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In Vivo Antiviral Properties of Biologically Active Compounds

II. Studies with Influenza and Vaccinia Viruses

ROBERT W. SIDWELL, GLEN J. DIXON, SARA M. SELLERS, AND FRANK M. SCHABEL, JR.
Virus Section, Southern Research Institute, Birmingham, Alabama 35205

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The in vivo anti-influenza virus and antivaccinia virus activity of 156 biologically active compounds was determined. One of two criteria was used for evaluating activity against the influenza virus. The criteria were increase in survivor number and mean survival time, and reduction in virus-induced lung consolidation in treated, infected Swiss mice. Increase in survivor number and mean survival time were the criteria for evaluation of antivaccinia virus activity. Several drug doses were tested against two virus concentrations to demonstrate antiviral activity more clearly. Two compounds were considered significantly active against the influenza virus: DL-noformicin (NSC 72942) and amantadine hydrochloride (NSC 83653). Eleven compounds had reproducible activity against vaccinia virus: isatin-β-thiosemicarbazone (NSC 721), 6-azauracil (NSC 3425), 9-α-fluoro-2α-methylhydrocortisone 21-acetate (NSC 12601), 5-[bis(2-chloroethyl)amino]uracil (NSC 34462), 5-iodo-2′-deoxyuridine (NSC 39661), streptonigrin (NSC 45383), *N*-methylisatin β-thiosemicarbazone (NSC 69811), cytovirin (NSC 91770), 9-β-D-arabinofuranosyladenine (NSC 404241), and 5-(mercaptomethyl)uracil (NSC 529351).

Effective viral disease chemotherapy has become increasingly feasible in recent years as a result of extensive screening studies and the apparent success of clinical trials with a few select compounds. The chemotherapy approach to viral disease control is of particular interest, since the most desirable alternative to this control, vaccination, is wholly effective only if the vaccines are used by the majority of a specific human population, and is ineffective if the disease to be controlled is caused by a broad spectrum of agents, such as is the case for the common cold. Because of the potential of antiviral chemotherapy, we have carried out a series of studies to evaluate the in vivo antiviral activity of a number of compounds. The compounds used were selected especially for their known biological activity in one or more in vivo tumor systems or their activity in vitro or in vivo in animal-virus systems, or because of their apparent chemical relationship to biologically active compounds. Viral agents used in these studies included Friend leukemia virus, Rous sarcoma virus, mouse salivary gland virus, influenza virus, vaccinia virus, and rabbitpox virus. In a previous communication (22), the results of studies with the Friend leukemia virus were described. The present report concerns the results of investigations with influenza, vaccinia, and rabbitpox viruses.

MATERIALS AND METHODS

Influenza virus. The strains of influenza virus used for primary screening experiments were the PR8 strain of influenza A virus (PR8) or the Japan 305 strain of Asian influenza virus (Asian). In certain follow-up studies, the Lee strain of influenza B virus (Lee) and the PR301 strain of influenza A virus (PR301) were also employed. The PR8 and PR301 viruses were obtained in mouse brain suspensions from Bernice Eddy, Laboratory of Virology and Rickettsiology, National Institutes of Health, Bethesda, Md.; the Asian agent was obtained in monkey kidney cell suspension from Wilton A. Rightsel of Parke, Davis & Co., Detroit, Mich.; and the Lee virus was received in chorioallantoic membrane suspension from Roslyn Robinson of the National Communicable Disease Center, Atlanta, Ga. We passed each virus at least three times through mice by intranasal inoculation prior to use in these studies. Virus stock was prepared from homogenized, infected mouse lung suspended in Hank's balanced salt solution. Dihydrostreptomycin in a concentration of 10 mg/ml of virus was added before virus was used to infect animals. The LD_{50} virus titers were calculated (17) from the numbers of intranasally inoculated mice that died within 12 days of inoculation.

Vaccinia and rabbitpox viruses. The International Health Division (IHD) or the Western Reserve (WR) strains of vaccinia virus were used in all primary screening experiments. When antiviral activity was suggested in at least one screening experiment, the Utrecht (Ut) strain of rabbitpox virus was often used

in a follow-up study. All three viruses were obtained in chorioallantoic membrane or mouse brain suspension from Arthur Brown, Fort Detrick, Frederick, Md. When received in our laboratory, the viruses were passed intracerebrally through ICR Swiss mice, and homogenized, infected mouse brains were suspended in Hank's balance salt solution. The LD_{50} virus titers were determined from the 12-day mortality of intracerebrally inoculated mice. All viruses were stored in sealed ampoules at about −70 C until used.

Mice. Random-bred ICR Swiss mice weighing 8 to 10 g were used in the majority of the influenza virus experiments. In certain follow-up experiments, 18- to 21-g mice were employed. For the vaccinia and rabbitpox virus experiments, 18- to 21-g mice were used. All animals were obtained from Southern Animal Farms, Prattville, Ala., and mice of the same sex were used in individual experiments. They were housed five to a cage.

Compounds tested. A total of 156 compounds were tested for antiviral activity. The Cancer Chemotherapy National Service Center (Washington, D.C.) supplied the majority of these compounds; hence, when each is described, the CCNSC (NSC) number is included. Among the compounds tested were antimetabolites, alkylating agents, antibiotics, hormone and hormonelike compounds, guanidines, thiosemicarbazones, semicarbazones, terephthalanilides, and other miscellaneous agents. Each compound was dissolved or suspended in the most appropriate medium, which was either sterile water, physiological saline, diluent E (a steroid-suspending vehicle containing 9% sodium chloride, 5% sodium carboxymethylcellulose 7LP, 0.4% polysorbate 80, and 0.9% benzyl alcohol in water), 1% $NaHCO_3$, 5% gum acacia in saline, or 0.4% carboxymethylcellulose in phosphate-buffered saline.

Influenza virus testing. Mice were inoculated intranasally with 0.06 ml of virus (10 or 32 LD_{50}) suspended in Hank's balanced salt solution. After virus inoculation, the animals were randomized and divided into groups of 10 for each drug dosage. Twenty animals were used as virus controls.

One of two drug treatment schedules was used in the primary screening evaluations. Schedule 1 consisted of twice daily drug injections beginning 1 day prior to virus inoculation and continuing for 9 days, and schedule 2 consisted of once daily drug injections starting 1 day after virus inoculation and continuing for 9 days. Drug doses used were the approximate LD_{10}, $LD_{10}/2$, $LD_{10}/4$, and $LD_{10}/8$ as based on previous toxicity tests in normal animals held 21 days from the beginning of treatment. The compounds were administered intraperitoneally on a milligram per kilogram basis, with each animal being weighed daily. Other treatment schedules were utilized in additional experiments with certain drugs; these treatment schedules were chosen in attempts to obtain a more positive demonstration of antiviral activity for compounds that appeared active in the screening studies and to elucidate prophylactic or therapeutic activity. These various schedules are indicated in the appropriate tables.

The initial criteria for evaluation of anti-influenza virus activity of each drug were increases in the time of mean survival and in the number of survivors 21 days after virus inoculation. Comparative experiments carried out during these studies indicated that the reduction of virus-induced lung consolidation of drug-treated animals on the 10th day after virus inoculation was an equally sensitive procedure and had the advantage of requiring less time. The former procedure was therefore used in approximately two-thirds of the experiments and the latter method was used in the remainder of the studies.

The results of the experiments in which the first procedure was used were statistically evaluated by comparing (by means of the t test) the mean survival time of drug-treated, virus-infected animals dying on or before day 21 with the mean survival time of the virus control animals. If the P value obtained was <0.05 but >0.001, the antiviral activity was considered questionable. If the P value was <0.001, the drug was considered to have possible antiviral activity. The data were questionable if a significant increase in mean survival time occurred in animals treated with low dosages of the drug, but did not occur in animals treated with higher nontoxic concentrations. The same consideration applied if activity was observed in mice infected with a high level of virus but not in those infected with lower virus doses. The number of surviving infected, treated animals was compared with the number of virus control mice surviving (if any) by means of the chi-square analysis technique. If the P values calculated by this method were <0.3 but >0.05 (approximate standard error), the antiviral activity was questionable, whereas values of <0.05 were indicative of possible antiviral activity.

