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Antiviral activities of 2′-deoxyribofuranosyl and arabinofuranosyl analogs of sangivamycin against retro- and DNA viruses

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Summary

Eight sugar-modified pyrrolopyrimidine nucleoside analogs related to the antibiotic sangivamycin were evaluated in cell culture against herpes simplex types 1 (HSV-1) and 2 (HSV-2), cytomegalovirus (CMV), adenovirus, and visna virus. Five of the compounds were highly active against most of the viruses with 50% inhibition (ED_{50}) values of 1–10 μM. The selectivity of the agents was low, with inhibition of uninfected cell proliferation occurring within 5-fold that of the virus ED_{50} for most of the viruses. The compounds did not possess RNA virus-inhibitory activity when evaluated against certain myxo-, paramyxo-, picorna-, reo-, rhabdo-, and togaviruses. Two of the nucleosides were tested further in a cell line persistently infected with Friend leukemia virus where they were inhibitory to both virus yield and cell proliferation at 4–5 μM. Several of the sangivamycin analogs were tested in animal models using a twice-a-day treatment regimen. They proved to be inactive against HSV-1, murine CMV and/or Friend leukemia virus infections in mice.

Herpes simplex virus; Cytomegalovirus; Adenovirus; Retrovirus; Pyrrolopyrimidine nucleoside

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Introduction

Several recent reports have appeared on the antiviral activities of sugar-modified pyrrolo [2,3-*d*]pyrimidine nucleosides (Fig. 1) (De Clercq and Robins, 1986; De Clercq et al., 1987; Turk et al., 1987). These compounds, which are commonly referred to as 7-deazaadenosine analogs, are derivatives of the naturally occurring nucleoside antibiotics tubercidin, toyocamycin and sangivamycin (Bergstrom et al., 1984). Of particular interest are certain 2′-deoxyribofuranosyl (or 2′-deoxy) and arabinofuranosyl (or ara-) derivatives of sangivamycin, since two of these compounds, 2′-deoxysangivamycin and ara-sangivamycin, were recently reported to be highly active in vitro against human cytomegalovirus (Turk et al., 1987). These two nucleosides have also been reported to inhibit other DNA viruses such as herpes simplex types 1 and 2 and vaccinia (De Clercq and Robins, 1986; De Clercq et al., 1987; Turk et al., 1987), and various RNA viruses (such as rhinovirus types 1-A, 1-B, and 9, coxsackievirus type B4, measles virus, parainfluenza virus type 3, reovirus type 1, and vesicular stomatitis virus) (De Clercq et al., 1986, 1987).

The 2′-deoxysangivamycin and ara-sangivamycin evaluated in the above cited studies were synthesized by conversion of the ribofuranosyl antibiotic sangivamycin (De Clercq et al., 1987; Maruyama et al., 1983). Recently, Ramasamy and coworkers at this Institute published total de novo syntheses of these and related nucleosides (Ramasamy et al., 1986, 1988). The advantages of these syntheses are twofold. First, each compound was prepared completely free of contaminating sangivamycin. Since sangivamycin is at least 100-fold more potent than 2′-deoxysangivamycin and ara-sangivamycin as an antiviral agent (De Clercq et al., 1986, 1987), trace amounts of sangivamycin in preparations of the sugar-modified analogs could contribute to their apparent virus-inhibitory properties. Second, de novo syntheses of these compounds allows for production of sufficient quantities to evaluate them in animals. To date, no reports of in vivo antiviral activities of 2′-deoxysangivamycin and ara-sangivamycin have appeared in the literature. A related compound, xylofuranosyltubercidin, was found to be effective against herpes infections in mice (De Clercq and Robins, 1986), however.

In this communication, we report the antiviral activities of 2′-deoxysangivamycin, ara-sangivamycin and six related compounds (which are modified in the carboxamide moiety of the pyrrolopyrimidine base) against several DNA viruses and visna virus. Visna retrovirus is our screening model for the human immunodeficiency (AIDS) virus (Frank et al., 1987). Furthermore, we show the effects of two

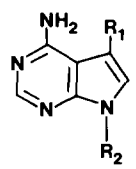

Fig. 1. Structure of 4-aminopyrrolo[2,3-*d*]pyrimidine. The modifications at R_1 and R_2 are given in Table 1.

of the compounds on Friend leukemia retrovirus in vitro. The efficacies of selected nucleosides in the series against herpes simplex, cytomegalo-, and Friend leukemia virus infections in mice are also presented.

