ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, July 1985, p. 84–89
0066-4804/85/070084-06$02.00/0

Vol. 28, No. 1

Antiviral and Antimetabolic Activities of Neplanocins

ERIK DE CLERCQ

Rega Institute for Medical Research, Katholieke Universiteit Leuven, B-3000 Leuven, Belgium

Received 26 February 1985/Accepted 29 April 1985

Of a series of carbocyclic analogs of adenosine, in which the ribose moiety was replaced by a cyclopentenyl ring, neplanocin A, or (−)-9-[*trans*-2, *trans*-3-dihydroxy-4-(hydroxymethyl)cyclopent-4-enyl]adenine proved particularly effective in inhibiting the multiplication of DNA viruses (i.e., vaccinia), (−)RNA viruses (i.e., parainfluenza, measles, and vesicular stomatitis), and double-stranded RNA viruses (i.e., reo) in vitro in cell culture. Depending on the cells used, the MIC of neplanocin A for these viruses ranged from 0.01 to 4 μg/ml, and depending on the parameter used to assess toxicity for the host cell, the specificity index of neplanocin A ranged from 50 to 4,000. As postulated before for other adenosine analogs, neplanocin A may owe its antiviral action to inhibition of *S*-adenosylhomocysteine hydrolase, hence perturbation of transmethylation reactions. In vivo, neplanocin A afforded only marginal protection against a lethal infection of mice with vesicular stomatitis virus.

In recent years several adenosine analogs have been described which share a unique antiviral activity spectrum that is primarily directed towards those viruses that require a methylated 5′-cap, e.g., $m^7Gpppm^6A^mpA^mp$, on their mRNAs. These compounds include (*S*)-DHPA [(*S*)-9-(2,3-dihydroxypropyl)adenine] (7), (D)-eritadenine [(2*R*,3*R*)-4-(adenin-9-yl)-2,3-dihydroxybutanoic acid] (17), c^3Ado (3-deazaadenosine) (1, 2), and the carbocyclic analogues of adenosine (C-Ado, aristeromycin), 3-deazaadenosine (C-c^3Ado,3-deazaaristeromycin) and 7-deazaadenosine (C-c^7Ado) (6, 11); they are particularly active against (−)RNA strand viruses (i.e., measles, parainfluenza, and vesicular stomatitis), double-stranded (±)RNA viruses (i.e., reo), and poxviruses (i.e., vaccinia). In particular, C-c^3Ado offers promise as a broad-spectrum antiviral agent, since it inhibits the replication of vesicular stomatitis, measles, parainfluenza, vaccinia, and reovirus at a concentration of 0.2 to 1 μg/ml but is not toxic for the host cells at a concentration of 400 μg/ml (11).

A common biochemical characteristic of (*S*)-DHPA (18, 22), D-eritadenine (18), c^3Ado (1, 14), C-Ado (5, 14), and C-c^3Ado (19) is that they all are potent inhibitors of *S*-adenosyl-L-homocysteine (AdoHcy) hydrolase, with K_i values ranging from 1 nM to 4 μM, depending on the compound and the assay system, e.g., nature and purity of the enzyme. AdoHcy hydrolase catalyzes the reversible hydrolysis of AdoHcy to adenosine and homocysteine. Inhibition of AdoHcy hydrolase results in the accumulation of AdoHcy, which is both the product and a feedback inhibitor of *S*-adenosylmethionine-dependent methylation reactions. Such methylation reactions are required for the 5′-capping of viral mRNAs, and therefore, inhibitors of AdoHcy hydrolase may be expected to inhibit the maturation of viral mRNAs and the production of progeny virus particles.

Recently, a novel class of carbocyclic analogs of purine nucleosides has been decribed in which the ribose moiety is replaced by a cyclopentene ring (15, 16, 21, 23, 24). These compounds were originally isolated from the culture broth of an actinomycete, *Ampullarilla regularis* A11079, and designated neplanocins. Neplanocins A, B, and C have antitumor activities in mice, with neplanocin A being more potent than the established antitumor drugs arabinosylcytosine, 5-fluorouracil, cyclophosphamide, 6-mercaptopurine, daunorubicin, doxorubicin, and mitomycin C in increasing the life span of mice inoculated with murine leukemia L1210 cells (21).

The prototype of the neplanocins, neplanocin A ((−)-9-[*trans*-2,*trans*-3-dihydroxy-4-(hydroxymethyl)cyclopent-4-enyl]adenine) has recently been described as a potent inhibitor of AdoHcy hydrolase with a K_i of 8.39 nM for the purified bovine liver enzyme (3). Neplanocin A was also found to inhibit vaccinia virus replication in murine L-929 cells at a concentration of 0.5 to 1.0 μM (3). In view of the striking parallelism between the antiviral activity of adenosine analogs and their inhibitory effects on AdoHcy hydrolase, it seemed imperative to further explore neplanocin A and its derivatives for their broad-spectrum antiviral properties.