The evaluation of antiviral activity on the basis of reduction of virus-induced lung consolidation was carried out by scoring each lung on a "blind" basis, i.e., the scorers did not know the history of the donors of the lungs. The lungs were graded according to the following scale: 5 = death with consolidation, 4 = 100% consolidation, 3 = ~75% consolidation, 2 = ~50% consolidation, 1 = ~25% consolidation, 0 = no consolidation. An average lung consolidation score was calculated by dividing the total grade of consolidation by the number of lungs graded. The data were statistically evaluated using White's modification of the Wilcoxon test (28). A P value of <0.05 calculated by this method indicated questionable antiviral activity, and a P value of <0.01 indicated possible antiviral activity. The same considerations of dose response and virus concentrations described above were also used in this method.

Any compound having questionable or possible antiviral activity when tested by either of the above procedures was retested for confirmation. If possible activity was again seen, additional experiments with other virus strains, different treatment schedules, or different methods for evaluation were then carried out.

Mice which had been exposed only to virus diluent were treated with identical drug dosages at the same time as the test animals. These animals were held 30 days after the end of treatment and served as drug toxicity controls.

Vaccinia virus testing. Mice were inoculated intracerebrally with 0.03 ml containing 10 or 32 LD_{50} of the virus suspended in Hank's balanced salt solution and were treated as described above for influenza

TABLE 1. *List of compounds and description of the tests[a] carried out against influenza and vaccinia viruses in vivo*

Class of compound[b] and NSC no.	Compound name[c]	Diluent[d]	Influenza virus experiments					Vaccinia virus experiments			
			No. of trials	Treatment schedule[e]	Dose range[f] (mg per kg per day)	Viruses[g] used	Evaluation method	No. of trials	Treatment schedule[e]	Dose range[f] (mg per kg per day)	Viruses[g] used
Alkylating agents											
750	Busulfan; myleran; 1,4-butanediol dimethanesulfonate	CMC	2	2	2.1–18.5	PR8	DP	1	2	8.3–66	IHD
762	Nitrogen mustard; 2,2 -dichloro-*N*-methyldiethylamine hydrochloride	S	1	2	0.02–0.17	PR8	DP	1	2	0.1–0.7	IHD
3052	1,9-Nonanediol dimethanesulfonate	CMC	1	2	1.5–11.9	PR8	DP	1	2	6–47.6	IHD
3088	Chlorambucil; 4-[*p*-[bis(2-chloroethyl)-amino]phenyl]-butyric acid	CMC	1	2	0.5–4	Asian	LG	1	2	1–8	IHD
6396	ThioTEPA; tris(1-aziridinyl)phosphine sulfide	S	1	2	0.1–0.9	Asian	LG	1	2	0.5–3.7	IHD
9698	Mannitol mustard; 1,6-bis[(2-chloroethyl)amino]-1,6-dideoxy-*d*-mannitol dihydrochloride	S	1	2	1–8	Asian	LG	1	2	4–32	IHD
9706	Triethylenemelamine; 2,4,6-tris-(1-aziridinyl)-*s*-triazine	S	1	2	0.02–0.17	PR8	DP	1	2	0.09–0.68	IHD
10107	Nitrogen mustard *N*-oxide; nitromin; 2,2′-Dichloro-*N*-methyldiethylamine, *N*-oxide hydrochloride	S	1	2	0.9–6.8	Asian	LG	2	2	3.4–27	IHD
14210	DL-Sarcolysin; 3-[*p*[bis(2-chloroethyl)-amino]phenyl]-DL-alanine hydrochloride	S	1	2	0.2–1.8	PR8	DP	1	2	0.9–7.2	IHD

17663	*N*,*N*-bis(2-chloroethyl)-DL-alanine hydrochloride	S	1	2	0.1–1.1	Asian	LG	1	2	0.7–4.5	IHD
18016	2-Chloroethyl methanesulfonate	S	1	2	3.4–26.8	Asian	LG	1	2	13.4–107	IHD
26271	Cyclophosphamide; cytoxan; 2-[bis(2-chloroethyl)-amino]-tetrahydro-2*H*-1,3,2-oxazaphosphorine 2-oxide hydrate	S	1	2	1.6–12.5	PR8	DP	1	2	6.3–50	IHD
26980	Mitomycin C	CMC	1	2	0.06–0.5	PR8	DP	1	2	0.3–2.1	IHD
34462	5-[Bis(2-chloroethyl)-amino]uracil	CMC	1	2	0.05–0.4	Asian	LG	2	1	0.05–0.4	IHD
								2	2	0.2–1.6	IHD
37905	Ethyl[bis(1-aziridinyl)phosphinyl]-carbamate	S	1	2	0.2–1.9	Asian	LG	1	2	0.9–7.5	IHD
50857	α-Vinyl-DL-1-aziridineethanol acetate	S	2	2	0.2–3.6	Asian	LG	1	2	0.9–7	IHD
56410	Porfiromycin	H_2O	1	1	1–8	PR8	DP	2	2	1–8	IHD
			6	2	0.3–4	PR8 (2), Asian (2), Lee (1), PR301 (1)	DP (3) LG (3)				
			1	3	5–40	Asian	LG				
			3	4	3.3–6.5	PR8	DP				

[a] The results of experiments with compounds considered to be active against influenza virus are presented in Tables 2–4. The results of experiments with compounds considered to be active against vaccinia virus are presented in Table 5.

[b] All compounds are listed in the most descriptive classification.

[c] Trivial name; chemical name. *Chemical Abstracts* nomenclature is used for all components.

[d] Diluents: CMC = 0.4% carboxymethylcellulose in phosphate-buffered saline; Dil. E = Diluent E, special suspending medium for steroids; H_2O = sterile distilled water; S = 0.85% NaCl in H_2O; $NaHCO_3$ = 1% $NaHCO_3$ in H_2O; Gum Ac = 5% gum acacia in saline.

[e] Treatment schedules: 1 = drug administered intraperitoneally (ip) twice daily from 24 hr pre- through 7 days post-virus inoculation; 2 = drug administered ip once daily from 24 hr post- through 9 days post-virus inoculation; 3 = drug administered ip in three injections: 15 min pre-, 6 hr post-, and 24 hr post-virus inoculation; 4 = drug administered ip in a single injection 24 hr post-virus inoculation; 5 = drug administered ip once daily from 24 hr pre- through 7 days post-virus inoculation; 6 = drug administered ip twice daily from 36 hr pre- through 7 days post-virus inoculation; 7 = drug administered ip in a single injection 2 hr pre-virus inoculation; 8 = drug administered ip in a single injection 24 hr pre-virus inoculation.

[f] Usually the dose range encompassed the approximate LD_{10}, $LD_{10}/2$, $LD_{10}/4$, and $LD_{10}/8$.

[g] Viruses: Asian = strain Japan 305 of Asian influenza virus; PR8 = strain PR8 of influenza A virus; PR301 = strian PR301 of influenza A virus; Lee = strain Lee of influenza B virus; IHD = strain IHD of vaccinia virus; WR = strain WR of vaccinia virus; Ut = strain Utrecht of rabbitpox virus; numbers in parentheses indicate the number of trials carried out with the indicated virus.

[h] Method: DP = death pattern; increase in survivor number and time of mean survival used as the criteria for antiviral activity. LG = lung grading; decrease in lung consolidation used as the criterion for antiviral activity.

TABLE 1.—*Continued*

Class of compound[b] and NSC no.	Compound name[c]	Diluent[d]	Influenza virus experiments					Vaccinia virus experiments			
			No. of trials	Treatment schedule[e]	Dose range[f] (mg per kg per day)	Viruses[g] used	Evaluation method[h]	No. of trials	Treatment schedule[e]	Dose range[f] (mg per kg per day)	Viruses[g] used
Antibiotics											
20088	Carzinophilin	$NaHCO_3$	1	2	0.8–6.4	PR8	DP	1	2	3.1–25	IHD
45383	Streptonigrin	$NaHCO_3$	1	2	0.03–0.2	Asian	LG	3	1	0.03–0.2	IHD
								1	2	0.1–0.8	IHD
52947	Pactamycin	CMC	1	2	0.05–0.38	PR8	DP	1	2	0.2–1.5	IHD
53396	Actinogan	CMC	1	2	0.01–0.09	PR8	DP	1	2	0.05–0.36	IHD
69856	Neocarzinostatin	S	2	2	7.9–63	Asian	LG	1	2	31.3–250	IHD
71901	Statolon	H_2O	1	2	15.6–125	PR8	DP	2	1	56.3–500	Ut
								2	2	62.5–500	IHD
72942	DL-Noformicin; *N*-(2-amidinoethyl)-5-imino-DL-2-pyrrolidinecarboxamide	CMC	2	1	0.3–10	PR8, Asian	DP	3	2	1.2–9.7	IHD
			7	2	0.3–2.4	PR8 (1), Asian (4), Lee (1), PR301 (1)	DP (4), LG (3)				
			5	3	2.5–60	PR8 (3), Asian (2)	DP (2), LG (3)				
			3	4	15–60	PR8 (2), Asian	DP (3)				
91770	Cytovirin	H_2O	1	2	0.06–0.5	Asian	LG	2	1	0.3–0.5	IHD
			1	3	0.8–6	Asian	LG	2	2	0.3–2	IHD
Antimetabolites											
Amino acid analogues											
8921	L-Canavanine; L-2-amino-4-(guanidinooxy)butyric acid	CMC	2	2	25–200	PR8	DP	1	2	100–800	IHD
57695	5-Methyl-DL-tryptophan	CMC	1	2	6.3–50	PR8	DP	1	2	25–200	IHD
521778	Hadacidin; *N*-formyl-*N*-hydroxyglycine sodium salt	CMC	1	2	62.5–500	PR8	DP	2	2	250–4000	IHD

