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9-(*trans*-2′,*trans*-3′-Dihydroxycyclopent-4′-Enyl)-Adenine and -3-Deazaadenine: Analogs of Neplanocin A Which Retain Potent Antiviral Activity but Exhibit Reduced Cytotoxicity

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Two synthetic analogs of neplanocin A, which were shown in a separate study to be inhibitors of *S*-adenosylhomocysteine hydrolase and devoid of substrate activity with adenosine kinase, were found in this study to inhibit vaccinia virus replication in murine L929 cells but to have reduced cytotoxicity compared with that of the parent compound. These results confirm that *S*-adenosylhomocysteine hydrolase is the molecular target which mediates the antiviral effects of neplanocin A and that transformation by cellular adenosine kinase mediates its cytotoxic properties.

S-Adenosylhomocysteine (AdoHcy) hydrolase (EC 3.3.1.1) has become an attractive target for the design of antiviral agents (4). This cellular enzyme catalyzes the hydrolysis of AdoHcy to adenosine and homocysteine, thus maintaining low cellular concentrations of this product inhibitor of *S*-adenosylmethionine-dependent methylations (11). Inhibition of cellular AdoHcy hydrolase, however, results in intracellular accumulation of AdoHcy, a significant increase in the intracellular AdoHcy/*S*-adenosylmethionine ratio, and subsequent inhibition of *S*-adenosylmethionine-dependent methylation reactions such as those essential for viral mRNA maturation (e.g., 5′-methylated cap structure) (2, 8, 10, 11). For example, our laboratory has reported that neplanocin A is a potent inhibitor of cellular AdoHcy hydrolase and an inhibitor of vaccinia virus replication in murine L929 cells (2, 7). The antiviral action of neplanocin A has been related to a decrease in viral mRNA methylation and to a subsequent suppression of viral protein synthesis (7). De Clercq and Cools (4) have also established a close correlation between the antiviral potency of several adenosine analogs, including neplanocin A, and their selective inhibitory effects on AdoHcy hydrolase.

Neplanocin A is of interest as a prototype antiviral agent because of its rather broad spectrum of antiviral effects (3), but the therapeutic utility of neplanocin A as an antiviral agent has been limited by its significant cytotoxicity (3, 12). However, evidence does exist to suggest that the antiviral effects of neplanocin A are mediated by its inhibition of AdoHcy hydrolase (2, 3, 7), whereas, its cytotoxic effects are mediated through its phosphorylation by adenosine kinase and subsequent conversion of the neplanocin nucleotides to *S*-neplanocylmethionine (5, 6, 9).

Therefore, we have designed neplanocin A analogs [9-(*trans*-2′,*trans*-3′-dihydroxycyclopent-4′-enyl)-adenine (analog 1) and -3-deazaadenine (analog 2) (Fig. 1)] that retain inhibitory effects toward cellular AdoHcy hydrolase but are devoid of substrate properties for cellular adenosine kinase and adenosine deaminase. In this brief communication, we have described the effects of neplanocin A and analogs 1 and 2 on vaccinia virus multiplication and murine L929 cell toxicity.

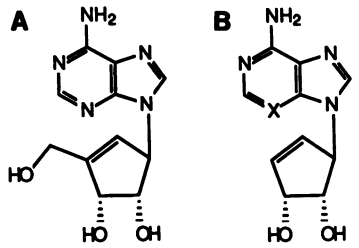

FIG. 1. Structures of neplanocin A (A) and analogs 1 and 2 (B). In analog 1, X=N; in analog 2, X=CH.

The antiviral effects of neplanocin A and analogs 1 and 2 were determined in L929 mouse cells infected with vaccinia virus. Experimental cultures for anti-vaccinia virus assays were plated in multiculture plates with Waymouth MB 752/1 medium (Hazleton) containing 4% calf serum. Nearly confluent cell monolayers were infected with vaccinia virus at about 200 PFU per well. After a 60-min virus adsorption period, the viral inoculum was diluted by the addition of culture medium and then completely removed by aspiration. Immediately after infection, each well containing a cell monolayer was overlaid with medium containing 0.1% methylcellulose to which was added medium containing test compounds in serial dilutions in 0.5-log increments. After incubation for 48 h at 37°C, cultures were washed with phosphate-buffered saline and stained with 0.1% crystal violet. The concentrations producing 50% inhibition of

TABLE 1. Antiviral activity and cytotoxicity of analogs 1 and 2 and of neplanocin A

Compound	Antiviral activity and index[a]		Cytotoxicity and index[b]		Antiviral effectiveness (ID_{50}/IC_{50})
	IC_{50} (μM)	IC_{50} (test compound)/ IC_{50} (NpcA)[c]	ID_{50} (μM)	ID_{50} (test compound) /ID_{50} (NpcA)	
1	0.28	3.5	17	34	61
2	0.95	11.9	56	112	59
NpcA	0.08	1	0.5	1	6

[a] Experiments conducted in duplicate.
[b] Experiments conducted in triplicate.
[c] NpcA, Neplanocin A.