MATERIALS AND METHODS

Test compounds. The neplanocins (neplanocin A, neplanocin C, neplanocin D, ara-neplanocin A, and 2′-deoxy-neplanocin A) were obtained from Toyo Jozo Co., Shizuoka-Ken, Japan. The structural formulae of the compounds are depicted in Fig. 1. The origin of the reference compounds was as follows: (*S*)-DHPA, A. Holý (Czechoslovak Academy of Sciences, Prague, Czechoslovakia); C-c^3Ado, J. A. Montgomery (Kettering-Meyer Laboratory, Southern Research Institute, Birmingham, Ala.); and ribavirin, ICN Nutritional Biochemicals, Cleveland, Ohio.

Viruses and cells. The origin of the viruses was as follows: herpes simplex virus type 1 (strain KOS), herpes simplex virus type 2 (strain G) (see reference 8); vaccinia virus, vesicular stomatitis virus, coxsackievirus type B-4, Sindbis virus, measles virus, and poliovirus type 1 (see reference 10); reovirus type 1 (ATCC VR-230), Semliki forest virus (ATCC VR-67), and parainfluenza virus type 3 (ATCC VR-93) (American Type Culture Collection, Rockville, Md.). The virus stocks were grown in primary rabbit kidney cells (herpes simplex types 1 and 2, vaccinia virus, and vesicular stomatitis virus), Vero cells (measles virus, reovirus, coxsackievirus, and Semliki forest virus), HeLa cells (polio virus), chicken embryo cells (Sindbis virus), or human embryonic lung cells (parainfluenza virus). The Vero and HeLa cell lines used in this study were regularly examined for mycoplasma contamination and found to be mycoplasma free.

Inhibition of virus-induced cytopathogenicity in vitro. Confluent cell cultures in microtiter trays were inoculated

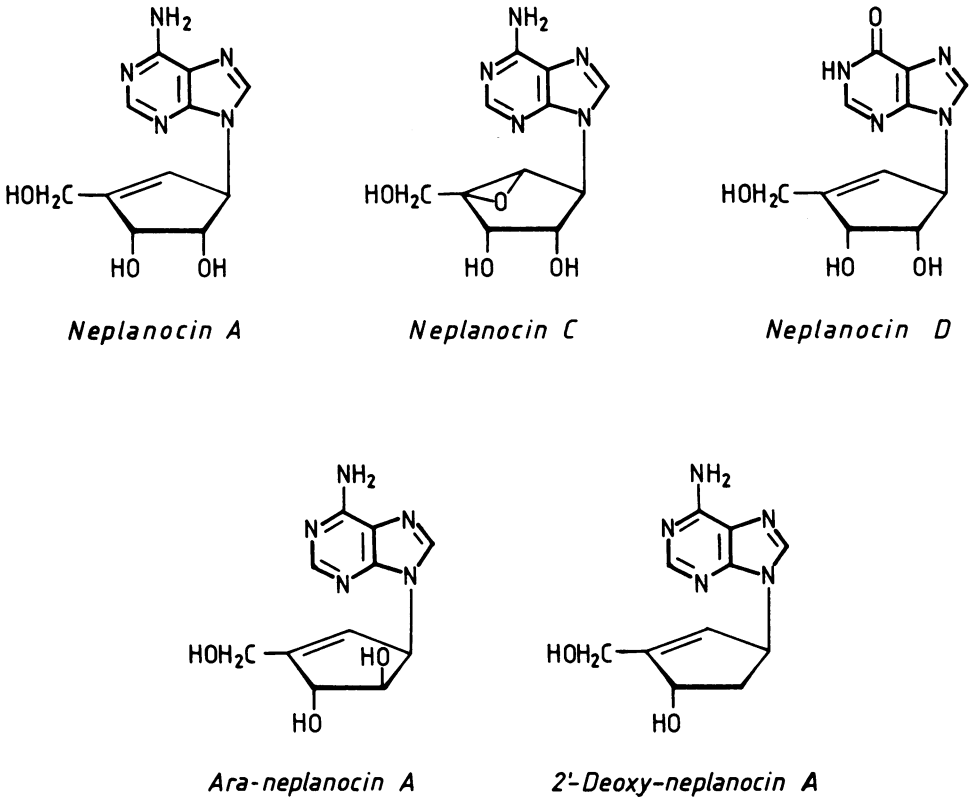

FIG. 1. Structural formulae of neplanocins.

with 100 $CCID_{50}$ (1 $CCID_{50}$ corresponding 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 minimal essential medium) containing 3% fetal calf serum 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; at 2 days for Semliki forest, coxsackie, and polio; at 2 to 3 days for vaccinia, herpes simplex types 1 and 2, and Sindbis; and at 6 to 7 days for reo, parainfluenza, and measles viruses. The antiviral activity of the compounds is expressed as the concentration required to inhibit viral cytopathogenicity by 50%.

