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In Vitro Effect of 1-β-D-Ribofuranosyl-1,2,4-Triazole-3-Carboxamide (Virazole, ICN 1229) on Deoxyribonucleic Acid and Ribonucleic Acid Viruses

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Virazole (1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide) is a highly soluble new synthetic nucleoside having significant, reproducible activity against a broad spectrum of deoxyribonucleic acid and ribonucleic acid viruses in vitro. The drug inhibited viral cytopathogenic effects in monolayers of cells infected for 3 days with type 3 adeno, types 1 and 2 herpes, myxoma, cytomegalo, vaccinia, infectious bovine rhinotracheitis, types 1A, 2, 8, 13, and 56 rhino, types 1 and 3 parainfluenza, vesicular stomatitis, subacute sclerosing panencephalitis, Semliki Forest, Newcastle disease, and measles viruses. Hemagglutinin production by influenza A_2, influenza B, and type 1 parainfluenza viruses in chicken embryo cells was reduced by Virazole treatment. Recoverable intra- and extracellular virus titers were reduced by the drug in experiments with type 1 herpes, vaccinia, type 3 parainfluenza, and vesicular stomatitis viruses. Plaque formation by type 1 herpesvirus was also inhibited by exposure of the infected cells to Virazole. Pretreatment of cells with the compound, followed by its removal before addition of type 1 herpesvirus, severely lessened the antiviral activity; the compound was still moderately effective in reducing the viral effects on the cells when added as long as 22 hr after the virus. Parallel experiments, in which the antiviral activity of a number of known active drugs was compared, indicated Virazole to have at least a comparable degree of activity, and it was also active against a wider variety of viruses than any of these known active materials. The $CCED_{50}$ of Virazole to chicken embryo cells was approximately 1,000 μg/ml, although concentrations as low as 10 μg/ml caused slight (15%) inhibition in total cellular protein after 72 hr of incubation.

The rapidly expanding knowledge of viruses and the mechanisms whereby they infect cells and cause disease has induced investigators in recent years to direct considerable effort toward discovering drugs for control of virus diseases. Despite such efforts, few antiviral drugs exist today which have clinical value, and these (e.g., methisazone, idoxuridine, adamantane, arabinosyladenine, arabinosylcytosine) are active only against a relatively small spectrum of deoxyribonucleic acid (DNA) or ribonucleic acid (RNA) viruses. An exception to this lack of broad-spectrum antiviral activity is the group of interferon inducers, none of which has yet been shown to have clinical usefulness. No synthetic compounds are known which have significant in vitro and in vivo antiviral activity against both DNA and RNA viruses.

The design and synthesis of a new chemical which appears to be one of the first synthetic, non-interferon-inducing broad-spectrum antiviral agents was recently reported by Witkowski et al. (11). A brief report summarizing the antiviral activity of this triazole nucleoside has been published by Sidwell et al. (10). The present series of papers describes the detailed quantitative in vitro and in vivo antiviral activity seen to date with this compound. The in vitro studies are presented in this report.

MATERIALS AND METHODS

Cell cultures. Monolayer cultures of human carcinoma of the nasopharynx (KB), rabbit kidney (RK-13), bovine embryonic trachea (EBTr), African green monkey kidney (Vero), human embryonic lung (WI-38), secondary mouse embryo (ME), and secondary chicken embryo (CE) cells were grown in Eagle minimal essential medium

(MEM; Grand Island Biological Co., Berkeley, Calif.), supplemented with 10% fetal bovine serum (FBS), penicillin (100 units/ml), and streptomycin sulfate (100 μg/ml).

