

## Lethal Mutagenesis of Picornaviruses with N-6-Modified Purine Nucleoside Analogues<sup>∇†</sup>

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Received 10 August 2007/Returned for modification 1 October 2007/Accepted 31 December 2007

**RNA viruses exhibit extraordinarily high mutation rates during genome replication. Nonnatural ribonucleosides that can increase the mutation rate of RNA viruses by acting as ambiguous substrates during replication have been explored as antiviral agents acting through lethal mutagenesis. We have synthesized novel N-6-substituted purine analogues with ambiguous incorporation characteristics due to tautomerization of the nucleobase. The most potent of these analogues reduced the titer of poliovirus (PV) and coxsackievirus (CVB3) over 1,000-fold during a single passage in HeLa cell culture, with an increase in transition mutation frequency up to 65-fold. Kinetic analysis of incorporation by the PV polymerase indicated that these analogues were templated ambiguously with increased efficiency compared to the known mutagenic nucleoside ribavirin. Notably, these nucleosides were not efficient substrates for cellular ribonucleotide reductase in vitro, suggesting that conversion to the deoxyribonucleoside may be hindered, potentially limiting genetic damage to the host cell. Furthermore, a high-fidelity PV variant (G64S) displayed resistance to the antiviral effect and mutagenic potential of these analogues. These purine nucleoside analogues represent promising lead compounds in the development of clinically useful antiviral therapies based on the strategy of lethal mutagenesis.**

Natural nucleotides exist as tautomers in solution, and tautomerization of the nucleobases in DNA has been recognized as a likely mutagenic mechanism ever since the double-helical structure of the DNA molecule was first deduced by Watson and Crick (33, 36). The keto (for G and U) and amino (for A and C) tautomers of the natural nucleotides are the predominant species with tautomeric constants ( $K_T$ ) on the order of  $10^5$ . However, tautomeric conversion to the rare enol or imino forms of the nucleobases can lead to altered hydrogen bonding specificity and thus mutagenesis through noncanonical base-pairing interactions. This has become known as the “rare tautomer” hypothesis of mutation (19, 29).

The tautomerization of bases to yield ambiguous base-pairing properties has also been exploited in the design of novel nucleoside drugs, including 5-hydroxy-2'-deoxycytidine (22) and 5-aza-5,6-dihydro-2'-deoxycytidine (KP-1212) (18, 25) (Fig. 1A). However, attempts to design clinically useful antiviral compounds around this premise have met with only limited success (13, 16, 18, 25).

Nucleobases exhibiting multiple conformations due to rotation or tautomerization have received attention as potential

antiviral agents acting through lethal mutagenesis (11, 22). Since the discovery that ribavirin (Fig. 1B) can act as a lethal mutagen (7), likely through rotation of the exocyclic carboxamide moiety, the concept of lethal mutagenesis as an antiviral strategy has received considerable attention (5, 11, 12).

Recently, we have demonstrated that the ribonucleoside analogue rP (Fig. 1C) can act as a potent mutagen of poliovirus (PV) in vitro (13). Tautomerization of the nucleobase of rP ( $K_T$ ,  $\sim 10$ ) allows it to be recognized as either cytosine or uracil by the PV RNA-dependent RNA polymerase (RdRp) 3D<sup>Po1</sup>, though its effectiveness as an antiviral molecule in vivo is hindered by the lack of accumulation of the active metabolite, rPTP (13).

The nucleobases K and Z have previously been examined as ambivalent purine analogues with tautomerization constants near unity (4). Deoxyribonucleosides containing these bases (dK and dZ) have been synthesized and were found to induce transition mutations in PCRs using *Taq* polymerase (4, 20). dK favors the imino (or “G-like”) tautomer by a ratio of 9:1, subsequently inducing transition mutations efficiently in *Escherichia coli* (20). Likewise, dZ favors the imino tautomer by a ratio of 4:1, though it is a less effective mutagen in vivo (4, 20). A library of N-6-substituted purines designed on this premise has recently been synthesized and evaluated for antimalarial properties (32).

Herein, we examine the corresponding ribonucleosides rK and rZ, (hereafter referred to as JA31 and JA26, respectively) and analogues thereof as lethal mutagens of picornaviruses. We demonstrate that the most potent of these analogues are able to reduce the titer of PV and coxsackievirus B3 (CVB3) in cell culture over 1,000-fold during a single passage. Treatment

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<sup>∇</sup> Published ahead of print on 7 January 2008.

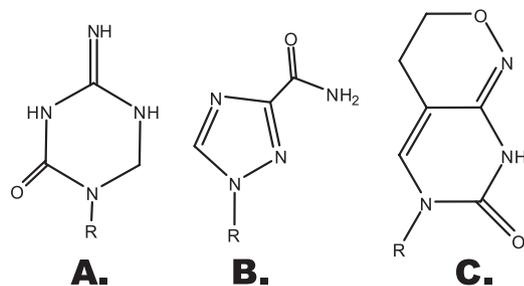


FIG. 1. Structures of mutagenic nucleoside analogs. (A) 5-Aza-5,6-dihydro-2'-deoxycytidine (KP-1212), imino tautomer. (B) Ribavirin. (C) P, imino tautomer. R denotes deoxyribose (KP-1212; dP) or ribose (ribavirin; rP).

with these analogues was able to increase the frequency of transition mutations by more than 50-fold over a single passage, as measured by a phenotypic assay, suggesting a role of lethal mutagenesis in the observed antiviral activity. Importantly, these nucleosides are substrates for cellular kinases, resulting in accumulation of the triphosphorylated species that is the necessary substrate for incorporation into viral genomic RNA. Furthermore, diphosphates of the active analogues were poor substrates of mammalian ribonucleotide reductase, suggesting that they may not be converted to intracellular deoxyribonucleotides, lessening the chance of permanent genetic damage to cells replicating their DNA in the presence of these nucleosides. Therefore, these purine analogues represent promising new lead compounds for the translation of mutagenesis-based antiviral strategies to clinical use.

#### MATERIALS AND METHODS

**Cells and viruses.** HeLa S3 cells (obtained from ATCC) were maintained in Dulbecco's modified Eagle's medium (DMEM)/F-12 medium supplemented with 2% dialyzed fetal bovine serum and 1× penicillin-streptomycin (Invitrogen). Cells were maintained at 37°C in a 5% CO<sub>2</sub> atmosphere. Antiviral activity assays were performed as previously described (16, 17). PV was generated by transfecting HeLa S3 cells with full-length genomic RNA transcribed using T7 RNA polymerase from a plasmid containing the viral cDNA (pMoRA) as previously described (1, 35). CVB3 was similarly produced using RNA transcribed from plasmid pSV-CVB3/0 (6) (a gift from N. Chapman and S. Tracy, University of Nebraska Medical Center). The guanidine resistance (*Gua*<sup>r</sup>) assay was performed as previously described (7). Statistical analysis was performed using Prism 4 for Windows (GraphPad Software, Inc.).