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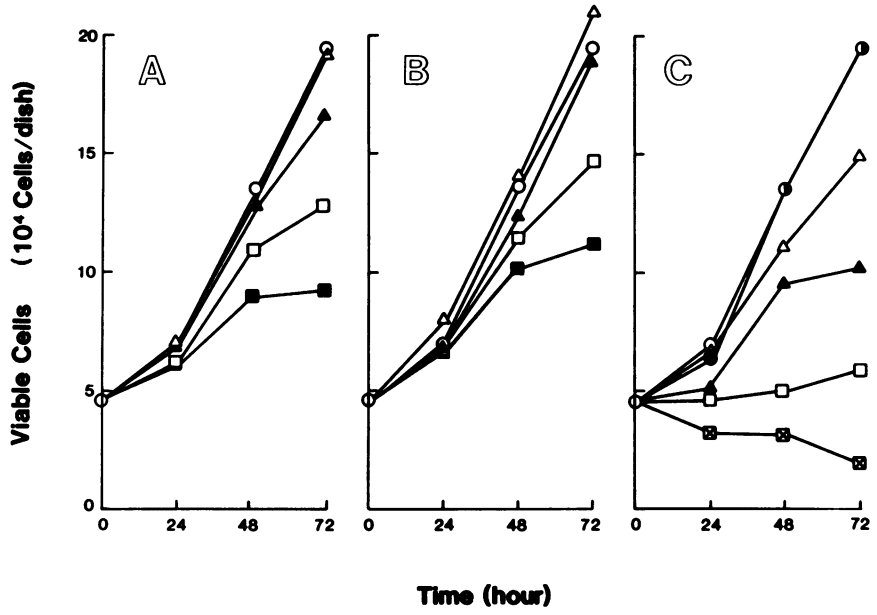
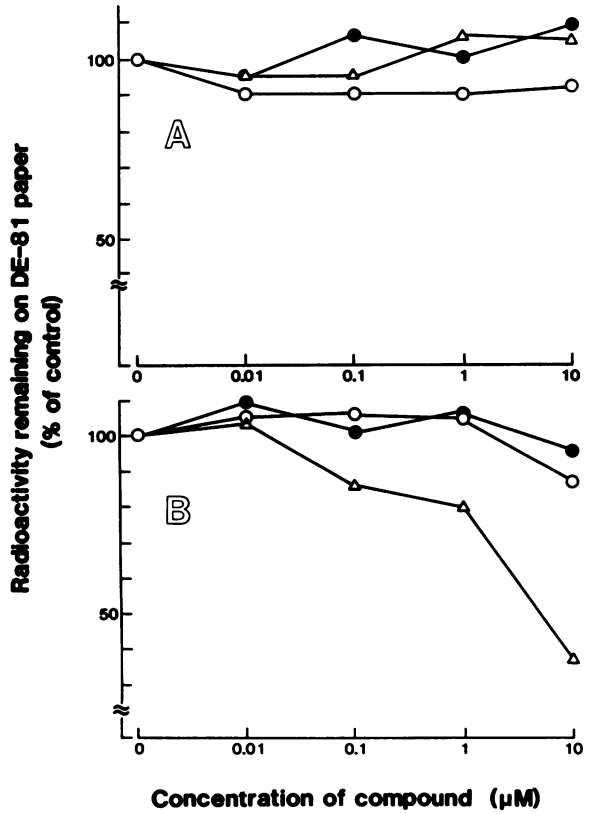


FIG. 2. Cytocidal and cytostatic effects of neplanocin A and analogs 1 and 2 on murine L929 cells. L929 cells were cultured in the absence of drug (○) or in the presence of drug at the following concentrations: ●, 0.01 μM; △, 0.1 μM; ▲, 1 μM; □, 10 μM; ⊠, 32 μM; ■, 100 μM. (A) Compound 1; (B) compound 2; (C) neplanocin A. At the indicated times, the numbers of viable cells were determined as described in the text.

plaque formation (IC_{50}) and the antiviral indices (ratio of IC_{50} for the test compound/IC_{50} for neplanocin A) are summarized in Table 1. For comparative purposes, the IC_{50} for ribavirin was determined in this assay to be 2.2 μM.