Viruses. Type 1 herpes simplex virus strain HF (HSV/1), myxoma virus strain Sanarelli (MV), vaccinia virus strain Lederle CA (VV), murine cytomegalovirus strain Smith (MCMV), type 1A rhinovirus strain 2060 (RV/1A), type 13 rhinovirus strain 353 (RV/13), type 3 parainfluenza virus strain C243 (PIV/3), type 1 coxsackievirus B strain Conn-5 (CV/1), type 2 poliovirus strain MEF-1 (PV/2), type A_2 influenza virus/Aichi/2/68 (IV/A_2), and type B influenza virus strain Lee (IV/B) were obtained from Frank M. Schabel, Jr. (Southern Research Institute, Birmingham, Ala.). Type 2 herpes simplex virus strain MS (HSV/2), infectious bovine rhinotracheitis virus strain LA (IBRV), Semliki Forest virus strain "Original" (SFV), measles virus strain Edmonston (MV), and vesicular stomatitis virus strain Indiana (VSV) were purchased from the American Type Culture Collection (Rockville, Md.). Type 3 adenovirus strain GB (AV/3) was supplied by David A. Fuccillo (National Institute of Neurological Disease and Stroke, Bethesda, Md.). Francis E. Payne (University of Michigan, Ann Arbor) provided the SSPE measles virus strain MUN-HT (SSPEV). Type 2 rhinovirus strain HGP (RV/2), type 8 rhinovirus strain CU-MRH (RV/8), and type 56 rhinovirus strain CH82 (RV/56) were furnished by C. A. Phillips (University of Vermont, Burlington). Type 1 parainfluenza virus strain Sendai (PIV/1) was obtained from Elva Minuse (University of Michigan, Ann Arbor). A. S. Kaplan (Albert Einstein Medical Center, Philadelphia, Pa.) supplied the pseudorabies virus, a derivative of the Aujeszky strain (PRV). K. W. Cochran (University of Michigan, Ann Arbor) provided type A influenza virus strain NWS (IV/A), and C. F. Fox (University of California, Los Angeles) provided Newcastle disease virus strain L-Kansas (NDV).

Primary antiviral evaluations. The initial experiments with all viruses were carried out in MicroTest II tissue culture plates (Falcon Plastics, Division of BioQuest, Oxnard, Calif.), with inhibition of viral cytopathic effect (CPE) determined microscopically 3 days after virus was added to the cells. In these experiments, 1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide (Virazole) dissolved in MEM, supplemented with 5% FBS, penicillin, and streptomycin sulfate, was added 10 to 20 min after virus exposure. CPE inhibition by a test compound was evaluated statistically by a modification (9) of the virus rating procedure originally described by Ehrlich et al. (2). Details of the complete protocol for this type of chemotherapy experiment have been previously described (9).

Virus titer reduction experiments. For total virus determinations, the cells were frozen and thawed with the original medium 3 days after addition of the virus and drug. The resulting lysate was collected, and pools were prepared from replicate cups at each level of Virazole treatment. These samples were diluted in test medium (MEM plus 5% FBS, 100 units of penicillin/ml, and 100 μg of streptomycin/ml). Material from each dilution was added to replicate cups of KB cells in microplates which were then incubated at 37 C for 1 hr. The samples were removed from the cups and the cells were washed once with test medium. Fresh test medium was placed in each cup and the plates were incubated at 37 C. After 3 days, the cells were examined microscopically for evidence of viral CPE. The virus titer was determined by the Reed-Muench method for estimating 50% end points (7).

For intra- and extracellular virus determinations, the medium was collected from replicate cups at each level of Virazole treatment, pooled, and clarified by low-speed centrifugation. The clarified supernatant fluids were collected and used as the extracellular virus samples. The cells remaining in the cups were washed three times with test medium. After the last wash, 0.2 ml of test medium was added back to each cup, and the cells were frozen and thawed. The resulting lysates were collected and used as the intracellular virus samples. Both intra- and extracellular samples were titered as described above.

HA titration. Since in our hands influenza viruses induced relatively low degrees of CPE, in experiments with these viruses, as well as PIV/1, hemagglutinin (HA) production in CE cells was used. The methodology for adding drug and virus to the cells was identical to that described above for the CPE inhibition experiments. The HA titer of the combined intra- and extracellular material was determined by using 0.5% guinea pig or chicken red blood cells in Ca- and Mg-free phosphate-buffered saline (PBS) containing 1% bovine albumin Fraction V, 35% sterile serological solution (ICN Nutritional Biochemicals Corp., Cleveland, Ohio). The HA titer was recorded after 45 min of incubation at room temperature. Antiviral activity in these experiments was expressed as the ratio, 50% cytotoxicity dose ($CCED_{50}$) divided by the drug dose inhibiting 50% of the viral HA (VED_{50}).

