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Broad-Spectrum Antiviral Activities of Neplanocin A, 3-Deazaneplanocin A, and Their 5′-Nor Derivatives

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The neplanocin A analogs, 3-deazaneplanocin A, 9-(*trans*-2′,*trans*-3′-dihydroxycyclopent-4′-enyl)adenine (DHCA), and 9-(*trans*-2′,*trans*-3′-dihydroxycyclopent-4′-enyl)-3-deazaadenine (DHCDA), all potent inhibitors of *S*-adenosylhomocysteine (AdoHcy) hydrolase, were studied for their broad-spectrum antiviral potential. 3-Deazaneplanocin A, DHCA, and DHCDA proved specifically effective against vesicular stomatitis virus, vaccinia virus, parainfluenza virus, reovirus, and rotavirus. Their selectivity was greater than that of neplanocin A, particularly against vesicular stomatitis virus and rotavirus. As could be expected from adenosine analogs that are directly targeted at AdoHcy hydrolase, 3-deazaneplanocin A, DHCA, and DHCDA were fully active in adenosine kinase-deficient cells, implying that their activity did not depend on phosphorylation by adenosine kinase. None of the AdoHcy hydrolase inhibitors showed selective activity against human immunodeficiency virus (type 1). 3-Deazaneplanocin A at a dose of 0.5 mg/kg per day conferred marked protection against a lethal infection of newborn mice with vesicular stomatitis virus.

Neplanocin A is a potent antiviral agent active against a broad spectrum of viruses belonging to the *Poxviridae*, *Rhabdoviridae*, *Paramyxoviridae*, and *Reoviridae* (6). It is particularly effective against vaccinia virus, vesicular stomatitis virus, parainfluenza virus, measles virus, reovirus (6), and human rotavirus (20). Neplanocin A is a potent inhibitor of *S*-adenosylhomocysteine (AdoHcy) hydrolase, a key enzyme in transmethylation reactions depending on *S*-adenosylmethionine as the methyl donor (2). As such, transmethylation reactions play an important role in the maturation (e.g., 5′ capping) of viral mRNA, and AdoHcy hydrolase has been considered to be an important target enzyme for broad-spectrum antiviral agents (7). In fact, a close correlation has been found between the antiviral effects of a series of adenosine analogs, including neplanocin A, and their inhibitory effects on AdoHcy hydrolase (4, 9).

Although antivirally active at concentrations which are well below its toxicity threshold for the host cells (6), neplanocin A is definitely cytotoxic for a number of tumor cells (18, 24). This cytotoxicity may be attributed to the fact that the compound is readily phosphorylated to its triphosphate (1, 24), which then interferes with host-cell RNA synthesis (18). Neplanocin A triphosphate could be further metabolized to *S*-neplanocylmethionine (14, 19), and this metabolite may also play an important role in the cytotoxic action of the compound (14). If the hypothesis is correct that the antiviral action of neplanocin A is due to an inhibitory effect of the compound per se on AdoHcy hydrolase, whereas its cytotoxic action depends upon phosphorylation to the corresponding triphosphate, it should be possible to design neplanocin A analogs that are endowed with antiviral properties while lacking cytotoxicity, thus achieving a better therapeutic index than that of neplanocin A itself.

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For this reason, 3-deazaneplanocin A and the "decapitated" analogs of neplanocin A and 3-deazaneplanocin A, referred to as DHCA [9-(*trans*-2′,*trans*-3′-dihydroxycyclopent-4′-enyl)adenine] and DHCDA [9-(*trans*-2′,*trans*-3′-dihydroxycyclopent-4′-enyl)-3-deazaadenine], were synthesized (3, 15, 22). Both DHCA and DHCDA were indeed more selective in their activity against vaccinia virus than neplanocin A was (16). In the initial study, the activity against only one virus (vaccinia) was determined and 3-deazaneplanocin A was not included for comparison. We have now extended these studies to a broad range of DNA and RNA viruses. In addition to DHCA, DHCDA, and neplanocin A, we included in our investigation 3-deazaneplanocin A, which is also assumed to be a potent inhibitor of AdoHcy hydrolase but with a lower degree of cytotoxicity than neplanocin A (13, 15). The structural formulae of the compounds are depicted in Fig. 1. The compounds were examined for both antiviral and antimetabolic activities in a variety of cell systems. The therapeutic potential of these compounds was also assessed in vivo by using newborn mice infected with a lethal dose of vesicular stomatitis virus.

MATERIALS AND METHODS

Test compounds. Neplanocin A (M_W 263.3) was obtained from Toyo Jozo Co. (Tagata-gun, Shizuoka-ken, Japan) through the courtesy of J. Murase. 3-Deazaneplanocin A (M_W 262.2) was synthesized as previously described (27) from the optically active cyclopentenyl moiety. DHCA and DHCDA (M_Ws 233.2 and 232.2) were synthesized by procedures described previously (3). Carbocyclic 3-deazaadenosine (C-c^{3}Ado) (M_W 300.7) (21) was obtained from J. A. Montgomery (Kettering-Meyer Laboratory, Southern Research Institute, Birmingham, Ala.). Tubercidin (M_W 266.3) was purchased from Sigma Chemical Company (St. Louis, Mo.). Xylotubercidin (12), sangivamycin, and toyocamycin

