Purine Analogs as Potential Anticytomegalovirus Agents* (34075)

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Cytomegalovirus (CMV) is the causal agent of cytomegalic inclusion disease, which has for many years been primarily associated with infants as a congenital infection (1). Recently, however, the virus has been repeatedly isolated from patients under treatment with immunosuppressive drugs, suggesting the possibility that such infections may be serious complications of intensive anticancer therapy (2) and of organ or tissue transplantation procedures (3, 4). A possible approach to this problem would be the chemotherapeutic treatment of these patients with drugs having activity against the virus. Limited studies of such anti-CMV treatments in human patients suggest this approach to be feasible (5).

Since relatively few compounds which have anti-CMV activity are known, a study was initiated in our laboratory in which a variety of biologically active compounds were tested for *in vitro* activity against the virus. Among the materials demonstrated to have significant anti-CMV activity were certain purine analogs. Additional experiments were carried out with these compounds to further quantitate and clarify their anti-CMV activity. These experiments are described in the present report.

Materials and Methods. Virus. A strain of CMV isolated from a human patient in 1965 by Dr. A. R. Casazza was used in these experiments. We obtained the virus after its eighty-eighth consecutive passage in human embryonic lung fibroblast (WI-38) (6) cells. The virus was maintained in our laboratory continuously in this cell line grown in Eagle's (7) basal medium (BME) containing twice the concentration of vitamins and amino acids and supplemented with 5% fetal calf serum. The virus causes WI-38 cells to become decidedly rounded and refractile within 7 days after inoculation, with the cells eventually being destroyed by the infection.

Compounds tested. Twenty-five biologically active purine analogs supplied by the Cancer Chemotherapy National Service Center, National Cancer Institute, were tested against CMV. These compounds, together with their National Service Center (NSC) reference numbers, are listed in Table I.

Procedures. In all primary chemotherapy experiments, growth medium was decanted from 5-day-old WI-38 cells, after which 0.1 ml of virus suspension and 2.0 ml of test medium (BME supplemented with 5% inactivated horse serum and twice the usual concentrations of vitamins and amino acids) containing test compound were added to the culture tubes. The tubes were incubated at 37° for 48 hr, then the medium was decanted from the cells and fresh medium containing test drug was added. This process was repeated 48 hr later. The cells were examined microscopically for cytopathogenic effect (CPE) 7 days post-virus inoculation. This CPE was graded from 0 (normal cells) to 4 (complete destruction of the cell layer). At least three and as many as seven drug concentrations were used in each experiment. These concentrations, declining by one-half log dilutions, were selected to range from cytotoxic to nontoxic levels. Controls for each experiment included cell controls (cells + test medium), drug cytotoxicity controls (cells + test medium + drug), and virus controls (cells + test medium + virus). Two to three virus levels were used in each experiment, each virus level varying from the next

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TABLE I. The *in Vitro* Anticytomegalovirus Activity of a Group of Purine Analogs.[a]

NSC no.	Compound	Degree of antiviral activity: Maximum virus rating[b]	Degree of antiviral activity: Maximum therapeutic index[c]
29422	2-Amino-9-β-D-ribofuranosyl-9*H*-purine-6-thiol	2.7	100
752	2-Aminopurine-6-thiol	2.7	100
404241	9-β-D-Arabinofuranosyladenine	1.4	32
755	Purine-6-thiol hydrate	1.4	32
95383	Purine-6-carboxaldehyde thiosemicarbazone	1.4	32
38887	2-Amino-6-[(1-methyl-4-nitroimidazol-5-yl)thio]purine	1.3	32
4911	9-β-D-Ribofuranosyl-9*H*-purine-6-thiol	1.1	32
43405	2-Amino-1-methylpurine-6(1*H*)-thione	0.8	10
11595	6-(Propylthio)purine	0.6	10
51095	Purine-6-yltrimethyl ammonium chloride	0.5	3.2
20105	6-(Methylthio)purine trihydrate	0.4	3.2
29421	6-(Benzylthio)purine	0.2	3.2
45150	8-Bromopurine	0.3	1.0
744	6-Chloropurine	0.2	1.0
14575	9-Ethyl-9*H*-purine-6-thiol	0.1	1.0
19487	9-Cyclopentyl-9-*H*-purine-6-thiol	0.1	1.0
19866	(Purine-6-yl-thio)acetonitrile	0.1	1.0
25740	2-(Methylthio)-9-β-D-ribofuranosyl-9*H*-purine	0.1	1.0
39084	6-[(1-Methyl-4-nitroimidazol-5-yl)thio]purine hydrate	0.1	1.0
4910	6-Chloro-9-β-D-ribofuranosyl-9*H*-purine	0	0
15747	2-Amino-6-(benzylthio)purine	0	0
19488	9-Butyl-9*H*-purine-6-thiol	0	0
30605	2-Fluoro-9-β-D-ribofuranosyladenine	0	0
40774	6-(Methylthio)-9-β-D-ribofuranosyl-9*H*-purine	0	0
83258	9-β-D-*Erythro*-pentafuranosyladenine	0	0