**Incorporation of nucleotide analogues by PV 3D<sup>pol</sup>.** Efficiency of incorporation by poliovirus polymerase (3D<sup>pol</sup>) opposite each of the four templating bases in assembled enzyme-symmetrical primer-template substrate (*s/s*) complexes was examined for each of the seven nucleotide analogues, ribavirin, the correct natural nucleotide, and the natural nucleotide giving rise to a transition mutation. Ribavirin triphosphate (RTP) was purchased from Moravak Biochemicals. PV 3D<sup>pol</sup> was expressed and purified as previously described (10). Extension assays utilizing symmetrical primer-template substrates (*s/s*) were performed as described (1). *s/s* RNAs were synthesized by Dharmacon, Inc. The sequences of RNA oligonucleotides were as follows (the templating base in the duplex substrate is indicated in boldface): *s/s*-U, 5'-GCAUGGGCCC-3'; *s/s*-C, 5'-GAUCGGGCCC-3'; *s/s*-A, 5'-GCUAGGGCCC-3'; and *s/s*-G, 5'-CAUGCCCGGG-3'. RNA oligonucleotides were 5'-end labeled using [ $\gamma$ -<sup>32</sup>P]ATP and T4 polynucleotide kinase according to the manufacturer's protocol (New England BioLabs). Annealing of <sup>32</sup>P-end-labeled and unlabeled *s/s* oligonucleotides to form primer/template duplex was accomplished by heating to 90°C for 1 min followed by cooling to 10°C at a rate of 5°C per min. 3D<sup>pol</sup> was allowed to preassemble with *s/s* duplex for 3 min at room temperature before initiation by addition of the nucleotide substrate. Reactions were performed at 30°C for 60 s or 330 s in 50 mM HEPES (pH 7.5), 10 mM 2-mercaptoethanol, 5 mM MgCl<sub>2</sub>, 60 μM ZnCl<sub>2</sub>, 2 μM 3D<sup>pol</sup>, 0.5 μM *s/s* duplex, and 0.5 mM NTP. Reaction products were

separated by denaturing polyacrylamide gel electrophoresis (PAGE), and gels were visualized using a PhosphorImager and quantified using ImageQuant software (Molecular Dynamics) as previously described (1).

**Chain termination assays.** Complexes of 3D<sup>pol</sup> and *s/s*-U duplex were preassembled as described above. Reaction conditions were as described above, except the reaction time was 180 s and reactions were initiated with either 0.5 mM ATP or nucleotide analogue or with 0.5 mM ATP or analogue and 10 μM UTP. Reaction products were separated, visualized, and quantified as described above.

**Cytotoxicity assays.** HeLa S3 cells were propagated in DMEM/F-12 (Invitrogen) supplemented with 2% fetal bovine serum (Invitrogen) and 1% penicillin-streptomycin (Invitrogen), with the cultures maintained between 20% and 80% confluence. Subconfluent monolayers of HeLa S3 cells were detached from the culture flasks by trypsin treatment and washed with 1× phosphate-buffered saline (Invitrogen), and the cell number was adjusted to 2 × 10<sup>3</sup> cells per 50 μl of DMEM/F-12. Fifty microliters of cell suspension was cultured in a 96-well plate, with one row serving as a negative control with no cells. The plate was incubated for 1 h at 37°C to allow cell adherence. Fifty microliters of the compound of interest diluted in DMEM/F-12 was added to the HeLa S3 cells, and the plate was incubated at 37°C. Six hours before a desired time point, 20 μl of the CellTiter-Blue reagent (Promega), resazurin, was added to triplicate series of wells. After 6 h of incubation, the A<sub>570</sub> and A<sub>590</sub> were measured using an HTSoft Bioassay plate reader (HTSoft). The 590-nm reading served as a reference wavelength to correct for the partial interference of unreacted reagent in the 570-nm reading. The following equation was used to determine the percentage of resazurin reduced: % reduced = A<sub>570</sub> - (A<sub>590</sub> × R<sub>0</sub>) × 100. The correction factor R<sub>0</sub> is the ratio of resazurin absorbances at different wavelengths: R<sub>0</sub> = AO<sub>570</sub>/AO<sub>590</sub>, where AO<sub>λ</sub> = (absorbance of medium + resazurin)<sub>λ</sub> - (absorbance of medium)<sub>λ</sub>.

**Detection of nucleotides in HeLa S3 cells.** Cell extracts were prepared and analyzed based on previously published methods (13, 30). HeLa S3 cells were adapted to suspension and maintained in spinner flasks at a density of 1 × 10<sup>6</sup> to 2 × 10<sup>6</sup> cells/ml in S-MEM (minimum essential medium modified for suspension cultures) containing 10% fetal bovine serum, 2 mM L-glutamine, 50 U/ml penicillin, and 50 μg/ml streptomycin (Invitrogen). Cell density was adjusted to 5 × 10<sup>6</sup> cells/ml immediately prior to treatment in S-MEM, to which was added 2% dialyzed fetal bovine serum, 2 mM L-glutamine, 50 U/ml penicillin, and 50 μg/ml streptomycin. Cells were treated with 2.5 μg/ml actinomycin D (Sigma) for 15 min at 37°C, and then nucleotide was added to the medium to a final concentration of 0.5 mM. One percent dimethyl sulfoxide (DMSO) was present in both treated and control samples. Samples were incubated for 2 h at 37°C in a Lauda circulating water bath with occasional mixing by inversion. Cells were collected by centrifugation at 2,000 × g for 4 min and washed with 2 volumes phosphate-buffered saline. Cells were then pelleted and resuspended in 1 volume ice-cold 0.6 M trichloroacetic acid (Sigma). The cell suspension was incubated on ice for 10 min and then centrifuged at 14,000 × g for 2 min at 4°C. The supernatant was collected and extracted with an equal volume of ice-cold 0.5 M triethylamine in 1,1,2-trichlorotrifluoroethane (Sigma). Samples were then vortexed for 30 s and centrifuged for 30 s at 14,000 × g and 4°C.

The upper (aqueous) layer of the cell extract was analyzed on a Hewlett Packard 1100 series instrument equipped with an Aquasil C<sub>18</sub> analytical column (4.6 × 250 mm, 5 μm; Keystone Scientific, Inc. [Thermo Electron Corp.]) running a mobile phase (flow rate of 1 ml/min) gradient of 1% to 15% CH<sub>3</sub>CN in KH<sub>2</sub>PO<sub>4</sub> buffer (pH 6.0, 10 to 20 min) and 15% to 80% CH<sub>3</sub>CN in KH<sub>2</sub>PO<sub>4</sub> buffer (20 to 30 min). All injections were 50 μl.

