Inhibition of RNA Virus Replication *in Vitro* by 3-Deazacytidine and 3-Deazauridine (36571)

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The pyridine nucleoside derivatives (Fig. 1), 3-deazacytidine (deaza CR) and 3-deazauridine (deaza UR), have recently been synthesized (1, 2). Subsequent experiments (3) have demonstrated that these compounds inhibit growth of *Streptococcus faecium, Escherichia coli*, Ehrlich ascites and leukemia L-1210 cells. Recent metabolic studies of Wang and Bloch (4) have shown that these pyrimidine nucleoside analogs are phosphorylated by extracts of Ehrlich ascites and intact leukemia L-1210 cells.

The present investigation was, therefore, undertaken to study the effect of these pyridine nucleoside derivatives on replication of various RNA viruses in mammalian or avian cell cultures.

Materials and Methods. Cell and growth media. Human carcinoma of the nasopharynx (KB), African green monkey kidney (Vero), and secondary cultures of chicken embryo (CE) cells were generally used. Primary cultures prepared from 9-day-old embryonated chicken eggs were used for the preparation of secondary cultures. Cells were seeded in 0.2 ml volume in each well of micro tissue culture plates (Falcon Plastics, Oxnard, CA) at a concentration of 1.8×10^5 cells/ml. Routinely, Eagle's minimum essential medium (MEM) containing 10% heat-inactivated fetal bovine serum (FBS) and 0.1% $NaHCO_3$ was used for cell culture and MEM containing 5% FBS and 0.25% $NaHCO_3$ (test medium) was used for virus propagation and other experiments.

Viruses. The viruses used were type 2 poliovirus (PoV); type 1A rhinovirus (RV), strain 2060; type 13 RV, strain 353; type 56 RV, strain CH 82; type 1 parainfluenza (Sendai) virus, strain murine; type 3 parainfluenza (HA-1) virus, strain C243; vesicular stomatitis virus (VSV), strain Indiana; type A_2 influenza virus, strain Aichi/2/68; and type B influenza virus, strain Lee. The type 1A and 13 RV, PoV, HA-1 virus, influenza A_2 and B viruses were provided by F. M. Schabel, Jr. (Southern Research Institute, Birmingham, AL), the type 56 RV was received from C. A. Phillips (The University of Vermont, Burlington); the Sendai virus was supplied by E. Minuse (University of Michigan, Ann Arbor); and VSV was obtained from the American Type Culture Collection (Rockville, MD). The HA-1 virus, RV and PoV were grown in KB cells. The VSV was passaged in mouse L-929 cells. Sendai and influenza A_2 viruses were propagated in the allantoic cavity of 9-day-old embryonated chicken eggs. The influenza B virus was the lung preparation from infected mice.

Chemicals. The 3-deazapyrimidine nucleosides were prepared at this Institute according to the procedure of Currie, Robins and Robins (2). The uridine and cytidine were obtained from ICN Nutritional Biochemicals, Cleveland, OH.

Testing procedure. An 18-hr monolayer of various cells on micro tissue culture plates was used for the test. The virus dilution containing a known amount of cell culture 50% infectious doses ($CCID_{50}$) in 0.1 ml volume was added to each well approximately 15 min before the addition of the compound. The virus dose per well was 3.2 $CCID_{50}$ for PoV, 32 $CCID_{50}$ for VSV, 100 $CCID_{50}$ for RV, 100 $CCID_{50}$ for influenza A_2 and B viruses, and 320 $CCID_{50}$ for Sendai and HA-1 viruses. The compound to be tested was diluted in the test medium in 0.5 log dilutions and 0.1 ml of these dilutions was added to each well so that the final

