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Novel Pyrazolo[3,4-*d*]Pyrimidine Nucleoside Analog with Broad-Spectrum Antiviral Activity

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A novel nucleoside analog, 4(5*H*)-oxo-1-β-D-ribofuranosylpyrazolo[3,4-*d*]pyrimidine-3-thiocarboxamide (N10169), was evaluated in cell culture and in animals for antiviral activity against DNA and RNA viruses. The compound was highly active against strains of adeno-, vaccinia, influenza B, paramyxo-, picorna-, and reoviruses, with 50% inhibition of virus-induced cytopathology at 1 to 10 μM. Lesser or no antiviral effects were observed against herpes simplex, cytomegalo-, corona-, influenza A, vesicular stomatitis, and visna viruses. Drug potency against certain viruses was highly cell line dependent (N10169 was highly active in HeLa cells but was much less potent in Vero cells). This was correlated, in part, to differences in levels of adenosine kinase activity in these cell lines, since adenosine kinase appears to phosphorylate N10169 to its active form. N10169 was inhibitory to proliferating cells at antiviral concentrations, whereas stationary-phase monolayers tolerated higher concentrations (≤100 μM). Exogenous uridine was able to reverse the virus-inhibitory effects of the compound, leading to the discovery that N10169 5′-monophosphate is a potent inhibitor of cellular orotidylate decarboxylase. N10169 was evaluated in mice that were infected intraperitoneally with banzi virus or inoculated intranasally with influenza B virus, and in hamsters that were infected intranasally with vaccinia virus. In each model, intraperitoneal injection of N10169 (100 to 300 mg/kg per day for 7 days) twice daily was ineffective, whereas intraperitoneal injection of ribavirin showed some benefit in the influenza B and banzi virus infection models.

In a recent report (3) a series of pyrazolo[3,4-*d*]pyrimidine nucleoside analogs possessing antiviral and antitumor properties was described. Additional studies of these compounds were undertaken to determine the spectrum of viruses which would be inhibited and to ascertain any therapeutic potential in animals. One compound, 4(5*H*)-oxo-1-β-D-ribofuranosylpyrazolo[3,4-*d*]pyrimidine-3-thiocarboxamide (N10169; Fig. 1), although similar in structure to the broad-spectrum antiviral agent ribavirin (18), was found to have an unusual spectrum of antiviral activity, degree of potency, and mode of action. Although the compound is related to purine nucleosides, it was found to be a potent inhibitor of pyrimidine biosynthesis. As the work proceeded it was determined that the properties of N10169 were remarkably similar to those of 6-azauridine (23). The compounds related to N10169 (3) did not exhibit the same degree of potency and spectrum of activity that N10169 did in vitro. In this report we summarize the results of cell culture studies and evaluate the antiviral activity of this novel compound in appropriate animal infection models.

MATERIALS AND METHODS

Cells. Human epithelioid carcinoma of the cervix (HeLa), human embryonic lung (MRC-5), human embryonic kidney, Madin-Darby canine kidney (MDCK), African green monkey kidney (Vero), and mouse connective tissue (L929) cells were purchased from the American Type Culture Collection, Rockville, Md. MA-104 cells (African green monkey kidney) were obtained from MA Bioproducts, Walkersville, Md. Sheep choroid plexus cells were started as primary cultures from a newborn lamb as described previously (22). Normal and adenosine kinase-deficient human B-cell leukemia (WI-L2) cell lines (24) were provided by Randall C. Willis, who brought them to this study from the Department of Medicine, University of California, San Diego. A continuous passage gerbil lung fibroblast cell line was started in our laboratory from a primary culture of lung tissue taken from a Mongolian gerbil (weight, 25g; purchased from Tumblebrook Farms, West Brookfield, Mass.).