**Preparation of *E. coli* R1 and R2.** The R1-containing plasmid was a kind gift of Jeff Baldwin. The *E. coli* R1 gene was amplified from the K-12 strain by colony PCR using primer 1 (5'-CATATGAATCAGAATCTGC; NdeI site in boldface) and primer 2 (5'-TACGCCGCATCCGGCATCTCGAGATCA; XhoI site in boldface). The 2,315-bp PCR product was purified by agarose gel electrophoresis followed by gel extraction using the Qiagen (Valencia, CA) QiaQuick system. Due to a second internal NdeI site in the gene, the restriction digest produced two fragments (996 bp and 1,300 bp). These fragments were both Qiagen kit gel purified and included in the ligation reaction with NdeI/XhoI-cut pET22b. After transformation of the plasmid (ligation mix) into the DH5α cell line, several colonies were selected from the culture plate and grown in LB-ampicillin (0.15 mg/ml) to an optical density (OD) of 1 to 2, and the cells were harvested for plasmid using the Promega (Madison, WI) Wizard mini-prep kit. Sequencing was then employed to find the plasmid with gene fragments in the correct orientation. Construction of plasmid pET22b-R2 has been described elsewhere, as have R2 expression and purification (27).

BL21(DE3) cells carrying the plasmid pET22b-R1 were grown at 37°C in rich LB broth containing 35 g/liter tryptone, 20 g/liter yeast extract, 5 g/liter NaCl, and 0.15 g/liter ampicillin to an OD<sub>600</sub> of 0.7 to 1.0 and then cooled on ice for 15 min,

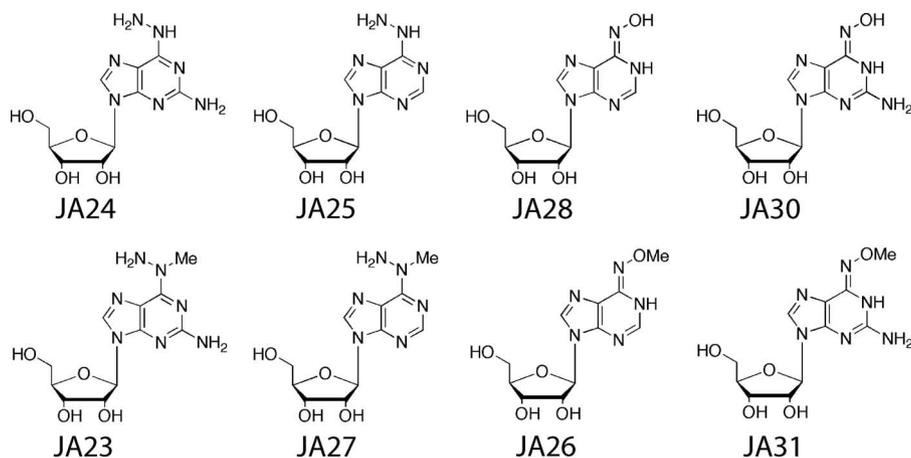


FIG. 2. Structures of N-6-modified purine nucleoside analogues.

induced by addition of 0.2 mM isopropyl- $\beta$ -D-thiogalactopyranoside (IPTG), and grown for 16 to 18 h at 15°C. The cells were then harvested by centrifugation, and the pellet was washed with buffer (25 mM Tris-Cl [pH 7.4], 70 mM NaCl, 4 mM KCl) prior to being frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$ . A typical yield produces  $\sim 20$  g of wet paste/liter of culture.

The frozen cells ( $\sim 60$  g wet paste) were thawed in a mixture of 50 mM Tris-Cl (pH 7.6), 5% (vol/vol) glycerol, 15 mM magnesium acetate, and 4 mM dithiothreitol (DTT) (buffer A) containing 0.25 mM phenylmethylsulfonyl fluoride and lysed in a French pressure cell at 16,000 lb/in $^2$ . Cell debris was removed by centrifugation at  $14,000 \times g$  for 10 min. Streptomycin sulfate was added to a final concentration of 1% (wt/vol). The precipitate was removed by centrifugation at  $13,900 \times g$  for 10 min. The supernatant was dialyzed against 4 liters buffer A and was diluted with an equal volume of buffer A containing 0.25 mM phenylmethylsulfonyl fluoride. The solution was then loaded onto a 1.25- by 37-cm ( $\sim 0.046$  liter) Affi-blue gel column (Bio-Rad) equilibrated in buffer A, and the protein was eluted with a 0.46-liter linear gradient of 0 to 0.5 M NaCl at a flow rate of 1 ml/min. Fractions containing R1 were pooled and concentrated by ultrafiltration in an  $\sim 350$ -ml Millipore (Billerica, MA) Amicon cell equipped with a YM30 membrane. The protein was dialyzed against 50 mM HEPES buffer (pH 7.6) containing 10% (vol/vol) glycerol, 15 mM magnesium acetate, and 4 mM DTT, flash-frozen in liquid nitrogen, and stored at  $-80^{\circ}\text{C}$ .

**Preparation of mouse R1 and R2.** Expression and purification of R2 were performed as previously described (38). Plasmid pMR1, which contains the mouse R1 gene inserted into the pET22b expression vector, was constructed as follows. The mouse R1 gene was amplified by PCR from the pET3 vector containing the mouse R1 gene. Primer 1 (5'-GGCGTGCATATGCATGTGATCAAGCGAGATGGCCGC-3'; NdeI site shown in boldface) and primer 2 (5'-CATCAACATGTGCTGCGGCCGTTTCAGCCACTTTACC-3'; EagI shown in boldface) were used to amplify a 527-bp fragment (fragment 1) and remove an endogenous NdeI site (ATG to GTG [underlined]). Fragment 1 was purified by agarose gel electrophoresis, extracted using the QiaQuick system, restricted with NdeI and EagI, and repurified. The second fragment (fragment 2) of the mouse R1 gene was PCR amplified using primer 3 (5'-GGTAAAGTGGCTGAACGGCCGCGAGCAGCATGTTGATG-3'; EagI site shown in boldface) and primer 4 (5'-CGCATATAGCTCGAGCTCAGGATCCACACATCAGGCACTCC-3'; XhoI shown in boldface). The 1,919-bp fragment 2 was gel purified, restricted with EagI and XhoI, repurified, and ligated to the cut fragment 1 and pET22b, which had been restricted with NdeI and XhoI and gel purified. BL21(DE3) cells were transformed with the pMR1 plasmid.

The purification method for mouse R1 was performed as described for *E. coli* R1, and it typically yielded  $\sim 0.5$  mg of protein per g of wet cell mass.