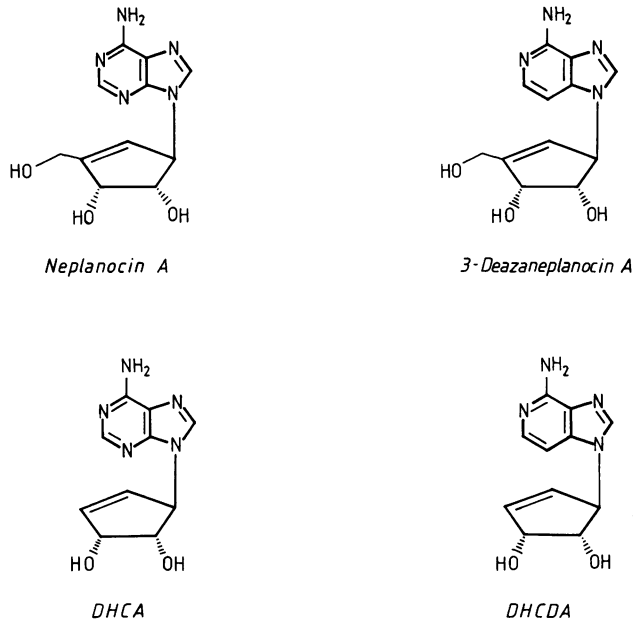

FIG. 1. Structural formulae of neplanocin A, 3-deazaneplanocin A, and their decapitated analogs DHCA and DHCDA.

(8) (M_Ws 266.3, 309.3, and 291.3, respectively) were obtained from M. J. Robins (Department of Chemistry, University of Alberta, Edmonton, Alberta, Canada; now at the Chemistry Department, Brigham Young University, Provo, Utah). Cordycepin (M_W 251.2) was from Sigma Chemical Company. Stock solutions of the compound were prepared in phosphate-buffered saline (PBS) or cell culture medium at a concentration of 1 mg/ml. At this concentration, all compounds were soluble in aqueous medium.

Viruses and cells. The origin of the viruses (herpes simplex virus type 1 KOS and type 2 G, vaccinia virus, vesicular stomatitis virus, coxsackievirus B4, poliovirus type 1, parainfluenza virus type 3, reovirus type 1, Sindbis virus, and Semliki Forest virus) and cells (primary rabbit kidney [PRK], HeLa, and African green monkey kidney [Vero]) has been described previously (6). For further details on the rotavirus MO strain (subgroup 2, serotype 3) and MA 104 cells (derived from embryonic rhesus monkey kidney), see Kitaoka et al. (20). Human immunodeficiency virus type 1 (HIV-1) was obtained from the supernatant of a persistently HIV-1-infected H9 cell line (H9/HTLV-III$_B$) which was kindly provided by R. C. Gallo (National Cancer Institute, Bethesda, Md.). Human T-lymphocyte MT-4 cells were kindly supplied by N. Yamamoto (Yamaguchi University, Yamaguchi, Japan). The cells were mycoplasma free. The AK$^+$ (adenosine kinase-positive) B-mix K-44/6 (Rous sarcoma virus-transformed) rat cells were originally obtained from J. Svoboda (Institute of Experimental Biology and Genetics, Prague, Czechoslovakia) (26). The AK$^-$ (adenosine kinase-deficient) cell line (clone D-4) was derived from the AK$^+$ parent line by selection of a cell population that remained resistant to an ara-A (vidarabine) concentration that was 100-fold greater than that tolerated by the AK$^+$ cells (25; N. B. Katlama, Ph.D. thesis, University of Michigan, Ann Arbor, 1984). Both the AK$^+$- and AK$^-$-B-mix K-44/6 cell lines were devoid of adenosine deaminase activity (25).

Inhibition of virus-induced cytopathogenicity. Confluent cell monolayers in microtiter trays were inoculated with 100 $CCID_{50}$ (50% confluent cell infective dose; 1 $CCID_{50}$ corresponds to the virus stock dilution that proved infective for 50% of the cell cultures). After 1 h of virus adsorption to the cells, residual virus was removed and replaced by cell culture medium (Eagle minimum essential medium) containing 3% fetal calf serum (horse serum for the experiments with the AK$^+$- and AK$^-$-B-mix K-44/6 cells) and various concentrations of the test compounds. Viral cytopathogenicity was recorded as soon as it reached completion in the untreated virus-infected cell cultures, i.e., at 1 to 2 days for vesicular stomatitis virus; at 2 days for Semliki Forest virus, coxsackievirus B4, and poliovirus type 1; at 2 to 3 days for vaccinia virus, herpes simplex virus types 1 and 2, and Sindbis virus; and at 6 to 7 days for reovirus type 1 and parainfluenza virus type 3. The antiviral activity of the compounds was expressed as the concentration required to inhibit viral cytopathogenicity by 50%.

Inhibition of rotavirus replication. Virus yield was measured as described previously (20), and the antiviral activity of the compounds was expressed as the concentration required to achieve a 90% reduction in the 24-h virus yield.

Inhibition of HIV replication. The method of the antiretroviral assays has been described previously (23). Briefly, MT-4 cells (5×10^5/ml) were suspended in fresh culture medium and infected with HIV-1 at 200 $CCID_{50}$ per ml of cell suspension. Then, 100 μl of the infected cell suspension was mixed with 100 μl of the appropriate dilution of test compound, transferred to microplate wells, and further incubated at 37°C for 5 days. Then the number of viable cells was determined for both virus-infected and mock-infected cell cultures.