Plaque formation. RK-13 cell monolayers in 60-mm (diameter) petri dishes (Falcon Plastics) were washed twice with sterile PBS. HSV/1 was diluted in test medium, and 0.5 ml was added to each test dish. Cell control dishes received 0.5 ml of test medium lacking virus. All dishes were placed at 37 C in a humidified CO_2 incubator (95% air, 5% CO_2) for 2 hr, after which the medium or virus preparation was removed. The cells were washed three times with test medium, and 5 ml of overlay material was added to each of the cell control and virus control dishes. The overlay consisted of test medium (twice concentrated) plus an equal volume of 3% methylcellulose (4,000 centipoise, laboratory grade, Fisher Scientific Co., Fair Lawn, N.J.) in distilled water. The same volume of overlay material, containing the indicated concentrations of Virazole, was added to the remaining

dishes. After a 4-day incubation at 37 C, the overlay material was gently rinsed off with several washes of Hanks balanced salt solution (HBSS). The cells were stained with Giemsa and the resulting plaques were counted.

Cytotoxicity determinations. To determine the effect of Virazole on cell viability, 3.0×10^5 RK-13 cells were seeded in 5-ml volumes in 60-mm plastic petri dishes (Falcon Plastics). After a 24-hr incubation at 37 C in a 5% CO_2 atmosphere, the medium was removed, and duplicate cultures were nourished with 2 ml of MEM containing 0.1% $NaHCO_3$, 5% FBS, and Virazole at concentrations of 1,000, 500, 100, 10, and 1 μg/ml. At 0, 24, 48, and 72 hr after addition of the drug, the medium was removed, 0.7 ml of 0.25% trypsin was added, and the cells were incubated at 37 C for 15 min. The cells were then gently scraped from the plates with a rubber policeman and suspended in 1.3 ml of PBS (0.02 M PO_4, pH 7.2; 0.15 M NaCl) containing 1% FBS. Each dish was also washed with 1.0 ml of the same saline suspending medium. Viable-cell counts were performed by use of the vital dye trypan blue. In this procedure, one part of 4.5% NaCl was mixed with four parts of 0.2% trypan blue, and an equal volume of this mixture was added to an equal volume of cell suspension. Those cells excluding the dye were counted in a hemacytometer.

Cytotoxicity was also determined by microscope examination of the drug-exposed cell layers, with numbers of floating cells, cell granulation, and alteration of cell shape used as indications of toxicity. The latter visual examination procedure was that used in the majority of the viral chemotherapy experiments.

Total cell protein was determined by the method of Oyama and Eagle (5). The cells were planted in plastic panels and allowed to grow overnight before being exposed to various concentrations of Virazole. The compound was left on the cells for 72 hr prior to the protein determination. The mean optical density of the treated cells (T) minus the mean optical density of the base line (C_0) divided by the mean optical density of the control cells (C) minus C_0 was plotted for each dose level of Virazole used. The slope of this line was utilized to determine the $CCED_{50}$.

RESULTS

The results of a typical experiment to determine the effect of Virazole on the growth of RK-13 cells are shown in Fig. 1. A definite decrease (from time zero) in number of viable cells was seen only at the 500 and 1,000 μg/ml levels of the drug, although the cell growth rate was inhibited in direct proportion to the Virazole concentration used at all levels tested.

The effect of Virazole treatment of KB cells infected with 320 and 3,200 $CCID_{50}$ (50% cell culture infectious doses) of HSV/1 is summarized in Table 1. Marked inhibition of viral CPE was seen at both virus levels, with the extent of CPE varying inversely with Virazole concentration. These CPE-inhibition data are supported by the titers of virus recovered from the same cells. The cytotoxicity indicated in Table 1 was determined visually and was evidenced primarily by enlargement of the cells in dosage levels as low as 32 μg/ml.