Folic acid antagonists											
740	Methotrexate; amethopterin; *N*-[*p*[[(2,4-diamino-6-pteridinyl)methyl]methylamino]benzoyl]-glutamic acid	CMC	2	1	0.1–2	PR8	DP	2	3	0.9–4.5	WR, Ut
3061	Daraprim; 2,4-diamino-5-(*p*-chlorophenyl)-6-ethylpyrimidine	CMC	1	2	1.6–12.5	PR8	DP	2	1	10–225	WR, Ut
Glutamine antagonists											
742	Azaserine; L-serine, diazoacetate (ester)	CMC	2	2	0.1–2.5	PR8	DP	1	2	1.1–8.4	IHD
								2	3	3.7–18.9	WR, Ut
7365	DON; 6-diazo-5-oxo-L-norleucine	S	1	2	0.0003–0.002	PR8	DP	1	2	0.006–0.05	IHD
Mitotic inhibitors											
757	Colchicine; 7-acetamido-6,7-dihydro-1,2,3,10-tetramethoxybenzol[*a*]-heptalen-9 (5*H*)-one	S	1	2	0.02–0.2	PR8	DP	2	2	0.1–1.2	IHD
49842	Vinblastine; vincaleukoblastine, sulfate hydrate	S	1	2	0.02–0.12	PR8	DP	1	2	0.06–0.48	IHD
Protein synthesis inhibitors											
185	Cycloheximide; 3-[2-(3,5-dimethyl-2-oxocyclohexyl)-2-hydroxyethyl]glutarimide	CMC	2	1	1.5–24	Asian, PR8	DP	1	1	3–24	IHD
								2	2,3	10.6–54	Ut
								1	3	10.6–54	WR
3053	Actinomycin D	CMC	1	1	0.008–0.06	PR8	DP	2	1	0.008–0.06	WR, Ut
			1	2	0.002–0.02	PR8	DP	3	2	0.003–0.06	IHD
31083	Actinobolin	CMC	1	2	28.9–231	PR8	DP	2	2	116–1856	IHD
59729	Sparsomycin	CMC	1	2	0.02–0.13	PR8	DP	1	2	0.06–0.5	IHD
525816	Tenuazonic acid; 3-acetyl-5-*sec*-butyl-4-hydroxy-3-pyrrolin-2-one sodium derivative	S	1	2	3.8–30	PR8	DP	1	2	15–120	IHD

TABLE 1.—*Continued*

Class of compound[b] and NSC no.	Compound name[c]	Diluent[d]	Influenza virus experiments					Vaccinia virus experiments			
			No. of trials	Treatment schedule[e]	Dose range[f] (mg per kg per day)	Viruses[g] used	Evaluation method[h]	No. of trials	Treatment schedule[e]	Dose range[f] (mg per kg per day)	Viruses[g] used
Purine antagonists or analogues											
743	2,6-Diaminopurine	CMC	1	1	5.8–46	PR8	DP	1	1	5.7–45.5	IHD
								1	2	22.8–182	IHD
749	8-Azaguanine; 5-amino-*v*-triazolo-[4,5-*d*]pyrimidin-7-ol	H_2O	1	2	3.9–31.5	PR8	DP	2	1	42.2–283.5	WR, Ut
752	6-Thioguanine; 2-aminopurine-6-thiol	CMC	2	2	0.3–2.6	PR8	DP	2	1	0.3–2.6	WR, Ut
								2	2	0.7–1.3	IHD, Ut
755	6-Mercaptopurine; purine-6-thiol hydrate	CMC	2	1	7.3–58	PR8	DP	3	3	25.5–131	WR, Ut
			1	2	0.9–7	Asian	DP				
1390	1*H*-Pyrazolo[3,4-*d*]-pyridin-4-ol	CMC	1	2	1–8.3	PR8	DP	2	2	4.1–66	IHD
1393	4-Amino-1*H*-pyrazolo-[3,4-*d*]-pyrimidine	CMC	1	1	1.1–8.8	PR8	DP	3	1	1.1–8.8	IHD, WR, Ut
4910	6-Chloropurine ribonucleoside; 6-chloro-9-β-D-ribofuranosyl-9*H*-purine	CMC	1	2	68.8–550	PR8	DP	1	2	68.8–550	IHD
4911	6-Mercaptopurine ribonucleoside; 9-β-D ribofuranosyl-9*H*-purine-6-thiol	CMC	1	1	7.5–60	PR8	DP	1	1	17.5–140	WR
7363	2,6-Diaminopurine ribonucleoside; 2,6-diamino-9-β-D-ribofuranosyl-9*H*-purine	S	1	2	5.2–41.3	PR8	DP	1	2	15.6–125	IHD
19487	9-Cyclopentyl-6-mercaptopurine; 9-cyclopentyl-9*H*-purine-6-thiol	CMC	1	2	1.9–15	PR8	DP	1	2	7.5–60	IHD
19488	9-n-Butyl-6-mercaptopurine; 9-butyl-9*H*-purine-6-thiol	CMC	1	2	2.9–23	PR8	DP	1	2	11.3–90	IHD

25650	6-(2,2-Dimethylhydrazino)-purine	S	2	2	1.4–11.2	PR8	DP	1	2	5.6–44.8	IHD
26273	6-Benzylthiopurine ribonucleoside; 6-(benzylthio)-9-β-D-ribofuranosyl-9*H*-purine	CMC	1	2	4.7–37.5	PR8	DP	1	2	2.3–18.8	IHD
29408	6-Hydrazinopurine ribonucleoside; 6-hydrazino-9-β-D-ribofuranosyl-9*H*-purine	H_2O	1	2	0.6–5	PR8	DP	1	2	2.5–20	IHD
29422	6-Thioguanosine; 2-amino-9-β-D-ribofuranosyl-9*H*-purine-6-thiol	CMC	1	2	0.2–1.3	PR8	DP	1	2	0.6–5	IHD
30605	2-Fluoroadenosine	H_2O	1	2	0.06–0.5	PR8	DP	1	2	0.3–2	IHD
31730	2-Amino-6-(benzylthio)-9-β-D-ribofuranosyl-9*H*-purine	Dil. E	1	2	7.3–50	Asian	LG	1	2	12.5–100	IHD
38845	8-(Benzylthio)purine	CMC	1	2	0.06–0.5	PR8	DP	1	2	0.3–2	IHD
39084	6-[(1-Methyl-4-nitroimidazol-5-yl)thio]-purine hydrate	CMC	1	2	0.8–6	PR8	DP	2	2	3.1–200	IHD
39367	6-(Ethylthio)-9-β-D-ribofuranosyl purine hydrate	S	1	2	2.5–20	Asian	LG	1	2	10–80	IHD
40774	6-Methylthiopurine ribonucleoside; 6-(methylthio)-9-β-D-ribofuranosyl-9*H*-purine	S	1	2	0.6–5	PR8	DP	1	2	2.5–20	IHD
56408	Tubercidin; 4-amino-7-β-D-ribofuranosyl-7*H*-pyrrolo-[2,3-*d*]-pyrimidine	S	1	2	0.01–0.1	PR8	DP	1	2	0.4–3	IHD
95012	6-Methylpurine, 1-oxide	S	1	2	1–8.3	Asian	LG	1	2	3.1–25	IHD
98014	1-Purin-6-yl-semicarbazide, 3-thiohydrate	H_2O	1	2	2.5–20	Asian	LG	1	2	10–80	IHD
404241	9-β-D-Arabinofuranosyladenine	CMC	1	2	7.9–63	Asian	LG	2	2	31.3–250	IHD