Cytotoxicity. Cytotoxicity measurements were based on the following parameters: (i) alteration of normal cell morphology, (ii) inhibition of host-cell protein synthesis, and (iii) reduction in the number of viable cells. To evaluate cell morphology (i), confluent cell cultures which had not been infected but were treated with various concentrations of the test compounds were incubated in parallel with the virus-infected cell cultures and examined microscopically at the same time as viral cytopathogenicity was recorded for the virus-infected cell cultures. A disruption of the cell monolayers, e.g., rounding up or detachment of the cells, was considered to be evidence for cytotoxicity. To determine host-cell protein synthesis (ii), the cells were seeded in 96-well microtest plates (Falcon; Becton Dickinson and Co., Lincoln Park, N.J.) at a density of 20,000 cells per well. After 24 h of incubation in a humidified, CO_2-controlled atmosphere at 37°C, the cells were refed with fresh Eagle minimum essential medium containing 3% fetal calf serum and the appropriate concentration of the test compound. After 72 h of incubation at 37°C, the cells were pulse-labeled for 3 h with L-[4,5-^{3}H]leucine (64 Ci/mmol; 0.5 μCi per well) (Amersham, Buckinghamshire, England). After the pulse-label period, the cells were washed with PBS and fixed with methanol. The proteins were precipitated with 10% trichloroacetic acid, and the trichloroacetic acid pellet was solubilized overnight with 1 N NaOH. The solubilized samples were then transferred to scintillation vials and, upon addition of scintillation fluid (Instafluor and Soluene; Packard Instruments, Groningen, The Netherlands), analyzed for radioactivity. Inhibition of the radioactivity counts by 50% was taken as the endpoint for cytotoxicity. To determine cell viability (iii), the cells were enumerated by a trypan blue dye exclusion assay (20), and the minimum cytotoxic concentration was defined as the concentration of test compound required to cause a 50% reduction in cell number (after 72 h

TABLE 1. Antiviral activity and cytotoxicity in PRK cell cultures

Compound	50% Effective concn[a] (μg/ml)				Minimum cytotoxic concn[b] (μg/ml)	
	Herpes simplex virus type 1 KOS	Herpes simplex virus type 2 G	Vaccinia virus	Vesicular stomatitis virus	Cell morphology	Protein synthesis[c]
Neplanocin A	7	10	0.02	0.02	≥400	2.7 ± 0.9
3-Deazaneplanocin A	>400	400	0.07	0.07	>400	≥117
DHCA	150	300	0.7	0.2	>400	≥200
DHCDA	>400	200	0.7	0.2	>400	61 ± 5
C-c^{3}Ado	>400	>400	2	0.2	>400	88 ± 30

[a] Required to inhibit virus-induced cytopathogenicity by 50%; average values for two to three experiments.
[b] Required to cause a microscopically detectable alteration of normal cell morphology or to inhibit protein synthesis (based on [4,5-^{3}H]Leu incorporation) by 50%.
[c] Average values ± standard deviations.

of incubation of the cells in the presence of the test compounds). The latter method for cytotoxicity evaluation was carried out specifically for the MA 104 cells (used in the rotavirus experiments).

Cytostatic activity. The cells were seeded in 96-well test plates (at 6,000 cells per well for the AK^+ and AK^- cells) and in 24-well test plates (at 16,000 cells per well for the PRK, Vero, and HeLa cells) in Eagle minimum essential medium containing 10% fetal calf serum (horse serum for the experiments with the AK^+ and AK^- cells), and 4 h later, various concentrations of the test compounds were added. The cells were allowed to proliferate for 72 h (for the Vero and AK^+- and AK^--B-mix K-44/6 cells) or 96 h (for the PRK and HeLa cells) at 37°C in a humidified, CO_2-controlled atmosphere. The growth of the cells was linear during this period. The cells were then trypsinized and enumerated in a Coulter counter. Cytostatic activity was expressed as the concentration of compound required to reduce the cell number by 50%.

Antiviral activity in vivo. The antiviral efficacy of the compounds was assessed in an experimental model infection that was previously developed to monitor the protective effects of interferon and interferon inducers (10). Newborn (2-day-old) NMRI (Naval Medical Research Institute) mice were inoculated subcutaneously with vesicular stomatitis virus (4 PFU [as determined in mouse L-929 cell cultures]/0.05 ml per mouse). The mice then received either a single intraperitoneal injection of the compound (in 0.1 ml of PBS) at 1 h postinfection or repeated intraperitoneal injections of the compound at 1 h postinfection (day 1) and the subsequent 4 days (days 2, 3, 4, and 5). Mortality was monitored for 20 days. The mortality rates of the different treatment groups were compared with that of the corresponding control group (which received either one or repeated injections of PBS), and the statistical significance of the differences was assessed by the χ^2 test with Yates' correction.

RESULTS

When examined for its activity against herpes simplex virus, vaccinia virus, and vesicular stomatitis virus in PRK cells (Table 1), neplanocin A showed 50% effective concentrations that were comparable to those reported previously (6). The novel neplanocin A analogs, 3-deazaneplanocin A, DHCA, and DHCDA, were inhibitory to vaccinia virus and vesicular stomatitis virus at concentrations which were only slightly higher than the 50% effective concentration of neplanocin A. In contrast to neplanocin A, its new congeners were much less toxic to host-cells (based on inhibition of host-cell protein synthesis) (Table 1). The new neplanocin A analogs were virtually inactive against herpes simplex virus type 1 or 2, and, in this sense, they behaved like C-c^{3}Ado (11) (included as the reference compound) and other adenosine analogs which have been previously recognized as AdoHcy hydrolase inhibitors (7).