The viral CPE-inhibitory activity of Virazole against all of the DNA and RNA viruses studied is summarized in Table 2. Only PRV-, PV/2-, CV-, and RV/2-induced CPE were not signifi-

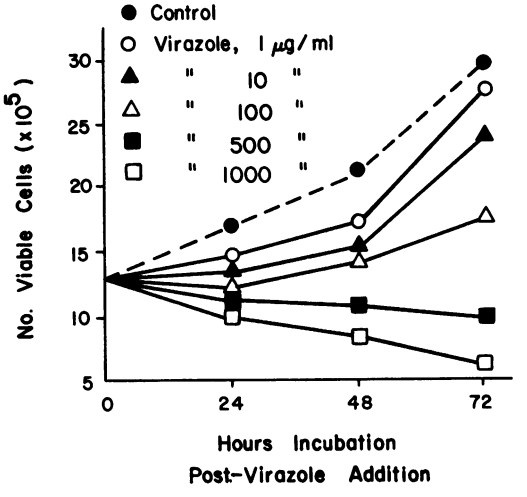

FIG. 1. *Effect of Virazole on growth of RK-13 cells.*

TABLE 1. *Effect of Virazole on type 1 herpes simplex virus in KB cells*

Virazole concn (μg/ml)	Visible cytotoxicity	Drug + 320 $CCID_{50}$ of virus		Drug + 3,200 $CCID_{50}$ of virus	
		CPE	Virus recovered[a]	CPE	Virus recovered[a]
1,000	++	0 0 0	10^0	0 0 0	10^0
320	+	0 0 0	$10^{0.5}$	0 0 0	$10^{1.5}$
100	+	0 0 0	$10^{1.0}$	0 0 0	$10^{1.0}$
32	+	1 1 1	$10^{2.0}$	1 1 1	$10^{2.5}$
10	−	1 1 1	$10^{4.0}$	3 3 2	$10^{6.0}$
3.2	−	1 1 1	$10^{3.5}$	4 4 4	$10^{5.6}$
1.0	−	2 2 2	$10^{4.5}$	4 4 4	$10^{7.5}$
0		3 4 4	$10^{4.7}$	4 4 4	$10^{7.5}$
Virus rating		1.4		0.9	

[a] Total intra- and extracellular virus recovered, expressed as $CCID_{50}$ per 0.2 ml.

TABLE 2. *Summary of the viral CPE-inhibitory activity of Virazole*

Virus[a]	Cells	No. of expt	Avg VR[b]	Median MIC[c] (μg/ml)
DNA viruses				
AV/3	KB	7	0.7	32
HSV/1	KB	21	1.1	1
HSV/1	RK-13	3	1.3	1
HSV/1	CE	2	1.4	0.32
HSV/1	Vero	2	0.4	100
HSV/1	WI-38	2	0.4	100
HSV/1	HeLa	1	1.4	0.32
HSV/2	KB	2	1.1	1
HSV/2	CE	1	0.4	100
HSV/2	RK-13	1	0.8	3.2
HSV/2	Vero	2	0.0	—
PRV	RK-13	3	0.0	—
PRV	Vero	1	0.2	1,000
MV	RK-13	2	1.5	3.2
MCMV	ME	1	0.9	3.2
VV	KB	3	1.0	3.2
VV	HeLa	1	0.6	32
VV	Vero	1	0.4	320
VV	CE	1	0.8	3.2
IBRV	EBTr	2	0.9	10
RNA viruses				
RV/1A	KB	3	0.9	10
RV/2	KB	1	0.4	100
RV/8	KB	1	0.8	32
RV/13	KB	15	0.7	32
RV/56	KB	3	0.9	10
PIV/1	KB	1	0.9	10
PIV/1	Vero	3	0.7	32
PIV/1	CE	3	0.9	3.2
PIV/3	KB	15	0.8	10
CV	KB	5	0.4	100
PV/2	KB	6	0.2	1,000
VSV	KB	4	0.8	32
VSV	RK-13	5	1.0	3.2
VSV	Vero	1	0.4	320
SSPEV	Vero	1	0.8	10
SFV	CE	2	0.6	32
NDV	CE	2	1.1	3.2
MeV	Vero	2	0.6	32

