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Antiviral and cytotoxicity evaluation of 3-nitro-3-deazauridine

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Summary

3-Nitro-3-deazauridine (3N-3DU) is a new synthetic nucleoside having activity against members of 5 RNA virus families including: paramyxoviruses (parainfluenza, PIV), picornaviruses (rhino-, RV), rhabdoviruses (vesicular stomatitis, VSV), togaviruses (Semliki Forest, SFV) and bunyaviruses (Punta Toro, PTV). In this report, we evaluate and compare its activity with the parent nucleoside, 3-deazauridine (3DU) and ribavirin as drug standards. Comparison of drug activities utilizes observations of antiviral indices, which are determined by the following formula: maximum tolerated dose (MTD)/minimum inhibitory concentration (MIC). The antiviral index (AI) of 3N-3DU (AI 15.3) was comparable to ribavirin and much higher than 3DU when evaluated against PIV. The 3N-3DU was the most active of the three when tested against RV (AI 24.1), SFV (AI 76.9) or VSV (AI 50). In contrast to the RV activity, 3N-3DU (AI 0.5) and 3DU (AI <0.1) were less active than ribavirin (AI 1.3) when evaluated against poliovirus, type 1 (PoV). Ribavirin (AI 10.0) was more active than 3N-3DU (AI 2.4) and 3DU (AI <0.1) against PTV. 3N-3DU exhibited comparable toxicity to ribavirin in KB cells, was 4-fold less toxic in WISH cells and 4-fold more toxic in LLC-MK_2 cells. Overall, 3N-3DU is markedly less toxic than its parent nucleoside, 3DU. It appears from this study that the structural modification of 3DU resulting from the addition of the nitro group in the 3 position of the base reduces toxicity and enhances the antiviral activity.

Anti-RNA virus activity; 3-Nitro-3-deazauridine; Ribavirin

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Introduction

3-Deazauridine [3DU, 4-hydroxy-1-β-D-ribofuranosyl-2 (1H)-pyridinone] synthesized by Robins and Currie (1968) has been shown to possess anticancer activity (Robins et al., 1969; Block et al., 1973; Wang and Block, 1972) and antiviral activity (Khare et al., 1972; Shannon et al., 1972, 1974). 3DU exhibited moderate activity against Gross leukemia virus, rhinoviruses and vesicular stomatitis virus and slight activity against influenza and parainfluenza viruses. The antiviral activity is reversed by uridine suggesting that the compound acts as an analogue of the natural pyrimidine nucleoside (Khare et al., 1972). 3DU was shown to exhibit activity in mice inoculated with L1210 leukemia cells (Helman and Slovik, 1976; Brockman et al., 1975). Subsequently, it has undergone human toxicity and efficacy evaluations. Although it has potential as an anticancer drug alone, the greatest interest has been directed toward its use in combination with cytosine arabinoside for treatment of leukemia (Moriconi et al., 1986).

The continuing exploration of the biological activity of 3DU has stimulated interest in the development of related derivatives. The subject of this report is the antiviral and cytotoxic evaluation of the most active member of a new series of derivatives, 3 nitro-3-deazauridine [3N-3DU, 4-hydroxy-3-nitro-1-β-D-ribofuranosyl-2 (1H)-pyridinone] and comparison of its activity with 3DU and ribavirin (1-β-D-ribofuranosyl-1, 2, 4-triazole-3-carboxamide) (Huffman et al., 1973) as standards.

Materials and Methods

Compounds

3DU [4-hydroxy-1-β-D-ribofuranosyl-2 (1H)-pyridinone] was purchased from Sigma. 3N-3DU[4-hydroxy-3-nitro-1-β-D-ribofuranosyl-2 (1H)-pyridinone], shown in Fig. 1 was synthesized at Warner Lambert/Parke Davis by D.J. McNamara and P.D. Cook by the method of McNamara et al. (in preparation). Ribavirin (1-β-D-ribofuranosyl-1, 2, 4-triazole-3-carboxamide) was obtained from Viratek, Division of ICN Pharmaceuticals. Stock solutions of compounds were prepared by weighing compounds, diluting in water and storing aliquots at −20°C.

