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INHIBITION OF REOVIRUSES IN VITRO BY SELECTED ANTIVIRAL SUBSTANCES

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The antiviral effect of 1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide (ribavirin), 3-deazaguanine (3-DG), 3-deazauridine (3-DU) and 9-(*S*)-(2,3-dihydroxypropyl)adenine ([*S*]-DHPA) on reovirus types 1, 2 and 3 replication and host cell functions are described. Inhibition of cytopathic effect (CPE) and of immunofluorescent cell counts (IFCC) were determined to assess antiviral activity in Madin–Darby bovine kidney (MDBK) cells. Ribavirin showed the strongest inhibitory effects with inhibition at 3.2 μg/ml. 3-DG and 3-DU had moderate activity and (*S*)-DHPA exhibited only weak effects. There was little difference in the degree of inhibition among reovirus serotypes. Antiviral effects were reversed by guanosine (for ribavirin and 3-DG), uridine (for 3-DU) and adenosine (for [*S*]-DHPA). Comparative studies with reovirus type 3 indicated both CPE and IFCC inhibition to be approximately equally sensitive parameters. In radiolabel uptake studies, each compound moderately inhibited uptake of radioactive precursor molecules at dosage levels where viral inhibition was observed, suggesting a lack of specific reovirus inhibitory effects.

reovirus ribavirin 3-deazauridine 3-deazaguanine 9-(*S*)-(2,3-dihydroxypropyl)adenine, antiviral

INTRODUCTION

Reovirus has become the prototype for a large group of animal, plant, fungal and bacterial viruses that contain segmented double-stranded (ds) RNA genomes [9, 12, 19]. The three serotypes of reovirus are now classified as a distinct genus within the family Reoviridae. They have been equivocally associated with a variety of clinical illnesses [2], but their exact role in the etiology of naturally occurring human diseases is still uncertain. They do cause death [25], teratogenic effects [7], and a juvenile onset diabetes syndrome in mice [16]. Reoviruses have the potential of being human pathogens, especially since they are associated with the respiratory and intestinal tract of man [22]. They represent a rather unique virus group with which to evaluate antiviral compounds.

There have been a few reports of attempts to inhibit reoviruses by antiviral substances. We evaluated 1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide (ribavirin) for efficacy against type 1 reovirus [23], with inhibition seen at 3.2 μg/ml in vitro. Reovirus type 3 has been inhibited in vitro by 6-azauridine [17] and methisazone [3]. The present

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study was run to determine the relative effects of four significant, known RNA virus inhibitors on the three reovirus serotypes. A particular effort has been made to define more fully the usefulness of available parameters for evaluating in vitro anti-reovirus activity and to determine biochemically whether the inhibition seen by the compounds was specific for these viruses.

MATERIALS AND METHODS

Antiviral compounds

Ribavirin, 3-deazaguanine (3-DG), and 3-deazauridine (3-DU) were obtained from ICN Pharmaceuticals, Inc. (Covina, CA). 9-(*S*)-(2,3-dihydroxypropyl)adenine ([*S*]-DHPA) was provided by Dr. Eric De Clercq, Rega Institute of the Catholic University of Leuven (Leuven, Belgium). Each was dissolved in cell culture medium at an initial concentration of 2000 μg/ml for use in these studies. Drug-containing medium was stored at 4°C for 2–3 months with no loss of antiviral activity.

Viruses

Reovirus type 1 (Lang strain), type 2 (Jones strain) and type 3 (Abney strain) were obtained from the Viral and Rickettsial Disease Laboratory, California State Department of Public Health (Berkeley, CA). Pools of each virus were prepared in bovine kidney (MDBK) cells and stored at -90°C. All viral stocks were made in the absence of fetal bovine serum and proteolytic enzymes [26].

Cells

Madin–Darby bovine kidney (MDBK) cells, obtained from the American Type Culture Collection (Rockville, MD) were used for experiments. Cells were grown in Eagle's minimum essential medium (MEM) supplemented with 10% fetal bovine serum, 0.19% $NaHCO_3$ and 50 μg/ml gentamycin. The above medium containing 5% serum was used in the antiviral tests. The cells were judged to be mycoplasma-free based upon selectivity media culture analysis done in our laboratory; selected samples were also evaluated and found free of mycoplasma contamination by Microbiological Associates (Bethesda, MD).

Antiviral experiments

Two methods were employed to determine antiviral activity. These were by inhibition of viral cytopathic effect (CPE), used for all three virus serotypes, and by reduction in actual numbers of virus-infected cells as determined by immunofluorescent cell counting (IFCC), used in studies with type 3 reovirus only. The CPE inhibition experiments were

run in 96-well disposable plastic microplates (Vangard International, Neptune, NJ), using the method of Sidwell and Huffman [20], and as described in a recent report [24]. The IFCC studies were run using the method of McClain et al. [13] in 24-well disposable microplates [23].