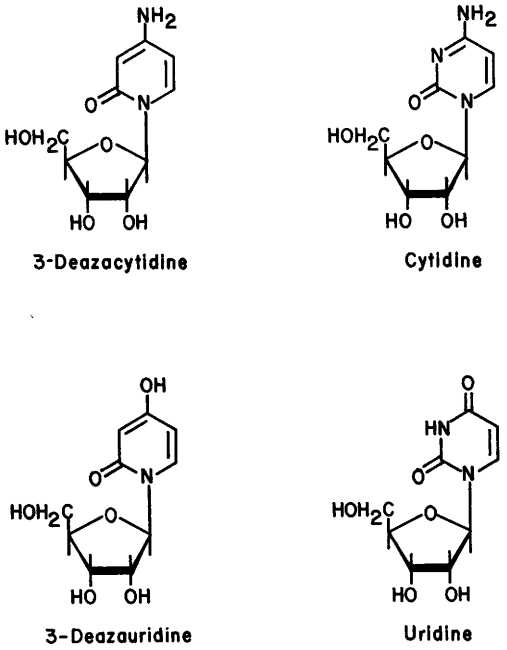

FIG. 1. Structural relationship of the two pyridine nucleosides and the respective pyrimidine nucleoside derivatives.

dilutions of the compound ranged from 1000 to 1.0 μg/ml. The plates were sealed with Saran Wrap (Dow Chemical Co., Midland, MI), and incubated at 33° for rhinoviruses and at 37° for other viruses. The readings on virus-induced cytopathogenic effect (CPE) were taken 3 days postinfection and the effectiveness of a particular compound against a given virus was determined statistically by the virus rating (VR) procedure (5). Each antiviral chemotherapy experiment also included the respective cell, virus and toxicity controls which were considered in the calculation of each VR. In our experience, a VR of > 0.5 denotes definite antiviral activity against the RNA viruses.

Hemagglutinin titration. The effectiveness of the compounds was also measured by the extent of ortho (influenza A_2 and B) and paramyxovirus (Sendai) hemagglutinin (HA) production in secondary cultures of CE cells during a 3-day incubation period using the testing procedure described above. The HA titer of the combined extra- and intracellular material was determined using 0.5% guinea pig red blood cells in phosphate buffered saline (0.02 *M* PO_4, pH 7.2; 0.15 *M* NaCl). The HA titer was recorded 45 min after incubation at room temperature.

Results. Virus rating. The results of the initial antiviral chemotherapy experiments are summarized in Table I. As shown, both the compounds, deaza CR and deaza UR, showed moderately significant antiviral activity against RV and VSV, but little or no antiviral activity against HA-1 virus and PoV type 2.

Hemagglutinin production. The HA experiments (Table II) indicated that both compounds were also active against the influenza and Sendai viruses. However, deaza CR had a greater degree of activity against influenza B and Sendai viruses than did deaza UR.

Cytotoxicity. Relative cytotoxicity studies of the two compounds in KB, Vero and CE cell cultures indicated a similar toxic effect at the 1000 μg/ml level of either compound, and approximately 50% cells were found floating in the medium within 3 days. At other concentrations, deaza CR induced slight cytoplasmic granularity down to 320 μg/ml, whereas deaza UR-exposed cells had a similar appearance down to the 32 μg/ml levels. Cell death or detachment was not observed at these concentrations.

Reversal of antiviral activity. Investigations were conducted to determine if antiviral activity of the two pyridine nucleosides could be reversed by simultaneous addition

TABLE I. Antiviral Activity of 3-Deazacytidine and 3-Deazauridine.[a]

Virus	Virus rating	
	Deaza-cytidine	Deaza-uridine
Poliovirus, type 2	0.2	0.0
Rhinovirus, type 1A	0.8	0.8
Rhinovirus, type 13	0.7	0.7
Rhinovirus, type 56	0.7	0.7
Parainfluenza virus, type 3	0.3	0.3
Vesicular stomatitis virus	0.5	0.8

[a] The extent of CPE inhibition in KB cells at 3 days postinfection was quantitatively determined by virus rating procedure (5).