**Mass spectrometry analysis of dNDP production by RNR catalysis.** The assay was performed as previously described (21). At  $22^{\circ}\text{C}$ , 8.8  $\mu\text{M}$  *E. coli* R2 was added to a 200- $\mu\text{l}$  reaction mixture consisting of 100 mM HEPES (pH 7.6), 1.7 mM dTTP, 1.8 mM 5'-dNDP analog, 15 mM magnesium acetate, 1 mM EDTA, 25 mM DTT, and 5  $\mu\text{M}$  *E. coli* R1. The reaction was quenched by filtration through an Amicon Microcon YM-3 filter (Millipore, Bedford, MA). The filtrate was then measured by direct injection onto a Waters Micromass ZQ 2000 mass spectrometer operating in ES $^{-}$  mode. For a zero-minute time, a portion of the total mix was filtered and measured prior to the addition of R2. The procedure for assaying mouse ribonucleotide reductase (RNR) for activity was the same as

that for *E. coli*, with the exception that eightfold more RNR was used (40  $\mu\text{M}$  mouse R1 and 86  $\mu\text{M}$  mouse R2).

## RESULTS

**Synthesis of N-6-substituted nucleoside and nucleotide analogues.** The synthesis of dK and dZ has previously been reported (4). The ribonucleotides containing the K and Z nucleobases, as well as the suite of related analogues shown in Fig. 2, were synthesized as reported previously (32). Furthermore, di- and triphosphorylated species (illustrated in Fig. S1 in the supplemental material) were synthesized. The results of biological and biochemical testing of these compounds are summarized in Table 1.

**Antiviral activity against PV and CVB3.** The purine ribonucleoside analogues were initially screened for antiviral activity in HeLa S3 cells against two picornaviruses, PV and CVB3.

TABLE 1. Biological and biochemical properties of nucleoside analogues<sup>a</sup>

Nucleoside	% Reduction in titer <sup>b</sup>		% Cell viability (HeLa S3) <sup>b</sup>	Fold Gua <sup>a</sup> increase <sup>c</sup>	% Triphosphate incorporation by PV 3D <sup>pol</sup>	
	PV	CVB3			vs U	vs C
RBV	98	98	75	74	0.13	— <sup>f</sup>
JA24	97	95	57	1.9	0.9	0.92
JA23	88	11	ND <sup>e</sup>	ND	0.54	0.1
JA25	99.7	99.9	56	1.8	ND	ND
JA27	35	0	ND	ND	0.54	0.02
JA28	98	97	61	35	0.92	0.94
JA26	81	22	ND	ND	0.79	0.18
JA30	99.97	99.997	37	65	0.89	0.65
JA31	45	0	ND	ND	0.92	0.31

<sup>a</sup> For antiviral and cytotoxicity studies, the JA25 and JA28 data shown are for 0.5 mM treatment. All other analogues are from the 2 mM treatment.

<sup>b</sup> Results are reported as percent reduction in titer or percent viability compared to untreated control. Cellular toxicity was measured via Alamar blue assay after 24 h of continuous exposure.

<sup>c</sup> Increase in guanine-resistant virions compared to untreated infection.

<sup>d</sup> Percent incorporation relative to the correct, natural nucleotide after 60 s.

<sup>e</sup> ND, not determined.

<sup>f</sup> —, below the limit of detection.

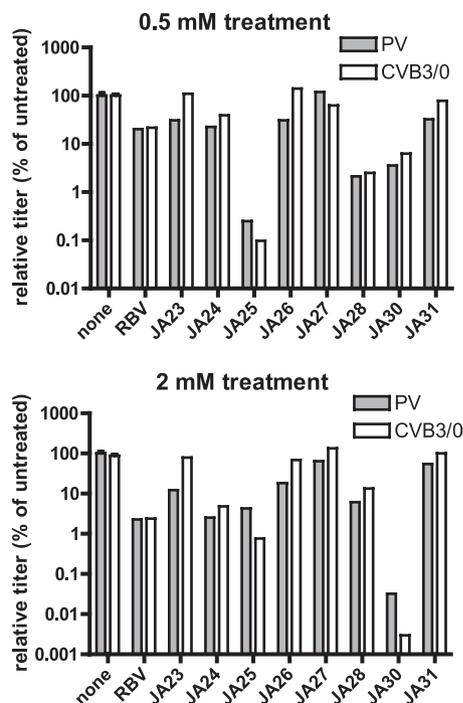


FIG. 3. Nucleoside analogues are antiviral against PV and CVB3. HeLa cells were pretreated with nucleoside analogs (at 0.5 or 2 mM) for 1 h prior to infection with PV or CVB3 at an MOI of 5. Infection was allowed to proceed for 6 h, at which time cell-associated virus was collected and the titer was determined. Data were normalized such that the titer of control sample with no nucleoside treatment was set to a value of 100.

HeLa S3 cells were pretreated with each nucleoside for 1 h prior to infection to allow for accumulation of intracellular metabolites. The known mutagenic nucleoside ribavirin was assayed in parallel for comparison. All nucleosides were evaluated at both 0.5 and 2 mM. Cells were then infected with either virus at a multiplicity of infection (MOI) of 5. Fresh medium containing nucleoside analogue was then added to the cells, and the infection was allowed to proceed for 6 h at 37°C. Cell-associated virus was collected after incubation, and the titer was determined on HeLa S3 monolayers.

The results of the antiviral activity screen are shown in Fig. 3. Ribavirin and four N-6-substituted purine analogues (JA24, JA25, JA28, and JA30) elicited a reduction in virus titer of greater than 90%. Addition of a methyl group to the N-6 substituent (see JA23, JA27, JA26, and JA31) reduced the antiviral properties of each nucleoside. Compared with JA24, JA25, and JA28, the presence of a methyl group in JA23, JA26, and JA27 reduced antiviral activity 3- to 5-fold against PV and 5- to 30-fold against CVB3 at the highest concentration tested (2 mM). Examination of JA25 and JA28 at the 0.5 mM treatment revealed that presence of the methyl group reduced antiviral activity 130- and 15-fold against PV and 160- and 60-fold against CVB3, respectively. The activity of JA30 was most highly impacted by the presence of a methyl group as in JA31, showing 2,000-fold and 40,000-fold reductions in activity against PV and CVB3, respectively, at the 2 mM treatment.