Viruses. Influenza A (Chile, Panama, and Victoria strains) and B (Texas and 72 strains) viruses were obtained from Vernon Knight, Baylor College of Medicine, Houston, Tex. Herpes simplex virus type 1 (KOS strain) was acquired from James North, Department of Microbiology, Brigham Young University, Provo, Utah. Visna virus (1413 strain) was obtained from Opendra Narayan, Johns Hopkins University, Baltimore, Md. All other viruses and strains listed in Tables 1 and 2 were purchased from the American Type Culture Collection.

The influenza B (72 strain) virus used in animal studies required extensive serial passage through mice to become virulent. The virulence of vaccinia virus (Elstree strain) in hamsters was enhanced by propagating the virus in roller bottle cultures of L929 cells. Banzi virus (H336 strain) was propagated in mouse brain before it was titrated in animals.

Antiviral compounds. N10169 and its 5′-monophosphate were synthesized by previously published procedures (3, 15). The 5′-monophosphate of ribavirin and [^{14}C]ribavirin (56.3 mCi/mmol) were obtained from Viratek, Costa Mesa, Calif. 6-Azauridine was purchased from ICN Biochemicals, Cleveland, Ohio.

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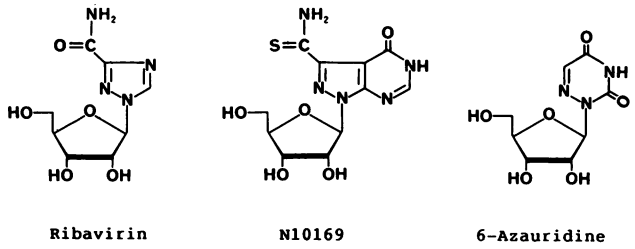

FIG. 1. Structures of ribavirin, N10169, and 6-azauridine.

Antiviral and cytotoxicity assays. Evaluation of virus-induced cytopathic effect and calculation of virus rating were performed by the method described by Sidwell and Huffman (17), except that concentrations of compounds were tested from 1 to 1,000 μM instead of 1 to 1,000 μg/ml. In this procedure uninfected toxicity controls were run in parallel with virus-infected cultures for each drug concentration. Cell toxicities in these uninfected stationary-phase monolayers were evaluated subjectively by microscopic examination. The 50% effective concentrations (ED_{50}s) were estimated to the nearest half-log_{10} value. Virus yield reduction assays were performed in two stages. Vaccinia virus, at 0.01 PFU per cell, was propagated in Vero and HeLa cells in the presence of various concentrations of N10169. When drug-free virus controls were completely destroyed by virus (at 48h), the 24-well plates were freeze-thawed once, and virus was plaque titrated in 6-well plates of Vero cells. Agarose (concentration, 0.5%; Sea Plaque; FMC Corp., Marine Colloids Div., Rockland, Maine) in culture medium was used to overlay cell monolayers. After 2 days the agar was removed, cells were fixed with 1% crystal violet in 20% ethanol, and plaques were counted by using a plaque viewer (Bellco Glass, Inc., Vineland, N.J.).

In experiments in which a reversal of antiviral effects was shown, natural purine and pyrimidine nucleosides (purchased from ICN Biochemicals) were added to the medium along with N10169. Results of these assays were evaluated by cytopathic effect inhibition in a manner similar to that used in other studies (20).

Effects of N10169 on the proliferation of cells were determined by incubating actively growing cells with N10169 for 4 days. Cells were harvested by trypsinization and counted by using a Coulter counter (Coulter Electronics, Inc., Hialeah, Fla.).

Mode of action studies. High-pressure liquid chromatography of [^{14}C]ribavirin nucleotides was performed by using methods described previously (19). The determination of adenosine kinase and orotidylate (orotidine 5'-monophosphate [OMP]) decarboxylase activities from uninfected cells was performed as follows. Cell lysates were prepared as described by Hershfield et al. (7), with modifications. Lysates were prepared by performing three freeze-thaw cycles (liquid nitrogen to 37°C) of cell pellets in 10 mM Tris hydrochloride–0.5 mM EDTA (pH 7.5), at a final cell density of 5×10^7 cells per ml, and by centrifugating the resulting suspension at 50,000 × *g* (4°C) for 1 h. Undialyzed extracts were used in the determination of enzyme activity. Protein was determined by the method described by Bradford (1). Adenosine kinase activity was determined by a filter binding assay (8). Orotidylate decarboxylase activity was measured by the conversion of [*carboxyl*-^{14}C]OMP to uridine 5'-monophosphate and $^{14}CO_2$ (9). Liberated CO_2 was collected as described by Willis and Seegmiller (25).