In HeLa cells, as in PRK cells, 3-deazaneplanocin A, DHCA, and DHCDA inhibited the replication of vesicular stomatitis virus at a concentration that was well below their cytotoxicity threshold, whether based on cell morphology or cellular protein synthesis (Table 2). However, the compounds were much less active in HeLa cells than in PRK cells. As could be expected from AdoHcy hydrolase inhibitors (7), neither 3-deazaneplanocin A, DHCA, nor DHCDA was inhibitory to the (+)RNA viruses poliovirus (Table 2), coxsackievirus (Tables 2 and 3), and Sindbis and Semliki Forest viruses (Table 3). However, they were markedly active against parainfluenza virus, a (−)RNA virus, and reovirus, a double-stranded (±)RNA virus (Table 3). In Vero cells (Table 3), 3-deazaneplanocin A, DHCA, and DHCDA were more inhibitory to protein synthesis than in PRK or HeLa cells, so that in Vero cells, their antiviral activity (against reovirus and parainfluenza virus) was

TABLE 2. Antiviral activity and cytotoxicity in HeLa cell cultures[a]

Compound	50% Effective concn (μg/ml)			Minimum cytotoxic concn (μg/ml)	
	Vesicular stomatitis virus	Coxsackievirus B4	Poliovirus type 1	Cell morphology	Protein synthesis
Neplanocin A	0.7	>10	>10	40	1.1 ± 0.5
3-Deazaneplanocin A	10	>400	>400	>400	≥129
DHCA	40	>400	>400	>400	91 ± 61
DHCDA	10	>400	>400	≥400	48 ± 11
C-c^{3}Ado	100	>400	>400	>400	103 ± 55

[a] See footnotes to Table 1.

TABLE 3. Antiviral activity and cytotoxicity in Vero cell cultures[a]

Compound	50% Effective concn (μg/ml)					Minimum cytotoxic concn (μg/ml)	
	Parainfluenza virus type 3	Reovirus type 1	Sindbis virus	Coxsackievirus B4	Semliki Forest virus	Cell morphology	Protein synthesis
Neplanocin A	0.2	0.7	>10	>10	>10	≥10	0.4 ± 0.1
3-Deazaneplanocin A	0.2	0.07	>400	400	>400	>400	0.3 ± 0.08
DHCA	2	0.7	>400	>200	>400	≥400	4.7 ± 2
DHCDA	2	2	>400	150	>200	≥400	3.6 ± 2.3
C-c^{3}Ado	0.2	1	>400	150	>400	>400	21 ± 12

[a] See footnotes to Table 1.

achieved at concentrations which were only slightly below the cytotoxicity threshold.

When neplanocin A and its congeners were examined for their inhibitory effects on the proliferation of PRK, HeLa, and Vero cells in their exponential growth phase, neplanocin A and 3-deazaneplanocin A were about equally inhibitory to PRK and HeLa cell growth, whereas 3-deazaneplanocin A was about fivefold more inhibitory to Vero cell growth (Table 4). Irrespective of the nature of the cells (Vero, HeLa, or PRK), the decapitated neplanocins DHCA and DHCDA were 10- to 100-fold less inhibitory than the parent compounds were (Table 4), which is consistent with the results reported previously for L-929 cells (16).

Among the double-stranded RNA viruses, rotaviruses are particularly sensitive to the inhibitory effects of AdoHcy hydrolase inhibitors (7, 20). This has now been confirmed for C-c^{3}Ado and neplanocin A (Table 5); in agreement with earlier findings (20), these compounds inhibited rotavirus replication at a concentration which was almost 100-fold below their cytotoxicity threshold. Also, 3-deazaneplanocin A, DHCA, and DHCDA proved inhibitory to rotavirus replication at a concentration that was at least 2 orders of magnitude lower than the cytotoxic concentration (Table 5). 3-Deazaneplanocin A inhibited rotavirus replication at a concentration of 0.04 μg/ml, while not being toxic up to a concentration of 400 μg/ml, thus achieving a selectivity index of 10,000, the highest selectivity index ever reported for a rotavirus inhibitor.

When evaluated for their inhibitory effects on the replication of HIV-1 in MT-4 cells, none of the compounds listed in Table 6 (i.e., neplanocin A, 3-deazaneplanocin A, DHCA, DHCDA, and C-c^{3}Ado) proved effective at nontoxic concentrations. In fact, neplanocin A and 3-deazaneplanocin A were highly toxic for MT-4 cells (50% cytotoxic doses, 0.016 and 0.025 μg/ml, respectively).

If the antiviral effects of neplanocin A and its congeners are mediated by inhibition of AdoHcy hydrolase, the agents do not need to be phosphorylated by AK to achieve their antiviral activity, and, therefore, they may be expected to be antivirally active in AK$^-$ cells. A paired AK$^+$-AK$^-$ cell system (5) was established to determine the role of AK in the antiviral activity of the adenosine analogs. As shown in Table 7, neplanocin A, 3-deazaneplanocin A, DHCA, and DHCDA were fully active against vaccinia virus and vesicular stomatitis virus in AK$^-$ cells and their 50% effective doses were equal to or only slightly higher than those recorded in AK$^+$ cells. In contrast, various other adenosine analogs such as tubercidin, xylotubercidin, sangivamycin, toyocamycin, and cordycepin, which are assumed to act primarily as their phosphorylated products and thus depend on AK for their metabolic conversion, were significantly less active in AK$^-$ than AK$^+$ cells. This differential activity was particularly striking for tubercidin, which was inhibitory to vaccinia virus and vesicular stomatitis virus in AK$^-$ cells at a concentration that was more than 1,000-fold higher than that required to inhibit the same viruses in AK$^+$ cells (Table 7).