Cytotoxicity. Cytotoxicity measurements were based on two parameters: (i) alteration of normal cell morphology and (ii) inhibition of host cell macromolecule (DNA, RNA, and protein) synthesis. To evaluate cell morphology, 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 monolayer, e.g., rounding up or detachment of the cells, was considered as evidence for cytotoxicity. To measure inhibition of host cell macromolecule synthesis, the cells were seeded in Linbro microtiter tray wells (at 300,000 to 400,000 cells per well) in Eagle minimal essential medium containing 10% fetal calf serum, various concentrations of the test compounds, and 2.5 μCi of [*methyl*-^{3}H]thymidine, [5-^{3}H]uridine, or [4,5-^{3}H]leucine per ml and allowed to proliferate for 16 h at 37°C. The cells were then treated with 5% ice-cold trichloroacetic acid, washed with 95% ethanol (five times), air dried, and counted for radioactivity in 7.5 ml of Lipoluma scintillation fluid.

Antiviral activity in vivo. In vivo assays were carried out with both vesicular stomatitis and coxsackievirus type B-4 in newborn (2-day-old) NMRI mice and with vesicular stomatitis virus in young weaned (25-day-old) NMRI mice. The NMRI mice were obtained from the Animal Production Center (Proefdierencentrum) of the Katholieke Universiteit Leuven. Newborn (2-day-old) mice were inoculated subcutaneously with vesicular stomatitis virus at 4 PFU/0.1 ml per mouse (PFU, as determined in mouse L-929 cell cultures) or coxsackievirus type B-4 at 5 $CCID_{50}$ per 0.1 ml per mouse ($CCID_{50}$, as determined in Vero cells). The mice then received either a single intraperitoneal injection of the compound (in 0.1 ml physiological saline) at 1 h postinfection or repeated intraperitoneal injections of the compound at 1 h and 1 and 2 days postinfection. Young (25-day-old) mice, weighing 11 to 13 g, were inoculated intranasally with vesicular stomatitis virus at 40 PFU/0.02 ml per mouse and then received either a single intraperitoneal injection of the compound (in 0.5 ml of physiological saline) at 1 h postinfection or repeated intraperitoneal injections of the compound at 1 h and 1, 2, 3, and 4 days postinfection.

RESULTS

The neplanocins were examined for their antiviral potential in a number of virus assay systems, each adapted to its optimal cell substrate. In primary rabbit kidney cells (Table 1), the antiviral assays were performed with herpes simplex

TABLE 1. Antiviral and cytotoxic effects of neplanocins in PRK cell cultures

Compound	Minimum antiviral concn[a] (μg/ml)				Minimum cytotoxic concn[b] (μg/ml)			
	Herpes simplex-1 (KOS)	Herpes simplex-2 (G)	Vaccinia	Vesicular stomatitis	Cell morphology	Macromolecule synthesis		
						[*methyl*-^{3}H]dThd	[5-^{3}H]Urd	[4,5-^{3}H]leucine
Neplanocin A	4	10	0.03	0.01	40	1.0 (0.5–1.7)[c]	1.4 (0.3–3.2)	13 (7–19)[c]
Neplanocin C	2	2	0.1	0.2	40	4.0 (2–7)	2.8 (2.3–3.4)	17 (12–23)
Neplanocin D	>400	>400	2	2	>400	>100	>100	>100
Ara-neplanocin A	>400	>400	20	20	>400	>100	>100	>100
2′-Deoxy-neplanocin A	100	>400	4	2	>400	>100	>100	>100
(*S*)-DHPA	>400	>400	20	20	>400	>100	>100	>100
C-c^{3}Ado	≥200	≥200	0.7	0.2	>400	>100	>100	>100
Ribavirin	>400	>400	20	100	>400	>100	>100	>100

[a] Required to inhibit virus-induced cytopathogenicity by 50%; average values for three or four experiments.
[b] Required to cause a microscopically detectable alteration of normal cell morphology (of stationary) cells or to inhibit by 50% macromolecule (DNA, RNA, or protein) synthesis in proliferating cells; average values for 6 to 10 experiments. Where appropriate, the range of the individual values is indicated in parentheses.
[c] Values higher than 100 μg/ml were noted in some experiments.