Animal experiments. To test whether N10169 had in vivo efficacy, three experiments in animals were developed. The first experiment was encephalitis caused by banzi virus (a flavivirus). Mice (weight, approximately 20 g each) were inoculated intraperitoneally with virus. The second experiment was a vaccinia virus infection in hamsters (weight, 50 to 60 g) that was induced by intranasal inoculation of the virus. This mode of virus administration consistently caused higher mortality than did intraperitoneal virus inoculation. The final experiment was respiratory disease and death induced by intranasal inoculation of mice (weight, 15 g) with influenza B virus (72 strain). The amount of virus used for each infection was approximately 10 times the 50% lethal dose. Intranasal inoculations were performed on animals anesthetized with Vetalar (ketamine hydrochloride; Parke, Davis & Co., Morris Plains, N.J.). The intranasal inoculum sizes were 20 and 50 μl for mice and hamsters, respectively. Swiss Webster mice were purchased from Bantin and Kingman Laboratories, Freemont, Calif. Syrian hamsters were obtained from Charles River Breeding Laboratories, Inc., Wilmington, Mass.

Animals were treated intraperitoneally with N10169, N10169 5'-monophosphate, ribavirin, or saline placebo (virus control) twice a day (half of the total daily dose was administered at 7 a.m. and 4 p.m.) for 7 consecutive days starting 2 h before virus inoculation. Deaths were recorded daily for 21 days.

In the banzi virus experiment, 16 mice per treatment group were used. Since death in the influenza B virus model can be quite variable from experiment to experiment, there were 20 mice per treatment group. In each treatment group there were 12 hamsters infected with vaccinia virus (fewer animals were used because we were limited in the amount of N10169 available to give to these larger animals). Toxicity control animals (five per treatment group for mice and four per treatment group for hamsters) were run with each experiment. Ribavirin-treated (300 mg/kg) mice gained weight more slowly than did saline-treated control animals. Otherwise, both compounds were well tolerated at the doses used.

The doses of ribavirin selected either were known to be effective in vivo (as in the influenza virus model [4]) or approached the limiting toxicity for the particular species. With N10169, the poor solubility of the compound was a factor which limited its utility to 300 mg/kg. Although the 5'-monophosphate of N10169 was more soluble, and so it could have been used at much higher doses, it was lethal to some mice at 800 mg/kg.

RESULTS

Antiviral and anticellular activities in vitro. Cell culture studies in which N10169 was compared with ribavirin were performed against a large number of DNA and RNA viruses (Tables 1 and 2, respectively). N10169 was most active against strains of adeno-, vaccinia, banzi, paramyxo-, picorna-, reo-, foamy, and influenza B viruses, with ED_{50} values of 1 to 10 μM. Curiously, the compound failed to inhibit influenza A viruses. The potency of N10169 exceeded that of ribavirin in many instances, but its effect was very cell line specific. For example, N10169 was highly active against adeno-, vaccinia, and picornaviruses in HeLa cells, but was less effective or inactive in other cell lines. Ribavirin also exerted its greatest potency in HeLa cells against the same viruses.