Materials and Methods

Antiviral compounds

2′-Deoxysangivamycin, ara-sangivamycin and compounds 3 through 8 defined in Table 1 were synthesized de novo by two published procedures (Ramasamy et al., 1987, 1988). Each compound was characterized by nuclear magnetic resonance (NMR) and ultraviolet (UV) spectroscopic methods. The purity of each compound was determined by elemental analysis and high pressure liquid chromatography. Ribavirin was from Viratek, Costa Mesa, CA. 5-Fluoro-arabinosylcytosine (5-fluoro-AraC) and 3′-azido-3′-deoxythymidine (AZT) were purchased from Sigma, St. Louis, MO. 5-Chloro-2′-deoxyuridine (5-chloro-dUrd) and arabinosyladenine (Ara-A) were bought from ICN Biochemicals, Cleveland, OH. The 5′-monophosphate and 2′-O-acetyl derivatives of compound 4 were prepared by unpublished procedures.

Cells and viruses

Continuously passaged African green monkey kidney (Vero), human embryonic lung (MRC-5), Madin Darby canine kidney (MDCK), human epidermoid carcinoma of the cervix (HeLa) and murine erythroleukemia (D1B) cells were bought from the American Type Culture Collection (ATCC), Rockville MD. Sheep choroid plexus (SCP) cells were started from primary culture by a published procedure (Sundquist and Larner, 1977). Mouse embryo fibroblast (MEF) cells, prepared as described previously (Freitas et al., 1985), were used after 1 to 3 passages from primary culture. The suspension cell line D1B was maintained in RPMI 1640 medium containing 10% fetal bovine serum (FBS). The other cells were maintained in Eagle's medium plus 10% FBS. Serum concentrations were reduced to 2% for experiments with confluent monolayers, and 50 μg gentamicin/ml was added to maintain sterility. The cell stocks were mycoplasma-free as determined by periodic testing.

Herpes simplex virus type 1 (KOS strain) was obtained from James North, Brigham Young University, Provo, UT. Friend leukemia virus (original strain) was from Robert Sidwell, Utah State University, Logan, UT. Influenza A (Chile strain) and influenza B (Texas strain) viruses were from Vernon Knight, Baylor College of Medicine, Austin, TX. Visna virus (1413 strain) was provided by Opendra Narayan, Johns Hopkins University, Baltimore, MD. The following were purchased from ATCC: adenovirus type 5 (Adenoid 75 strain), herpes simplex virus type 2 (MS strain), human cytomegalovirus (AD-169 strain), murine cytomegalovirus (Smith strain), vervet monkey cytomegalovirus (CSG strain), coxsackievirus type B1 (Conn-5 strain), Coxsackievirus type B4 (JVB strain), poliovirus type 1 (Chat strain), rhinovirus type 1-A (2060 strain), rhinovirus type 2 (HGP strain), para-

influenza virus type 3 (C243 strain), reovirus type 3 (Dearing strain), Semliki Forest virus (original strain), and vesicular stomatitis virus (Indiana strain).

Antiviral and cytotoxicity assays

Initially, compounds were evaluated in 96-well microplates (Sidwell and Huffman, 1971) to determine their inhibitory properties against many DNA and RNA viruses. The adeno- and picornaviruses were tested in HeLa cells, influenza A and B viruses in MDCK cells (using 5 μg trypsin/ml in the medium as described by Nerome and Ishida, 1978), and visna virus in SCP cells. The rest of the viruses which were evaluated in this assay were screened in Vero cells. Half-$\log_{10}$ dilutions of each compound were evaluated from 1000 to 1 μM, with uninfected drug toxicity control wells run in parallel. The plates were evaluated when 100% virus-induced cytopathology was achieved, which was generally in 3 days, except for influenza A and visna viruses (requiring 5–6 days).

Those DNA viruses that were markedly inhibited by the compounds were further evaluated by plaque reduction methods (Freitas et al., 1985; Smee et al., 1983). Briefly, virus at 100–150 plaque forming units/ml was adsorbed to monolayers in 6-well plates 1.25 h. Then each test compound at 6 different concentrations (in two-fold dilution increments) in 0.5% SeaPlaque agarose (FMC Corp., Bar Harbor, ME) was applied after aspirating off the virus-containing medium. Three wells were used for each drug dilution and 4 wells for the untreated virus control. The basic medium for the overlays was Eagle's containing 2% fetal bovine serum. All experiments except human cytomegalovirus (HCMV) were treated as follows. The time to develop plaques varied with the kind of virus: adenovirus type 5 (Ad-5), 6 days; herpes simplex virus types 1 and 2 (HSV-1 and HSV-2), 2 days, murine cytomegalovirus (MCMV), 3 days, and vervet monkey cytomegalovirus (VCMV), 4 days. Occasionally the plaques might take a day longer to develop. After the formation of viral plaques, a solution of 10% buffered formalin was applied on top of the agar overlays for 15 minutes, then the overlays were removed. Cell monolayers were stained with 1% crystal violet in 20% ethanol. For HCMV, a second overlay of test compound in agarose was required on day 4. A final overlay containing neutral red dye (0.002%) was applied on day 7, and HCMV plaques were counted on day 8. Ad-5 virus was plaqued in HeLa cells, HSV-1 and HSV-2 in Vero cells, HCMV and VCMV in MRC-5 cells, and MCMV in MEF cells. Plaques were counted at 13 to 17 × magnification using a Plaque Viewer (Bellco Glass Co., Vineland, NJ).