**Cytotoxicity of analogues exhibiting antiviral activity.** While reduction in titer may be indicative of specific antiviral activity,

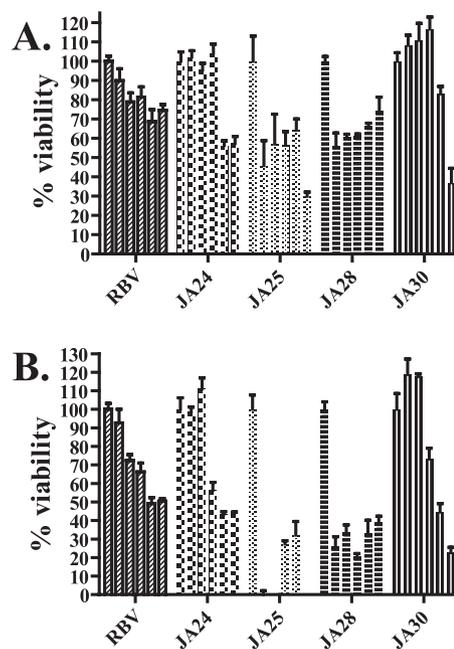


FIG. 4. Cytotoxicity of nucleoside analogs in HeLa cells. Cytotoxicity was measured using an Alamar blue-based assay as described in Materials and Methods. Cells were incubated in the presence of the nucleoside of interest, and Alamar blue reagent was added 6 h prior to measuring absorbance (A) 24 (A) or 48 (B) h postexposure. Data were normalized such that 100% viability was set equal to the percentage of Alamar blue reagent reduced by untreated cells. Data are presented as the means and standard deviations from three independent wells. For each series, the bars represent, from left to right, 0, 0.05, 0.1, 0.5, 1, and 2 mM treatments.

treatment of cells with nucleoside analogues may also have toxic effects that can cause an apparent, yet nonspecific, reduction in titer. Evaluation of cytotoxicity using a typical MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide]-based assay was complicated by cross-reactivity of some analogues with the assay reagent (data not shown). Instead, an assay based on the reduction of resazurin (Alamar blue) as a measure of the metabolic capacity of cells was used. HeLa S3 cells were treated with medium containing 0.05 to 2 mM of each nucleoside, and reduction of resazurin was measured after 24 or 48 h of incubation at 37°C (Table 1 and Fig. 4). The data were normalized such that resazurin reduction by cells treated only with vehicle (DMSO) was set to 100%. JA24, JA28, and JA30 all exhibited a cytostatic effect (50% inhibition at 24 h), whereas JA25 had more marked toxicity.

**Guanidine resistance assay to estimate transition mutation frequency in PV.** If these purine analogues are ambiguous and base pair efficiently with either of the natural pyrimidines due to their rapid tautomerization, an increased frequency of transition mutations for virus grown in their presence should be observed. Consequently, the antiviral activity of these nucleosides may be attributable to lethal mutagenesis.

To test this hypothesis, we utilized a previously established assay for guanidine resistance in PV (7). PV replication is blocked in the presence of 3 mM guanidine hydrochloride due to inhibition of the 2C protease; however, resistance can be conferred by a single transition mutation which arises naturally

TABLE 2. Guanidine resistance frequency of PV populations exposed to nucleoside analogues

Nucleoside	Gua <sup>r</sup> frequency/ 10 <sup>6</sup> PFU <sup>a</sup>
None	5.4 ± 2.3
RBV	400 ± 120
JA24	10 ± 3.1
JA25	9.6 ± 6.0
JA28	190 ± 42
JA30	350

<sup>a</sup> Guanidine resistance was measured as previously described (7). Cells were treated with 2 mM nucleoside analogue prior to infection as described in the text. Monolayers were infected with  $1 \times 10^6$  PFU of PV and incubated in the presence of 3 mM guanidine hydrochloride, and the number of plaques was determined 3 days later via crystal violet staining. Data are presented as means ± standard deviations from 3 independent samples. For JA30,  $n = 2$  and standard deviation was not calculated.

in PV populations at a frequency of approximately  $10^{-5}$  (3). If the mechanism of action of these nucleoside analogues is lethal mutagenesis, the frequency of Gua<sup>r</sup> variants should increase in PV populations treated with the analogues.

Guanidine resistance frequency was measured in virus populations treated with 2 mM JA24, JA25, JA28, or JA30 (Table 2). The known mutagen ribavirin was included as a control, and treatment with ribavirin increased the frequency of Gua<sup>r</sup> variants approximately 75-fold. JA24 and JA25 proved to be only mildly mutagenic, increasing the Gua<sup>r</sup> frequency only twofold. However, JA28 and JA30 both showed significant increases in Gua<sup>r</sup> frequency (35- and 65-fold, respectively).

**Incorporation of nucleoside triphosphate analogues in vitro by PV 3D<sup>pol</sup>.** To determine whether the mutagenic activity of the analogues JA24, JA28, and JA30 could be due to incorporation of the monophosphate of each into RNA and subsequent induction of mutagenesis through ambiguous base pairing, the 5'-triphosphorylated species of each was synthesized (see Fig. S1A in the supplemental material). The nucleosides that lacked substantial antiviral activity (JA23, JA26, JA27, and JA31) were also triphosphorylated as controls. JA25 was not further investigated due to its acute cellular toxicity. These nucleotides, in addition to RTP, were evaluated as substrates of the PV RNA-dependent RNA polymerase, 3D<sup>pol</sup>, using a primer extension assay which utilizes the s/s RNA duplex (1). Nucleotides were examined as purine analogues by measuring in vitro incorporation opposite uridine or cytidine as previously described (1). Each s/s duplex was end labeled with <sup>32</sup>P and preincubated with PV 3D<sup>pol</sup> for 3 min at room temperature to allow for assembly of stable extension complex. The reaction was then initiated by addition of nucleotide and incubated at 30°C for 60 or 330 s before quenching with EDTA. Product was separated via denaturing PAGE, and product was quantitated via PhosphorImager as previously described (1). Each nucleotide was also evaluated as a pyrimidine analog by utilizing the appropriate adenine and guanine s/s templates. No significant incorporation was detected opposite the natural purines (data not shown).

The results of incorporation of nucleotides via PV 3D<sup>pol</sup> are shown in Table 3. Results were normalized so that the amount of extended product formed at a given time when the correct, natural substrate was added was set to a value of 1. All nucleotides tested were incorporated more rapidly than the known ambiguous substrate RTP when uridine was employed as the

TABLE 3. Nucleotide analogue incorporation into RNA by PV 3D<sup>pol</sup> in vitro

Nucleotide or analogue	Relative incorporation with templating base <sup>a</sup> :			
	Uridine		Cytidine	
	60 s	330 s	60 s	330 s
JA23-TP	0.54	0.96	0.1	0.15
JA24-TP	<b>0.9</b>	<b>0.92</b>	<b>0.92</b>	<b>0.9</b>
JA26-TP	0.79	0.79	0.18	0.25
JA27-TP	0.54	0.89	0.02	0.01
JA28-TP	<b>0.92</b>	<b>0.82</b>	<b>0.94</b>	<b>0.9</b>
JA30-TP	<b>0.89</b>	<b>0.8</b>	<b>0.65</b>	<b>0.65</b>
JA31-TP	0.92	0.79	0.31	0.65
RTP	0.13	0.57	—	0.6
ATP	1	1	—	—
GTP	0.13	0.61	1	1

<sup>a</sup> Incorporation of analogues was measured by primer extension using PV 3D<sup>pol</sup>. For each substrate, incorporation of the correct nucleotide was set to 1. —, no extension detected. Results for analogues capable of inducing transition mutations (Table 2) are shown in bold.

templating base, indicating that all nucleotides examined mimic adenosine as substrates for 3D<sup>pol</sup>. However, the analogues containing a methyl substituent at the N-6 position (JA23, JA26, JA27, and JA31) showed a reduced capacity as substrates when templated by cytidine. Importantly, both JA24-TP and JA28-TP showed a high capacity to act as ambiguous substrates; that is, after 60 s of incubation, they were templated approximately equally well by either uridine or cytidine, and the amount of product formed under these conditions was at least 90% of the amount of product formed with the correct nucleotide. JA30-TP also showed rapid and ambiguous incorporation characteristics, although it was templated by cytidine at only 65% of the efficiency of GTP after 60 s of incubation.