Cell Cultures

Monolayer cultures of WISH, KB, and LLC-MK_2 cells were grown in Eagle's minimal essential medium (MEM) supplemented with 10% fetal bovine serum (FBS), 1 mM N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (HEPES), and 50 μg/ml gentamicin with pH being adjusted with sodium hydroxide and/or sodium bicarbonate. For antiviral assays, cells were diluted to the appropriate concentrations and dispensed in 0.2 ml volumes into wells of 96 well microtiter plates. The concentrations of cells prepared for use in microtiter plates were about 0.6 ×

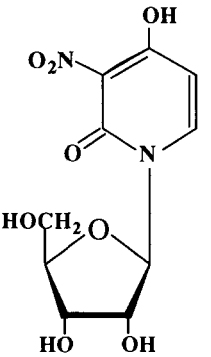

Fig. 1. [4-hydroxy-3-nitro-1-β-D-ribofuranosyl-2 (1H)-pyridinone], 3-nitro-3-deazauridine.

10^5 WISH cells/ml, 0.8×10^5 KB cells/ml, and 1.0×10^5 LLC-MK_2 cells/ml. Medium for antiviral experiments was similar to growth medium except that FBS was reduced to 2%.

Viruses

The viruses used in this study were parainfluenza virus, type 3 (PIV), rhinovirus, type 34 (RV), poliovirus, type 1 (PoV, strain Bruhnhilda), vesicular stomatitis virus (VSV), Semliki Forest virus (SFV), and Punta Toro virus (PTV). PIV, VSV and SFV were obtained from Dr. Robert W. Sidwell, Utah State University. RV, PoV and PTV were obtained from the American Type Culture Collection, PIV, PoV, SFV and VSV were propagated in KB cells, RV in WISH cells and PTV in LLC-MK_2 cells.

Antiviral evaluations

In general, the initial antiviral and cytotoxicity evaluations were performed by the methods of Sidwell and Huffman (1971). In the experiments, serial two-fold dilutions (4 wells/concentration) of drugs were added to 18–24 h monolayers in microtiter plates. Within 15–30 min, 100–320 times the tissue culture 50% infectious dose ($TCID_{50}$) of virus was added. Two additional wells per concentration containing drug and media only were used for observation of drug cytotoxicity. Experimental controls included: cell controls (cells plus medium) and virus controls (cells plus medium and virus). Microtiter plates were then wrapped with Saran Wrap and incubated at the temperature and time appropriate for the virus/cell system. These included: 33°C for 3 days for RV; 37°C for 3 days for PTV, PIV, PoV and SFV; and 37°C for 2 days for VSV. Virus cytopathic effect (CPE) and drug cytotoxicity were then observed microscopically and scored on a 0–4 basis with 4 representing total cell destruction. The concentration of drug that inhibited CPE by 50% in comparison to the virus controls was considered the minimum inhibitory concentration (MIC). When necessary, these concentrations or 50% end-points

were calculated using a linear regression technique from the actual percent inhibition at various concentrations.

In order to determine the effect of the drugs on the yield of virus, the standard microtiter plate assay was performed using Semliki Forest virus, the plate read and frozen at −70°C. The plate was thawed, the medium from the 4 replicate wells was pooled, mixed and one-log dilutions prepared in test medium. The dilutions were placed into wells (0.2 ml) of microtiter plates (4 wells per dilution), and incubated for 2 h. Medium was removed and replaced with test medium. The plates were incubated, observed for CPE and titers calculated by the Reed-Muench (1938) technique.

Experiments were performed using SFV to determine if natural nucleosides would reverse the antiviral activity of 3N-3DU. In these experiments, test medium was prepared with dialyzed serum in order to minimize the contribution of nucleosides in the serum. In the initial experiments, each nucleoside was added to the test medium and this was used for preparing dilutions of the 3N-3DU, such that each concentration of 3N-3DU also contained a constant concentration of natural nucleoside. Activity of 3N-3DU in the presence of nucleoside was compared to drug alone in test medium containing dialyzed serum.