The effects of neplanocin A and analogs 1 and 2 on cell growth were determined by trypan blue staining of L929 cells grown in various concentrations of the test compounds. L929 cells were plated at 4.5×10^5 cells per dish (30 mm) and grown in Waymouth MB 752/1 medium containing 4% calf serum. After a 6-h incubation, the cells were fed with fresh medium with or without the test compounds. After incubation for 24, 48, or 72 h, the cells were dispersed by trypsin treatment and suspended in phosphate-buffered saline containing 0.04% trypan blue, and viable cells were counted with a hemacytometer. The 50% cell growth inhibitor concentrations (ID_{50}s) and the cell growth inhibitor indices (ratio of ID_{50} for the test compound/ID_{50} for neplanocin A) are summarized in Table 1. Cultures treated with concentrations of analogs 1 and 2 as high as 100 μM produced only cytostatic effects (Fig. 2). In contrast, concentrations of neplanocin A as low as 32 μM produced cytocidal effects.

The cytotoxic effects of the drugs were also assessed by measuring their effects on DNA and RNA synthesis. Cultures plated in 24-well dishes were incubated in modified Waymouth medium containing 1% calf serum with or without various concentrations of the test compounds at 37°C for 12 h and then pulse-labeled for 2 h with [*methyl*-^{3}H]thymidine or [5,6-^{3}H]uridine. The radio-active culture medium was removed by aspiration, and the monolayers were lysed with solution containing 1% sodium dodecyl sulfate, 0.1 EDTA, 0.1 M Tris hydrochloride (pH 9.0), and 10 mM vanadyl ribonucleoside complex (1). The lysate was trans-

FIG. 3. Effect of various concentrations of analogs 1 or 2 or of neplanocin A on L929 cell DNA and RNA synthesis. L929 cells were treated with various concentrations of analog 1 (○) or 2 (●) or neplanocin A (△). Cultures were incubated at 37°C for 12 h and then pulse-labeled with [*methyl*-^{3}H]thymidine (final concentration, 0.5 μCi/ml) (A) or [5,6-^{3}H]uridine (final concentration, 1.0 μCi/ml) (B) for 2 h. The incorporation of radioactivity into RNA and DNA was determined as described in the text.

ferred onto a DE-81 disk. After being washed five times with 5% Na_2HPO_4 and two times with distilled water, each disk was placed in a scintillation vial to which was added 10 ml of 3a70 scintillation cocktail, and the radioactivity remaining on the disk was measured by liquid scintillation spectrometry.

DNA synthesis as measured by incorporation of [*methyl*-^{3}H]thymidine was not inhibited by any of the test compounds at concentrations of up to 10 μM (Fig. 3). RNA synthesis as measured by incorporation of [5,6-^{3}H]uridine was unaffected by analogs 1 and 2 up to concentrations of 10 μM. However, treatment of cells with neplanocin A produced a concentration-dependent reduction in [5,6-^{3}H]uridine incorporation, indicating an inhibition of RNA synthesis.

In separate studies, we have shown that analogs 1 and 2 are potent inhibitors of purified bovine liver AdoHcy hydrolase (D. R. Borcherding, S. A. Scholtz, S. R. Narayanan, M. Hasobe, J. McKee, B. Keller, and R. T. Borchardt, Fed. Proc. **46**:860, 1987), and they inhibit AdoHcy hydrolase in L929 cells, producing significant increases in the intracellular ratio of AdoHcy/*S*-adenosylmethionine, but they are not metabolized by L929 cell adenosine kinase or adenosine deaminase (M. Hasobe, J. McKee, D. Borcherding, B. Keller, and R. T. Borchardt, Fed. Proc. **46**:93, 1987).

Thus, by removing the 4′ hydroxymethyl group of neplanocin A, we have generated analogs that retain inhibitory effects toward AdoHcy hydrolase and that have antiviral effects. However, these analogs are devoid of substrate activity toward adenosine kinase and thus have substantially reduced cellular toxicity. These data suggest we have achieved partial separation of the antiviral and cytotoxic effects of neplanocin A. This separation of the antiviral and cytotoxic effects can be illustrated by comparing the antiviral effectiveness values (ID_{50}/ divided by IC_{50}) for neplanocin A and analogs 1 and 2, which are 6, 61, and 59, respectively. The increased antiviral effectiveness of analogs 1 and 2 primarily reflects the reduction in cellular toxicity.

It is also interesting to note that compounds 1 and 2 do not inhibit RNA synthesis at concentrations of up to 10 μM. These results strongly suggest that inhibition of RNA synthesis by neplanocin A is not related to the effect of the drug on AdoHcy hydrolase, but instead results from its transformation to phosphorylated products or *S*-neplanocylmethionine or both, as suggested by Glazer et al. (5, 6).

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