[a] Full names and sources of viruses are given in Materials and Methods.
[b] Virus rating.
[c] Minimal inhibitory concentration.

cantly influenced by this drug in any experiment, although in later experiments, when Virazole was added to the cells at least 1 hr prior to PRV or PV/2, moderate CPE inhibition (virus rating, 0.4 to 0.5) was demonstrated.

Inhibition of IV/A_2, IV/B, and PIV/1 HA production by Virazole treatment is shown in Table 3. In these experiments, the highest degree of activity was seen against PIV/1. The cytotoxicity of Virazole in this study was determined both by total protein level (Fig. 2) and by microscope examination of the toxicity controls; both findings are indicated in Table 3. Although the cells appeared normal visually, slight reductions in total protein were apparent at drug concentrations of 10 and 3.2 μg/ml. The $CCED_{50}$ of Virazole in chicken embryo cells appeared to be approximately 1,000 μg/ml. Treatment of cells infected with VV, PIV/3, and VSV with various concentrations of Virazole resulted in reduction of

TABLE 3. *Effect of Virazole on hemagglutinin (HA) production in influenza and parainfluenza virus-infected chicken embryo cells*

Drug concn (μg/ml)	Cytotoxicity		HA titer/0.1 ml		
	Protein[a]	Visual[b]	IV/A_2[c]	IV/B	PIV/3
1,000	48	++	1:4	<1:4	<1:2
320	40	+	1:4	<1:4	<1:2
100	32	+	1:8	<1:4	1:2
32	24	±	1:8	1:4	1:2
10	15	−	1:16	1:4	1:4
3.2	8	−	1:16	1:4	1:8
1	0	−	1:16	1:8	1:8
0			1:16	1:8	1:16
$CCED_{50}$ /VED_{50}			30	300	1,000

[a] Approximate percent cytotoxicity based on total protein level, determined from the slope shown in Fig. 2.
[b] Microscope examination of cells, with cytotoxicity manifested as granulation and alteration of cell shape.
[c] Full names of viruses are given in Materials and Methods.

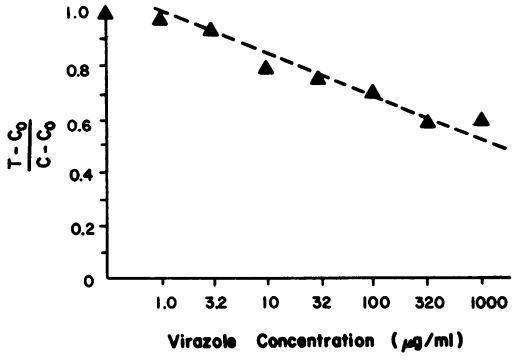

FIG. 2. *Effect of Virazole on chicken embryo cell growth as determined by total protein levels. Data expressed as the slope, where T = treated cell optical density, C_0 = base line control optical density, and C = untreated (control) cell optical density.*

both intra- and extracellular virus titer (Fig. 3–5). Concentrations of the drug as low as 10 μg/ml were effective in reducing the number of plaques produced by HSV/1 in RK-13 cells (Table 4).

In a timed experiment, Virazole significantly inhibited HSV/1 CPE when added as late as 22 hr after exposure of KB cells to the virus (Table 5). The activity of the compound began to decline at these later time intervals, however. If the cells were incubated with Virazole for 2 hr, the drug was removed, and the cells were washed prior to addition of the virus, the antiviral activity was markedly reduced.

A comparison of the in vitro antiviral activity of several previously reported antiviral compounds with the activity of Virazole is summarized in Table 6. All cytotoxicity, virus ratings, and $CCED_{50}/VED_{50}$ values were obtained in our laboratory under similar conditions for each compound.