TABLE 1.—*Continued*

Class of compound[b] and NSC no.	Compound name[c]	Diluent[d]	Influenza virus experiments					Vaccinia virus experiments			
			No. of trials	Treatment schedule[e]	Dose range[f] (mg per kg per day)	Viruses[g] used	Evaluation method[h]	No. of trials	Treatment schedule[e]	Dose range[f] (mg per kg per day)	Viruses[g] used
Pyrimidine antagonists or analogues											
3425	6-Azauracil; *as*-triazine-3,5-(2*H*,4*H*)-dione	CMC	1	2	31.3–250	PR8	DP	2	2	125–1000	IHD
19893	5-Fluorouracil	CMC	1	2	1.2–9.5	PR8	DP	1	2	3.8–30	IHD
19940	5-Bromouracil	CMC	1	2	17.3–138	PR8	DP	1	1	553–1106	IHD
								2	2	69.1–1106	IHD
23519	5-Diazouracil hydrate	S	1	2	0.3–2	Asian	LG	1	2	1–8	IHD
27640	2-Deoxy-5-fluorouridine	CMC	1	1	2.0–16	PR8	DP	2	2	16–128	IHD
32074	6-Azauridine; 2-β-D-ribofuranosyl-*as*-triazine-3,5(2*H*,4*H*)-dione	CMC	1	2	15–120	PR8	DP	1	2	59.8–478	IHD
38297	5-Bromo-2′-deoxyuridine	CMC	2	2	14.4–115	PR8	DP	3	2	56.3–450	IHD
39661	5-Iodo-2′-deoxyuridine	CMC	1	2	9.4–75	PR8	DP	3	1	9.4–300	IHD
								2	2	37.5–300	IHD
63878	Cytosine arabinoside; 1-β-D-arabinofuranosylcytosine·HCl	S	1	2	1.5–11.8	PR8	DP	1	2	5.9–47	IHD
								1	4	500	IHD
								1	8	500	IHD
73753	5-(2,4-Dichlorophenoxy)-2-thiouracil	CMC	1	2	4.8–38	PR8	DP	2	1	9.4–75	IHD
								3	2	18.8–600	IHD
76460	5-(2-Chloroacetamido)-1,3-dimethyluracil	CMC	1	2	6.3–50	Asian	LG	1	2	25–200	IHD
407413	8-Amino-7-chlorotetrazolo-[1,5-*c*]-pyrimidine	CMC	1	2	6.3–50	PR8	DP	1	2	12.5–100	IHD
524767	6-Azacytidine	S	1	2	7.9–63	Asian	LG	1	2	31.3–250	IHD
527083	2′-Deoxy-5-iodocytidine	CMC	1	2	7.9–63	Asian	LG	1	2	31.3–250	IHD
529351	3-(Mercaptomethyl)-uracil	S	1	2	2.5–20	Asian	LG	1	2	10–80	IHD

Vitamin antagonists or analogues											
3063	Deoxypyridoxine·HCl; 5-hydroxy-4,6-dimethyl-3-pyridinemethanol hydrochloride	CMC	1	2	6.3–50.5	PR8	DP	1	2	25.3–202	IHD
21206	6-Aminonicotinamide	CMC	1	2	0.3–2	Asian	LG	2	1	0.3–2	IHD
								3	2	1.5–25	IHD
87609	Isonicotinic acid (*m*-sulfobenzylidene)-hydrazide sodium salt tetrahydrate	S	1	2	6.3–50	Asian	LG	1	2	25–200	IHD
Guanidines											
1516	1-[(*p*-Methoxybenzylidene)amino]-3-nitroguanidine	CMC	1	2	6.3–50	PR8	DP	2	2	25–400	IHD
1517	1-(Benzylideneamino)-3-nitroguanidine	CMC	1	2	6.3–50	PR8	DP	1	2	25–200	IHD
1518	1-(Cinnamylideneamino)-3-nitroguanidine	CMC	1	2	6.3–50	PR8	DP	1	2	25–200	IHD
1519	1-[(2,4-Dichlorobenzylidene)amino]-3-nitroguanidine	CMC	1	2	6.3–50	PR8	DP	1	2	25–200	IHD
1520	1-[(3,7-Dimethyl-2,6-octadienylidene)-amino]-3-nitroguanidine	S	1	2	6.3–50	PR8	DP	1	2	25–400	IHD
1525	1-[(*o*-Methoxybenzylidene)amino]-3-nitroguanidine	CMC	1	2	6.3–60	PR8	DP	1	2	25–400	IHD
1529	1-Nitro-3-[(4-pyridylmethylene)amino]-guanidine	CMC	1	2	25–200	PR8	DP	2	2	25–200	IHD
1530	1-Nitro-3-[(2-pyridylmethylene)amino]-guanidine	CMC	1	2	6.3–50	PR8	DP	1	2	25–200	IHD
1531	1-Nitro-3-[(3-pyridylmethylene)amino]-guanidine	CMC	1	2	6.3–50	PR8	DP	1	2	25–200	IHD
7295	Guanidine nitrate	CMC	2	2	11.4–182	PR8	DP	2	2	45.4–762	IHD

TABLE 1.—*Continued*

Class of compound[b] and NSC no.	Compound name[c]	Diluent[d]	Influenza virus experiments					Vaccinia virus experiments			
			No. of trials	Treatment schedule[e]	Dose range[f] (mg per kg per day)	Viruses[g] used	Evaluation method[h]	No. of trials	Treatment schedule[e]	Dose range[f] (mg per kg per day)	Viruses[g] used
22185	Methylglyoxal bis-(guanylhydrazone)-sulfate sesquihydrate; 1,1′-[(methylethane-diylidene)dinitrilo]-diguanidine sulfate sesquihydrate	CMC	2	2	1.3–10.5	PR8	DP	1	2	5.3–42	IHD
32946	1,1′-[(Methylethane-diylidene)dinitrilo]-diguanidine dihydrochloride hydrate	CMC	1	2	1.4–11	PR8	DP	1	2	5.6–45	IHD
Hormones and hormonelike compounds											
741	17-Hydroxycorticosterone 21-acetate; cortisol 21-acetate	Dil. E	1	2	0.3–2.5	PR8	DP	1	2	1.3–10	IHD
3070	α,α′-Diethyl-4,4′-stilbenediol	Dil. E	1	2	4.9–39.5	Asian	LG	1	2	9.9–79	IHD
9166	Testosterone propionate; 17β-hydroxyandrost-4-en-3-one, propionate	Dil. E	1	2	31.3–250	PR8	DP	1	2	125–1000	IHD
9895	17β-Estradiol; estradiol	Dil. E	1	2	0.3–2	Asian	DP	1	1	250–500	IHD
								3	2	125–1000	IHD
12601	9-α-Fluoro-2α-methylhydrocortisone 21-acetate; 9-fluoro-11β,17,21-trihydroxy-2α-methylpregn-4-ene-3,20-dione 21-acetate	Dil. E	1	2	6.3–50	PR8	DP	1	1	6.3–50	IHD
								2	2	25–200	IHD
49420	Cortisone 21-acetate	Dil. E	1	2	0.4–3	PR8	DP	1	2	1.4–11	IHD
92222	4-Aza-5α-cholestane	Dil. E	1	2	0.6–5	Asian	LG	2	2	2.5–40	IHD

Nitrosoureas											
23909	1-Methyl-1-nitrosourea	S	1	2	0.8–6	PR8	DP	1	2	3–24	IHD
409962	1,3-Bis(2-chloroethyl)-1-nitrosourea	S	6	2	0.2–2	PR8 (3), Lee (2) PR301 (1)	DP (4), LG (2)	1	1	1.5–12	WR
			1	3	3.1–25	Asian	LG				
Semicarbazones, thiosemicarbazones, thiocarbohydrazides											
695	1-(3-Pyridylmethylene)-3-thiocarbohydrazide	CMC	1	2	1.7–13.5	PR8	DP	1	2	8–64	IHD
712	*p*-Anisaldehyde thiosemicarbazone	CMC	1	2	6.3–50	PR8	DP	1	2	25–200	IHD
721	Isatin β-thiosemicarbazone; indole-2,3-dione 3-(thiosemicarbazone)	CMC	2	2	7.8–62.5	Asian, PR8	DP	4	1	31.3–250	IHD (2), WR, Ut
								3	2	31.3–250	IHD, WR, Ut
1604	Salicylaldehyde semicarbazone	CMC	1	2	6.3–50	PR8	DP	1	2	25–200	IHD
9936	*p*-Isopropylbenzaldehyde thiosemicarbazone	CMC	1	2	0.6–5	Asian	DP	2	2	2.5–20	IHD
69405	2,3-Butanedione bis(thiosemicarbazone)	CMC	1	2	2–16	PR8	DP	1	2	8–64	IHD
69811	*N*-Methylisatin β-thiosemicarbazone; 1-methylindole-2,3-dione-3-(thiosemicarbazone)	CMC	1	2	1.8–14	PR8	DP	5	1	28.5–250	IHD (3), WR, Ut
								2	2	3.6–114	IHD
								1	7	42.8–342	IHD
								1	8	42.8–342	IHD
83459	Isatin β-(4,4-dimethyl-3-thiosemicarbazone); indole-2,3-dione 3-(4,4-dimethyl-3-thiosemicarbazone)	CMC	1	2	6.3–50	PR8	DP	1	2	25–200	IHD
92856	3,4-Dihydro-7-methoxy-2(1*H*)-naphthalenone thiosemicarbazone	CMC	1	2	4–32	Asian	LG	1	1	16–128	IHD
								1	2	8–64	IHD