Visna virus inhibition was quantified by assaying yield of reverse transcriptase in virions released from drug-treated infected SCP cell cultures. Compounds were diluted in two-fold increments near the estimated 50% effective dose as was done for the plaque reduction assays. The visna virus experiments were run in 24-well plates by pooling 4 wells together for each concentration of inhibitor. The virus was allowed to propagate for 6 days until complete cytopathology was noted in drug-free control wells. Samples were pelleted by ultracentrifugation and then assayed for reverse transcriptase exactly as described by Frank et al. (1987). We have found that this assay very closely approximates results obtained by plaque reduction.

To determine the activities of compounds on the proliferation of uninfected HeLa, MEF, MRC-5, SCP and Vero cells, 2×10^3 cells were seeded into each well of 96-well microplates and allowed to grow for 1 day. Then test compounds were added and diluted in half $\log_{10}$ increments using 100 μM as the top concentration for most experiments. Some retests were performed using up to 1 mM of the less toxic inhibitors. Each concentration of compound was evaluated in three microwells per experiment. After 5 days of growth at 37°C, cells were trypsinized and the cell suspensions counted using a Coulter counter (Coulter Electronics, Hialeah, FL).

The effects of compounds on Friend leukemia virus yield and D1B cell proliferation were performed as follows. D1B cells, which are persistently infected with Friend virus, were diluted in 10 ml volumes of fresh media containing nucleoside inhibitors at a concentration of 5×10^4 cells/ml and placed in T-25 flasks. The concentrations of each compound used are presented in Table 5. One flask was used per drug dilution and two flasks used for the untreated cell control. After 3–4 days incubation at 37°C, cell numbers were determined by counting trypan blue-excluding viable cells using an hemocytometer. At toxic drug concentrations, cells were pelleted and resuspended in 0.1 ml volumes to get accurate counts. The supernatants from these cells were initially frozen at −80°C. Thawed supernatants were pelleted at 100000 × *g* for 2 h, then the pellets were resuspended in 0.2 ml of solubilization buffer (33 mM Tris chloride, pH 8.0, 500 mM KCl, 2.5 mM dithiothreitol, 0.33% Triton X-100).

The assay for reverse transcriptase was slightly modified from that used for visna virus (Frank et al., 1987). The reaction mixture was identical to that used previously except it also contained 5 mM $MnCl_2$. Because of the high amount of Friend leukemia virus released from the D1B cells, each preparation of enzyme required a 50-fold dilution in buffer to produce a linear reaction with substrate during 30 min at 37°C. Twenty microliters of enzyme were used in a 100 μl total reaction volume. The capture of acid precipitable counts of radioactivity was performed by first adding 50 μl of yeast RNA (ICN Biochemicals) followed by 150 μl 5% trichloroacetic acid (TCA)/1 mM pyrophosphate (PPi) to each tube. Duplicate 140 μl volumes from each tube were spotted onto 0.22 micron cellulose nitrate squares which were filtered dry, allowed to set for 2–3 min, and then batch washed 4 times in TCA/PPi before counting.

An evaluation was done to ascertain the effects of nucleosides on T-lymphocyte functions of the immune system using a mixed lymphocyte reaction (MLR) assay. Peripheral blood lymphocytes from human volunteers were isolated from whole blood using Lymphoprep (Robbins Scientific, Mountain View, CA). Cultures of 10^5 lymphocytes from two different donors were combined in equal proportions and treated with compounds for 4 days at 37°C. Six dilutions of each inhibitor varying by two-fold increments were run in each experiment. [^{3}H]thymidine (ICN Radiochemicals, Irvine, CA) was then added at 20 μCi/ml in drug-containing medium for 18 h. Harvested, lysed cells were collected on glass fiber filters then were counted by liquid scintillation spectroscopy. Under these conditions about 20000 counts of radioactivity were detectable in untreated cultures.

For the above assays, fifty percent inhibition of virus plaque numbers or of virus yield (ED_{50}), or fifty percent inhibition of cell proliferation or of the incorporation of [^{3}H]thymidine in lymphocytes (ID_{50}) were estimated by plotting results on semi-log paper. Each reported value represents an average of 2 separate experiments for all determinations, except for the data reported in Table 5 which represent a single experiment.