TABLE II. Comparative Efficacy of 3-Deazacytidine and 3-Deazauridine on Hemagglutinin Production in Ortho- or Paramyxovirus-Infected Chicken Embryo Cell Cultures.[a]

Compound conc (μg/ml)	Hemagglutinin titer/0.1 ml					
	Deazacytidine			Deazauridine		
	Influenza A_2	Influenza B	Sendai	Influenza A_2	Influenza B	Sendai
1000	1:4	<1:4	1:2	1:4	<1:4	1:4
320	1:4	<1:4	1:2	1:4	<1:4	1:4
100	1:4	1:4	1:8	1:4	1:4	1:16
32	1:4	1:4	1:8	1:8	1:4	1:16
10	1:8	1:4	1:16	1:8	1:8	1:16
3.2	1:16	1:8	1:16	1:8	1:16	1:16
1.0	1:16	1:8	1:16	1:16	1:16	1:32
0.	1:16	1:16	1:32	1:16	1:16	1:32

[a] Hemagglutination was performed at day 3 postinfection.

of the respective analogous naturally occurring nucleosides. The deaza UR and uridine or deaza CR and cytidine in MEM with 2% dialyzed FBS was added in 0.1 ml volume to type 56 RV-infected KB cell culture wells within 15 min after infection. The final 0.5 log dilution of either analog ranged from 1000 to 1 μg/ml along with 0, 100, 200 or 400 μg/ml concentrations of the reversal agent. The VR determined at the end of the 3-day postinfection period is presented in Fig. 2. The reversal of deaza UR or deaza CR antiviral activity ranged from approximately 80 to 47% at 400 to 100 μg/ml concentrations of uridine or cytidine, respectively. It was therefore dependent on the concentration of the reversal agent used in the postinfection medium.

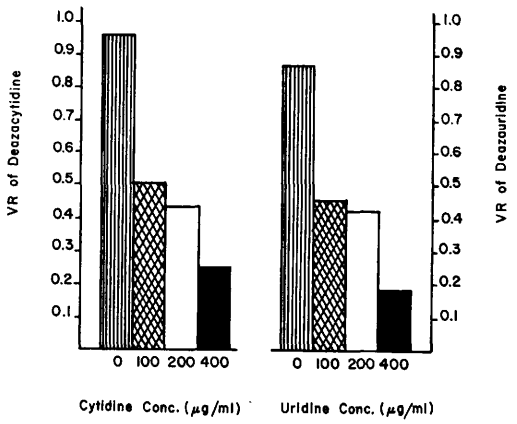

FIG. 2. Reversal of anti-type 56 rhinovirus activity in KB cells by simultaneous addition of : (A) varying concentrations of uridine with deaza UR, and (B) varying concentrations of cytidine with deaza CR. The antiviral activity was determined at 3 days postinfection by the virus rating procedure.

These data indicate that the two 3-deazapyrimidine nucleoside analogs have antiviral activity against a broad spectrum of RNA viruses. Experiments were, therefore, designed to study the mode of antiviral action of these compounds.

Virucidal study. To determine if the antiviral activity seen was a result of a virucidal action, 1000 $CCID_{50}$ of Sendai virus in MEM was incubated at 37° for 30 min with 500 or 100 μg/ml of each compound. The residual viral infectivity as measured by CPE development at 72 hr postinfection in Vero cell culture was not reduced by either compound. This suggested that they were not virucidal and must be affecting some process during virus infection of susceptible cells.

Sequential addition of deaza UR or deaza CR. In a series of experiments, KB cells were exposed to various dilutions of deaza UR or deaza CR at 1 hr pre-, 15 min post- and 4 hr postinfection with 100 $CCID_{50}$ of type 1A or 13 RV. The VR calculated for each time interval at 3 days postinfection had values similar to those described in Table I for deaza UR and deaza CR against RV types IA and 13, indicating no alteration in antivi-

ral activity even though addition of the compounds was delayed until 4 hr postinfection.

Another experiment was therefore performed wherein 320 $CCID_{50}$ of Sendai virus was allowed to adsorb onto CE cells for 1 hr. The unadsorbed virus was removed, the cell sheet was washed three times with MEM and 0.1 ml of the test medium was added to each virus-infected or toxicity control cell culture well. The 0.1 ml of test medium with or without deaza CR (640 μg/ml) was added at varying time intervals and CPE development recorded at 72 hr postinfection. An almost 100% inhibition in CPE occurred if the compound was added up to 12 hr postinfection (Fig. 3). This CPE development was considerably decreased if the compound was added at 24 and 48 hr postinfection.