The cytotoxicity of N10169 in various cell lines was determined by cell proliferation assays. Concentrations of

TABLE 1. Anti-DNA virus activities of N10169, ribavirin, and 6-azauridine

Virus (strain)	Cell line	Anti-DNA virus activity of:					
		N10169		Ribavirin		6-Azauridine	
		VR[a]	ED_{50} (μM)	VR	ED_{50} (μM)	VR	ED_{50} (μM)
Adenovirus type 2 (Adenoid 6)	HeLa	1.9	3	0.7	320	1.8	3
	MA-104	0.0	>1,000	0.0	>1,000		
	MRC-5	0.0	>1,000	0.0	>1,000	0.0	>1,000
Adenovirus type 5 (Adenoid 75)	HeLa	2.0	1	0.6	320		
	MA-104	0.0	>1,000	0.0	>1,000		
	MRC-5	0.7	100	0.5	320		
Adenovirus type 11 (Slobitski)	HeLa	1.5	3	1.0	100		
Adenovirus type 31 (1315/63)	HeLa	0.0	>1,000	0.6	320		
Mouse adenovirus (FL)	L929	0.0	>1,000	0.4	320		
Simian adenovirus type 18 (676)	MA-104	1.7	1	1.1	100		
Herpes simplex virus type 1 (KOS)	MA-104	0.4	1,000	0.7	320		
Herpes simplex virus type 2 (MS)	MA-104	0.0	>1,000	0.3	1,000	0.0	>1,000
Human cytomegalovirus (AD-169)	MRC-5	0.0	>1,000	0.0	>1,000		
Vervet monkey cytomegalovirus (CSG)	MRC-5	0.0	>1,000	0.0	>1,000		
Vaccinia virus (Elstree)	HeLa	2.2	1	1.5	32	2.0	3
	L929	0.0	>1,000	0.4	1,000		
	MA-104	0.2	1,000	0.7	320	0.0	>1,000
	Vero	0.2	1,000	0.0	>1,000		

[a] VR, Virus rating. By convention VRs of ≥1.0 indicate marked antiviral activity, VRs of 0.5 to 0.9 indicate moderate activity, and VRs of <0.5 indicate weak or no activity.

compound inhibiting the replication of HeLa, MA-104, and WI-L2 cells by 50% were 3, 2.2, and 0.5 μM, respectively (Table 3). In a WI-L2 cell line that was deficient in adenosine kinase activity, N10169 was not inhibitory to cell proliferation at ≤100 μM. This is an indication that adenosine kinase plays a role in phosphorylating N10169 to its biologically active form.

Although the anticellular concentrations given above were approximately the same as those required to inhibit some of the viruses (indicating a lack of preferential inhibition of virus replication in these cultures), nonproliferating cells were more resistant to the agent. In stationary-phase monolayers of all the cell types used for viral assays (Tables 1 and 2), the morphologically discernable toxicity (granularity and cell enlargement, with minimal detachment of cells from the monolayer) of N10169 varied slightly from experiment to experiment but was consistently evident at 320 to 1,000 μM. Ribavirin and 6-azauridine were toxic at nearly the same concentrations. Monolayers of HeLa cells appeared to be the most affected by each compound, and occasionally, the toxicities of N10169 and 6-azauridine were manifest down to of 32 μM. With concentrations of inhibitors in infected cultures of 320 to 1,000 μM, it was still possible to discern virus-induced cell destruction or antiviral activity.

Mode of action studies. Experiments were conducted to determine the manner in which N10169 exerts its antiviral and cytostatic effects. In an initial experiment the activities of the compound were measured in the presence of naturally occurring purine and pyrimidine nucleosides. Uridine and, to a lesser extent, cytidine were able to reverse or neutralize the antiviral activity of N10169 and to rescue uninfected cells from growth inhibition (Table 3). Other natural nucleosides such as adenosine, guanosine, inosine, xanthosine, purine 2′-deoxynucleosides, and pyrimidine 2′-deoxynucleosides exerted no effect on the action of N10169. The effect of cytidine in HeLa cells, i.e., to reverse the activity of N10169, probably is a consequence of cytidine deaminase activity, which would convert the nucleoside to uridine.