[a] Compounds given in order of decreasing activity.

[b] Virus rating: A weighted measurement of antiviral activity, determined by a modification of the method of Ehrlich *et al.* (8). 1.0 or $>$ = Definite antiviral activity; 0.5–0.9 = moderate or questionable antiviral activity; <0.5 = no apparent antiviral activity.

[c] Therapeutic index: $\frac{\text{Highest nontoxic drug dose}}{\text{Lowest effective drug dose}}$.

by a factor of 10. Since CMV is relatively labile to freezing, the virus could not be pretitered effectively. Therefore, varying dilutions of virus suspensions which were estimated to have titers of 10, 100, or 1000 cell culture 50% infectious doses/ml ($CCID_{50}$/ml) were used. The actual titer determinations were then carried out in parallel with the experiment. Each virus level at all drug dosages was tested in duplicate tubes.

Evaluation of antiviral activity. The percentage of reduction of virus-induced CPE caused by the test drug was determined and then evaluated statistically using a modification of the virus rating (VR) method described by Ehrlich *et al.* (8). To determine this VR, the value assigned to the CPE of the treated, infected cells at each drug level was subtracted from that of the virus controls, and the differences, C-T, were added. If the drug was slightly toxic at one or more levels, the C-T at those levels was divided by 2. If three virus concentrations were used, the total C-T at the highest virus level was tripled, and at the next virus level was doubled. The C-T total of all virus levels was divided by the number of levels, and this figure was divided by 10. From our experience, a VR of >1.0 is usually indicative of definite antiviral activity, whereas a VR of

0.5 – 0.9 is indicative of moderate or questionable antiviral activity, and a VR of <0.5 suggests no apparent antiviral activity. In addition to the VR, a therapeutic index was also calculated for each active compound by dividing the highest nontoxic drug dose by the lowest active drug dose.

Toxicity was determined primarily by cellular anomalies apparent when the toxicity control cells were examined microscopically. In addition, the medium containing the test drug was decanted from the toxicity control cells. The cells were washed, resuspended in growth medium, and incubated at 37°. Those cells from the highest nontoxic drug level, as determined microscopically, grew at a normal rate. Aliquots of these normally growing cells supported CMV replication when the virus was added.

Determination of extra- or intracellular virus. The concentration of "extracellular" virus was determined by centrifuging the supernate from the infected cells at low speed to remove cellular debris and adding serial $\log_{10}$ dilutions of the centrifugate to 7-day-old WI-38 cells. "Intracellular" virus titers were determined by scraping supernate-free infected monolayers into fresh medium, homogenizing these cells in a Servall Omnimix homogenizer (Ivan Sorvall, Inc., Norwalk, Conn.) run at maximum speed 1½ min, and adding dilutions of this material to WI-38 cells. Demonstrable CPE in these cells within 7 days was considered indicative of infectious virus.

Results. The results of the primary chemotherapy experiments carried out against CMV are summarized in Table I. Each compound is listed in the order of maximum activity as determined by its therapeutic index and VR. Nineteen compounds were considered to have a degree of anti-CMV activity in this study, but only seven had marked activity, with VR's of >1.0 and therapeutic indices of 32 or greater. These seven compounds were: 2-amino-9-β-D-ribofuranosyl-9*H*-purine-6-thiol (6-thioguanosine, TGS), 2-aminopurine-6-thiol (6-thioguanine, TG), 9-β-D-arabinofuranosyladenine (ara-A), purine-6-thiol hydrate (6-mercaptopurine, MP), 9-β-D-ribofuranosyl-9*H*-purine-6-thiol (6-mercaptopurine ribonucleoside, MPR), 2-amino-6-[(1-methyl-4-nitroimidazol-5-yl)thio] purine (azathiopurine, NITG), and purine-6-carboxaldehyde thiosemicarbazone (PCTC). These data were derived from two or more experiments similar to that shown in Table II, which is an example using 6-thioguanine.