The role of AK in the biological activity of the adenosine analogs was further explored by direct comparison of their 50% inhibitory doses (ID_{50}s) for the growth of AK$^+$ and AK$^-$ cells. In all cases, the ID_{50} for AK$^-$ cell growth was higher than the ID_{50} for AK$^+$ cell growth (Table 8), but,

TABLE 4. Inhibitory effects on the growth of PRK, HeLa, and Vero cells

Compound	50% Cytotoxic concn[a] (μg/ml)		
	PRK	HeLa	Vero
Neplanocin A	0.12 ± 0.08	0.17 ± 0.1	0.25 ± 0.17
3-Deazaneplanocin A	0.14 ± 0.05	0.31 ± 0.2	0.05 ± 0.03
DHCA	9.0 ± 6.5	18 ± 15	1.1 ± 0.6
DHCDA	8.9 ± 2.9	5.6 ± 4	2.2 ± 2
C-c^{3}Ado	17 ± 10	1.3 ± 0.7	0.33 ± 0.1

[a] Required to inhibit cell growth (after 3 days for Vero cells and 4 days for PRK and HeLa cells) by 50%. The data represent average values ± standard deviations for four or more experiments.

TABLE 5. Activity against rotavirus in MA 104 cell cultures

Compound	50% Effective concn[a] (μg/ml)	Minimum cytotoxic concn[b] (μg/ml)	Selectivity index[c]
Neplanocin A	0.2	14	70
3-Deazaneplanocin A	0.04	400	10,000
DHCA	0.5	>100	>200
DHCDA	1.0	>100	>100
C-c^{3}Ado	1.4	>100	70

[a] Required to effect a 90% reduction in virus yield. The virus strain used was MO (subgroup 2, serotype 3).
[b] Required to reduce viable cell counts by 50%.
[c] Ratio of minimum cytotoxic concentration to minimum (antivirally) effective concentration.

TABLE 6. Activity against HIV-1 in MT-4 cell cultures

Compound	50% Effective concn[a] (μg/ml)	50% Cytotoxic concn[b] (μg/ml)	Selectivity index[c]
Neplanocin A	>0.016	0.016	<1.0
3-Deazaneplanocin A	>0.025	0.025	<1.0
DHCA	>0.9	0.9	<1.0
DHCDA	>3.3	3.3	<1.0
C-c^{3}Ado	>1.1	1.1	<1.0

[a] Required to inhibit virus-induced cytopathogenicity by 50%.
[b] Required to reduce the number of living MT-4 cells by 50%.
[c] Ratio of 50% cytotoxic concentration to 50% (antivirally) effective concentration.

while the ratio of the ID_{50} for AK^- cell growth to the ID_{50} for AK^+ cell growth did not exceed 1 order of magnitude for the neplanocin A congeners, it went up to 3 orders of magnitude for tubercidin, toyocamycin, and cordycepin. Again, these results point to the need for phosphorylation of the latter adenosine analogs by AK, whereas such phosphorylation is of lesser importance for the biological activity of the neplanocin A analogs.

Because of their potency against vesicular stomatitis virus in cell culture (Table 1), the neplanocin A analogs were further investigated for their activity against a lethal vesicular stomatitis virus infection in mice, i.e., newborn mice inoculated subcutaneously with vesicular stomatitis virus. In this model (10), neplanocin A does not offer any protection (6). 3-Deazaneplanocin A, however, conferred a highly significant reduction in the mortality rate when administered as a single dose of 0.5 or 2.5 mg/kg per day (1 h postinfection) or repeated doses of 0.5 mg/kg per day (1 h and 2, 3, and 4 days postinfection) (Fig. 2). At lower doses (0.1 mg/kg per day) and higher doses (2.5 mg/kg per day [five times], 10 mg/kg per day, and 50 mg/kg per day), it was not protective (Table 9). At these higher doses, 3-deazaneplanocin A was also toxic to the mice (data not shown). DHCA offered some protective activity, but only at a dose of 0.5 mg/kg per day, given singly or repeatedly (Table 9). At a dose of 2.5 mg/kg per day (or higher) it was toxic (data not shown). For DHCDA there was only a hint of activity, if administered at a dose of 0.5 mg/kg per day (Table 9).

TABLE 7. Antiviral activity in AK^+- and AK^--B-mix K-44/6 cell cultures

Compound	50% Effective concn[a] (μg/ml)			
	Vesicular stomatitis virus		Vaccinia virus	
	AK^+ cells	AK^- cells	AK^+ cells	AK^- cells
Neplanocin A	0.007	0.002	0.002	0.007
3-Deazaneplanocin A	0.04	0.2	0.007	0.07
DHCA	0.2	1	0.2	0.2
DHCDA	0.7	4	0.7	0.7
Tubercidin	0.007	10	0.004 (tox)	7
Xylotubercidin	1 (tox)	>400	>4 (tox)	>100
Sangivamycin	0.1 (tox)	10 (tox)	0.1 (tox)	0.7
Toyocamycin	0.4 (tox)	4 (tox)	0.1 (tox)	0.7
Cordycepin	0.7	>200	0.1 (tox)	>200

[a] Required to inhibit virus-induced cytopathogenicity by 50%. The values are averages for two to three experiments. Where the indicated value is followed by (tox), microscopically visible cytotoxicity was observed at the minimum (antivirally) effective concentration.