virus type 1 and type 2 as well as vaccinia virus and vesicular stomatitis virus as the challenge viruses. Neplanocins A and C inhibited herpes simplex virus type 1 and type 2 at a concentration that was similar to the MIC required to inhibit DNA or RNA synthesis in the uninfected host cells. The other neplanocins and the reference compounds (*S*)-DHPA, C-c^{3}Ado, and ribavirin did not interfere with herpes simplex virus replication. Neplanocin C and, in particular, neplanocin A were highly inhibitory to vaccinia virus and vesicular stomatitis virus; neplanocin A was 20 times more potent than C-c^{3}Ado and 10^3 to 10^4 times more potent than ribavirin or (*S*)-DHPA. Neplanocin D, ara-neplanocin A, and 2′-deoxy-neplanocin A were also inhibitory to vaccinia virus and vesicular stomatitis virus but much less so than neplanocin A. Neplanocin D and 2′-deoxy-neplanocin A inhibited vesicular stomatitis virus replication at 2 μg/ml; neither compound disturbed normal cell morphology at 400 μg/ml, the highest concentration tested. Of the three parameters (DNA, RNA, and protein synthesis) monitored to evaluate the effects of the neplanocins on normal PRK (primary rabbit kidney) cell metabolism, DNA and RNA synthesis appeared to be more sensitive than protein synthesis. Neplanocins A and C were inhibitory to DNA and RNA synthesis at a concentration of 1 to 4 μg/ml, that is 10- to 100-fold higher than their MIC for vesicular stomatitis virus.

The neplanocins were then examined for their inhibitory effects on reovirus, coxsackievirus, Sindbis virus, Semliki forest virus, parainfluenza virus, and measles virus in Vero cells (Table 2). Again, neplanocins A and C turned out to be efficient inhibitors of these viruses; the potency of neplanocin C was comparable to that of C-c^{3}Ado, whereas neplanocin A was at an average fivefold more potent. Neplanocin A by far exceeded ribavirin and (*S*)-DHPA in potency against any of the viruses assayed in Vero cells. As compared with ribavirin, (*S*)-DHPA, and C-c^{3}Ado, neplanocin A and neplanocin C were also more cytotoxic since they altered normal cell morphology at a concentration of 10 μg/ml, whereas the reference compounds failed to do so at concentrations up to 400 μg/ml. Neplanocin D was inhibitory to parainfluenza virus at a concentration of 2 μg/ml but was noncytotoxic at 400 μg/ml. Neplanocin A and neplanocin C were less potent antimetabolites for Vero cells (Table 2) than for PRK cells (Table 1). Neplanocin A was not inhibitory to DNA, RNA, or protein synthesis at a concentration of 100 μg/ml; neplanocin C was only inhibitory to protein synthesis at a concentration of about 50 μg/ml. Of the reference compounds, only ribavirin interfered with host cell DNA and RNA synthesis at a concentration below 100 μg/ml.

In HeLa cells (Table 3), three viruses were evaluated for

TABLE 2. Antiviral and cytotoxic effects of neplanocins in Vero cell cultures

Compound	Minimum antiviral concn[a] (μg/ml)						Minimum cytotoxic concn[b] (μg/ml)			
	Reovirus type 1	Coxsackie virus type B-4	Sindbis	Semliki forest	Parainfluenza type 3	Measles	Cell morphology	Macromolecule synthesis		
								[*methyl*-^{3}H]dThd	[5-^{3}H]Urd	[4,5-^{3}H]leucine
Neplanocin A	0.2	0.04	2	1	0.07	0.04	10	>100	>100	>100
Neplanocin C	2	2	7	2	0.2	0.4	10	>100	>100	52 (29–74)
Neplanocin D	150	7	>400	300	2	20	>400	>100	>100	>100
Ara-neplanocin A	70	100	>400		10	300	>400	>100	>100	>100
2′-Deoxy-neplanocin A	20	40	>400		40	150	>400	>100	>100	>100
(*S*)-DHPA	70	100	>400	150	20	40	>400	>100	>100	>100
C-c^{3}Ado	1	2	7	10	0.7	0.7	>400	>100	>100	>100
Ribavirin	70	200	40	70	40	70	>400	25	22	>100

[a] Required to inhibit virus-induced cytopathogenicity by 50%; average values for three or four experiments.
[b] Required to cause a microscopically detectable alteration of normal cell morphology (of stationary cells) or to inhibit by 50% macromolecule (DNA, RNA, or protein) synthesis in proliferating cells; average values for 6 to 10 experiments. Where appropriate, the range of the individual values is indicated in parentheses.