Animal experiments

Ten 50% lethal doses of HSV-1 were inoculated intraperitoneally into 20 gram mice. Compounds in saline were administered by the same route twice a day for 7 days starting 2 h pre-virus inoculation. Deaths were recorded for 21 days. The same protocol was used to evaluate compounds against a lethal MCMV infection, except that weanling mice (11–13 grams) were used. There were 12 mice per treatment group and 4 mice in uninfected toxicity control groups.

Another experiment involved intraperitoneal inoculation of 20 gram mice with diluted spleen homogenates from mice infected with Friend leukemia virus, resulting in splenomegaly in the newly infected animals (Sidwell et al., 1975). The virus pool was pretitrated in animals to produce a 10-fold or greater spleen enlargement in most of the infected animals. Compounds in saline were administered intraperitoneally twice a day for 14 days starting 2 h pre-virus inoculation. At 21 days, spleens from all animals were removed and carefully weighed. Spleens from uninfected mice were weighed at the same time. There were 16 mice in each treatment group and 4 mice in uninfected toxicity control groups.

The doses of antiviral substances run in the experiments were selected based upon amounts of available compounds, preliminary toxicity indications (weight loss), and published results (as was the case for ribavirin; Sidwell et al., 1975). The compounds were not overtly toxic since uninfected mice treated in parallel with the infected ones did not die. However, mice treated with the higher doses of sangivamycin analogs gained weight slower than did saline-treated animals.

Swiss-Webster mice, purchased from Bantin and Kingman Labs, Freemont, CA were used in all animal experiments. They were specific virus pathogen-free.

Results

In vitro studies

Figure 1 shows the general structure of the pyrrolo[2,3-*d*]pyrimidine heterocycle. The eight compounds tested have various 'R' groups as defined in Table 1. These compounds were evaluated in cytopathic effect inhibition assays against several DNA and RNA viruses. In these experiments we were unable to show that any of the compounds inhibited the following RNA viruses to any degree at any concentration from 1 to 1000 μM: coxsackievirus type B1, coxsackievirus type B4, influenza A virus, influenza B virus, parainfluenza virus type 3, poliovirus type 1, reovirus type 3, rhinovirus type 1-A, rhinovirus type 2, Semliki Forest virus, and vesicular stomatitis virus. Most of the compounds affected the morphology of cell

TABLE 1

Antiviral and cell-inhibitory activities of sangivamycin analogs

Compound number and structure[c]	Virus-inhibitory ED_{50}[a] (μM)/Cell-inhibitory ID_{50}[b] (μM)						
	Ad-5/ HeLa	HSV-1/ Vero	HSV-2/ Vero	HCMV/ MRC-5	MCMV/ MEF	VCMV/ MRC-5	Visna/ SCP
1[d] R_1= $CONH_2$	7/1.5	12/12	28/12	0.6/1	15/7	3/1	0.1/0.6
R_2= 2′-deoxy[e]	(0.2)[f]	(1.0)	(0.4)	(1.7)	(0.5)	(0.3)	(6.0)
2[g] R_1= $CONH_2$	8/15	8/40	28/40	1.6/1.6	8/9	3/1.6	0.3/4
R_2= Ara[h]	(1.9)	(5.0)	(1.4)	(1.0)	(1.1)	(0.5)	(13.3)
3 R_1= $CSNH_2$	1/3	6/4	6/4	4/14	11/4	3/14	0.3/2
R_2= 2′-deoxy	(3.0)	(0.7)	(0.7)	(3.5)	(0.4)	(4.7)	(6.7)
4 R_1= $CSNH_2$	1/2.4	1.5/1	1.5/1	1.5/3	2.6/1.7	1/3	0.3/1.3
R_2= Ara	(2.4)	(0.7)	(0.7)	(2.0)	(0.7)	(3.0)	(4.3)
5 R_1= $C(NH)NH_2$	6/14	6/11	9/11	5/16	11/17	2/16	0.3/13
R_2= 2′-deoxy	(2.3)	(1.8)	(1.2)	(3.2)	(1.5)	(8.0)	(43.3)
6 R_1= $C(NH)NH_2$	>100/>100	>100/>100	>100/>100	35/>100	120/235	>100/>100	6/300
R_2= Ara				(>2.9)	(2.0)		(50.0)
7 R_1= $C(NOH)NH_2$	8/90	60/70	75/70	9/20	110/85	2/20	2/35
R_2= 2′-deoxy	(11.3)	(1.2)	(0.9)	(2.2)	(0.8)	(10.0)	(17.5)
8 R_1= $C(NOH)NH_2$	20/175	80/>100	>100/>100	22/>100	120/175	15/>100	15/>100
R_2= Ara	(8.8)	(>1.3)		(>4.5)	(1.5)	(>6.7)	(>6.7)

[a] Determined by plaque reduction assays for all but visna virus, which was assessed by virus yield reduction.