TABLE 8. Cytostatic activity against AK^+- and AK^--B-mix K-44/6 cells

Compound	50% Inhibitory concn[a] (μg/ml)		Ratio[b]
	AK^+ cells	AK^- cells	
Neplanocin A	0.0054 ± 0.0013	0.02 ± 0.012	3.7
3-Deazaneplanocin A	0.036 ± 0.011	0.40 ± 0.08	11
DHCA	1.3 ± 0.8	8.8 ± 2.2	6.8
DHCDA	3.3 ± 0.9	20.6 ± 7.8	6.2
Tubercidin	0.036 ± 0.016	14 ± 7.3	389
Xylotubercidin	3.7 ± 1.2	>500	>135
Sangivamycin	0.031 ± 0.015	5.2 ± 0.9	168
Toyocamycin	0.0030 ± 0.0005	2.6 ± 0.8	867
Cordycepin	0.030 ± 0.019	72 ± 19	2,400

[a] Required to inhibit cell growth (after 3 days) by 50%. The data represent average values ± standard deviations for at least four separate determinations.
[b] Ratio of 50% inhibitory concentration for AK^- cell growth to 50% inhibitory concentration for AK^+ cell growth.

DISCUSSION

3-Deazaneplanocin A, DHCA, and DHCDA were synthesized with the intent of developing agents with greater selectivity as AdoHcy hydrolase inhibitors or antiviral agents (13, 16). This expectation was fulfilled in that 3-deazaneplanocin A, DHCA, and DHCDA were indeed more selective in their antiviral action than neplanocin A was. This higher selectivity is particularly evident when the concentrations required for inhibition of vesicular stomatitis virus replication are compared with those that are needed to inhibit cellular protein synthesis (Tables 1 and 2). On the basis of these data, 3-deazaneplanocin A, DHCA, and DHCDA would attain a selectivity index (ratio of the minimum cytotoxic concentration to the 50% effective dose) of 3 orders of magnitude (Table 1). Even more remarkable is the selectivity of 3-deazaneplanocin A against rotavirus, for which it achieves a selectivity index of 4 orders of magnitude (Table 5).

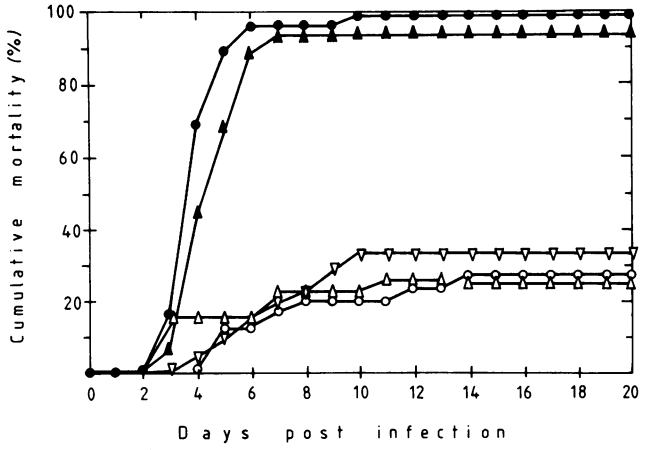

FIG. 2. Inhibitory effect of 3-deazaneplanocin A on the mortality of NMRI mice inoculated subcutaneously when 48 h old with vesicular stomatitis virus. 3-Deazaneplanocin A was administered intraperitoneally at the following dosage regimens: 2.5 mg/kg at 1 h postinfection (△); 0.5 mg/kg at 1 h postinfection (▽); and 0.5 mg/kg at 1 h (day 1) and on days 2, 3, 4, and 5 (○). Control PBS, One injection at 1 h postinfection (▲) or five injections at 1 h (day 1) and on days 2, 3, 4, and 5 (●).

TABLE 9. Effects of 3-deazaneplanocin A, DHCA, and DHCDA on the mortality rate of NMRI mice infected subcutaneously when 48 h old with vesicular stomatitis virus

Compound[a]	Dose (mg/kg per day)	Time of treatment[b] (h or days postinfection)	Mortality[c]	Significance[d]
3-Deazaneplanocin A	50	1, 2, 3, 4, 5	10/10[e]	NS
		1	10/10[e]	NS
	10	1, 2, 3, 4, 5	10/10[e]	NS
		1	9/10[e]	NS
	2.5	1, 2, 3, 4, 5	18/20[e]	NS
		1	10/40	$P < 0.001$
	0.5	1, 2, 3, 4, 5	8/30	$P < 0.001$
		1	10/30	$P < 0.001$
	0.1	1, 2, 3, 4, 5	9/10	NS
DHCA	50	1, 2, 3, 4, 5	10/10[e]	NS
		1	10/10[e]	NS
	10	1, 2, 3, 4, 5	10/10[e]	NS
	2.5	1, 2, 3, 4, 5	10/10[e]	NS
		1	19/20[e]	NS
	0.5	1, 2, 3, 4, 5	25/30	$P < 0.02$
		1	31/40	$P < 0.05$
	0.1	1, 2, 3, 4, 5	20/20	NS
		1	20/20	NS
DHCDA	10	1	9/10[e]	NS
	2.5	1, 2, 3, 4, 5	19/20[e]	NS
		1	19/20[e]	NS
	0.5	1, 2, 3, 4, 5	17/20	$P < 0.05$
		1	16/20	NS
Placebo		1, 2, 3, 4, 5	69/70	
		1	47/50	

[a] Injected intraperitoneally.
[b] If treatment consisted of only one injection, it was administered 1 h postinfection (indicated by 1); if treatment consisted of five repeated injections, they were administered at 1 h postinfection (day 1) and on days 2, 3, 4, and 5 (indicated by 1, 2, 3, 4, 5).
[c] Mortality was monitored until day 20 postinfection.
[d] As compared to the corresponding control (placebo group) which received either one injection of PBS or five injections of PBS. Significance was assessed by the χ^2 test with Yates' correction. NS, Not significant.
[e] Mortality due to toxicity of the compound.