**Nucleotide analogues are not “chain terminators.”** Incorporation of nonnatural nucleotides into RNA may affect subsequent polymerization by blocking further addition of nucleotides to the 3' end of the nascent RNA (“chain termination”). Additional primer extension assays were employed to determine whether this mechanism might contribute to the observed antiviral activity of these nucleoside analogues. PV 3D<sup>pol</sup> was incubated with s/s-U substrate to form active elongation complexes. The reaction was then initiated by addition of the nucleotide analogue of interest combined with 10 μM UTP. The s/s-U primer/template substrate contains an adenosine in the second template position, allowing UTP to “chase” the +1 RNA reaction product to a +2 (12-mer) product if further primer elongation is possible (Fig. 5). For each of the nucleotide analogues tested, quantitative conversion of the +1 product to +2 product was observed, indicating that incorporation of these nucleotides does not prevent incorporation of the next templated nucleotide. Thus, chain termination was not observed after a single incorporation of any of these nucleotide analogues.

**HPLC analysis of intracellular metabolites of JA28.** A nucleoside analog must be metabolized to its 5'-triphosphate in order to be incorporated into viral RNA. To assess whether these purine analogues might be acting via lethal mutagenesis, we attempted to identify the triphosphorylated species in HeLa S3 cells treated with the nucleoside. We chose to work with a potent and efficiently incorporated analogue, JA28. HeLa S3

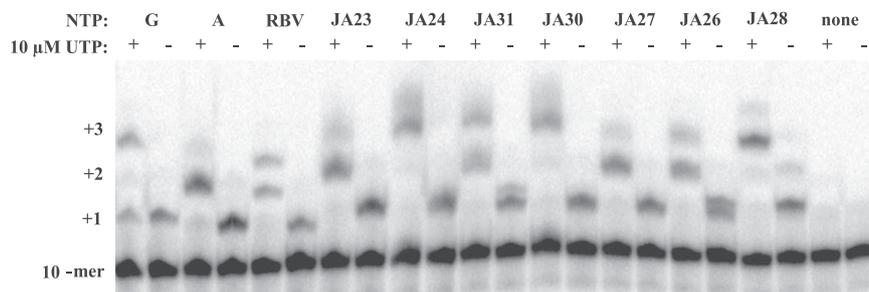


FIG. 5. Nucleotide analogues are not chain terminators. Complexes of  $3D^{pol}$  and s/s-U were preassembled and then mixed with either 500  $\mu$ M nucleotide (– lanes) or with 500  $\mu$ M nucleotide and 10  $\mu$ M UTP (+ lanes). The reaction was allowed to proceed at 30°C for 180 s, followed by quenching with EDTA and separation via denaturing PAGE. NTP added is indicated above each lane. The positions of 10-mer substrate and extended products are indicated to the left.

cells were grown in suspension and treated with actinomycin D (2.5  $\mu$ g/ml) to prevent incorporation of ribonucleotides into cellular RNA. Cells were then treated with 0.5 mM JA28 for 2 h and extracted as previously described (13, 30). Reversed-phase high-performance liquid chromatography (HPLC) analysis of the cell extracts indicated that a substantial amount of the triphosphorylated species was present (Fig. 6). No mono- or diphosphorylated species were observed. Retention time and characteristic UV absorbance trace identified the peak as JA28-TP compared to HPLC analysis of synthesized material (compare Fig. 6A to C). The characteristic peak was not present in the untreated control (Fig. 6B). Thus, JA28 is recognized by cellular kinases and converted to detectable amounts of intracellular JA28-TP within 2 h of exposure.

**Enzymatic 2'-dehydroxylation of nucleoside analogue diphosphates by RNR.** Intracellular accumulation of triphosphorylated forms of nucleoside analogues is essential for lethal mutagenesis via direct incorporation into viral genomic RNA. However, it is also possible for a nucleoside analogue to be utilized as a substrate for cellular DNA polymerases if the ribonucleoside is deoxygenated at the 2' position by cellular metabolic processes to yield the 2'-deoxyribonucleoside-5'-triphosphate (dNTP). Conversion to the deoxyribonucleotide may allow for incorporation into host cell DNA, increasing the possibility of nonreversible genetic mutation to the host cell.

The most likely pathway for conversion to the 2'-deoxyribonucleotide is through ribonucleotide reductase, a cellular enzyme which removes the 2'-hydroxyl from a ribonucleoside-5'-diphosphate (rNDP) (26). However, the substrate specificity of these enzymes is largely undefined. As such, we have developed an assay to measure the conversion of rNDPs to dNDPs by monitoring substrate formation and product disappearance by mass spectrometry in the presence of active RNR from either *E. coli* or mouse. We synthesized the ribonucleoside diphosphates of JA24 (JA53), JA28 (JA51), and JA30 (JA52) (hereafter referred to as JA24-DP, JA28-DP, and JA30-DP; see Fig. S1B in the supplemental material), as well as ribavirin diphosphate (RDP) for comparison.

Recombinant mouse and *E. coli* RNRs differed greatly in their capacity to convert the nucleotide analogues (Table 4). Although both are in the class I RNR family, mouse RNR was not able to produce detectable amounts of dRDP or dJA28-DP, whereas the *E. coli* RNR catalyzed production of both, albeit much more slowly than dGDP. RDP was converted at

approximately 46% the rate of rGDP by the *E. coli* RNR; JA28-DP was converted only 7% as efficiently as rGDP. Presumably this discrimination by mouse RNR also exists in other mammalian systems, including humans, and extends a certain defense against miscoding. JA24-DP was unstable under these assay conditions. The JA24-DP mass peak signal decayed without any concomitant production of a deoxy-JA24-DP signal in the presence or absence of RNR. Thus, the conversion of this analogue could not be established with the current assay. Notably, neither mouse nor *E. coli* RNR catalyzed conversion of JA30-DP to detectable levels.