Cytotoxicity evaluation

As a part of the antiviral evaluation, cells treated with drug and medium were observed for drug induced cytotoxicity. This information was used to determine concentrations to be evaluated in growth inhibition assays. In these experiments, cells were planted in a volume of 1 ml per well in 24-well plates at half the concentration used in antiviral experiments and incubated for 18–24 h. Five concentrations of each compound (200, 50, 12.5, 3.13, 0.78 μg/ml) were diluted in growth medium. One milliliter of each drug concentration was placed into 4 wells. The same number of wells received only growth medium and served as untreated cell controls. The cells in one plate were used to determine the baseline concentration of cells at the beginning of the treatment period. The plates were covered, wrapped, and incubated at 37°C for 72 h.

At the end of the incubation period, medium was decanted from the plate and cells were rinsed twice with calcium and magnesium-free Hanks balanced salt solution (HBSS) and 0.5 ml of 0.25% trypsin plus 2 μg/ml EDTA was added to each well. The trypsin/EDTA solution was left on the cells for 1 min and decanted. The wells were then incubated 5 min at 37°C and 1 ml of growth medium containing trypan blue was added to each well. The cells were then suspended in the medium containing trypan blue and live cells counted on a hemocytometer. The highest concentration of compound which did not kill cells, but allowed a slight increase (about 10%) in cell number was considered the maximum tolerated dose (MTD).

Calculation of the antiviral index

In order to compare the overall effectiveness of the compounds, the antiviral index (AI) was calculated using the following formula: AI = MTD/MIC.

Evaluation of drug combinations

Since 3N-3DU and ribavirin were active in several of the same systems, we decided to evaluate the effect of the drugs together. Seven dilutions of 3 ratios of the two drugs (5:1, 1:1, 1:5 3N-3DU:ribavirin) were tested against PIV. As controls, each individual drug was reevaluated on the same plate as each ratio of the combination. The lowest concentration of drugs in combination that reduced virus-induced CPE by $\geq 50\%$ (with the aid of the other drug) was again considered the MIC. The types of drug interaction (synergism, additive effects, indifference, antagonism) were evaluated by a numeric method involving the fractional inhibitory concentration (FIC) index (Allen et al., 1982). The FIC index is calculated and interpreted as follows:

$$\text{FIC index} = \frac{(\text{MIC drug A in comb.})}{(\text{MIC drug A alone})} + \frac{(\text{MIC drug B in comb.})}{(\text{MIC drug B alone})}.$$

The FIC index is interpreted as follows: <0.5 = significant synergism, 0.5–0.9 = suggestive of synergism, ~ 1 = effects are additive, 1.1–1.9 = indifference or partial antagonism, and ≥ 2 = antagonism.

Results

Cytotoxicity evaluations

MTD values were determined in experiments in which 4 replicates were used per concentration of drug evaluated. Of the materials tested, 3DU was consistently the most toxic with an MTD of 3.1 μg/ml in each cell line. Ribavirin (MTD 50.0 μg/ml) was 4-fold more toxic than 3N-3DU (MTD 200.0 μg/ml) in WISH cells. In contrast, 3N-3DU (MTD 12.5 μg/ml) was 4-fold more toxic than ribavirin (MTD 50.0 μg/ml) in LLC-MK_2 cells. 3N-3DU and ribavirin (MTD 200.0 μg/ml) exhibited comparable levels of toxicity in KB cells.