Because preliminary results showed that N10169 suppresses intracellular levels of pyrimidine nucleotides (unpublished data), a chemically synthesized N10169 5′-monophosphate was evaluated as an inhibitor of the UMP synthetase complex. This nucleotide was found to be a competitive inhibitor of the conversion of OMP to UMP catalyzed by the orotidylate decarboxylase activity. When the activity from WI-L2 cell lysates was used, the inhibitor constant (K_i) for N10169 5′-monophosphate was found to be <1 nM.

It became evident from the results of the mode of action studies described above that N10169 inhibits de novo pyrimidine biosynthesis and, therefore, acts in a manner similar to that of another known antiviral agent, 6-azauridine (23). We decided to compare the two compounds to determine what the correlation is between them. When tested against several viruses (Tables 1 and 2), the spectrum of activity and the degree of potency of the two agents were remarkably similar. Both compounds shared the peculiar quality of inhibiting influenza B virus but not influenza A virus. Also, both compounds exerted a pronounced effect against adeno-, vaccinia, and picornaviruses in HeLa cells but were less effective in other cells. Some differences were evident in the activities of the two compounds. Against measles virus in MA-104 cells, N10169 was active at a lower dose than 6-azauridine, whereas in Vero cells 6-azauridine was more potent. This could reflect differential phosphorylation of the two compounds in different cell lines, since 6-azauridine is known to be phosphorylated by uridine kinase (16), whereas N10169 appears to be phosphorylated by adenosine kinase.

Finally, studies were conducted to attempt to allow us to understand why N10169 works more effectively in some cell lines than in others against viruses such as vaccinia, adeno-, and picornaviruses. Vaccinia virus was selected as a model to show the differential effects of N10169 and ribavirin on virus yield in HeLa and Vero cells. This assay was run to rule out the possibility that N10169 might suppress virus-induced cytopathology but might not directly inhibit virus

TABLE 2. Anti-RNA virus activities of N10169, ribavirin, and 6-azauridine

Virus (strain)	Cell line	Anti-RNA virus activity of:					
		N10169		Ribavirin		6-Azauridine	
		VR[a]	ED_{50} (μM)	VR	ED_{50} (μM)	VR	ED_{50} (μM)
Banzi virus (H336)	HeLa	1.6	1	0.6	100		
San Angelo virus	GLF[b]	0.9	100	1.1	32		
Semliki Forest virus (original)	Vero	0.0	>1,000	0.4	320		
Influenza A virus (Chile)	MDCK	0.0	>1,000	1.5	32	0.0	>1,000
Influenza A virus (Panama)	MDCK	0.0	>1,000	1.0	100		
Influenza A virus (Victoria)	MDCK	0.0	>1,000	1.8	10		
Influenza B virus (Texas)	MDCK	2.2	3	1.8	10	1.8	10
Measles virus (Edmonston)	MA-104	1.9	3	0.8	100	1.4	32
	Vero	1.5	32	0.7	100	1.3	3
Parainfluenza virus type 3 (C243)	MA-104	2.5	1	1.7	32	2.1	1
	Vero	1.3	32	1.1	100	1.9	3
Respiratory syncytial virus (Long)	MA-104	2.4	1	1.7	32		
Coxsackievirus type B1 (Conn-5)	HeLa	0.0	>1,000	0.0	>1,000		
Poliovirus type 1 (Chat)	HeLa	2.4	1	1.0	100	2.3	1
	MA-104	0.1	>1,000	0.1	>1,000		
Rhinovirus type 1-A (2060)	HeLa	2.2	1	0.6	320	1.4	3
	L929	0.8	320	0.8	320	0.5	1,000
	MA-104	1.6	10	0.3	1,000		
	MRC-5	1.1	100	0.6	320		
Rhinovirus type 1-B (B632)	HeLa	1.3	10	0.8	320		
	MRC-5	0.9	100	0.9	100		
Rhinovirus type 2 (HGP)	HeLa	1.4	3	0.8	100	1.2	3
	MRC-5	0.2	1,000	0.6	320		
Rhinovirus type 29 (5582)	HeLa	1.7	3	1.0	100		
	MRC-5	0.9	100	0.8	320		
Rhinovirus type 31 (140F)	HeLa	1.3	10	1.0	100		
	MRC-5	1.2	32	0.9	100		
Simian picornavirus type SA-4 (L-79-C2)	MA-104	2.2	1	0.2	1,000		
Simian picornavirus type SV-4 (1715UWB)	MA-104	1.6	3	0.1	>1,000		
Reovirus 3 (Dearing)	MA-104	1.8	3	1.4	32	1.4	3
Simian rotavirus (SA-11)	MA-104	1.3	10	0.9	100	1.2	10
Simian foamy virus type 6 (Pan 1)	HEK[c]	2.4	1	1.7	32	2.2	1
Visna virus (1513)	SCP[d]	0.0	>1,000	0.0	>1,000		
Vesicular stomatitis virus (Indiana)	MA-104	0.0	>1,000	0.5	320	0.0	>1,000