TABLE 3. Antiviral and cytotoxic effects of neplanocins in HeLa cell cultures

Compound	Minimum antiviral concn[a] (μg/ml)			Minimum cytotoxic concn[b] (μg/ml)			
	Poliovirus type 1	Coxsackie virus type B-4	Vesicular stomatitis	Cell morphology	Macromolecule synthesis		
					[*methyl*-^{3}H]dThd	[5-^{3}H]Urd	[4,5-^{3}H]leucine
Neplanocin A	>10	>10	0.07	20	>100	100	6.6 (5–8)
Neplanocin C	>10	>10	0.4	10	>100	3.8 (2.3–5.4)	3.3 (2.8–4.6)
Neplanocin D	>400	>400	20	>400	>100	>100	>100
Ara-neplanocin A	>400	>400	150	>400	>100	>100	>100
2′-Deoxy-neplanocin A	>400	>400	20	>400	>100	>100	>100
(*S*)-DHPA	>400	>400	150	>400	>100	>100	>100
C-c^{3}Ado	>400	>400	0.4	>400	>100	>100	>100
Ribavirin	20	20	10	>400	>100	>100	>100

[a] Required to inhibit virus-induced cytopathogenicity by 50%; average values for three or four experiments.
[b] Required to cause a microscopically detectable alteration of normal cell morphology (of stationary cells) or to inhibit by 50% macromolecule (DNA, RNA, or protein) synthesis in proliferating cells; average values for 6 to 10 experiments. Where appropriate, the range of the individual values is indicated in parentheses.

their sensitivity to the neplanocins. Poliovirus and coxsackievirus were totally resistant to the inhibitory effects of neplanocins A and C. This is more surprising for coxsackievirus, since in Vero cells this virus was readily inhibited by neplanocin A. Of the compounds tested, ribavirin was the only one capable of inhibiting poliovirus and coxsackievirus replication in HeLa cells. Ribavirin also inhibited vesicular stomatitis virus replication in HeLa cells, but in this respect neplanocins A and C and C-c^{3}Ado were much more potent than ribavirin. Neplanocins A and C inhibited the replication of vesicular stomatitis virus at concentrations of 0.07 and 0.4 μg/ml, respectively; that is 100- or 10-fold lower than their MIC for protein synthesis (6.6 and 3.3 μg/ml, respectively). Neplanocin D and 2′-deoxy-neplanocin A were inhibitory to vesicular stomatitis virus at 20 μg/ml but were nontoxic for the host cells at 400 μg/ml.

Since vesicular stomatitis virus ranked among the viruses that were most susceptible to the inhibitory effects of the neplanocins, its susceptibility to neplanocins A and C was further examined in a variety of cell lines (Table 4). In all cell lines, neplanocin A and neplanocin C inhibited vesicular stomatitis virus replication at a concentration well below their minimal cytotoxic concentration. The ratio of the minimal cytotoxic concentration to the minimal antiviral concentration of neplanocin A ranged from 10 (in CV-1 or BHK 21 cells) to 4,000 (in PRK cells). For neplanocin C the ratio of the minimal cytotoxic to the minimal antiviral concentration fell between 5 (Vero cells) and 200 (PRK cells).

Based on their potency against vesicular stomatitis virus in cell culture, neplanocins A and C 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 and young weaned mice inoculated intranasally with vesicular stomatitis virus. Neplanocins A and C were themselves lethal if administered intraperitoneally at 20 μg per mouse to newborn mice or 500 μg per mouse to young weaned mice (11 to 13 g). Thus, the doses of neplanocins A and C used in these mice had to be adjusted to maximally 5 μg per mouse for the newborn mice and 100 μg per mouse for the young weaned mice. In the newborn mice the neplanocins did not offer any protection against vesicular stomatitis virus infection (Table 5). Neither did they protect newborn mice against a lethal coxsackievirus type B-4 infection (data not shown). In the young weaned mice, neplanocin A and neplanocin C effected a partial reduction in the mortality rate (Table 5), but this reduction was not statistically significant.

DISCUSSION

Neplanocins A and C proved effective against a broad range of viruses, in particular (−)RNA strand viruses (i.e., vesicular stomatitis virus, parainfluenza virus, and measles virus), but they were also effective against (±)RNA viruses (i.e., reovirus) and poxviruses (i.e., vaccinia virus). Other viruses, i.e., herpes simplex virus and the (+)RNA viruses (togavirus and picornavirus) were also affected but to a significantly lesser extent. Thus, the antiviral activity spectrum of neplanocins A and C is remarkably similar to that of the other adenosine analogs (*S*)-DHPA and C-c^{3}Ado (6).