Antiviral evaluations

A summary of antiviral experiments composed of mean values derived from at least 3 experiments are presented in Table 1. The usual range of MIC values of 3N-3DU was from 2.6 μg/ml for SFV to 13.1 μg/ml for PIV with the exception of PoV (MIC 400 μg/ml). The antiviral indices for 3N-3DU ranged from 0.5 for PoV

TABLE 1

Comparison of antiviral activity of 3N-3DU, 3DU and ribavirin

Virus	Cells	3N-3DU			3DU		Ribavirin	
			(μg/ml)	AI*	(μg/ml)	AI	(μg/ml)	AI
Rhino-	WISH	MTD	200.0		3.1		50.0	
		MIC	8.3	24.1	57.1	<0.1	20.5	2.4
Polio-	KB	MTD	200.0		3.1		200.0	
		MIC	400.0	0.5	150.7	<0.1	84.5	2.4
Parainfluenza-	KB	MTD	200.0		3.1		200.0	
		MIC	13.1	15.3	107.8	<0.1	13.5	14.8
Vesicular stomatitis-	KB	MTD	200.0		3.1		200.0	
		MIC	4.0	50.0	2.0	1.6	18.7	10.6
Semliki Forest	KB	MTD	200.0		3.1		200.0	
		MIC	2.6	76.9	3.2	1.0	34.9	5.7
Punta Toro	LLC-MK_2	MCC	12.5		3.1		50.0	
		MIC	5.2	2.4	181.7	<0.1	5.0	10.0

*Antiviral indices = maximum tolerated dose/minimum inhibitory concentration.

to 76.9 for SFV. The lowest MICs for 3DU were against VSV (2.0 μg/ml) and SFV (3.2 μg/ml) while the high MIC (181.7 μg/ml) was seen with PTV. Since 3DU is relatively toxic, the highest AI was a low value of 1.6. Although ribavirin was more active against PTV and PoV than 3N-3DU, it had comparable activity to 3N-3DU against PIV with an MIC of 13.5 μg/ml and an AI of 14.8. Ribavirin was less active by about 5- to 10-fold than 3N-3DU when evaluated against RV, VSV and SFV.

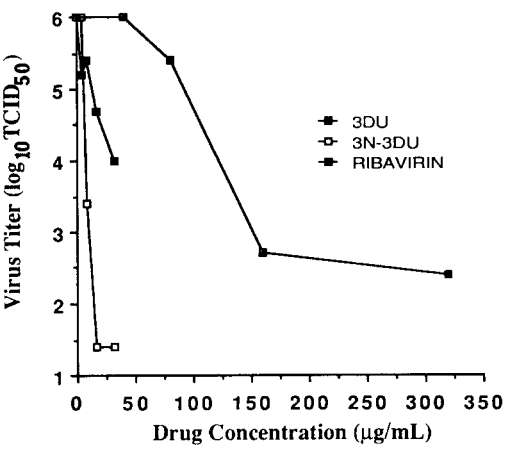

Fig. 2. Effect of 3DU, 3N-3DU and ribavirin on Semliki Forest virus yield in KB cells.

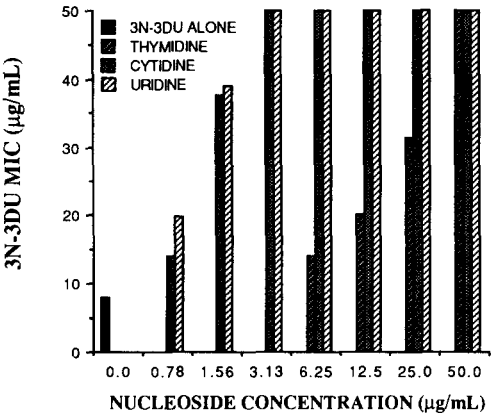

Fig. 3. Effect of pyrimidine nucleosides on the anti-SFV activity of 3N-3DU.

The three drugs were evaluated for their ability to reduce yield of SFV (Fig. 2). 3N-3DU reduced the concentration of virus to levels below the inoculum concentration at 32 and 16 μg/ml and reduced the concentration by 2.5 $\log_{10}$ at 8 μg/ml but had no effect at lower concentrations. The titer was only decreased by 3DU by 2 $\log_{10}$ units at 32 μg/ml, the highest concentration tested. Ribavirin maintained the virus concentration at inoculation levels at 320 and 160 μg/ml. A reduction of less than 1 $\log_{10}$ unit was seen with 80 μg/ml of ribavirin.