TABLE 1.—*Continued*

Class of compound[b] and NSC no.	Compound name[c]	Diluent[d]	Influenza virus experiments					Vaccinia virus experiments			
			No. of trials	Treatment schedule[e]	Dose range[f] (mg per kg per day)	Viruses[g] used	Evaluation method[h]	No. of trials	Treatment schedule[e]	Dose range[f] (mg per kg per day)	Viruses[g] used
95010	1,2,3,4-Tetrahydro-2,4-dioxo-5-pyrimidinecarboxaldehyde, 5-thiosemicarbazone	CMC	1	2	7.8–62.5	Asian	LG	1	2	31.3–250	IHD
95383	Purine-6-carboxaldehyde thiosemicarbazone	CMC	1	2	0.8–6.3	Asian	LG	2	2	3.1–25	IHD
95669	Dihydro-3(2*H*)-thiophenone thiosemicarbazone	CMC	3	2	2.5–80	Asian	LG	1	2	10–80	IHD
95670	Tetrahydro-4*H*-thiopyran-4-one thiosemicarbazone-1,1-dioxide	CMC	1	2	7.8–62.5	Asian	LG	1	2	13.3–250	IHD
Terephthalanilides											
35843	4′,4″-Di-2-imidazolin-2-ylterephthalanilide dihydrochloride	CMC	1	2	0.6–5	PR8	DP	1 3	1 2	20–40 2.5–40	IHD IHD
38280	2-Chloro-4′,4″-di-2-imidazolin-2-ylterephthalanilide dihydrochloride	CMC	1	2	0.6–5	PR8	DP	2	2	1.3–21	IHD
53212	4′,4″-Di-2-imidazolin-2-yl-isophthalanilide dihydrochloride	H_2O	1	2	4.2–33.3	PR8	DP	1	2	16.6–133	IHD
57155	*N*′,*N*″-Bis[*p*-(methylamidino)phenyl]-terephthalamidine tetrahydrochloride	H_2O	8	2	1.5–12	PR8 (2), Asian (2), Lee (3), PR301 (1)	DP (6), LG (2)	1 2	1 2	5.9–47 6.5–52	Ut IHD, Ut
60339	2-Chloro-4′-4″-di-2-imidazolin-2-ylterephthalanilide	CMC	1	2	1.4–11.5	PR8	DP	2	2	5.8–46	IHD
Miscellaneous compounds											
697	1-(2-Quinolylmethylene)-3-thiocarbohydrazide	CMC	1	2	6.3–50	PR8	DP	1	2	25–200	IHD

746	Urethan; ethyl carbamate	CMC	1	1	85.4–683	PR8	DP	1	2	400–800	Ut
					21.4–171	PR8	DP	2	3	280.7–1545.5	Ut, WR
1026	1-Aminocyclopentanecarboxylic acid	S	1	2	2.3–18.5	Asian	LG	1	2	9.3–74	IHD
1553	4-[(*o*-Methoxybenzylidene)amino]phenol	CMC	1	2	6.3–50	PR8	DP	2	2	25–400	IHD
1555	2,2′-(Methylidynenitrilo)di-	CMC	1	2	2–16	PR8	DP	2	2	8–64	IHD
1562	*p*-[(*o*-Nitrobenzylidene)-amino]phenol	CMC	1	2	6.3–50	PR8	DP	1	2	25–200	IHD
1576	*p*-[(6-Hydroxy-3,4-xylyl)-azo]benzoic acid	CMC	1	2	6.3–50	PR8	DP	1	2	25–200	IHD
2582	2,3,5-Triiodobenzoic acid	S	1	2	2–16	Asian	LG	1	2	8–64	IHD
2594	2,4,6-Triiodophenol	S	1	2	6.3–50	Asian	LG	2	2	25–200	IHD
3051	*N*-Methylformamide	CMC	2	2	7.1–57	PR8	DP	2	1	46.9–375	Ut
								1	3	23.5–188	IHD
								1	4	114–228	IHD
3094	3,3-Dimethyl-1-phenyltriazene	S	2	2	6.3–50	PR8	DP	1	2	25–200	IHD
3095	1,5-Diaminobiuret; imidodicarboxylic acid dihydrazide	CMC	1	2	2.3–18.3	PR8	DP	1	1	4.6–36.5	IHD
4646	2,3-Dimercapto-1-propanol	H_2O	2	2	2–16	PR8	DP	2	2	8–64	IHD
4730	2-(Ethylamino)-1,3,4-thiadiazole	CMC	1	2	4.1–33	PR8	DP	2	2	8.2–131	IHD
5354	*o*-Phenylenediamine	CMC	1	2	3.6–29	PR8	DP	1	2	14.4–115	IHD
13875	Hexamethylmelamine	Gum Ac.	1	2	3.7–29.3	Asian	LG	1	2	14.6–117	IHD
14574	Miracil D; 1-[(2-diethylaminoethyl)-amino]-4-methylthioxanthen-9-one hydrochloride	H_2O	1	1	2.5–20	PR8	DP	1	2	9.8–78	IHD
			6	2	2.5–20	PR8 (3), Asian (2), PR301 (1)	DP (4) LG (2)				
			1	3	46.3–390	Asian	LG				
			5	4	32–64	PR8	DP				
32065	Hydroxyurea	CMC	1	1	4–32	PR8	DP	2	2	16–128	IHD
			2	2	4–32	PR8	DP				
38721	1,1-Dichloro-2-(*o*-chlorophenyl)-2-(*p*-chlorophenyl)ethane	CMC	1	2	7–28	PR8	DP	2	2	28.1–450	IHD
42415	D-Xylononitrile tetraacetate	CMC	1	2	0.6–5	PR8	DP	1	2	2.5–20	IHD

TABLE 1.—*Concluded*

Class of compound[b] and NSC no.	Compound name[c]	Diluent[d]	Influenza virus experiments					Vaccinia virus experiments			
			No. of trials	Treatment schedule[e]	Dose range[f] (mg per kg per day)	Viruses[g] used	Evaluation method[h]	No. of trials	Treatment schedule[e]	Dose range[f] (mg per kg per day)	Viruses used[g]
43841	4,4′-Biphenyldiglyoxyaldehyde dihydrate	CMC	1	2	2.5–20	PR8	DP	1	2	25–200	IHD
46015	4,5-Dicarboxytetrahydro-6-methylenepyran-2-succinic acid dianhydride, α-6-β-polymer	S	1	2	7.9–63	Asian	LG	1	2	31.3–250	IHD
57197	3,4-Dihydroxycinnamic acid	CMC	1	2	6.3–50	Asian	LG	1	2	25–200	IHD
83653	Amantadine hydrochloride; 1-adamantanamine hydrochloride	S	2	1	0.3–13	Asian	DP, LG	1	2	6.3–50	IHD
			2	2	1.6–25	PR8, Asian	DP, LG				
			2	3	12.5–50	Asian	DP				
			1	5	1.6–12.5	PR8	DP				
			1	6	0.9–7	PR8	DP				
			1	7	17.9–63	Asian	LG				
84531	4-Amino-1,2,5-selenadiazole-3-carboxylic acid	CMC	1	2	0.06–0.5	Asian	LG	1	2	0.3–2	IHD
84963	4-Amino-1,2,5-selenadiazole-3-carboxamide	CMC	1	2	0.06–0.5	Asian	LG	1	2	0.3–2	IHD
86047	4-Amino-*N*-butyl-1,-2,5-selenadizaole-3-carboxamide	CMC	1	2	0.3–2	Asian	LG	1	2	1–8	IHD
89020	Hydroxy(α-phenyl-*p*-toluoyl)methanesulfonic acid sodium salt	CMC	1	2	2–16	PR8	DP	1	2	8–64	IHD
89022	(Ethylenedi-*p*-phenylene)diglyoxal dihydrate	CMC	1	2	6.3–50	PR8	DP	1	2	25–200	IHD
89024	(Oxydi-*p*-phenylene)-diglyoxal dihydrate	CMC	1	2	2–16	PR8	DP	1	2	8–64	IHD