[b] The inhibition of actively growing uninfected cell populations was determined.

[c] Refer to Figure 1 for the structure of the heterocycle and positions of the "R" groups.

[d] 2′-Deoxysangivamycin.

[e] 2′-Deoxy-β-*D*-ribofuranose.

[f] Selectivity index, determined by dividing the cell ID_{50} by the virus ED_{50}.

[g] Arabinofuranosylsangivamycin.

[h] β-*D*-arabinofuranose.

monolayers above 100 μM, indicating some toxicity. But even at those concentrations the various RNA viruses were able to replicate and destroy the cells. The viruses that were inhibited in these preliminary assays included Ad-5 and HSV-2 (which are DNA viruses) and visna virus.

Plaque reduction assays were performed to determine more precisely the antiviral activities of the compounds against adenovirus and herpes simplex viruses, whereas the effects against visna virus yield was quantified by a reverse transcriptase assay (Table 1). We also tested various cytomegaloviruses by plaque reduction assays since activity against HCMV was known against compounds 1 and 2 (Turk et al., 1987). In these experiments compounds 1–5 and 7 were inhibitory to most of the viruses at $\leq$10 μM. Compounds 6 and 8 were somewhat less active against the majority of viruses. Curiously, analog 6 was inhibitory principally to visna virus.

In uninfected cells, the compounds inhibited the proliferation of most cell lines at low concentrations (Table 1). Compounds 1 through 8 also impaired the functions of mixed human T-lymphocytes (as measured by [^{3}H]thymidine incorporation) at 1, 5, 6, 5, 15, 150, 1.5, and 15 μM, respectively. By comparing the 50% inhibitory concentration of virus (ED_{50}) to inhibition of the respective cell line (ID_{50}) in which the virus was assayed, one observes selectivity indices of 5 or less against most of the viruses (Table 1). Compounds 7 and 8 were the most selective against adenovirus. Several of the inhibitors showed >10-fold selectivity against visna virus, with compounds 5 and 6 being particularly selective. Against MCMV, six of the eight nucleosides inhibited cell proliferation at concentrations lower than those required to inhibit virus plaque formation by 50%, indicating a negative selectivity. In these antiviral assays confluent monolayers were not disrupted or destroyed by the compounds at concentrations where plaque numbers were completely inhibited, so this did not affect our ability to accurately quantify the plaques.

Animal experiments

Selected compounds were evaluated in mouse models to ascertain their in vivo potential against herpes simplex virus, cytomegalovirus and Friend leukemia virus.

TABLE 2

Effects of compounds on a herpes simplex virus type 1 infection in mice

Compound	Dose[a] (mg/kg)	Survivors/ total (%)	Mean survival time[b] (days)
Saline	–	1/12 (8)	12.3 ± 3.0[c]
3	80	2/12 (17)	12.2 ± 3.5
5′-phosphate of 4	80	1/12 (8)	12.7 ± 1.7
5′-phosphate of 4	40	4/12 (25)	13.3 ± 3.0
Ara-A	250	10/12 (83)[d]	10.5 ± 2.2

[a]Half-daily doses were administered twice a day for 7 days starting 2 h before virus inoculation.
[b]Of mice that died. Survivors lived through 21 days.
[c]Standard deviation.
[d]Statistically significant ($P<0.002$), determined by the two-tailed Fisher exact test.

TABLE 3

Effects of compounds on a murine cytomegalovirus infection in weanling mice

Expt. No.	Compound	Dose[a] (mg/kg)	Survivors/ total (%)	Mean survival time[b] (days)
1	Saline	–	3/12 (25)	6.1 ± 0.9[c]
	3	80	0/12 (0)	5.1 ± 1.4
	3	40	2/12 (17)	6.5 ± 1.1
	3	20	3/12 (25)	6.2 ± 0.8
	5	80	0/12 (0)	6.5 ± 1.2
	5	40	2/12 (17)	6.2 ± 0.8
	5	20	0/12 (0)	6.3 ± 1.2
2	Saline	–	3/12 (25)	6.2 ± 1.1
	3	100	1/12 (8)	5.5 ± 1.9
	5	100	1/12 (8)	5.5 ± 1.0
	8	100	1/12 (8)	6.0 ± 2.0
3	Saline	–	7/12 (58)	6.6 ± 0.9
	5′-phosphate of 4	80	0/12 (0)[d]	3.7 ± 0.7[e]
	5′-phosphate of 4	40	1/12 (8)[d]	5.9 ± 0.8
	2′-*O*-acetyl of 4	80	3/12 (25)	6.3 ± 1.4

[a] Half-daily doses were administered twice a day for 7 days starting 2 h before virus inoculation.
[b] Of mice that died. Survivors lived through 21 days.
[c] Standard deviation.
[d] Statistically significant ($P<0.05$), determined by the two-tailed Fisher exact test.
[e] Statistically significant ($P<0.001$), determined by two-tailed *t*-test.