TABLE II. Effect of 6-Thioguanine on Cytomegalovirus *in Vitro*.[a]

Drug concentration[b] (μg/ml)	Toxicity controls	CPE[c]: Drug + 10 $CCID_{50}$ of virus	% CPE reduction	CPE: Drug + 100 $CCID_{50}$ of virus	% CPE reduction	CPE: Drug + 1000 $CCID_{50}$ of virus	% CPE reduction
320.0	sl. toxic	p p	—	p p	—	p p	—
100.0	v. sl. toxic	0 0	100	0 0	100	2 2	33
32.0	0	0 0	100	0 0	100	2 2	33
10.0	0	0 0	100	1 1	50	2 2	33
3.2	0	1 0	50	1 1	50	3 3	33
1.0	0	0 1	50	1 2	25	3 3	0
0.32	0	0 1	50	2 2	0	3 3	0
0 (virus controls)		1 1 1		2 2 2		3 3 3	

VR[d] = 1.5; therapeutic index = 100

[a] "Primary" chemotherapy experiments, in which drug and virus were added simultaneously to WI-38 cells and fresh drug was added every 48 hr until the degree of CPE was determined on Day 7.

[b] Drug concentration when the drug–virus mixture was in contact with the cells.

[c] CPE: Cytopathogenic effect, graded from 0 (normal cells) to 4 (complete destruction of cells). p = CPE discernible, but somewhat obscured due to partial toxicity of the drug.

[d] Virus rating; see Materials and Methods.

TABLE III. The Effect of a Group of Purine Analogs on Intra- and Extracellular Titers of Cytomegalovirus.[a]

Compound	Maximum non-cytotoxic drug concentration[b] (μg/ml)	Lowest effective concentration[c] (μg/ml)	Titer of virus used ($CCID_{50}$/ml)	CPE inhibition at maximum drug concentration[d] (%)	Virus titer of treated cells (log_{10})		Virus titer of virus control cells (log_{10})	
					Intracellular virus	Extracellular virus	Intracellular virus	Extracellular virus
2-Amino-9-β-D-ribofuranosyl-9*H*-purine-6-thiol	10	0.1	100	50	<1.0	<1.0	4.5	3.5
2-Aminopurine-6-thiol	32	0.32	320	100	<1.0	<1.0	3.0	2.0
9-β-D-Arabinofuranosyladenine	<32	<3.2	320	50	<1.0	<1.0	4.0	3.5
Purine-6-thiol hydrate	320	0.32	100	50	<1.0	<1.0	4.5	3.5
Purine-6-carboxaldehyde thiosemicarbazone	<100	<10	1000	75	<1.0	<1.0	4.0	3.5
2-Amino-6-[(1-methyl-4-nitroimidazol-5-yl)thio]purine	<10	<1	100	50	<1.0	<1.0	4.5	4.5
9-β-D-Ribofuranosyl-9*H*-purine-6-thiol	<320	<3.2	320	50	<1.0	<1.0	4.3	3.5
2-Amino-1-methylpurine-6(1*H*)-thione	<32	<10	320	50	<1.0	<1.0	4.0	3.0
6-(Propylthio)purine	<32	<0.1	320	50	2.0	2.5	4.0	4.0

[a] Drug and virus were added simultaneously to WI-38 cells and fresh drug was added every 48 hr until Day 7.

[b] Drug concentration when in contact with the cells. < = Drugs were insoluble, hence exact concentrations cannot be stated.

[c] Effective as determined by discernible virus titer reduction.

[d] CPE determined microscopically on Day 7.

An experiment was carried out in which the effects of the nine most active compounds on intra- and extracellular titers of cytomegalovirus were determined. The results of these experiments are summarized in Table III. All of the compounds tested markedly reduced the virus titers, usually to below detectable limits.