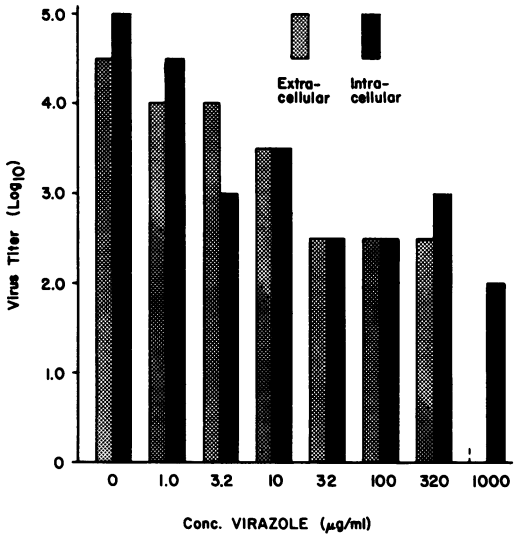

FIG. 3. *Effect of Virazole on intra- and extracellular vaccinia virus titers in KB cells.*

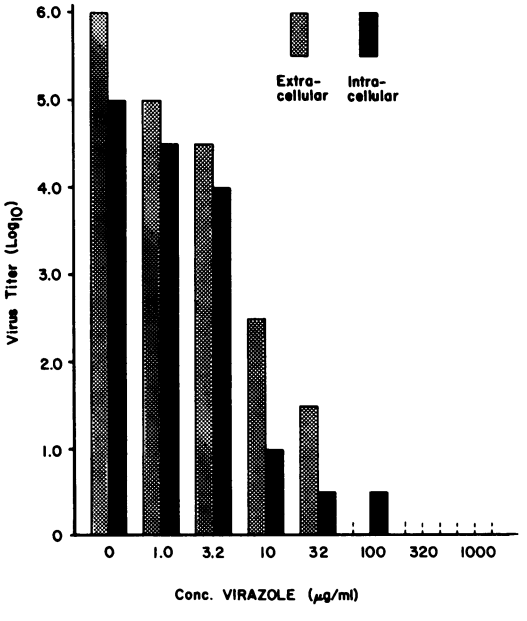

FIG. 4. *Effect of Virazole on intra- and extracellular type 3 parainfluenza virus titers in KB cells.*

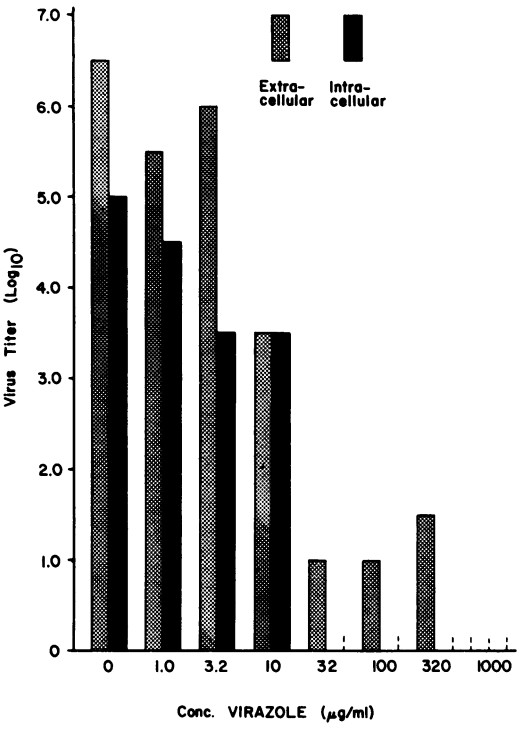

FIG. 5. *Effect of Virazole on intra- and extracellular vesicular stomatitis virus titers in KB cells.*

TABLE 4. *Effect of Virazole on plaque formation by type 1 herpesvirus in RK-13 cells*

Virazole concn (μg/ml)	No. of plaques per plate
1,000	0
320	0
100	0
32	6
10	112
3.2	>400
0	>400

DISCUSSION

The discovery of a synthetic nucleoside which exerts readily demonstrable inhibitory effects against a wide variety of both DNA and RNA viruses is most significant. In the present report, Virazole was highly active against 13 RNA and 7 DNA viruses, as determined in repeated experiments. This activity was discernible with a variety of cell systems and parameters for measuring antiviral activity. Comparative studies run in parallel with other known active antiviral materials imply that Virazole has as great, if not a greater, degree of activity against the pertinent viruses as each of the known active drugs.