As compared with C-c^{3}Ado, neplanocin A was slightly more potent in its antiviral activity but was also more cytotoxic. It achieved an inhibition of vaccinia virus and vesicular stomatitis virus replication at a concentration as low as 0.02 to 0.04 μg/ml (Table 1). Our results thus confirm

TABLE 4. Antiviral activity of neplanocin A and neplanocin C against vesicular stomatitis virus in different cell lines

Cell culture	Minimum antiviral concn[a] (μg/ml)	
	Neplanocin A	Neplanocin C
PRK fibroblast[b]	0.01 (40)	0.2 (40)
Vero fibroblast	0.4 (10)	2 (10)
HeLa[b]	0.07 (20)	0.4 (10)
Human embryonic skin-muscle (E_6SM) fibroblast	0.07 (40)	0.2 (10)
Human kidney	0.02 (40)	0.2 (10)
HEp-2	0.02 (10)	0.07 (10)
Rabbit kidney (RK-13) fibroblast	2 (100)	7 (40)
Monkey kidney (CV-1) fibroblast	4 (40)	1 (40)
Monkey kidney (BSC-1A)	2 (40)	2 (40)
BHK-21 fibroblast	1 (10)	1 (10)
Mouse embryo BALB-c 3T3 fibroblast	0.7 (10)	0.7 (10)

[a] Required to inhibit virus-induced cytopathogenicity by 50%; average values for three experiments. Listed in parentheses are the minimal cytotoxic concentrations required to cause a microscopically detectable alteration of normal cell morphology (stationary cells).
[b] For PRK fibroblast and HeLa cell cultures the data are taken from Tables 1 and 3, respectively.

the findings of Borchardt et al. (3) that neplanocin A is a potent inhibitor of vaccinia virus replication. For those viruses that were most susceptible to the antiviral activity of neplanocin A, i.e., vaccinia, vesicular stomatitis, parainfluenza, and reovirus, the specificity index (ratio of minimum cytotoxic concentration to minimal antiviral concentration) ranged from 50 to 4,000, depending on the choice of the virus and parameter used to assess cytotoxicity. The specificity index of the reference compound C-c^{3}Ado fell in the range of 500 to 2,000. Neplanocin C was, in general, equally cytotoxic but less potent than neplanocin A. If for each cell system (Tables 1 through 3), the lowest minimum cytotoxic concentrations were compared to the lowest minimum antiviral concentrations (Table 6), the specificity indexes obtained for neplanocin A were 100, 250, and 94 in PRK, Vero, and HeLa cells, respectively; and for neplanocin C they were 28, 50, and 8.2 in PRK, Vero, and HeLa cells, respectively. Thus, neplanocin C was not only less potent but also less specific in its antiviral action than neplanocin A.

The antiviral potency of neplanocin A was drastically reduced upon modification of its *trans*-2-hydroxyl group to a hydrogen, as in 2'-deoxy-neplanocin A, or *cis*-2-hydroxyl, as in ara-neplanocin A. Neplanocin D was also less potent as an antiviral agent than neplanocin A, but since it was absolutely nontoxic for the host cells, its specificity index for some viruses, i.e., vaccinia, vesicular stomatitis, and parainfluenza, was >50. That deamination of neplanocin A resulted in a marked loss of antiviral potency is consistent with previous findings that hypoxanthine derivatives are, as a rule, less potent antiviral agents than their adenine counterparts, i.e., hypoxanthine arabinoside versus adenine arabinoside (4, 12, 20), formycin B versus formycin A (13) and 9-(2,3-dihyroxypropyl)hypoxanthine versus 9-(2,3-dihydroxypropyl)adenine (9).

TABLE 5. Effects of neplanocins on the mortality rate of mice infected subcutaneously or intranasally with vesicular stomatitis virus