For each antiviral method, activity was expressed as minimum inhibitory concentration (MIC) in which 10% or greater inhibition of CPE or IFCC-stained virus-infected cells was considered indicative of positive antiviral effect, and by virus rating (VR), a numerical indication of antiviral activity which takes into account the degree of inhibition of viral CPE and any cytotoxicity seen [20]. In previous experiments, we have considered a virus rating of <0.5 to indicate slight or no antiviral effect, 0.5–0.9 to indicate moderate antiviral effect, and 1.0 or greater to be indicative of marked antiviral effect. Since biochemical parameters used in the present studies have increased the sensitivity of detecting cytotoxicity, the interpretation of VR has been modified as follows: $\leqslant 0.3$, weak antiviral effect; 0.4–0.7, moderate antiviral effect; and $\geqslant 0.8$, marked antiviral effect.

Reversal studies were conducted using CPE inhibition in 96-well plates and evaluated as described above. In these experiments, the medium also contained guanosine (for ribavirin and 3-DG), uridine (for 3-DU), or adenosine (for [*S*]-DHPA) in concentrations varying by one-half $\log_{10}$ increments from 32–1000 μg/ml.

Extracellular virus titer reductions were determined by first growing reovirus type 3 in the presence of ribavirin and placebo. Inoculum virus was aspirated off wells after 1.5 h absorption, wells washed once with Earle's balanced salt solution, and fresh medium applied so as to remove unadsorbed inoculum virus. Fluids were removed from wells when placebo controls exhibited 100% CPE, then were titrated by serial dilution onto fresh monolayers of MDBK cells in 96-well plates, four wells being used for each dilution. Virus titers were calculated by 50% end-point determination method [18].

Biochemical cytotoxicity determinations

To further elucidate possible cytotoxic or cytostatic effects of the various compounds studied the effects of each compound on cellular macromolecule synthesis were determined. Experiments were run in 24-well disposable microplates using four wells for each drug concentration and eight wells for the drug-free control. Uptake of [^{3}H]thymidine, [^{3}H]uridine and [^{3}H]leucine were used for studies of DNA, RNA and protein synthesis, respectively. [^{3}H]Adenosine was used in RNA experiments with 3-DU, since that compound markedly inhibits [^{3}H]uridine uptake into cells [24]. All radiolabeled materials were obtained from ICN Chemical and Radioisotope Division (Irvine, CA). Procedures for quantifying radioactivity of acid-soluble and acid-insoluble fractions of cells were described previously [24]. Modifications of that procedure used in the present study are as follows. The cells were labeled with 2 μCi of [^{3}H]precursor/ml, the trichloroacetic acid (TCA)-soluble sample was assayed by counting 0.1 ml of solution/well after precipitation of cells with TCA, and cell homogenates labeled with [^{3}H]adenosine (3-DU

studies) were treated with 200 Kunitz/ml DNAase 1 (Sigma Chemical Corp., St. Louis, MO) for 2 h at 37°C prior to precipitation.

RESULTS

Table 1 shows virus ratings and minimal inhibitory concentrations for each antiviral compound against the three reovirus serotypes. Ribavirin exerted the strongest inhibitory effect and (*S*)-DHPA the least, using the CPE inhibition parameter. 3-DG and 3-DU showed a moderate viral inhibition. For ribavirin, 3-DG and 3-DU there was little variation of antiviral activity among reovirus strains. Reovirus type 2 appeared less sensitive to (*S*)-DHPA than the other serotypes in repeated studies. Comparison of CPE inhibition and IFCC reduction indicated both methods to be nearly equally sensitive as antiviral indicators. The IFCC assay was less sensitive in evaluating 3-DG.

The appropriate natural nucleoside added to the culture medium reversed the antiviral activities of all four test agents (Table 2). For ribavirin, 3-DU and (*S*)-DHPA a progressive loss of activity was observed as the concentration of natural nucleoside increased. The activity of 3-DG was completely reversed by 32 μg guanosine/ml or greater concentration.

Since ribavirin was the most active antiviral substance, it was evaluated in two further studies. Fig. 1 demonstrates the effect of ribavirin on extracellular reovirus titers for the three serotypes. A marked inhibition of virus production was observed at 32 or greater μg of drug/ml. The reovirus serotypes varied little in their relative sensitivities to the compound when evaluated by this method. In studies run to determine the effect of time of addition of ribavirin to virus-infected cells, the agent showed moderate anti-reovirus activity (VR of 0.6, MIC of 32 μg/ml) even when applied 6 h post-infection. The greatest efficacy was observed when drug was applied 1 h before or at the time of infection.