[a] VR, Virus rating. See footnote *a* to Table 1 for details.
[b] GLF, Gerbil lung fibroblast cell.
[c] HEK, Human embryonic kidney cell.
[d] SCP, Sheep choroid plexus cell.

replication. N10169 and ribavirin inhibited vaccinia virus yield in a dose-dependent manner (Table 4), and both compounds were more effective in HeLa than in Vero cells.

Because the differential virus inhibition reported above might be linked to differences in the amounts of important enzymes found in HeLa and Vero cells, crude cell extracts were analyzed for the adenosine kinase and OMP decarboxylase activities that were present (Table 5). There was essentially no difference in the amount of OMP decarboxylase activities in the two cell lines, so it could be predicted that N10169 5′-monophosphate, at a given intracellular concentration, should be equally inhibitory to Vero and HeLa cell enzymes. There was, however, about 5.4-fold more adenosine kinase present in HeLa cells compared with

TABLE 3. Reversal of antiviral and cytostatic effects of N10169 by natural nucleosides

Nucleoside[a]	% Virus-induced cytopathology in:			Proliferation ID_{50} (μM) of the following cells[b]:		
	Adenovirus type 2[c]	Rhinovirus type 1-A[c]	Respiratory syncytial virus[d]	HeLa	MA-104	WI-L2
None	100	100	100			
N10169	0	0	0	3.0	2.2	0.5
Uridine-N10169	100	100	100	>32	>32	>32
Cytidine-N10169	100	100	25	>32	1.8	0.6

[a] For virus experiments N10169 was present in cell culture medium at 32 μM. Uridine or cytidine was present at 320 μM. In cell proliferation assays, uridine and cytidine concentrations were 100 μM, and N10169 concentrations ranged from 0.3 to 32 μM in half-log_{10} increments.
[b] ID_{50}, Concentration of compound inhibiting cell replication by 50%.
[c] Virus assays were performed in HeLa cells.
[d] Virus assays were performed in MA-104 cells.

TABLE 4. Effects of N10169 and ribavirin on vaccinia virus yield in two cell lines[a]

Drug concn (μM)	Log_{10} PFU/ml in the following cells treated with the indicated compounds:			
	HeLa		Vero	
	N10169	Ribavirin	N10169	Ribavirin
1,000	<1.0	<1.0	8.8	9.7
320	<1.0	<1.0	9.0	9.7
100	<1.0	2.1	9.7	9.7
32	1.8	6.7	9.7	9.7
10	3.9	7.2	9.7	9.7
3	4.1	7.3	9.7	9.7
1	7.0	7.3	9.7	9.7
0	7.3	7.3	9.7	9.7

[a] Virus was propagated in the presence of various concentrations of N10169 until extensive cytopathology was observed in drug-free control wells. Supernatant virus was plaque titrated in Vero cells.