**A high-fidelity PV variant is resistant to the mutagenic effects of JA24, JA28, and JA30.** Previously, a PV variant (PV-G64S) was isolated that demonstrated increased polymerase fidelity and resistance to the antiviral effects of ribavirin due to a single amino acid substitution in the  $3D^{pol}$  coding region (2, 28). This mutation also conferred resistance to 5-azacytidine, another known mutagenic nucleoside analogue (28). If the antiviral activity of a nucleoside analogue is due to lethal mutagenesis via incorporation of the nucleotide triphosphate into viral genomic RNA by the viral polymerase  $3D^{pol}$ , PV-G64S may exhibit resistance to the analogue relative to the wild-type virus (PV-WT).

At the 2 mM concentration, PV-G64S showed resistance to each of the four nucleotides tested, with titers 1.5- to 3-fold higher than that of PV-WT when normalized to the untreated control, which is similar to the resistance seen for ribavirin treatment at this concentration (Fig. 7A). The treated virus populations were then assayed for guanidine resistance. PV-G64S treated with nucleoside analogue demonstrated markedly fewer transition mutations in progeny virions (as estimated by *Gua*<sup>r</sup> frequency) than the wild-type polymerase (Fig. 7B). The frequency of *Gua*<sup>r</sup> variants was two- to sixfold lower for G64S virus than for PV-WT under identical treatment. These results suggest that the mechanism of antiviral activity of these nucleosides affects a specific virus target (the viral RNA-dependent RNA polymerase) and that the activity is mediated, at least in part, by replication fidelity.

## DISCUSSION

Although the N-6-substituted purine analogues described herein showed more favorable incorporation kinetics than ribavirin (see Table 2), they were less effective mutagens based

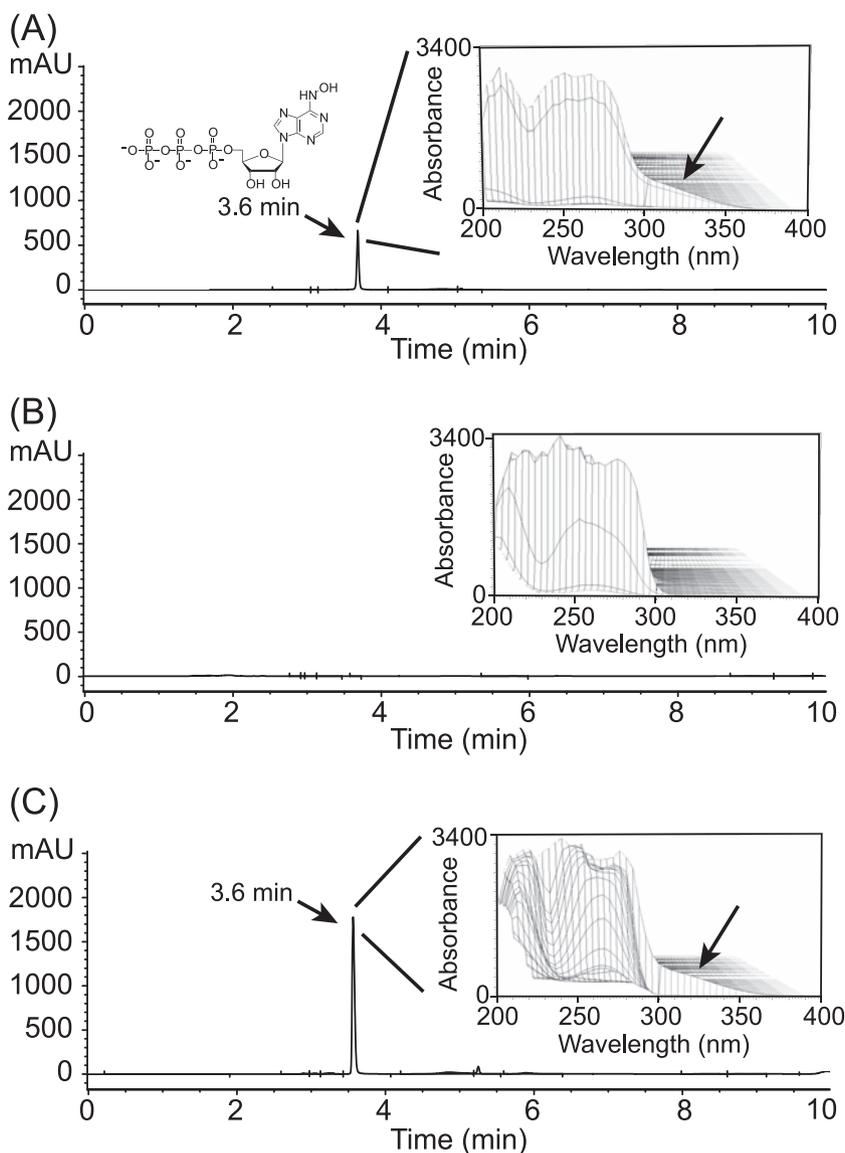


FIG. 6. Analysis of extracts of HeLa S3 cells by reverse-phase HPLC. (A) Analysis of pure JA28-TP including the full UV spectrum of the JA28-TP peak at 3.6 min (inlay). mAU, milli-absorbance units. (B) Analysis of extracts of untreated HeLa S3 cells including the full UV spectrum of the eluent at 3.6 min. (C) Analysis of extracts of HeLa S3 cells treated with JA28 (0.5 mM) including the full UV spectrum of the eluent at 3.6 min (inlay). The long-wavelength absorption characteristic of JA28-TP is illustrated by the arrows to the right. Peaks were detected by  $A_{325}$ .

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TABLE 4. Conversion of nucleoside diphosphates by RNR

Substrate	Activator	Organism	Conversion rate/min
GDP	dTTP	<i>E. coli</i>	10.5
		Mouse	0.3
RDP	dTTP	<i>E. coli</i>	4.8
		Mouse	<0.005
JA24-DP	dTTP		Unstable in DTT
JA28-DP	dTTP	<i>E. coli</i>	0.75
		Mouse	<0.005
JA30-DP	dTTP	<i>E. coli</i>	<0.005
		Mouse	<0.005

on  $Gua^r$  frequency. There are a number of possible explanations for this observation. First, ribavirin monophosphate is a known inhibitor of cellular inosine monophosphate dehydrogenase, which should cause a reduction in cellular purine nucleotide pools (most notably GTP). By reducing the concentration of competitor nucleotide, RTP may be able to more effectively compete with the natural nucleotides present in the cell. The ability of the JA nucleosides to influence cellular nucleotide pools by targeting enzymes of cellular nucleotide metabolism has not yet been investigated. Inosine monophosphate dehydrogenase inhibition alone has been demonstrated to cause an antiviral effect and likely contributes to the mutagenic activity of ribavirin (9, 24, 31, 37).