In the first experiment analog 3 and the 5′-phosphate of 4 (the nucleoside has very poor solubility in water) were administered for 7 days to mice inoculated intraperitoneally with HSV-1 (Table 2). In this test neither pyrrolopyrimidine nucleoside was able to decrease mortality or extend survival times significantly, although the 5′-phosphate of compound 4 at 40 mg/kg may have provided some benefit. Ara-A at its effective dose protected most of the mice from this lethal infection.

In a set of experiments, animals inoculated with MCMV were treated with various analogs of sangivamycin. Therapy with compounds 3, 5, and 8 for seven days provided no protection compared to saline (Table 3). Although not statistically significant, more mice died when treated with compounds 3 and 5 at 80 and 100 mg/kg in two separate experiments. A decrease in time to death at 80–100 mg/kg for compound 3 and at 100 mg/kg for analog 5 was also evident.

A less virulent infection was achieved when evaluating the 5′-phosphate and 2′-*O*-acetyl derivatives of compound 4 (Table 3) against MCMV. In that test, the 5′-phosphate caused a statistically significant potentiation of the disease. There was an increase in mortality at 40 and 80 mg/kg, as well as decreased time to death at 80 mg/kg. It was apparent that the 2′-*O*-acetyl derivative of 4 also increased mortality, but this was not statistically significant. Uninfected toxicity control animals run in parallel did not die from drug administration although they failed to gain weight.

A final experiment was conducted to determine efficacies of compounds against a Friend leukemia virus infection in mice (Table 4). A known antiviral agent (ri-

TABLE 4

Effects of compounds against Friend leukemia virus-induced splenomegaly in mice

Compound	Dose[a] (mg/kg/day)	Average spleen weight[b] (grams)
Uninfected	–	0.10 ± 0.01[c]
Saline	–	1.54 ± 0.54
Ribavirin	75	0.74 ± 0.51[d]
Ribavirin	25	1.48 ± 0.62
5-Fluoro-AraC	25	0.55 ± 0.39[d]
5-Chloro-dUrd	25	1.05 ± 0.55[d]
2	75	1.82 ± 0.55
2	25	1.28 ± 0.67
7	75	1.21 ± 0.74
7	25	1.56 ± 0.73

[a] Half-daily doses were administered twice a day for 14 days starting 2 h pre-virus inoculation.
[b] Determined 21 days after virus inoculation.
[c] Standard deviation.
[d] Statistically significant ($P<0.01$), determined by a two-tailed t-test.

bavirin) and two antitumor compounds (5-fluoro-AraC and 5-chloro-dUrd) were run in parallel for comparison. The antiviral activity was determined as a function of average spleen weight of infected mice. The spleen weights of mice treated with compounds 2 and 7 were within 80 to 120% of the saline-treated control, indicating ineffective therapy. The other three compounds were all efficacious in reducing the splenomegaly, with 5-fluoro-AraC being the most active.

TABLE 5

Effects of compounds on cell proliferation and on Friend leukemia virus yield from D1B cells

Drug concentration (μM)	Compound 2		Compound 7		Ribavirin		AZT	
	Cells/ml[a]	% Virus[b]	Cells/ml	% Virus	Cells/ml	% Virus	Cells/ml	% Virus
0	4.2 (100)	100	4.2 (100)	100	2.4 (100)	100	2.4 (100)	100
0.3	3.9 (93)	98	4.1 (98)	101	2.4 (100)	75	2.2 (92)	77
1	3.3 (79)	93	3.3 (79)	90	1.9 (79)	58	1.3 (54)	57
3	2.4 (57)	69	2.7 (64)	92	0.7 (29)	21	1.0 (42)	46
10	1.4 (33)	27	1.7 (40)	37	0.3 (13)	1	0.4 (17)	24
30	0.1 (2)	2	0.2 (5)	3	0.0[c] (0)	0	0.2 (8)	9
ID_{50} or ED_{50} (μM)	4	5	5	7	2	1.3	2	1.7

[a] Viable cells/ml (trypan blue-excluding) × 10^{-6} after 3–4 days of replication. There were 5 × 10^4 cells/ml at the start of the experiment in 10 ml volumes. The values in parentheses are percents of the drug-free control.
[b] Supernatant virus from cells was quantified by a reverse transcriptase assay. The values are percents of the drug-free control.
[c] Less than 10^2 viable cells/ml.