TABLE 5. *Effect of time of addition of Virazole on antiherpesvirus activity in KB cells*

Virazole addition[a]	Virus rating	MIC[b] (μg/ml)
2 hr pre, removed prior to virus	0.6[c]	32
2 hr pre	1.4	1.0
1 hr pre	1.4	1.0
0 hr	1.4	1.0
1 hr post	1.4	1.0
2 hr post	1.4	1.0
4 hr post	1.4	1.0
6 hr post	1.2	3.2
8 hr post	1.1	3.2
10 hr post	0.8	10
22 hr post	0.8	10

[a] Time of addition of Virazole relative to exposure of cells to virus.

[b] Minimal inhibitory concentration.

[c] Discernible cytotoxicity eliminated by removal of the compound in this test.

In our own experience, as well as that of other investigators (4, 6), it is important that the activity of an antiviral compound be determined in a number of cell systems, since that activity can be quite varied in dissimilar systems. The lesser degree of Virazole anti-DNA virus activity in Vero cells confirms this. In spite of the susceptibility of these cells to many viruses (3), the DNA virus inhibitory activity of Virazole may not have been discovered had Vero cells been used as the primary evaluation system. It is unclear why the drug is less active in these cells, although it may be speculated that they are producing a metabolite which at least partially blocks or reverses the antiviral activity of the compound.

The mode of antiviral action of Virazole was not fully explored in the present study. Other experiments have indicated that the drug is apparently not virucidal and that it is not an inducer of interferon (10). The timing experiments indicate that the drug may be inhibiting an early stage of viral replication, although the effect on virus adsorption to, or penetration into, the cell was not explored in these experiments. The compound apparently must remain in contact with the cell to exert its antiviral action, since its removal from the medium surrounding the cell reverses its activity.

It is recognized that in vitro antiviral activity cannot be used as a major criterion to judge the eventual clinical applicability of a test chemical, since other investigations (1, 8) have demonstrated that a material active in cell culture may not be active in animal systems. It is significant, then, that the antiviral activity of Virazole has been confirmed in a variety of animal virus ex-

TABLE 6. *Comparison of the antiviral activity of Virazole and other known antiviral compounds*

Compound	Virus rating							$CCED_{50}/VED_{50}$		
	HSV/1[a]	VV	MV	PRV	PIV/3	RV/13	PV/2	IV/A2	IV/B	PIV/1
Virazole	1.2	1.0	1.5	0.0	0.8	0.7	0.2	30	300	1,000
9-β-D-Arabinofuranosyladenine	1.1	0.9	0.8	1.1	0.1	0.1	0.0	0.0	0.0	0.0
1-β-D-Arabinofuranosylcytosine	1.3	0.8	0.6	0.4	0.1	0.1	0.0	0.0	0.0	0.0
5-Iodo-2′-deoxyuridine	1.4	1.3	0.8	0.6	0.0	0.0	0.0	0.0	0.0	0.0
1-Adamantanamine·HCl	0.2	0.3	0.0	0.1	0.0	0.4	0.0	30	10	30
2-Methyl-4[5-methyl-5H-*as*-triazino(5,6-b)indol-2-yl amino]-2-butanol	0.2	0.0	0.0	0.0	0.0	0.7	0.0	300	3	10
Guanidine·HCl	0.0	0.0	0.0	0.0	0.0	0.0	0.6	0.0	0.0	0.0

[a] Full virus names are given in Materials and Methods.

periments (10). Subsequent reports will further describe this in vivo activity.

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