The effects of compounds on cellular macromolecule synthesis are presented in Table 2. Ribavirin inhibited the incorporation of [^{3}H]leucine, [^{3}H]uridine and, to a lesser extent, [^{3}H]thymidine into TCA soluble pools, and corresponding inhibitions were observed in the TCA-insoluble fractions. Both 3-DG and 3-DU inhibited [^{3}H]thymidine incorporation into the TCA precipitate but not into the soluble portion, which suggests that the compounds inhibit cellular DNA synthesis. 3-DU and (*S*)-DHPA inhibited uptake of [^{3}H]leucine into both the acid-soluble and the acid-insoluble fractions. By comparing minimal virus-inhibitory drug concentrations in Table 1 to cytotoxic or cytostatic concentrations in Table 2, one notes that cellular and viral-inhibitory effects were evident at the same dosage levels.

DISCUSSION

The results obtained in this study show that each of the four known anti-RNA virus inhibitors inhibited the reoviruses of the three serotypes, although the effects of (*S*)-DHPA were marginal. Ribavirin was the most viral-inhibitory of the compounds tested. The agent

TABLE 1

Inhibition of reovirus-induced CPE[a] and IFCC[b] in MDBK cells by antiviral substances, and reversal of activity by natural nucleosides

Reovirus type[c]	Assay method	Natural nucleoside[d] in medium (μg/ml)	Ribavirin		3-DG		3-DU		(*S*)-DHPA	
			VR[e]	MIC[f]	VR	MIC	VR	MIC	VR	MIC
1	CPE	0	0.9	3.2	0.6	10	0.6	10	0.2	320
2	CPE	0	1.0	3.2	0.6	10	0.6	10	0.1	1000
3	CPE	0	1.0	3.2	0.7	10	0.6	10	0.4	100
3	IFCC	0	0.9	3.2	0.3	100	0.5	32	0.4	100
3	CPE	32	0.9	10	0	> 1000	0.7	10	0.2	320
3	CPE	100	0.8	10	0	> 1000	0.6	10	0.1	1000
3	CPE	320	0.6	32	0	> 1000	0.4	100	0.1	1000
3	CPE	1000	0.3	100	0	> 1000	0.3	100	0.1	1000

[a] Cytopathic effect inhibition.

[b] Reduction in numbers of immunofluorescing cells across diameter of microwell.

[c] Virus concentrations at infection (IFCC/0.1 ml): Reo 1, $10^{3.0}$; Reo 2, $10^{3.5}$; Reo 3, $10^{3.0}$.

[d] Guanosine in medium with ribavirin and 3-DG, uridine with 3-DU, and adenosine with (*S*)-DHPA.

[e] Virus rating.

[f] Minimum inhibitory concentration (μg/ml).

TABLE 2

Effect of antiviral substances on [^{3}H]leucine, [^{3}H]uridine, [^{3}H]adenosine, and [^{3}H]thymidine uptake and incorporation into MDBK cells[a,b]

Drug	Minimum inhibitory concentration[c]					
	[^{3}H]leucine		[^{3}H]uridine or [^{3}H]adenosine		[^{3}H]thymidine	
	Acid-soluble	Acid-insoluble	Acid-soluble	Acid-insoluble	Acid-soluble	Acid-insoluble
Ribavirin	3.2	3.2	3.2	3.2	1000	1000
3-DG	> 1000	> 1000	> 1000	> 1000	> 1000	10
3-DU	10	10	> 1000	> 1000	> 1000	32
(*S*)-DHPA	32	32	> 1000	> 1000	> 1000	> 1000[d]

[a] [^{3}H]Adenosine used for 3-DU experiments only. Cell homogenates were treated with DNAase prior to precipitation and processing for liquid scintillation analysis.

[b] 18-h established monolayer treated with compound 24 h, followed by a 1 h pulse with radioactive molecule.

[c] That concentration of drug inhibiting uptake or incorporation by at least 20% of concomitantly run controls.

[d] Enhanced uptake or incorporation seen at (*S*)-DHPA concentrations of $\geq$ 32 μg/ml.

