-thio-FaraDAP was also somewhat lower than that of the corresponding 4′-thio arabinofuranosyl nucleosides, but these 2′-fluoro-4′-thio derivatives still retained anti-HCMV activity equipotent to cidofovir and approximately 3-fold more potent than ganciclovir. 4′-Thio-FaraG had the greatest selectivity for HCMV among the arabinosyl nucleosides tested. AraG and araDAP had only marginal antiviral activity, but were more inhibitory to cell proliferation. In contrast, 4′-thio and 2′-fluoro-4′-thio substitution gave no significant influence on biological activities of araA, except that 2′-fluoro-4′-thio-araA (4′-thio-FaraA) showed relatively potent activity against HSV-1 and HCMV and was less inhibitory to cell growth than araA.

3.3. Activity of 4′-thio-FaraG and 4′-thio-FaraDAP against clinical isolates of HCMV

4′-Thio-FaraG and 4′-thio-FaraDAP exhibited potent antiviral activity against all seven clinical isolates of HCMV tested (Fig. 2). The mean ED_{50} values of 4′-thio-FaraG and 4′-thio-FaraDAP were 0.43 and 0.33 μM, respectively, and that of ganciclovir was 2.8 μM. The mean ED_{50} values of 4′-thio compounds were 6- to 7-fold lower than that of ganciclovir, even when the ED_{50} for ganciclovir-resistant isolate was excluded. Only a limited variability in the sensitivities of these isolates to 4′-thio-FaraG and 4′-thio-FaraDAP was observed, whereas the susceptibilities to ganciclovir

Table 2
Anti-herpesvirus and antiproliferative activities of arabinofuranosyl purine nucleosides and control compounds

Compound	Anti-herpesvirus activities (ED_{50}, μM)[a]				IC_{50} for cell growth (μM)	
	HSV-1	HSV-2	VZV	CMV	CCRF-HSB-2[b]	HAIN-55[c]
Arabinofuranosyl purine						
araA	64	25	4.4	24	52	<12
araDAP	174	354	33	46	6.0	79
araG	194	275	36	59	9.9	65
4′-Thioarabinofuranosyl purine						
4′-Thio-araA	65	48	6.5	4.8	52	33
4′-Thio-araDAP	1.74	1.34	0.37	0.073	0.67	2.1
4′-Thio-araG	1.64	1.97	0.37	0.033	0.97	2.2
2′-Fluoro-4′-thioarabinofuranosyl purine						
4′-Thio-FaraA	5.6	10.8	10.7	4.9	189	26
4′-Thio-FaraDAP	0.019	0.17	0.33	0.22	7.0	4.1
4′-Thio-FaraG	0.030	0.21	0.32	0.26	11.9	5.4
Control compounds						
Acyclovir	0.62	1.02	12.1	31	>444	>444
Ganciclovir	0.062	0.15	12.1	0.74	67	47
Cidofovir	ND[d]	ND	ND	0.25	ND	ND

[a] Determined by the plaque reduction method; the value is the average for two of three experiments.
[b] Determined by the MTT method; data from a single experiment for many test compounds except some key compounds for which two experiments were conducted.
[c] Data from a single experiment.
[d] Not determined.

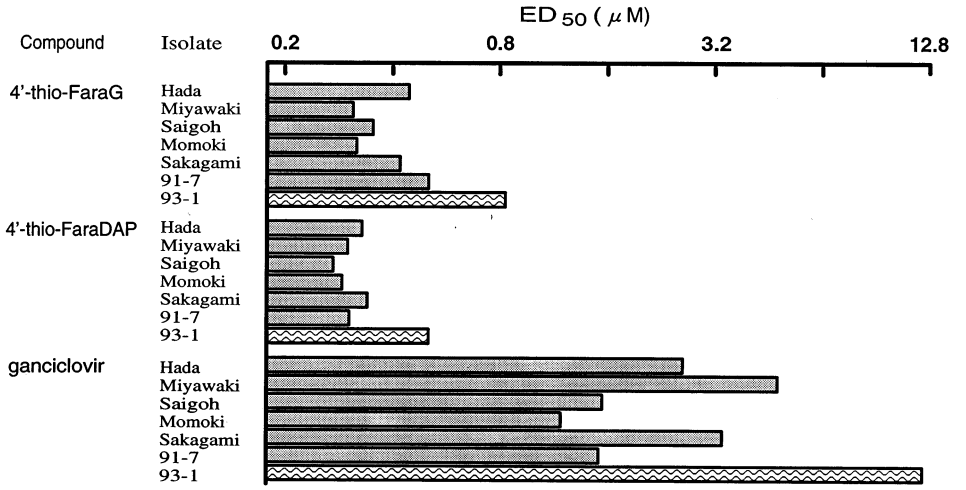

Fig. 2. Antiviral effect of 4′-thio-FaraG, 4′-thio-FaraDAP, and ganciclovir against clinical isolates of HCMV. The 93-1 strain, which is indicated by a bar shaded with waved lines, was isolated as a ganciclovir-resistant strain (Harada et al., 1997). The indicated ED_{50} values are the average of three to four separate experiments.