93157	5-Amino-3-phenyl-isoxazole	CMC	1	2	4–32	Asian	LG	1	2	8–64	IHD
93169	4-Amino-*N*-methyl-1,2,5-selenadiazole-3-carboxamide	CMC	1	2	0.2–1.5	Asian	LG	1	2	0.8–6	IHD
95437	6-Hydroxy-4-methoxy-5-benzofuranacrylic acid, 8-lactone	CMC	1	2	7.8–62.5	Asian	LG	1	2	31.3–250	IHD
95668	Tetrahydro-4*H*-thiopyran-4-one, 1,1-dioxide	S	1	2	10.3–82.5	Asian	LG	1	2	31.3–250	IHD
400525	*N*-Acetyl-D-glucosamine	S	1	2	7.9–63	Asian	LG	1	2	31.3–250	IHD
401575	5,6-Dichloro-1-β-D-ribofuranosylbenzimidazole	CMC	7	2	3.8–242	PR8 (2), Asian (1), Lee (3), PR301 (1)	DP (4), LG (3)	1	2	15.1–121	IHD
			1	3	75.6–605	Asian	LG				

virus. Increases in time of mean survival and number of survivors were the criteria used for evaluation of antivaccinia virus activity.

RESULTS

A total of 248 experiments were carried out with influenza virus and 263 experiments with vaccinia virus in the evaluation of 156 compounds. The compounds tested, diluents, number of trials, treatment schedules, dose range, and viruses tested are summarized in Table 1. Each compound is classified according to structure or probable mode of action. If a compound could be placed in more than one category, it was listed under the most descriptive classification.

Two compounds were considered active against influenza virus. These were amantadine hydrochloride (NSC 83653) and DL-noformicin (NSC 72942). The results of the death-pattern experiments with these two compounds are summarized in Table 2, and the results of lung consolidation experiments with these compounds are indicated in Table 3. DL-Noformicin was tested against the PR8 virus by means of reduction in hemagglutination (HA) titers in lung suspensions of treated, infected mice as an additional criterion for evaluation. The results of this experiment are shown in Table 4. The methods of Ginsberg and Horsfall (8), and modifications of these methods as indicated in Table 4, were used to determine the HA titers. Marked reductions in HA titers were observed after treatment in this experiment, indicating that therapy with DL-noformicin reduced the virus titer in the lungs of the infected mice. Neither amantadine hydrochloride nor DL-noformicin was considered active in every test carried out, although the activity was reproducible when identical viruses and treatment schedules were used in confirming experiments. The former compound was more active against the Asian virus than against the PR8 virus, and was usually more effective when administered by a treatment schedule beginning prior to virus inoculation. DL-Noformicin was active in one or more experiments against all the influenza viruses tested and when used in a variety of treatment schedules, including intraperitoneal administration in a single injection 24 hr after virus inoculation. The use of larger mice seemed to be more efficacious for evaluating this compound.

Eleven compounds were considered active against vaccinia virus or the related rabbitpox virus. These included isatin-β-thiosemicarbazone (NSC 721), 6-azauracil (NSC 3425), 9-α-fluoro-2α-methylhydrocortisone 21-acetate (NSC 12601), 5-[bis(2-chloroethyl)amino]uracil (NSC 34462), 5-iodo-2′-deoxyuridine (NSC 39661),

TABLE 2. *Summary of the results of death pattern experiments with compounds active[a] against in vivo influenza virus infections[b]*

Name	Virus[c]	Dose range (mg per kg per day)	Treatment schedule[d]	10 LD_{50} of virus				32 LD_{50} of virus			
				Max survivors, T-C (%)	Survivors, P[e]	Max mean survival time increase (days)	Increase, P[f]	Max survivors, T-C (%)	Survivors, P	Max mean survival time increase (days)	Increase, P
DL-Noformicin (NSC 72942)	PR301	0.3–2.4	2	0	—	1.1	<0.05	0	—	0.1	>0.05
	PR8	0.3–2.4	1	0	—	0.8	>0.05	0	—	1.6	<0.05
	PR8	0.3–2.4	2	0	—	0.7	>0.05	0	—	1.0	<0.05
	PR8	15–60	4	0	—	1.0	<0.05	5	>0.3	0.2	>0.05
	PR8	15–60[g]	4	0	—	3.1	<0.05	0	—	3.4	<0.05
	PR8	15–60[g]	3	35	<0.3	1.5	<0.05	0	—	1.8	>0.05
	Asian	0.3–2.4	2	5	>0.3	0.3	>0.05	0	—	0.0	—
	Asian	0.3–2.4	2	5	>0.3	2.5	<0.05	0	—	0.0	—
	Asian	10–40[g]	3	40	<0.05	3.6	<0.05	0	—	4.0	<0.001
	Asian	15–60[g]	4	30	<0.3	2.4	<0.05	40	<0.05	0.2	>0.05
	Asian	0.3–10[g]	1	10	>0.3	3.3	<0.001				
Amantadine hydrochloride (NSC 83653)	PR8	1.6–12.5	5	0	—	0.6	<0.05	10	>0.3	1.6	<0.001
	PR8	1.6–12.5	2	0	—	0.3	>0.05	10	>0.3	0.0	—
	PR8	0.9–7	6	10	>0.3	0.0	—	0	—	0.0	—
	Asian	12.5–50	3	10	>0.3	2.8	<0.001	30	<0.3	2.8	<0.05
	Asian	0.3–2.4	1	30	<0.3	2.5	<0.001	30	<0.3	2.2	<0.05

[a] Compounds were considered active if treatment of virus-infected mice resulted in dose-responsive significant increases in mean survival time or in number of survivors.

[b] ICR Swiss mice were inoculated intranasally with 0.06 ml of influenza virus; 8- to 10-g mice were used unless otherwise indicated.

[c] Viruses: *see* footnote *g*, Table 1.

[d] Treatment schedules: *see* footnote *e*, Table 1.

[e] P = probability that any increase in the number of survivors in the virus-infected, treated groups compared to the virus control group was due to chance, as determined by chi-square analysis. $P < 0.3$ = significant, $P < 0.05$ = highly significant.

[f] P = probability that any observed increase in mean survival time of virus-infected, treated groups compared to the virus-control group was due to chance, as determined by t test. $P < 0.05$ = significant, $P < 0.001$ = highly significant.

[g] In these instances, 18- to 21-g mice were used.

streptonigrin (NSC 45383), *N*-methylisatin-β-thiosemicarbazone (NSC 69811), 5-(2,4-dichlorophenoxy)-2-thiouracil (NSC 73753), cytovirin (NSC 91770), 9-β-D-arabinofuranosyladenine (NSC 404241), and 5-(mercaptomethyl)uracil (NSC 529351). The results of the experiments with these compounds are summarized in Table 5. In addition to the experiments indicated, one of the two active thiosemicarbazone compounds was used as a positive control for the majority of the vaccinia virus chemotherapy experiments carried out. In every experiment, treatment of vaccinia virus-infected mice with these compounds resulted in marked increases in number of survivors and life span.

The results of the influenza and vaccinia virus experiments with the other compounds listed in Table 1 have been omitted to conserve space, since these other compounds were not reproducibly active against either virus by the methods employed.

DISCUSSION

The chemotherapy experiments which were carried out with the influenza virus system indicated that few of the compounds tested were reproducibly active against the virus, although most of the compounds have been reported to have biological activity in one or more in vivo tumor systems or against one or more animal viruses in vitro.