The antiviral activity spectrum of 3-deazaneplanocin A, DHCA, and DHCDA is similar to that of neplanocin A (6), C-c^{3}Ado (11), and the other adenosine analogs that have been previously recognized as AdoHcy hydrolase inhibitors (7). This spectrum includes, in addition to the viruses that were found to be susceptible to the neplanocin A analogs in the present study (vaccinia, vesicular stomatitis, parainfluenza, reo-, and rotaviruses), African swine fever, measles, rabies, and several plant viruses. It seems imperative to further examine 3-deazaneplanocin A, DHCA, and DHCDA for their activity against these viruses.

In contrast, neither neplanocin A nor 3-deazaneplanocin A, DHCA, DHCDA, or C-c^{3}Ado caused a selective inhibition of HIV replication in MT-4 cells. Yet, treatment of MT-4 cells with neplanocin A (1 to 10 μM) increased intracellular AdoHcy levels 5- to 10-fold and reduced AdoHcy hydrolase activity to 1 to 2% (data not shown). Also, DHCA, DHCDA, and C-c^{3}Ado are assumed to achieve their antiviral and anticellular effects through inhibition of AdoHcy hydrolase. Since none of these compounds proved to be active against HIV, one may infer that the replication of HIV, unlike the reduction of the other viruses mentioned above, is not influenced by an increase in AdoHcy levels. Thus, AdoHcy hydrolase does not appear to be an appropriate target for the design of anti-HIV agents.

3-Deazaneplanocin A, DHCA, and DHCDA are potent inhibitors of AdoHcy hydrolase (13, 17, 22), and their antiviral effects can be attributed to inhibition of the enzyme. With a series of four compounds [(*S*)-9-(2,3-dihydroxypropyl)adenine, (*RS*)-3-adenin-9-yl-2-hydroxypropanoic acid, C-c^{3}Ado, and neplanocin A], a close correlation ($r = 0.986$) was found between antiviral potency (against vesicular stomatitis virus) and inhibitory effects on (bovine liver) AdoHcy hydrolase (9). With an extended number of compounds, now also including adenosine dialdehyde and 3-deazaneplanocin A, an even better correlation ($r = 0.993$) was found between potency against vaccinia virus and inhibitory effects on L-929 cell AdoHcy hydrolase (4). This close correlation points to AdoHcy hydrolase as the target for the antiviral action of these adenosine analogs.

From a mechanistic viewpoint, the present results reinforce the concept that neplanocin A and its congeners owe their antiviral activity to inhibition of AdoHcy hydrolase. The fact that the decapitated analogs DHCA and DHCDA, which lack the 4′-hydroxymethyl group that would otherwise make them substrates for AK, were more selective in their antiviral action than neplanocin A was clearly indicates that the compounds are active per se and do not have to be phosphorylated to acquire antiviral activity. Also, the neplanocin A analogs did not lose much of their antiviral or cytostatic potential in AK$^-$ cells relative to the corresponding AK$^+$ cells (Tables 7 and 8), which again indicates that any phosphorylation by the AK would be superfluous for their antiviral or cytostatic action.

From a therapeutic viewpoint, it is evident that 3-deazaneplanocin A, DHCA, and DHCDA should be further pursued for their in vivo efficacy in the appropriate animal models. In one such model, in which mortality of newborn mice was monitored after a lethal infection with vesicular stomatitis virus, 3-deazaneplanocin A provided a highly significant protection (Fig. 2) over a narrow dosage range (0.5 to 2.5 mg/kg per day) (Table 9). With DHCA and DHCDA, little efficacy was noted at the subtoxic dose (0.5 mg/kg per day). To obtain a better insight into the clinical potential of the compounds, these studies should be extended to other treatment regimens (e.g., treatment started late postinfection) and other animal models (e.g., rota and rabies).

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LITERATURE CITED

1. **Bennett, L. L., Jr., P. W. Allan, L. M. Rose, R. N. Comber, and J. A. Secrist III.** 1986. Differences in the metabolism and metabolic effects of the carbocyclic adenosine analogs, neplanocin A and aristeromycin. Mol. Pharmacol. **29**:383–390.
2. **Borchardt, R. T., B. T. Keller, and U. Patel-Thombre.** 1984. Neplanocin A. A potent inhibitor of *S*-adenosylhomocysteine hydrolase and of vaccinia virus multiplication in mouse L929 cells. J. Biol. Chem. **259**:4353–4358.
3. **Borcherding, D. R., S. A. Scholtz, and R. T. Borchardt.** 1987. Synthesis of analogues of neplanocin A: utilization of optically active dihydroxycyclopentenones derived from carbohydrate. J. Org. Chem. **52**:5457–5461.