An experiment was carried out to determine if those compounds which had significant antiviral activity were also active prophylactically against CMV. In this experiment, varying concentrations of each drug mixed with test medium were incubated with monolayers of WI-38 cells at 37° for 1 hr. At the end of this incubation, the medium was decanted and the cell layers rinsed a single time with 10 ml of sterile phosphate-buffered saline. Virus in a titer of approximately 100 $CCID_{50}$ suspended in test medium was then added to each tube of cells. On Days 3 and 5, the medium was decanted and fresh medium was added; on Day 7 the degree of virus-induced CPE was compared with nondrug-exposed virus controls. The results of this study are summarized in Table IV. Definite prophylactic activity was exhibited by two drugs, TGS and TG, which were also the most active compounds demonstrated in the antiviral experiments summarized in Table I. A maximum of 33% CPE inhibition was seen in prophylactic experiments with MP, NITG, and MPR.

Each of the above antiviral compounds was also tested for virucidal activity. In this experiment, dilutions of each compound were incubated with 1000–3200 $CCID_{50}$ of CMV at 37° for 1 hr. Each mixture was then diluted 1:10 in test medium and added to about 5-day-old cell monolayers in tubes. The tubes were incubated at 37° for 7 days, with the media decanted and fresh media added on Days 3 and 5. Virucidal activity was considered if the virus-induced CPE was

TABLE IV. Prophylactic[a] and Virucidal[b] Effects of Purine Analogs on Cytomegalovirus *in Vitro*.

Compound	Prophylactic experiments		Virucidal experiments	
	Drug concentration range[c] (μg/ml)	Maximum inhibition of CPE (%)	Drug concentration range[d] (μg/ml)	Maximum inhibition of CPE (%)
2-Amino-9-β-D-ribofuranosyl-9*H*-purine-6-thiol	10–1000	100	1–100	0
2-Aminopurine-6-thiol	10–1000	100	1–1000	0
9-β-D-Arabinofuranosyladenine	<100–<1000	0	<100–<1000	0
Purine-6-thiol hydrate	10–1000	33	1–100	0
Purine-6-carboxaldehyde thiosemicarbazone	<100–<1000	0	<32–<320	0
2-Amino-6-[(1-methyl-4-nitroimidazol-5-yl)thio]purine	<10–<1000	33	<1–<32	0
9-β-D-Ribofuranosyl-9*H*-purine-6-thiol	<100–<10,000	33	<32–<1000	0
2-Amino-1-methylpurine-6(1*H*)-thione	<10–<1000	0	<10–<320	0
6-(Propylthio)purine	<100–<1000	0	<32–<1000	0

[a] Cells were pretreated 1 hr at 37° with drug and rinsed prior to addition of 100 $CCID_{50}$ virus. The culture medium was decanted and fresh medium was added every 48 hr, and the degree of CPE determined on Day 7.

[b] Virus incubated with drug for 1 hr at 37°, then diluted 1:10 and added to cells. The culture medium was decanted and fresh medium was added every 48 hr, and the degree of CPE determined on Day 7.

[c] Drug concentration when in contact with cells. < = indicates the drugs were relatively insoluble.

[d] CPE inhibition observed at the highest nontoxic drug level.

[e] Drug concentration when in contact with virus.

inhibited in the test cells to a greater degree than in cells receiving the virus and similar concentrations of the drug without prior incubation. None of the compounds was considered to have virucidal activity (Table IV).

Discussion. These data indicate that certain purine analogs, particularly certain thiopurines, ara-A, and PCTC, have significant activity against CMV *in vitro*. This activity was demonstrable as both inhibition of virus-induced CPE, and as reduction of intra- and extracellular virus titers. Such antiviral activity appears to differ from that reported to be exhibited in the same virus system by the pyrimidine analog 5-fluorodeoxyuridine, which apparently interferes with viral production but not with CPE caused by the virus (9). In our experiments, the degree of inhibition of virus production usually declined at the same drug concentration in which inhibition of CPE also decreased.

Purine analogs have been considered as potential antivirus drugs by several investigators (10, 11), so it was therefore expected that a number of the known biologically active compounds investigated in the present study would be active against the DNA-containing CMV. Certain of these compounds, particularly MP, are known to inhibit the interconversion of purine nucleotides (12) and to inhibit purine synthesis *de novo* (13), suggesting that in the present virus studies these compounds may be inhibiting the synthesis of the immediate precursors of nucleic acid necessary for viral replication.