Since the initial reports on the antiviral activity of the compounds in 1964 (5, 10; R. R. Grunert et al., Federation Proc. **23**:387, 1964; C. E. Hoffman, R. E. Haff, and E. M. Neumayer, Federation Proc. **23**:387.), much has been pub-

TABLE 3. *Summary of the results of lung consolidation experiments with compounds active[a] against in vivo influenza virus infections[b]*

Name	Virus[c]	Dose range (mg per kg per day)	Treatment schedule[d]	Max survivors, T-C (%)	Survivors P^e	Max reduction in lung consol. score[f]	Max reduction, P^g
DL-Noformicin (NSC 72942)	Asian	0.3–2.4	2	15	>0.3	1.3	<0.01
	Asian	0.3–2.4	2	5	>0.3	1.2	<0.01
	Asian	10–40[h]	3	60	<0.001	1.5	<0.01
	Lee	0.3–2.4	2	30	<0.3	0.5	>0.05
	PR8	10–40	3	10	>0.3	0.5	>0.05
	PR8	2.5–20[h]	3	10	>0.3	0.8	<0.05
Amantadine hydrochloride (NSC 83653)	Asian	1.6–13	1	10	>0.3	0.2	>0.05
	Asian	7.9–63	7	15	>0.3	0.5	>0.05
	Asian	3.1–25	2	10	>0.3	0.6	<0.05
	Asian	12.5–50[h]	3	50	<0.05	1.5	<0.01

[a] Compounds were considered active if treatment of virus-infected mice resulted in dose-responsive significant increases in the number of survivors or reductions in lung consolidation.

[b] ICR Swiss mice were inoculated intranasally with 0.06 ml of influenza virus; 8- to 10-g mice were used unless otherwise indicated.

[c] Virus: *see* footnote *g*, Table 1.

[d] Treatment schedules: *see* footnote *e*, Table 1.

[e] P = probability that any observed increase in number of survivors in the virus-infected, treated groups compared to the virus-control group was due to chance. Determined by chi-square analysis.

[f] Lung consolidation score: total grade of consolidation/number of animals. Lungs were graded according to the following scale: 5 = death with consolidation; 4 = 100% consolidation; 3 = ~75% consolidation; 2 = ~50% consolidation; 1 = ~25% consolidation; 0 = no consolidation.

[g] P = probability that decrease in lung consolidation score of drug-treated, infected animals compared to the virus-control group was due to chance. Determined by White's modification of the Wilcoxon test.

[h] In these instances, 18- to 21-g mice were used.

lished describing the anti-influenza virus activity of amantadine hydrochloride. These reports have generally indicated that this drug is most effective when administered prophylactically against the Asian influenza virus. Our studies confirm these observations, although the antiviral activity of the compound was not spectacular in any experiment.

DL-Noformicin was originally reported to be active against the influenza virus by McClelland (14), and by Schabel and Skipper (20) in separate investigations. Our experiments indicate that the compound is active to a degree against the PR8, Asian, and Lee influenza viruses, but, as was the case for amantadine hydrochloride, this activity was not considered marked in any experiment. In the original reports, the noformicin used was produced biosynthetically by *Nocardia formica*. The material used in the present experiments, however, was chemically synthesized, and a definite difference between the in vivo toxicity of the synthetic compound and the recorded toxicity of the naturally occurring material has been observed. These data suggest that a chemical difference exists between the natural and synthetic products. None of the original natural product was available for the present studies, so concomitant comparisons could not be made. DL-Noformicin had a greater degree of antiviral activity, that is, a higher therapeutic index (25), when large mice were used. We have found that the large (18 to 21 g) mice will apparently tolerate up to four times the amount of drug per unit weight that the small (8 to 10 g) mice can be given; hence, if the drug has a low therapeutic index, and is consequently active only at a nearly toxic dose, this activity may be masked by using the smaller animals. Because of this observation, all compounds in these influenza virus studies which had a suggestion of antiviral activity when tested in the small mice were retested in the larger animals. With the exception of DL-noformicin, no consistent antiviral activity of significance was observed. Treatment with 1,3-bis(2-chloroethyl)-1-nitrosourea (NSC 409962) resulted in slight increases in the life span of PR8-infected mice, as we reported previously (21), but these increases were not statistically significant.

The vaccinia virus chemotherapy experiments confirmed the reported in vivo antiviral activity of isatin-β-thiosemicarbazone (2, 27), 5-iodo-2'-deoxyuridine (12), *N*-methylisatin-β-thiosemi-

TABLE 4. *Anti-influenza virus activity of* DL*-noformicin as evaluated by reduction of hemagglutinin titers in the lungs of infected treated*[a] *mice*

Drug dose (mg per kg per day)	Hemagglutinin titers[b] of lungs removed 5 days post-virus inoculation					
	Test 1[c]		Test 2[d]		Text 3[e]	
	0.25% chick RBC	0.4% guinea pig RBC	0.25% chick RBC	0.4% guinea pig RBC	0.25% chick RBC	0.4% guinea pig RBC
20	40	40	<20	40	<20	<20
10	40	80	20	160	<20	20
5	80	320	160	320	<20	20
2.5	80	320	160	320	40	40
0 (virus controls)	80	320	160	320	40	40
0 (normals)	<20	<20	<20	<20	<20	<20

[a] Swiss mice (18 to 21 g) were infected intranasally with 10 LD_{50} of influenza A (strain PR8) virus. Drug was administered intraperitoneally in three injections: 15 min pre-, 6 hr post-, and 24 hr post-virus inoculation.

[b] Reciprocals of antibody titers.

[c] Lungs were homogenized in phosphate-buffered saline (PBS) centrifuged at low speed, and twofold dilutions of the supernatant fluid were mixed with the appropriate red blood cells (RBC). The supernatant fluid and RBC were incubated at room temperature for up to 2 hr, and the degree of hemagglutination was determined by the sedimentation pattern.

[d] Lungs were homogenized in phosphate-buffered saline, treated with receptor-destroying enzyme (RDE) for 2 hr at 37 C, centrifuged at low speed, and twofold dilutions of the supernatant fluid were made in 2.5% sodium citrate. Each dilution was then mixed with the appropriate RBC (suspended in PBS), incubated and the degree of hemagglutination determined as in test 1.

[e] Lungs were homogenized in PBS, treated with RDE for 16 hr at 20 C and 2 hr at 37 C, centrifuged at low speed, and twofold dilutions of the supernatant fluid were made in 2.5% sodium citrate. Each dilution was then mixed with the appropriate RBC (suspended in 2.5% sodium citrate), incubated, and the degree of hemagglutination determined as in test 1. This method was similar to that described by Ginsberg and Horsfall (8).

carbazone (2, 27) and 5-(2,4-dichlorophenoxy)-2-thiouracil (1, 15, 26). 9-β-D-Arabinofuranosyladenine reportedly has antivaccinia virus activity in vitro (6, 7), but our studies are the first to demonstrate that the compound has marked in vivo activity against this virus. We consider the in vivo activity of this compound to be approximately equivalent to the antiviral activity of isatin-β-thiosemicarbazone. None of the other compounds listed in Table 5 was considered as active against the vaccinia virus as the thiosemicarbazones and 9-β-D-arabinofuranosyladenine.

Four uracil derivatives were active against in vivo vaccinia virus infections. 5-(2,4-Dichlorophenoxy)-2-thiouracil reportedly has a protective effect against intracerebrally or intranasally inoculated vaccinia virus in mice (1, 26); this activity was confirmed in the present studies. Smejkal et al. (24) found that 6-azauracil is active against the virus in vitro. The compound had moderate antivaccinia virus activity in the present study. It is apparent from our studies that the related compounds 5-[bis(2-chloroethyl)amino]-uracil and 5-(mercaptomethyl)uracil are also active against the virus in mice to a limited extent. The supply of 5-(mercaptomethyl)uracil was sufficient to carry out a single experiment only, but the activity observed was dose-responsive.

Treatment of vaccinia virus-infected mice with a cortisone derivative, 9-α-fluoro-2α-methylhydrocortisone 21-acetate, was effective in prolonging the mean survival time. This observation was interesting, since a number of reports (3, 9, 16) indicated that cortisone treatment usually enhances the infectious process.

5-Iodo-2′-deoxyuridine is known to be active against herpes and vaccinia viruses, both deoxyribonucleic acid agents in animals (11, 12). In our studies, the drug was apparently inactive against vaccinia virus infections in mice inoculated with the virus by the intracerebral route, but was effective in prolonging the mean survival time if the mice were inoculated with the virus by the intranasal route. These data suggest that this drug does not cross the blood-brain barrier, but is effective against infections in other parts of the body. In contrast, isatin-β-thiosemicarbazone and cytovirin were apparently less active against the intranasally inoculated agent. Streptonigrin, *N*-methylisatin β-thiosemicarbazone, and 5(2,4-

dichlorophenoxy)-2-thiouracil were essentially as active against both intracerebrally and intranasally inoculated vaccinia viruses.