Second, the kinetic flux through the cellular nucleotide metabolism pathway may differ between nucleotides. Hence, if

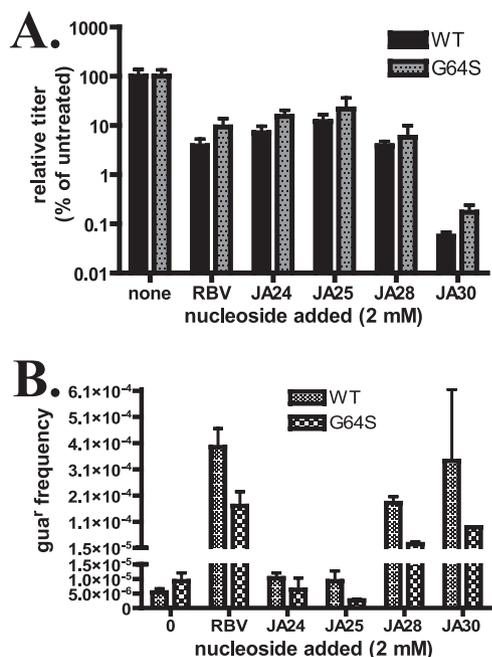


FIG. 7. Antiviral activity of nucleoside analogues against a high-fidelity PV variant (PV-G64S). Infections were performed at an MOI of 5 and treatment with 2 mM nucleoside analogue. Infection was allowed to proceed for 6 h at 37°C, and cell-associated virus was collected by freeze-thaw. (A) The titer of virus was determined on HeLa S3 cell monolayers. Data were normalized for each virus such that the virus titer resulting from untreated control infection was set equal to 100. Data are plotted as the means and standard deviations from three independent samples. (B) Virus was assayed for the GUA<sup>+</sup> phenotype as described in the text. Results are reported as means and standard deviations from three independent samples. Due to the small amount of viable virus recovered from JA30-treated samples,  $n = 2$  for wild-type (WT) PV and  $n = 1$  for PV-G64S for this treatment only.

ribavirin is more rapidly converted to the corresponding triphosphate by cellular metabolism machinery, it may act as a more potent mutagen due to the higher intracellular concentration of the active metabolite. Finally, the unnatural nucleobases of these analogues may be modified through the action of other cellular metabolic enzymes to yield bases that have diminished or absent ambiguous base-pairing capacity. Such metabolic conversion would reduce the ability of the mutagenic nucleotide to accumulate within the cell.

The differences observed in antiviral activity between nucleosides may also be due to subtle differences in the kinetics of incorporation. In the present study, nucleotide incorporation prior to 60 s was not examined, and thus quantitative information on the rate of incorporation is currently unknown, as in some cases the incorporation had reached completion prior to 60 s. However, we can conclude that JA24, JA28, and JA30 were all incorporated more rapidly than ribavirin in the context of either templating pyrimidine.

Modified nucleosides can often exert activity through inhibition of viral or cellular polymerases by acting as low-efficiency substrates. Low rates of incorporation of nonnatural nucleosides can result in polymerase “stalling” (16), chain termination, or lowered rates of replication from templates containing modified nucleosides (23). Thus, the observed antiviral

activity may result, at least in part, from a reduction in RNA replication kinetics caused by inhibition of the viral RdRp. However, this effect is likely minor considering the fairly rapid kinetics of incorporation of these analogs relative to ribavirin (Table 3).

While the concentrations of nucleoside analogue used in these studies may be considered high for therapeutic application, the proposed mechanism of action for these analogues (i.e., lethal mutagenesis) implies competition with the intracellular pools of natural nucleotides. Particularly, these analogues act as general purine analogues, indicating they would need to compete against the extraordinarily high intracellular concentrations of ATP and GTP (approximately 3 mM total purine nucleotides) (34). Furthermore, the mutagenic effect we have documented at these concentrations is substantial, and these analogues may in fact provide relevant antiviral effects at lower concentrations, because continuous exposure should lead to accumulation of mutations over multiple rounds of genome replication. With regard to therapeutic relevance, the only clinically employed nucleoside analogue proposed to act via antiviral lethal mutagenesis is ribavirin, which is dosed at extraordinarily high concentrations during treatment (in the range of 1 g per day) and can accumulate to concentrations approaching 0.5 mM in the liver (7).

While we have demonstrated that the active nucleoside analogues identified in this work are not efficient substrates for RNR *in vitro*, conversion to the deoxyribonucleotide may still be possible through nucleotide phosphorolysis, liberating the nucleobase to be available as a substrate for phosphoribosyl transferase and allowing for generation of the deoxyribonucleosides through nucleobase salvage. Furthermore, the ribonucleosides themselves may exert toxicity through inhibition of, or incorporation by, cellular RNA polymerase II or mitochondrial polymerases. Further exploration of the interaction of mutagenic nucleosides with cellular polymerases is necessary to understand the clinical implications of treatment with antiviral lethal mutagens.

Recently, modified bases of JA23 and JA27 were shown to inhibit hepatitis C virus replication when attached to a 2'-C-methyl ribose (14, 15). However, those compounds were found not to function as substrates for adenosine kinase, with administration of a cyclic monophosphate prodrug increasing efficacy significantly. While the templating specificity of these 2'-C-methyl ribonucleotide analogues has not been directly elucidated, some variability in the nucleobase can be tolerated without loss of antiviral activity (8). Therefore, the use of nucleobases with ambiguous hydrogen bonding properties, such as those described herein, may allow chain termination to be exerted in the context of two or more templating bases. Furthermore, the lack of phosphorylation activity observed by Gunic et al. (14) may explain, at least in part, the lack of activity of related analogues in the present study.

In summary, we have identified nucleoside analogues which can act as antiviral lethal mutagens against picornaviruses. An increase in the frequency of transition mutations was observed, presumably due to tautomerization of the modified nucleobases leading to two distinct hydrogen bonding acceptor/donor surfaces which can interact favorably with either of the natural pyrimidines. Furthermore, these nucleosides had favorable properties in terms of polymerase incorporation and cellular metabolism and appeared not to be recognized as substrates

for mammalian ribonucleotide reductase. Thus, these modified nucleosides represent important lead compounds in the development of antiviral lethal mutagens for clinical use.

#### ACKNOWLEDGMENTS

Financial support was provided by the National Institutes of Health (AI054776 to C.E.C., B.R.P., and J.M.B.), the MRC Development Gap Fund (financial assistance to K.T.), and the American Heart Association (established investigator award 0340028N to C.E.C. and predoctoral fellowships to J.P.E. and D.A.H.).

We thank Danny Yun and Jeff Baldwin for cloning and purifying RNR using the BL21(DE3)/pET22b expression system. We thank Nora Chapman and Steven Tracy (University of Nebraska Medical Center) for providing plasmids encoding CVB3 cDNA.

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