TABLE 5. Comparative enzymatic activities in extracts of HeLa and Vero cells

Cell line	Enzymatic activity of:		
	OMP decarboxylase[a]	Adenosine kinase[a]	Ribavirin metabolism[b]
HeLa	456 ± 36	630 ± 18	1,017
Vero	484 ± 27	116 ± 10	85

[a] Picomoles per minute per milligram of protein ± standard deviation.
[b] Picomoles of ribavirin triphosphate formed per 10^6 cells at 6 h. The extracellular ribavirin concentration was 100 μM.

that in Vero cells. The low amount of adenosine kinase in Vero cells would result in the limited phosphorylation of N10169 and ribavirin to their biologically active forms. Evidence for this was obtained by treating intact Vero and HeLa cells with [^{14}C]ribavirin (radioactive N10169 was not available). Nearly 12-fold more ribavirin triphosphate was formed in HeLa cells than in Vero cells after a 6-h incubation with drug (Table 5), with adenosine kinase being the rate-limiting step to ribavirin triphosphate accumulation. From these results, one explanation for the lesser antiviral potency of N10169 (and ribavirin) in Vero cells is the low amount of adenosine kinase activity that is present in those cells.

Animal experiments. Three different animal models were employed to assess the in vivo utility of N10169. Mice inoculated intraperitoneally with banzi virus died of encephalitis at 8 ± 0.6 days. Mice treated with ribavirin (300 mg/kg per day) lived 9.6 ± 1.2 days ($P < 0.001$), but lower doses were ineffective. N10169-treated mice (300 mg/kg per day) died in 7.7 ± 0.7 days ($P > 0.05$), indicating that the compound has no effect on the outcome of the disease. There were no survivors of the infection in any treatment group.

In a second experiment hamsters were inoculated intranasally with vaccinia virus. Animals underwent a disseminated viral infection with up to 10^8 PFU of virus per ml recovered from the brain, liver, and kidney. Neither ribavirin (100 mg/kg per day) nor N10169 (300 mg/kg per day) prevented death nor increased mean survival times. The overall mortality rate in this experiment was 92%.

In mice infected with influenza B virus, two experiments were conducted. In the first experiment with this virus, survival was 0% (virus-infected control), 50% (ribavirin, 100 mg/kg per day), and 0% (N10169, 300 mg/kg per day). A second study gave survival results of 30% (virus-infected control), 100% (ribavirin, 100 mg/kg per day), 20% (N10169, 150 mg/kg per day), and 35% (N10169, 75 mg/kg per day). Only the ribavirin treatments caused statistically significant increases in numbers of survivors ($P < 0.002$). Mean survival times were not extended by N10169 treatment of mice that died from the infection.

In analyzing the lack of the in vivo efficacy of N10169, the possibility existed that the compound was not in a form that was appropriate for maximum bioavailability. At a dose of 300 mg/kg per day the nucleoside was administered as a thick suspension in saline. N10169 5′-monophosphate, which is highly water soluble, was evaluated at a dose of 400 mg/kg per day against influenza B virus in a manner similar to those in the experiments reported above. In this final experiment mortality rates were 100% (virus control), 80% (N10169 5′-monophosphate), and 20% (ribavirin, 100 mg/kg per day; $P < 0.001$). The effect of N10169 5′-monophosphate on mortality was not statistically significant, nor did the nucleotide increase the mean survival times of mice that died compared with those of the virus controls.

DISCUSSION

In these studies, N10169 was shown to be a potent inhibitor of many unrelated DNA and RNA viruses in certain cell lines. The compound was active against representatives of respiratory viruses (adeno-, rhino-, paramyxo-, and influenza B viruses) which are important human pathogens, with the exception of influenza A and human coronaviruses. The potency of N10169 exceeded that of ribavirin in many instances, but was nearly identical to that of 6-azauridine. The similarity of action of N10169 and 6-azauridine was not surprising since both compounds are inhibitors of OMP decarboxylase (23; this study).