Mice and compound	Dose[a] (μg/mouse)	Time of treatment postinfection	Median survival time[b] (days)	Mortality rate[c]
Newborn[d]				
Neplanocin A	5	+ 1 h	4.7 (2–10)	20/20
	5	+ 1 h, 1 d[e], 2 d	3.5 (3–5)	10/10
Neplanocin C	5	+ 1 h	4.3 (3–6)	20/20
	5	+ 1 h, 1 d, 2 d	3.9 (3–5)	10/10
Control		+ 1 h	4.2 (3–8)	20/20
25-day old[f]				
Neplanocin A	100	+ 1 h	7.7 (6–10)	6/10
	20	+ 1 h	8.5 (6–11)	9/10
	20	+ 1 h, 1 d, 2 d, 3 d, 4 d	8 (6–10)	6/10
Neplanocin C	100	+ 1 h	8 (6–10)	6/10
	100	+ 1 h, 1 d, 2 d, 3 d, 4 d	8 (6–9)	5/10
	20	+ 1 h	7.7 (6–9)	6/10
	20	+ 1 h, 1 d, 2 d, 3 d, 4 d	9 (7–11)	5/10
Control		+ 1 h, 1 d, 2 d, 3 d, 4 d	8.1 (5–12)	17/20

[a] The neplanocins were administered intraperitoneally at the maximum tolerated dose which did not cause mortality in uninfected mice.
[b] Range of days to death is indicated in parentheses.
[c] Mortality was followed until the day 20 postinfection. None of the treated groups showed a mortality rate that was significantly different from the mortality rate of the control group (as assessed by the χ^2 test with Yates correction).
[d] Inoculated subcutaneously with vesicular stomatitis virus.
[e] d, day.
[f] Inoculated intranasally with vesicular stomatitis virus.

TABLE 6. Specificity indices[a] of neplanocins in vitro

Compound	Cells		
	PRK	Vero	HeLa
Neplanocin A	100	250	94
Neplanocin C	28	50	8.2
Neplanocin D	>50	>50	>5
Ara-neplanocin A	>5	>10	>0.7
2'-Deoxy-neplanocin A	>50	>5	>5
(*S*)-DHPA	>5	>5	>0.7
C-c^{3}Ado	>500	>150	>250
Ribavirin	>5	0.5	>10

[a] Determined as the ratio of the minimum cytotoxic concentration (required to inhibit DNA, RNA, or protein synthesis or to affect normal cell morphology, whatever gave the lowest value) to the minimum antiviral concentration (for any of the viruses tested, whatever gave the lowest value).

That the antiviral activity of adenosine analogs may be related to an inhibition of AdoHcy hydrolase was originally postulated for c^{3}Ado (1) and (*S*)-DHPA (7, 22). AdoHcy hydrolase has also been proposed as a target for the antiviral action of C-c^{3}Ado (19), and it also seems to serve as the target for the antiviral activity of the neplanocins. With a K_i of about 0.003 μg/ml (3), it is one of the most potent inhibitors of AdoHcy hdydrolase, and as recently established by De Clercq and Cools (E. De Clercq and M. Cools, Biochem. Biophys. Res. Commun., in press), there appears to be a direct correlation between the antiviral potency of the adenosine analogs and their inhibitory effects on AdoHcy hydrolase.

As demonstrated in Table 4, the antiviral activity of neplanocins A and C depended to a large extent on the choice of the cell system. The differences in neplanocin susceptibility of a particular virus when assayed in various cell lines, as well as differences in susceptibility of different viruses when tested in the same cell line, may be related to differences in AdoHcy hydrolase activity and concomitant transmethylation reactions under these varying conditions. To unequivocally assess the role of AdoHcy hydrolase as target for the antiviral action of the neplanocins, it would appear necessary to directly measure the AdoHcy activity (or AdoHcy/*S*-adenosylmethionine ratio) in virus-infected cells under the same conditions as those used for monitoring the inhibitory effects of the compounds on virus multiplication.

Despite their potent activity in vitro, neplanocins A and C were only weakly effective in vivo. They did not protect newborn mice against a lethal infection with vesicular stomatitis virus, and in young mice inoculated intranasally with vesicular stomatitis virus, they offered only marginal protection. The reasons for the poor performance of neplanocins A and C in vivo remain to be elucidated. Possibly, neplanocins A and C are rapidly deaminated in vivo; neplanocin D, the deaminated product of neplanocin A, is, as shown in Tables 1 through 3, markedly less potent as an antiviral agent than is neplanocin A.

Neplanocins A and C were lethal for mice at a fairly low dose (20 to 40 mg/kg). These findings, together with the

limited antiviral protection afforded by the neplanocins at sublethal doses (Table 5), cast doubt on the therapeutic potentials of these compounds as antiviral drugs.

ACKNOWLEDGMENTS

This work was supported by Krediet no. 3.0040.83 from the Belgian Fonds voor Geneeskundig Wetenschappelijk Onderzoek.

The excellent technical assistance of Anita Van Lierde, Lizette Van Berckelaer, Ria Van Berwaer, Marleen Verstreken, and Willy Zeegers is gratefully acknowledged.