4. **Cools, M., and E. De Clercq.** 1989. Correlation between the antiviral activity of acyclic and carbocyclic adenosine analogues in murine L929 cells and their inhibitory effect on L929 cell S-adenosylhomocysteine hydrolase. Biochem. Pharmacol. **38:** 1061–1067.
5. **Cools, M., E. De Clercq, and J. C. Drach.** 1987. Role of adenosine kinase in the biological (antiviral and anticellular) activities of adenosine analogues. Nucleosides Nucleotides **6:** 423–424.
6. **De Clercq, E.** 1985. Antiviral and antimetabolic activities of neplanocins. Antimicrob. Agents Chemother. **28:**84–89.
7. **De Clercq, E.** 1987. *S*-Adenosylhomocysteine hydrolase inhibitors as broad-spectrum antiviral agents. Biochem. Pharmacol. **36:**2567–2575.
8. **De Clercq, E., J. Balzarini, D. Madej, F. Hansske, and M. J. Robins.** 1987. Nucleic acid related compounds. 51. Synthesis and biological properties of sugar-modified analogues of the nucleoside antibiotics tubercidin, toyocamycin, sangivamycin, and formycin. J. Med. Chem. **30:**481–486.
9. **De Clercq, E., and M. Cools.** 1985. Antiviral potency of adenosine analogues: correlation with inhibition of S-adenosylhomocysteine hydrolase. Biochem. Biophys. Res. Commun. **129:** 306–311.
10. **De Clercq, E., and P. De Somer.** 1968. Protective effect of interferon and polyacrylic acid in newborn mice infected with a lethal dose of vesicular stomatitis virus. Life Sci. **7:**925–933.
11. **De Clercq, E., and J. A. Montgomery.** 1983. Broad-spectrum antiviral activity of the carbocyclic analog of 3-deazaadenosine. Antiviral Res. **3:**17–24.
12. **De Clercq, E., and M. J. Robins.** 1986. Xylotubercidin against herpes simplex virus type 2 in mice. Antimicrob. Agents Chemother. **30:**719–724.
13. **Glazer, R. I., K. D. Hartman, M. C. Knode, M. M. Richard, P. K. Chiang, C. K. H. Tseng, and V. E. Marquez.** 1986. 3-Deazaneplanocin: a new and potent inhibitor of *S*-adenosylhomocysteine hydrolase and its effects on human promyelocytic leukemia cell line HL-60. Biochem. Biophys. Res. Commun. **135:**688–694.
14. **Glazer, R. I., and M. C. Knode.** 1984. Neplanocin A. A cyclopentenyl analog of adenosine with specificity for inhibiting RNA methylation. J. Biol. Chem. **259:**12964–12969.
15. **Glazer, R. I., M. C. Knode, C. K. H. Tseng, D. R. Haines, and V. E. Marquez.** 1986. 3-Deazaneplanocin A: a new inhibitor of *S*-adenosylhomocysteine synthesis and its effects in human colon carcinoma cells. Biochem. Pharmacol. **35:**4523–4527.
16. **Hasobe, M., J. G. McKee, D. R. Borcherding, and R. T. Borchardt.** 1987. 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. Antimicrob. Agents Chemother. **31:**1849–1851.
17. **Hasobe, M., J. G. McKee, D. R. Borcherding, B. T. Keller, and R. T. Borchardt.** 1988. Effects of 9-(*trans*-2′,*trans*-3′-dihydroxycyclopent-4′-enyl)adenine and -3-deazaadenine on the metabolism of *S*-adenosylhomocysteine in mouse L929 cells. Mol. Pharmacol. **33:**713–720.
18. **Inaba, M., K. Nagashima, S. Tsukagoshi, and Y. Sakurai.** 1986. Biochemical mode of cytotoxic action of neplanocin A in L1210 leukemic cells. Cancer Res. **46:**1063–1067.
19. **Keller, B. T., R. S. Clark, A. E. Pegg, and R. T. Borchardt.** 1985. Purification and characterization of some metabolic effects of *S*-neplanocylmethionine. Mol. Pharmacol. **28:**364–370.
20. **Kitaoka, S., T. Konno, and E. De Clercq.** 1986. Comparative efficacy of broad-spectrum antiviral agents as inhibitors of rotavirus replication in vitro. Antiviral Res. **6:**57–65.
21. **Montgomery, J. A., S. J. Clayton, H. J. Thomas, W. M. Shannon, G. Arnett, A. J. Bodner, I.-K. Kim, G. L. Cantoni, and P. K. Chiang.** 1982. Carbocyclic analogue of 3-deazaadenosine: a novel antiviral agent using *S*-adenosylhomocysteine hydrolase as a pharmacological target. J. Med. Chem. **25:**626–629.
22. **Narayanan, S. R., B. T. Keller, D. R. Borcherding, S. A. Scholtz, and R. T. Borchardt.** 1988. 9-(*trans*-2′,*trans*-3′-Dihydroxycyclopent-4′-enyl) derivatives of adenine and 3-deazaadenine: potent inhibitors of bovine liver *S*-adenosylhomocysteine hydrolase. J. Med. Chem. **31:**500–503.
23. **Pauwels, R., E. De Clercq, J. Desmyter, J. Balzarini, P. Goubau, P. Herdewijn, H. Vanderhaeghe, and M. Vandeputte.** 1987. Sensitive and rapid assay on MT-4 cells for detection of antiviral compounds against the AIDS virus. J. Virol. Methods **16:** 171–185.
24. **Saunders, P. P., M.-T. Tan, and R. K. Robins.** 1985. Metabolism and action of neplanocin A in Chinese hamster ovary cells. Biochem. Pharmacol. **34:**2749–2754.
25. **Shipman, C., Jr., S.-L. Tong, S. H. Smith, N. B. Katlama, and J. C. Drach.** 1983. Establishment of a murine cell line resistant to arabinosyladenine and devoid of adenosine deaminase activity. Antimicrob. Agents Chemother. **24:**947–949.
26. **Svoboda, J., P. Vesely, and L. Donner.** 1967. Characterization of highly polyploid clones isolated from lines of RSV-transformed rat cells. Folia Biol. (Prague) **13:**40–43.
27. **Tseng, C. K. H., and V. E. Marquez.** 1985. An improved method of synthesis of neplanocin and related cyclopentenyl-containing nucleosides. Tetrahedron Lett. **26:**3669–3672.