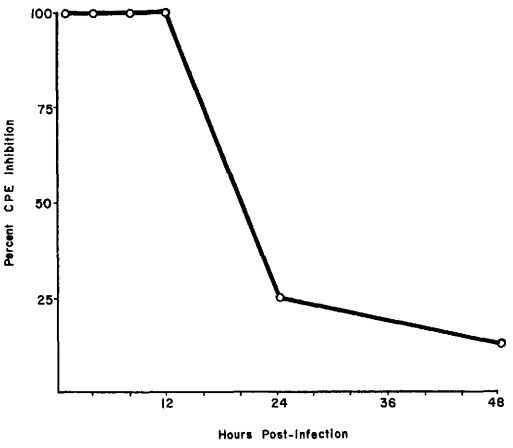

FIG. 3. Effect of sequential addition of 3-deazacytidine on inhibition of Sendai virus replication in chicken embryo cell cultures at 72 hr postinfection.

Discussion. During recent years considerable attention has been focused on the biological activity and therapeutic use of various pyrimidine nucleoside analogs (6, 7), especially 5-azacytidine and 6-azauridine. The data included in this report suggest that 3-deazapyrimidine nucleoside analogs (8), such as 3-deazacytidine and 3-deazauridine have a wide spectrum of anti-RNA viral activity in various cell cultures. Shannon, Arnett and Schabel (personal communication), have independently arrived at similar conclusions with regard to the antiviral activity of deaza UR. It is important to note that the anti-RV type 13 activity of 2-methyl-4-[(5-methyl-5H-*as*-triazino[5,6-b]-indol-3-yl)amino]-2-butanol (SK & F 30097), appears to be essentially the same (5) as that of deaza CR and deaza UR. However, SK & F 30097 has not been reported to possess any anti-influenza virus or parainfluenza virus activity (9). In the absence of a good anti-RNA agent for epidemiologically significant viral diseases, it is of interest to note that these two deazapyrimidine nucleoside derivatives possess substantial value as being potential anti-rhinoviral and anti-influenza agents. Comparison of deaza CR and deaza UR reveals that deaza CR has better antiviral activity against influenza B and Sendai viruses. The cellular granularity noticed as very slight cytotoxic manifestations in these experiments is of little significance since no visible cell death was ever noted at concentrations other than the highest level of the analogs. It is of considerable interest that deaza UR, having significant anticancer activity, is currently being considered for clinical trials as an anticancer agent (personal communication, Harry B. Wood, Jr., National Cancer Institute, Bethesda, MD).

The mode of action of these compounds against RNA viruses is still obscure and requires additional studies. However, it appears that these compounds do not exert a virucidal effect when placed with a known amount of Sendai virus and incubated at 37° for 30 min. Rather, these analogs require a virus infective process for their antiviral activity, since Sendai viral CPE production can be completely blocked even when deaza CR is added up to 12 hr after infection of CE cell culture. Reversal studies indicate that the antiviral activity of deaza CR and deaza UR could be effectively reversed by cytidine and uridine, respectively. The reversal of antiviral activity based on virus rating was found to be dependent on the concentrations of uridine or cytidine used. It appears that these analogs are competing with the pyrimidine nucleosides in their utilization in cellular or viral biosynthetic processes. This result correlates with previous data (3) on the reversibility of the analog-inhibitory activity in *E. coli* by their corresponding natu-

rally occurring pyrimidine nucleosides.

Summary. Investigations conducted on *in vitro* antiviral activity of 3-deazacytidine and 3-deazauridine indicated that these nucleosides possess significant antiviral activity against rhino 1A, 13 and 56; influenza A and B; parainfluenza 1 (Sendai) and vesicular stomatis viruses. 3-Deazacytidine was found to possess superior antiviral activity against influenza B and Sendai viruses. Preliminary studies performed on the mode of antiviral action showed that the analogs did not have any virucidal property, and exerted their inhibitory action during the viral infective process. The antiviral activity of the two 3-deazapyrimidine nucleosides could be reversed by the corresponding naturally occurring pyrimidine nucleosides.

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