Reversal of antiviral activity of 3N-3DU

Adenosine, guanosine, thymidine, cytidine and uridine were evaluated for effects on the 3N-3DU MIC values against SFV. In this assay system, where the medium was prepared with dialyzed FBS from a different source, the MIC of 3N-3DU was 8.0 μg/ml which is higher than that (2.6 μg/ml) seen in the other assays. Adenosine and guanosine did not reverse the activity of 3N-3DU. In fact, adenosine exhibited some anti-SFV activity and cell toxicity and therefore lowered the MIC and toxicity levels of 3N-3DU in a concentration dependent manner. Thymidine, cytidine and uridine all reversed the antiviral activity of 3N-3DU as evidenced by an increase in the MIC values (Fig. 3). Thymidine was the least effective in reversing the activity of 3N-3DU, raising the MIC to >50 μg/ml only at 50 μg/ml. At doses of ≥3.13 μg/ml cytidine and uridine raised the MIC for 3N-3DU to >50 μg/ml. At concentrations of ≤1.56 μg/ml, uridine was slightly more effective than cytidine in reversing the activity of 3N-3DU and raising the MIC values.

Combination experiments

In the PIV combination study, 3N-3DU:ribavirin ratios had the following FIC indices: 5:1–1.023; 1:1–1.024 and 1:5–1.300. The first two values suggest additive interactions and the latter indifference or partial antagonism.

Discussion

We have demonstrated that 3N-3DU exhibits antiviral activity against members of 5 RNA virus families in CPE inhibition assays. Further, the compound has been shown to prevent the increase in concentration of virus when evaluated against Semliki Forest Virus.

The mode of action of 3DU has been extensively investigated. 3DU or its metabolites are known to interfere with at least 6 enzymes in the pyrimidine biosynthetic pathway. These enzymes include carbamoyl phosphate synthetase, cytidine deaminase, cytidine triphosphate synthetase, cytidine/uridine phosphatase, ribonucleotide reductase and deoxycytidine monophosphate deaminase (Karle and Cysyk, 1984; Lockshin et al., 1984; Moriconi et al., 1986). Considering the inhibition of these enzymes and the overall effect on DNA and RNA synthesis, the toxicity of 3DU is not surprising. Since 3N-3DU is structurally related to 3DU, the structural changes which resulted in 3N-3DU would probably produce a molecule that would have effects on some of the same enzymes. At this time, one can only speculate on the mode of action of 3N-3DU. As suggested by the reversal of activity by uridine, cytidine and to a lesser extent thymidine, 3N-3DU appears to affect processes involved in pyrimidine metabolism as does 3DU.

In contrast to 3DU, ribavirin has been found to predominately affect purine metabolic processes (Robins, 1986). The antiviral activity of ribavirin or its metabolites is attributed to the inhibition of inosine monophosphate dehydrogenase, RNA polymerase, guanylyl transferase and N7-methyl transferase. These activities result in the reduction of guanosine triphosphate pools, inhibition of RNA polymerase (reduced production of viral RNA) and inhibition of guanosine capping of messenger RNA. Consequently, viral RNA is either not transcribed or is nonfunctional.

When compounds having different modes of action are combined in antimicrobic assays, the combination should exhibit synergy. Since this did not occur when 3N-3DU and ribavirin were combined, the findings might be interpreted to suggest that there are some similarities between the mode of action of ribavirin and 3N-3DU.

When comparing the activities of 3N-3DU to the parent compound, it had MIC values much lower than 3DU against RV, PIV and PTV, but was found to have similar MIC values against VSV and SFV. In contrast, 3DU had a lower MIC than 3N-3DU when evaluated against polio, but neither was appreciably effective. In toxicity assays using dividing cells, 3DU was 4- to 60-fold more toxic than 3N-3DU. These differences suggest that the structural modification involving the addition of the nitro group to the carbon 3 of deazauridine results in an appreciable alteration in the antiviral activity of the molecule accompanied by a significant reduction in toxicity. In consideration of these findings, we feel that 3N-3DU merits additional in vitro and in vivo evaluation.

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