N10169 is unique in that it is an antiviral purine analog which is activated by phosphorylation by adenosine kinase and then inhibits de novo pyrimidine biosynthesis. Recently, other inhibitors of de novo pyrimidine biosynthesis have been reported which inhibit dihydroorotate dehydrogenase (2, 11). Certain metabolites of allopurinol are also known inhibitors of orotidylate decarboxylase, such as allopurinol riboside 5′-monophosphate and oxopurinol riboside 5′-monosphosphate. These compounds, however, are activated differently (5) than N10169.

The first published report of the antiviral and cytotoxic activities of N10169 also showed the biological effects of other similar pyrazolo[3,4-*d*]pyrimidine nucleosides (3). These other nucleoside analogs have been examined in detail in our laboratory for antiviral properties. N10169, which has a $CSNH_2$ at the 3 position of the heterocyclic ring, was by far the most active of the series. Compounds with CN or $CONH_2$ at the 3 position inhibited adeno- and rhinoviruses, but at concentrations of ≥30 μM (and only in HeLa cells). The CN compound was also evaluated against influenza B virus in mice in the same experiment in which N10169 5′-monophosphate was tested, and it was inactive. The only compound in this series which is a potent and broad-spectrum virus inhibitor in vitro is N10169. It is of interest to point out that the less-active compounds were also less-potent inhibitors of OMP decarboxylase.

N10169 is believed to exert its antiviral effects by lowering pyrimidine nucleotide pools in the cell to the point at which virus-specific polymerases can no longer operate (or else operate inefficiently). This is analogous to the effect of ribavirin and mycophenolic acid, which inhibit certain vi-

ruses as a result of suppression of guanosine nucleotide pools (14). Of course, ribavirin inhibits some viral polymerases and RNA capping enzymes directly as the triphosphate (21).

We were able to detect only the monophosphate form of N10169 in treated cells using ion-exchange high-performance liquid chromatographic methods. Since the inhibition of the cellular enzyme OMP decarboxylase appears to be the main target of the activity of the compound, the antiviral mode of action of N10169 is not virus specific per se. Whatever specificity toward virus inhibition is achieved with the compound is confined to resting cells, which normally are not in a state of high metabolic activity.

Results of in vivo studies indicate that N10169 is not a good candidate for further drug development as an antiviral agent. The various aspects of drug metabolism, distribution, and elimination play a role in the efficacy of any compound since a drug must reach and persist at the site of virus replication to be effective. At this time the pharmacokinetic parameters of N10169 in laboratory animals are not known. The fact that the compound is only active in certain cell lines could be deleterious, especially if the virus replicates in cells of the animals, in which N10169 would not be phosphorylated or otherwise have no effect.

Another general problem which may play a role in neutralizing the efficacy of N10169 in vivo is the reversal of antiviral activity by dietary uridine (10). If this were to occur with N10169, one might wonder why a compound such as ribavirin, which inhibits guanosine triphosphate levels in cells (14), is still active in vivo, even when guanosine is coadministered with the drug (13). One important difference between the two nucleosides is that ribavirin 5′-triphosphate has virus-specific targets that it inhibits (21), in addition to the effect of ribavirin 5′-monophosphate on guanosine nucleotide depletion. The importance of virus-specific targets is borne out by the fact that certain viruses are still inhibited by ribavirin in vitro, even when exogenous guanosine is present (19, 26).

Other compounds that only inhibit nucleotide pools have not been effective antiviral agents in vivo, such as mycophenolic acid (14); tiazofurin (6, 12); selenazofurin (6, 12); and, most relevant to this study, 6-azauridine (23). It is our hypothesis that if the primary mode of antiviral action of an agent is via nucleotide pool depletion, that compound will not be a very effective drug in vivo.

In summary, although N10169 was found to be a potent inhibitor of many unrelated DNA and RNA viruses in cell culture, the compound had no therapeutic effect on rodents. The compound may serve as a useful tool for studying pyrimidine biosynthesis.

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