Effects of compounds on D1B cell proliferation and Friend leukemia virus yield.

At the time the Friend leukemia virus experiment was conducted in animals, we did not have a cell culture system to preliminarily evaluate compounds against the virus. Thus, compounds 2 and 7 were tested blindly on the assumption that inhibitors of visna virus should be active against Friend leukemia virus as well. When the two sangivamycin analogs failed to suppress Friend virus-induced splenomegaly (Table 4), we were left wondering whether the compounds were active against the virus in the first place. Subsequently, we obtained a cell line, designated D1B, that was suitable for assaying compounds against Friend leukemia virus in vitro. The cells chronically excrete the virus, which can be quantified by a reverse transcriptase assay.

Table 5 shows the results of a single experiment where ribavirin, AZT, and compounds 2 and 7 inhibited Friend leukemia virus yield and the proliferation of D1B cells in culture. AZT, a highly selective anti-retrovirus agent active against the closely related Rauscher murine leukemia virus in vitro and in mice (Ruprecht et al., 1986), was evaluated as a control for these studies. It is interesting that all compounds, including AZT, inhibited virus yield at approximately the same concentration as they inhibited cell replication. Also evaluated but not depicted on the table was 5-fluoro-AraC. That compound inhibited virus yield and cell replication by 50% at 0.05 μM. These data pertain to the potency of 5-fluoro-AraC in mice against Friend virus-induced splenomegaly (Table 4). Since compounds 2 and 7 were inhibitory to virus yield/cell proliferation at low concentrations, the lack of response of Friend disease to these substances in mice must be attributed to factors other than poor antiviral or anti-proliferative effects.

Discussion

In this report several 2′-deoxy and arabinofuranosyl analogs of sangivamycin were shown to inhibit various DNA viruses and visna virus at 1–10 μM. These results substantiate and extend the work of others (De Clercq and Robins, 1986; De Clercq et al., 1987) who reported only on compounds 1 and 2 (2′-deoxysangivamycin and ara-sangivamycin, respectively). The antiviral properties of compounds 3 through 8 have never been reported.

Analogs 1 and 2 have been found active against Coxsackie, parainfluenza, reo-, rhino-, and vesicular stomatitis virus at concentrations that were below the cytotoxic concentrations (De Clercq et al., 1986, 1987). For most of the test systems, the types of viruses and cell cultures we used were comparable to those of the other investigators, and the compounds were screened at concentrations 10 times higher than those used in the former studies. In our hands, however, the 2′-deoxy and arabinofuranosyl sangivamycin analogs did not show RNA virus inhibitory activity, with the exception of visna (and Friend leukemia virus for those compounds which were tested) which has a DNA replication phase in its life cycle. Whether the discrepancies between our results and those reported previously relate to types

of assays, strains of viruses, or cells employed is unknown.

As was evident from the cytotoxicity data, these compounds are not very selective in their anti-DNA virus activity, although greater selectivity was achieved against visna virus. Most of the substances were cell growth-inhibitory at or near antiviral concentrations. Compounds 1 and 2 appeared to be more cell inhibitory in our systems than others have reported (De Clercq et al., 1986; De Clercq and Robins, 1986; Turk et al., 1987). This can be explained by the fact that we used replicating cells to assay cytostatic or cytotoxic activities whereas the other groups reported microscopic changes in morphology of confluent monolayers as an indication of toxicity. These microscopic changes were recorded at the same time as viral cytopathogenicity.

Because of the anti-visna virus activities of these nucleosides in cell culture, we were prompted to send the more selective ones through the U.S. Army testing program to determine effects against the related human immunodeficiency (AIDS) virus. None of the submitted compounds were active against the human virus. We speculate that these nucleosides may inhibit the human immunodeficiency virus, but also inhibit the cells at or near antiviral concentrations.

Even though most of these antiviral agents were potent inhibitors of DNA viruses, visna virus and Friend leukemia virus (where tested) in vitro, they proved to be ineffective in mice. Compound 1 (2′-deoxysangivamycin) was not evaluated in any animal model, so we can only surmise that it too would have been ineffective in vivo. The toxicity of these compounds in cell culture appeared to translate into deleterious effects in animals, especially with regard to the treatment of an MCMV infection. Three of the four compounds that were used in that model were more inhibitory to cells than they were to the virus in vitro. In addition, 7 of the 8 nucleosides had inhibitory effects on mixed T-lymphocyte reactions at low concentrations, which would have deleterious consequences on the host's ability to fight an infection in vivo. Further work will need to be done to completely rule out that these compounds have no beneficial effect in animals. In particular, changes in dosing schedules may improve the performance of the compounds.