Actinomycin D (NSC 3053), 6-aminonicotinamide (NSC 21206), 5-fluoro-2'-deoxyuridine (NSC 27640), and 5-bromo-2'-deoxyuridine (NSC 38297) have been shown by others (13, 18, 19, 24) to be active against vaccinia virus in cell culture, and in our studies treatment with each prolonged the life spans of vaccinia virus-infected mice in one or more experiments. This activity was not readily reproducible, however, so the compounds were not considered to have acceptable antiviral activity. 1-β-D-Arabinofuranosylcytosine has been reported by Renis and Johnson (Bacteriol. Proc., p. 140, 1962) and Buthala (4) to have antivaccinia virus activity in embryonated eggs and in cell culture, but no activity was seen in the present in vivo studies. Studies in L1210 leukemia systems have indicated that this compound is effective when given over a period of time which encompasses one or more doubling times of the leukemia cells (23). This finding prompted an attempt to demonstrate antiviral activity by treating infected mice constantly over a 24-hr period by use of a perfusion machine, but no activity was demonstrated. A single treatment 24 hr after virus inoculation was likewise ineffective.

A number of thiosemicarbazones and semicarbazones were evaluated in these studies, but only the two thiosemicarbazones cited above had consistent antivaccinia virus activity.

In vivo screening systems such as those described in this report for the evaluation of potential antiviral compounds seem to have real value for the demonstration of compounds having unequivocal activity against viruses. It is possible that such systems present a more severe challenge to the test compound than in vitro systems; hence, unless a compound is markedly active against the virus, this activity may not be demonstrable in vivo. Many investigators have reported a variety of compounds as having antiviral activity. Additional studies with these compounds often fail to confirm this activity unequivocally, particularly when in vivo systems are used. We feel that several criteria should be met before a compound is considered to have significant and acceptable antiviral activity. These criteria include demonstrable antiviral activity in a reasonable and orderly dose response, decreasing antiviral activity when an effective dose regimen of compound is used against progressively increasing concentrations of the infectious agent, readily reproducible antiviral activity in subsequent experiments carried out in an identical manner, and antiviral activity when acceptably nontoxic doses of the compound are used. No protocol for evaluating in vivo antiviral activity has achieved complete acceptance in the scientific community, but the procedures used in the present studies appear to be as sensitive and reproducible as any other procedures now known.

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Table 5. *Summary of experiments with compounds active[a] against in vivo vaccinia virus infections[b]*

Name	Virus[c]	Dose range (mg per kg per day)	Treatment scheduled[d]	10 LD_{50} of virus				32 LD_{50} of virus			
				Max survivors, T-C (%)	Survivors, P[e]	Max mean survival time increase (days)	Increase, P[f]	Max survivors, T-C (%)	Survivors, P[e]	Max mean survival time increase (days)	Increase, P[f]
Isatin β-thiosemicarbazone (NSC 721)	Ut	31.3–250	2	100	<0.001	>15.0	<0.001	100	<0.001	>15.0	<0.001
	Ut	125–250	1	100	<0.001	>15.0	<0.001	100	<0.001	>15.0	<0.001
	IHD	125–250	2	70	<0.001	4.5	<0.001	80	<0.001	3.5	<0.001
	IHD	125–250	1	80	<0.001	0.0	—	70	<0.001	1.5	<0.05
	WR	31.3–250	2	80	<0.001	0.9	>0.05	60	<0.001	1.9	<0.001
	WR	31.3–250	1	70	<0.001	0.9	>0.05	70	<0.001	1.8	<0.05
	IHD[g]	31.3–64.5	1	40	<0.05	0.7	>0.05				
6-Azauracil (NSC 3425)	IHD	125–1000	2	0	—	0.8	<0.05	10	>0.3	0.8	<0.05
	IHD	125–1000	2	0	—	0.4	>0.05	10	>0.3	0.6	<0.05
9α-Fluoro-2α-methylhydrocortisone 21-acetate (NSC 12601)	IHD	25–200	2	0	—	0.7	<0.05	0	—	1.4	<0.01
	IHD	50–100	2	0	—	1.1	<0.05	0	—	1.1	<0.05
	IHD	6.3–50	1	0	—	1.6	<0.001				
5-[Bis(2-chloroethyl)amino]uracil (NSC 34462)	IHD	0.2–1.6	2	0	—	1.1	<0.05	0	—	0.7	>0.05
	IHD	0.8–1.6	2	0	—	1.0	<0.001	0	—	0.0	—
	IHD	0.05–0.4	1	55	<0.05	2.2	<0.05				
5-Iodo-2′-deoxyuridine (NSC 39661)	IHD	37.5–300	2	10	>0.3	0.7	>0.05	0	—	0.3	>0.05
	IHD	37.5–300	2	10	>0.3	0.0	—	0	—	0.8	<0.05
	IDH[g]	37.5–75	1	50	<0.05	0.8	<0.05				
	IHD[g]	9.4–75	1	10	>0.3	1.6	<0.05	10	>0.3	0.7	<0.05
	IHD	37.5–300	1	0	—	0.4	>0.05	10	>0.3	0.1	>0.05
Streptonigrin (NSC 45383)	IHD	0.1–0.8	2	0	—	1.7	<0.001	0	—	1.3	<0.001
	IHD[g]	0.03–0.2	1	5	>0.3	1.2	<0.05	15	>0.3	0.8	<0.05
	IHD	0.03–0.05	1	0	—	0.0	—				
N-Methylisatin-β-thiosemicarbazone (NSC 69811)	Ut	31.3–250	1	80	<0.001	0.0	—	60	<0.001	0.5	>0.05
	IHD	7.1–57	2	50	<0.05	2.6	<0.001	60	<0.001	2.7	<0.05
	IHD	3.6–114	2	100	<0.001	>15.0	<0.001	90	<0.001	>15.0	<0.001

[a] Compounds were considered active if treatment of virus-infected mice resulted in dose-responsive significant increases in mean survival time or in number of survivors.

[b] ICR Swiss mice were infected intracerebrally with 0.03 ml of vaccinia virus, unless otherwise indicated.

[c] Viruses: *see* footnote *g*, Table 1.

[d] Treatment schedules: *see* footnote *e*, Table 1.

[e] P = probability that any increase in the number of survivors in the virus-infected, treated groups compared to the virus control group was due to chance, as determined by chi square analysis. $P < 0.03$ = significant, $P < 0.05$ = highly significant.

[f] P = probability that any observed increase in mean survival time of virus-infected, treated groups compared to the virus-control group was due to chance, as determined by t test. $P < 0.05$ = significant, $P < 0.001$ = highly significant.

[g] Virus was inoculated intranasally in these instances.

TABLE 5.—*Continued*

	IHD	28.5–57	1	90	<0.001	>15.0	<0.001			
	IHD	28.5–57	1	90	<0.001	>15.0	<0.001			
	IHD	42.8–342	7	30	<0.3	1.4	<0.05	30	<0.3	1.8 <0.05
	IHD	42.8–342	8	50	<0.05	0.5	>0.05	50	<0.05	1.0 <0.05
	WR	31.3–250	1	75	<0.001	1.8	<0.001	65	<0.001	1.6 <0.001
	IHD[a]	28.5–57	1	90	<0.001	>15.0	<0.001			
5-(2,4-Dichlorophenoxy)-2-thiouracil (NSC 73753)	IHD	37.5–300	2	35	<0.3	2.4	<0.001	0	—	0.5 >0.05
	IHD	75–600	2	10	>0.3	0.4	<0.05	10	>0.3	1.0 <0.001
	IHD	18.8–150	2	20	>0.3	0.1	<0.05	25	<0.3	1.2 <0.05
	IHD	18.8–37.5	1	45	<0.05	2.9	<0.001	15	>0.3	0.9 <0.05
	IHD[a]	9.4–75	1	65	<0.05	2.1	<0.001	15	>0.3	0.9 <0.05
Cytovirin (NSC 91770)	IHD	0.3–2	2	0	—	1.5	<0.001	5	>0.3	0.8 <0.05
	IHD	0.3–2	2	0	—	0.9	<0.001	5	>0.3	0.4 >0.05
	IHD[a]	0.3–0.5	1	10	>0.3	0.0	—			
	IHD[a]	0.3–0.5	1	5	>0.3	0.0	—			
9-β-D-Arabinofuranosyladenine (NSC 404241)	IHD	31.3–250	2	75	<0.001	2.1	<0.05	50	<0.05	2.1 <0.05
	IHD	31.3–250	2	50	<0.05	1.6	<0.05	50	<0.05	2.5 <0.001
5-(Mercaptomethyl)uracil (NSC 529351)	IHD	10–80	2	0	—	1.3	<0.05	0	—	1.1 <0.05

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