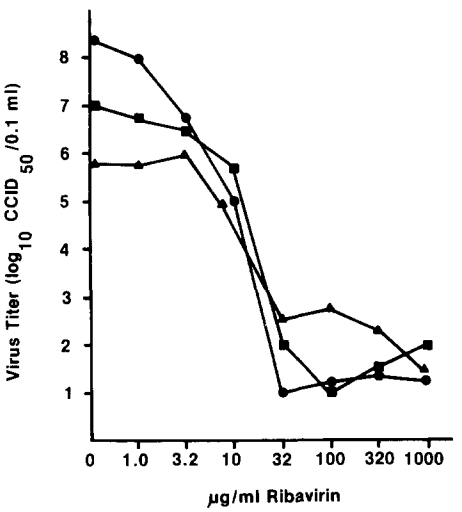

Fig. 1. Effect of ribavirin on infectious reovirus released from MDBK cells. Reo 1 (■—■). Reo 2 (▲—▲). Reo 3 (●—●).

inhibited reovirus-induced CPE even when added to cells as late as 6 h post-infection. Others have observed this same effect using ribavirin against different viruses [8]. The reovirus serotypes varied little in their relative sensitivities to each antiviral compound. The minimal inhibitory doses of drugs against reoviruses compared well to the degrees of inhibition seen by these same agents against other viruses [1, 6, 8, 10, 24].

The fact that certain natural nucleosides reverse the antiviral activities of the test compounds has been reported previously for ribavirin [28] and 3-DU [10]. The activity of 3-DG was completely reversed at a low dose of guanosine. This is evidence that the active form of the drug may be as the ribofuranosyl [27], and that the conversion of 3-DG to 3-deazaguanosine or to phosphorylated derivatives is not likely to occur in the presence of excess guanosine.

Much emphasis has recently been placed on more sensitive methods for measurement of possible cytotoxicity, with increasing use being reported of the radiolabeled metabolic precursors [4, 11, 14]. Of importance was our use of confluent cell monolayers in contrast to rapidly dividing cells used in similar studies by others [11, 15]. We used cell monolayers, exposed for a relatively long period of time to the test compounds prior to pulsing, in order to more closely duplicate conditions of the antiviral experiments. Our studies indicate that 3-DG and 3-DU inhibit DNA synthesis in MDBK cells. Ribavirin, 3-DU and (*S*)-DHPA inhibited incorporation of [^{3}H]leucine into the cytoplasmic pool, with corresponding inhibition observed in the acid-insoluble fraction. Ribavirin also influenced the uptake of [^{3}H]uridine and [^{3}H]thymidine into the soluble and insoluble pools. Many of the earlier studies with ribavirin [11, 15] do not report on the TCA-soluble fractions, so one cannot determine the exact nature of macromolecular synthesis inhibition. Canonico and colleagues [5] reported a reduced [^{3}H]uridine uptake in both acid-soluble and acid-insoluble fractions in ribavirin-treated BHK-21 cells. Because the degree of inhibition was nearly the same for both fractions, they concluded that the compound did not inhibit cellular RNA synthesis per se, but rather reduced the level of the intracellular soluble uridine pool. By the same rationale, the results of these studies suggest that the inhibition of [^{3}H]uridine uptake by ribavirin and the inhibition of [^{3}H]leucine by ribavirin, 3-DU and (*S*)-DHPA is a result of a shift in soluble cytoplasmic levels of uridine or leucine. The significance of these shifts in cytoplasmic pool sizes is not presently understood, but may possibly suggest a slowdown in cellular metabolism due to cytostatic or cytotoxic drug effects.

De Clercq et al. [6] found that (*S*)-DHPA in concentrations as high as 200 μg/ml did not significantly reduce DNA, RNA or protein synthesis in HeLa, Vero, and primary rabbit kidney cells when incubated with the radiolabel and drug for 20 h. They did not show their complete data and made no comment about TCA-soluble levels, however. An interesting result was our finding that (*S*)-DHPA enhanced incorporation of [^{3}H]thymidine into cellular DNA, an enhancement we have also observed in MA-104 cells [24]. This enhancement was over 350% of controls at 1000 μg/ml. A more definitive assay will need to be performed to ascertain whether the observed increase in radiolabeled precursor incorporation was the result of an absolute increase in DNA synthesis or simply synthesis of macromolecules with higher specific activity.

Since each drug influenced cellular processes at doses where antiviral activity also occurred, specific antiviral effects were not achieved in these experiments. The apparent cytostatic or cytotoxic effects, however, may not correlate well with the toxicity of drug in man or animals. From animal studies conducted by us and reported by others, ribavirin [21] and (*S*)-DHPA [6] are well tolerated in mice, whereas 3-DG is 20–40 times more toxic when delivered over several days. 3-DU has not been tested in our in vivo systems. Further studies in the mouse model may establish the usefulness of these agents in treating reovirus infections in animals.

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