Of the 3 animal models that were run, we expected the sangivamycin analogs to have been active in treating the Friend leukemia virus infection. This is because either an antiviral (ribavirin) or an antitumor (5-fluoro-AraC) compound can suppress the extent of splenomegaly (Table 4). The results in Table 5 show that compounds 2 and 7 were active in inhibiting virus yield and D1B cell proliferation at concentrations where ribavirin and AZT showed similar activities, suggesting that these sangivamycin analogs had potential for in vivo activity. Considering the cell culture and in vivo results, these data strongly suggest that the lack of response of the disease to treatment by compounds 2 and 7 was due to unfavorable (and as yet undefined) pharmacological parameters.

As a side issue to these studies, new insight was gained of the way anti-retrovirus agents most likely inhibit Friend leukemia and related leukemia virus diseases in mice. We showed that D1B erythroleukemia cells, which are spleen cells that have been transformed in vivo and are persistently infected by the Friend leukemia virus, are inhibited by these compounds at or near antiviral concentrations

(Table 5). These cultures represent the host cells for virus replication in vivo. Splenomegaly can be induced in mice by inoculating either the supernatant from D1B cells into mice or by injecting the cells themselves (into allogeneic DBA mice) (unpublished). From the results presented in Table 5, we hypothesize that the proliferation of virus-transformed spleen cells in culture or in mice (resulting in splenomegaly) is linked to the replication of the virus, and the two events cannot be separated by antiviral chemotherapy. This means that the inhibition of virus replication will lead to an inhibition of cell proliferation, and the inhibition of cell proliferation will decrease overall virus production. These combined effects will decrease the extent of splenomegaly and virus recovery, the endpoints that are measured in antiviral experiments with the Friend and Rauscher leukemia virus.

With regard to the mode of action and selectivity of AZT as it relates to the above discussion, although AZT can be shown to have a high degree of virus selectivity if assayed against a murine leukemia virus applied to fresh monolayer cells prior to cell transformation (Ruprecht et al., 1986), the same degree of selectivity does not exist in cells already transformed by the virus (Table 5). In the report of the activity of AZT against Rauscher murine leukemia virus in mice (Ruprecht et al., 1986), therapy was started 4 days after virus inoculation, or after many cells were already transformed by the virus. Under these conditions the compound would be acting in three ways: to inhibit virus production in the transformed cells (which would slow the spread of the virus to new cells), to inhibit the proliferation of the transformed cells, and to prevent the transformation of new cells by intracellular inhibition of virus replicative events required for integration of viral genomes into host cell chromosomes. All of these effects would decrease the extent of splenomegaly and reduce the amount of recoverable virus. A general statement which would explain the mode of action of both antiviral and antitumor compounds in murine leukemia virus infection in vivo models is that the inhibition of splenomegaly and suppression of virus are due to a combination of antiviral and antiproliferative activities. This may apply to other murine retrovirus animal models as well.

From these studies we have shown that analogs of 2′-deoxysangivamycin and arasangivamycin, which are modified carboxamide forms of these nucleosides, retain antiviral and anticellular properties in cell culture (Table 1). Thio and amino substitutions (compounds 3 and 5) of the carboxamide on a 2′-deoxyribose were equivalent to 2′-deoxysangivamycin (compound 1) in terms of biological activity. The hydroxyamino-substituted carboxamide on a 2′-deoxyribose (compound 7) was somewhat less effective. When these carboxamide substitutions were made on arabinosyl nucleosides, the amino and hydroxyamino compounds (compounds 6 and 8) lost antiviral potency, although the thio derivative (compound 4) retained activity (and even became more cytotoxic) relative to ara-sangivamycin (compound 2).

It appeared that changes in the carboxamide moiety provided moderate increases in the selectivity ratios of some compounds against particular viruses (Table 1). For example compounds 7 and 8 were more selective than compounds 1 and 2 against adeno 5 virus. Analog 5 retained potency against visna virus but was

less cytotoxic to cells than compound 1. Then there are examples where the modified carboxamide heterocycles gained selectivity but lost potency as virus inhibitors, such as compound 6 against visna virus and compound 8 versus HCMV (the counterpart structure to analogs 6 and 8 being compound 2). In general, all of the highly active nucleosides were still inhibitory to cell proliferation at low concentrations, probably because they are too closely related in structure to their natural counterpart (which is most likely 2′-deoxyadenosine). Whether modifications of the carboxamide moiety of heterocycles attached to acyclic side chains would yield more selective antiviral compounds is a subject that merits investigation.

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