varied from strain to strain. The clinical isolate, strain 93-1, isolated after long-term treatment with ganciclovir from the same patient from which the 91-7 strains had been isolated, was ganciclovir-resistant (Harada et al., 1997). In our assay, the 93-1 strain had 8-fold lower susceptibility to ganciclovir than had the 91-7 strain. In contrast, the 93-1 strain was very susceptible to 4′-thio-FaraG and 4′-thio-FaraDAP. There was only small difference (1.7-fold in terms of ED_{50}) in susceptibility between the 93-1 and 91-7 strains.

4. Discussion

Replacement of the 4′-oxygen of the sugar ring of nucleosides is one of the chemical modifications applied in searching for novel antiviral nucleosides. Such a replacement has given rise to carbocyclic and 4′-thio nucleosides, which are resistant to degradation by phosphorylytic enzymes due to the absence of the labile glycosidic linkage between base and sugar. The replacement of 4′-oxygen provides analogues with a metabolic profile more favorable than that found for the 4′-oxo nucleosides (Rahim et al., 1996; Van Draanen et al., 1996). Some 4′-thio and 4′-methylene substituted analogues of naturally occurring deoxynucleosides such as thymidine and 2′-deoxyguanosine exhibited relatively potent antiviral properties (Shealy et al., 1983, 1984; Dyson et al., 1991). Usually, carbocyclic analogues of antiviral pyrimidine nucleosides, however, such as 5-iodo-2′-deoxyuridine, 5-ethyl-2′-deoxyuridine and BVDU, do not have increased activity against HSV-1 and/or HSV-2 relative to the corresponding traditional 4′-oxo nucleosides (Shealy et al., 1983; Herdewijn et al., 1985; Shealy et al., 1986). Carbocyclic FMAU is also two orders of magnitude less active than FMAU against HSV-1 and does not exhibit anti-HSV-2 activity (Borthwick et al., 1990).

In the present study, we compared the anti-herpesvirus activities of 4′-thio substituted arabinofuranosyl nucleosides with those of the corresponding 4′-oxo nucleosides to determine the influence of the 4′-thio substitution. The influence on the biological activity imparted by the 4′-thio replacement was different in purine and pyrimidine nucleosides. Many 5-substituted 4′-thio arabinosyluracils and 2′-fluoro-arabinosyluracils had an activity profile similar to that of the corresponding 4′-oxo nucleosides or sometimes reduced anti-herpesvirus activity, except for 4′-thio-EtaraU, which had higher anti-HSV activity than the corresponding 4′-oxo nucleoside. Particularly,

the 4′-thio derivatives of BV-araU and its analogues showed a great reduction in anti-VZV and anti-HSV-1 activity, as compared to the parent compounds. These findings are different, in part, from those of some 4′-thio-2′-deoxyribo-pyrimidine nucleosides: 4′-thio-BVDU is only slightly less potent than BVDU against HSV-1 and VZV (Dyson et al., 1991) and 4′-thio-5-ethyl-2′-deoxyuridine has significant activity against HSV and VZV (Rahim et al., 1996).

Opposite trends were observed upon 4′-thio substitution of araG and araDAP, but not of araA. 4′-Thio substituted araG and araDAP were two to three orders of magnitude more active against herpesviruses than the 4′-oxy counterparts and had the most potent anti-HCMV activity, but on the other hand, also possessed significant cytotoxicity. In contrast to 4′-thio-araG and 4′-thio-araDAP, 4′-thio-2′-deoxyguanosine and its analogues were extremely potent against hepatitis B virus and HCMV, but only moderately active against HSV-1 and VZV (Van Draanen et al., 1996). This is not surprising because the base guanine is unique in tolerating a wide variety of sugar modifications, including oxo and carbocyclic nucleosides with a four-membered ring structure, while retaining potent anti-herpes activity (Nishiyama et al., 1989; Borthwick et al., 1991).

Further substitution of the 4′-thio purine nucleosides with 2′-fluoro potentiated the anti-HSV-1 and anti-HSV-2 activities, while the anti-proliferative action was decreased 2-fold (HAIN-55) to 10-fold (CCRF-HSB-2). 4′-Thio-FaraG and 4′-thio-FaraDAP were the most potent against HSV-1 and HSV-2 among the arabinosyl nucleosides tested, and equipotent to the structurally very similar carbocyclic arabinofuranosylguanine (Borthwick et al., 1991). In addition, the 4′-thio analogues were equally active to cidofovir and about 3-fold more potent than ganciclovir against HCMV AD-169 strain. They showed marked antiviral activity against clinical isolates of HCMV. Furthermore, the difference in the susceptibilities to 4′-thio-FaraG and 4′-thio-FaraDAP was less than 2-fold between the ganciclovir-resistant 93-1 and ganciclovir-sensitive 91-7 strains, indicating that the 4′-thio nucleosides are not cross-resistant with ganciclovir. Thus, these compounds are likely candidates as new anti-HCMV agents, and further studies, including their in vivo efficacy and toxicity, are needed to assess their true therapeutic potential.