All of the active thiopurines also markedly inhibited the unrelated RNA-containing Friend leukemia virus *in vivo* (14), but were inactive against infections of vaccinia and influenza viruses in animals (15).

The purine nucleoside ara-A and the thiosemicarbazone derivative PCTC may have somewhat different mechanisms of action against CMV than the other active compounds since neither of these compounds was prophylactically active against the virus. Ara-A reportedly inhibits both DNA and protein synthesis (16), based on studies with bacteria. Furth and Cohen (17) have reported data which indicate that ara-A may act in animal cells via its triphosphate to inhibit the animal cell DNA polymerase, although the ara-A triphosphate may also act by inhibiting ribonucleotide reductase. Ara-A is also active *in vitro* against other DNA viruses, including vaccinia, herpes (18–20), adeno, and varicella, and possibly against Rous sarcoma virus, an RNA virus requiring cell DNA synthesis (19, 20). The drug is also markedly active *in vivo* against vaccinia (15, 20, 21) and herpes viruses (20, 22–24).

Since the original report by Hamre *et al.* (25) on the anti-vaccinia virus activity of derivatives of benzaldehyde thiosemicarbazone, numerous investigations with these agents have been described. Derivatives produced by alkylation in the 1 position of isatin β-thiosemicarbazone have particularly been found effective against the poxviruses (26), but these compounds have not been reported active against cytomegalovirus, a member of the herpesvirus group. In the present study, the anti-CMV activity of PCTC, a combination of a purine and a thiosemicarbazone, was therefore unique. The thiosemicarbazones are generally considered to be most active against poxviruses when used prophylactically (27), but PCTC apparently exerted no such action against CMV in the present study.

Incubation of each of the CMV-active purine analogs with CMV for 1 hr had no apparent effect on the virus, indicating the drugs were not virucidal and did not affect the ability of the virus to attach to the host cell.

Studies with benzimidazole derivatives demonstrated that those derivatives containing β-D-ribofuranosyl moieties were usually more active at lower concentration and had higher therapeutic indices against influenza virus in cell culture (28). Such a conclusion may not be valid in the present studies, however, since most of the most active compounds as well as a number of the inactive drugs had β-D-ribofuranosyl groups as a portion of their structure.

An anomaly becomes apparent when one considers the basic purpose of this investigation, that purpose being to find compounds which may prove active against human CMV infections, particularly in immunosuppressed patients. The irregularity exists in the fact

that the thiopurines, which were among the most active compounds demonstrated in this study, have immunosuppressive properties. Some of these compounds, MP particularly, were often being used in cancer patients when the reported disseminated CMV infections developed (29). Such an inconsistency may possibly be explained in two ways. First, it is a long stride from *in vitro* antiviral activity to activity against the same virus in man; many compounds exist today which have apparent *in vitro* activity and yet are not demonstrably active *in vivo* (15). A second possible explanation for this problem is that the thiopurines demonstrated in these experiments to be the most active against CMV were more effective when used prophylactically. The CMV infections arising as complications to immunosuppression have been considered to be possible activated latent infections (30), which would imply that the virus was therefore present in the host before treatment with the drugs.

Limited studies carried out to date do not indicate ara-A to be immunosuppressive (20, 22); no data are known regarding PCTC in this respect. Additional studies with these two compounds may therefore be warranted to determine their value as potential anti-CMV drugs.

Summary. The activity of 25 purine analogs was determined against human cytomegalovirus *in vitro*. Nineteen compounds were considered to have a degree of antiviral activity with seven of them markedly inhibiting both virus-induced cytopathogenic effects in WI-38 cells and the development of detectable virus. These seven compounds were 2-amino-9-β-D-ribofuranosyl-9*H*-purine-6-thiol, 2-aminopurine-6-thiol, 9-β-D-arabinofuranosyladenine, purine-6-thiol hydrate, purine-6-carboxaldehyde thiosemicarbazone, 2-amino-6-[(1-methyl-4-nitroimidazol-5-yl) thio] purine, and 9-β-D-ribofuranosyl-9*H*-purine-6-thiol. The active thiopurines protected the cells from virus infection if allowed to incubate with the cells 1 hr prior to addition of the virus. None of the compounds had demonstrable virucidal activity.

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