Acknowledgements

We thank Dr K. Kodama of Yamasa Corporation for supporting this study and M. Nishitani for her excellent technical assistance in the biological assays.

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Keywords: Anti-herpesvirus activity; 4′-Thioarabinosyl purine nucleosides; 4′-Thioarabinosyl uracil nucleosides; HCMV; HCMV isolates

1. Introduction

Chemical modification of nucleosides can confer anti-cancer and antiviral properties, and the modified nucleoside analogues act as anti-metabolites in nucleic acid synthesis. Therefore, modifications made in the sugar moiety, as well as in the base residue, are targets of drug design in the search for novel effective antiviral agents (Herdewijn, 1992). Nucleoside derivatives, in which the oxygen of the sugar ring of the nucleosides is replaced with a methylene group, are known as carbocyclic nucleosides, and a number of carbocyclic nucleosides have potent antiviral activity (Montgomery, 1989). Replacement of the 4′-oxygen of furanose with sulfur (4′-thio nucleosides) is another approach for modifying the sugar of nucleosides with a five-membered ring structure. There are, however, only a few reports of antiviral activity in 4′-thio nucleosides. Dyson et al. (1991) reported antiviral activity of 4′-thio analogues of thymidine and 5-(*E*-2-bromovinyl)-2′-deoxyuridine (BVDU). Recently, synthesis and antiviral activities of a wide variety of 2′-deoxy-4′-thio purine and pyrimidine nucleosides were reported (Rahim et al., 1996; Van Draanen et al., 1996): a number of 5-substituted 4′-thio-2′-deoxyuridines were active or moderately active against herpesvirus type 1 (HSV-1) and varicella-zoster virus (VZV), and some purine analogues had potent antiviral activities against hepatitis B virus and human cytomegalovirus (HCMV).

We have synthesized a series of anti-tumor and antiviral 4′-thio nucleosides, and found, interestingly, that 1-β-D-2′-fluoro-4′-thioarabinofuranosylcytosine and 4′-thio-2′-deoxy-2′-methylidenecytidine exhibit anti-tumor effects that are enhanced by the 4′-thio replacement (Miura et al., 1996; Yoshimura et al., 1997a,b,c). A great number of 5-substituted arabinofuranosyluracils exhibit potent and selective anti-herpesvirus activities (Machida and Sakata, 1993) and 9-β-D-arabinofuranosyladenine (araA) has potency against a wide range of DNA viruses; thus it is important to understand how introduction of a 4′-thio replacement modifies the antiviral activities of arabinofuranosyl nucleosides. In this report, we describe anti-herpesvirus and anti-proliferative activities of novel 4′-thio- and 2′-fluoro-4′-thioarabinofuranosyl purine and 5-substituted uracil nucleosides in comparison with those of the corresponding 4′-oxo arabinosyl nucleosides. 9-β-D-2′-Fluoro-4′-thioarabinofuranosylguanine (4′-thio-FaraG) and 9-β-D-2′-fluoro-4′-thioarabinofuranosyl-2,6-diamino-purine (4′-thio-FaraDAP), which have potent and selective activity against a laboratory strain of HCMV, were also tested for antiviral effects against clinical isolates of HCMV.

2. Materials and methods

2.1. Cells and viruses

A human embryonic lung (HEL) cell line, HAIN-55, a gift from Dr Okumura, National Institute of Health of Japan, Tokyo, and HSV-1 VR-3 strain, herpes simplex virus type 2 (HSV-2) MS strain, VZV Oka strain, and HCMV AD169 strain were used to test the antiviral activity of the test compounds. The origin of these viruses has been described previously (Machida, 1990). Seven clinical isolates of HCMV were used for the antiviral test of 4′-thio-FaraG, 4′-thio-FaraDAP, and ganciclovir. Two isolates, strains 91-7 and 93-1, originated from a patient with aplastic anemia complicated with CMV retinitis and encephalitis, and the ganciclovir-resistant 93-1 strain was isolated after long-term ganciclovir-treatment (Harada et al., 1997). Five other isolates originated from the urine of independent patients with congenital HCMV infections or transplanted organs. Virus stock of the isolates was prepared from infected cells by sonication and low speed centrifugation, except for two cell-free virus-producing isolates for which viral stock was prepared from culture media of infected cells. The latter procedure was also followed for preparing the stock of the AD169 strain. Human T-cell acute lymphoblastoid leukemia cells, CCRF-HSB-2, kindly provided by