LITERATURE CITED

1. **Bader, J.P., N. R. Brown, P. K. Chiang, and G. L.Cantoni.** 1978. 3-Deazaadenosine, an inhibitor of adenosylhomocysteine hydrolase, inhibits reproduction of Rous sarcoma virus and transformation of chick embryo cells. Virology **89**:494–505.
2. **Bodner, A. J., G. L. Cantoni, and P. K. Chiang.** 1981. Anti-viral activity of 3-deazaadenosine and 5′-deoxy-5′-isobutylthio-3-deazaadenosine (3-deaza-SIBA). Biochem. Biophys. Res. Commun. **98**:476–481.
3. **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.
4. **Bryson, Y. J., and J. D. Connor.** 1976. In vitro susceptibility of varicella zoster virus to adenine arabinoside and hypoxanthine arabinoside. Antimicrob. Agents Chemother. **9**:540–543.
5. **Chiang, P. K., A. Guranowski, and J. E. Segall.** 1981. Irreversible inhibition of S-adenosylhomocysteine hydrolase by nucleoside analogs. Arch. Biochem. Biophys. **207**:175–184.
6. **De Clercq, E., D. E. Bergstrom, A. Holý, and J. A. Montgomery.** 1984. Broad-spectrum antiviral activity of adenosine analogues. Antiviral Res. **4**:119–133.
7. **De Clercq, E., J. Descamps, P. De Somer, and A. Holý.** 1978. (S)-9-(2,3-Dihydroxypropyl)adenine: an aliphatic nucleoside analog with broad-spectrum antiviral activity. Science **200**: 563–565.
8. **De Clercq, E., J. Descamps, G. Verhelst, R. T. Walker, A. S. Jones, P. F. Torrence, and D. Shugar.** 1980. Comparative efficacy of antiherpes drugs against different strains of herpes simplex virus. J. Infect. Dis. **141**:563–574.
9. **De Clercq, E., and A. Holý.** 1979. Antiviral activity of aliphatic nucleoside analogues: structure-function relationship. J. Med. Chem. **22**:510–513.
10. **De Clercq, E., M. Luczak, J. C. Reepmeyer, K. L. Kirk, and L. A. Cohen.** 1975. Fluoroimidazoles as antiviral agents and inhibitors of polynucleotide biosynthesis. Life Sci. **17**:187–194.
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. **Gephart, J. F., and A. M. Lerner.** 1981. Comparison of the effects of arabinosyladenine, arabinosylhypoxanthine, and arabinosyladenine 5′-monophosphate against herpes simplex virus, varicella-zoster virus, and cytomegalovirus with their effects on cellular deoxyribonucleic acid synthesis. Antimicrob. Agents. Chemother. **19**:170–178.
13. **Giziewicz, J., E. De Clercq, M. Luczak, and D. Shugar.** 1975. Antiviral and antimetabolic activities of formycin and its N_1-, N_2-,2′-O- and 3′-O-methylated derivatives. Biochem. Pharmacol. **24**:1813–1817.
14. **Guranowski, A., J. A. Montgomery, G. L. Cantoni, and P. K. Chiang.** 1981. Adenosine analogues as substrates and inhibitors of S-adenosylhomocysteine hydrolase. Biochemistry **20**: 110–115.
15. **Hayashi, M., S. Yaginuma, N. Muto, and M. Tsujino.** 1980. Structures of neplanocins, new antitumor antibiotics. Nucleic Acid Res. Symp. Ser. **8**:s65–s67.
16. **Hayashi, M., S. Yaginuma, H. Yoshioka, and K. Nakatsu.** 1981. Studies on neplanocin A, new antitumor antibiotic. II. Structure determination. J. Antibiot. **34**:675–680.
17. **Holý, A., I. Votruba, and E. De Clercq.** 1982. Synthesis and antiviral activity of stereoisomeric eritadenines. Coll. Czech. Chem. Commun. **47**:1392–1407.
18. **Merta, A., I. Votruba, J. Vesely, and A. Holý.** 1983. *S*-Adenosyl-L-homocysteine hydrolase from mouse leukemic cells: isolation and properties. Coll. Czech. Chem. Commun. **48**:2701–2708.
19. **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.
20. **Shipman, C., Jr., S. H. Smith, R. H. Carlson, and J. C. Drach.** 1976. Antiviral activity of arabinosyladenine and arabinosylhypoxanthine in herpes simplex virus-infected KB cells: selective inhibition of viral deoxyribonucleic acid synthesis in synchronized suspension cultures. Antimicrob. Agents Chemother. **9**:120–127.
21. **Tsujino, M., S. Yaginuma, T. Fujii, K. Hayano, T. Matsuda, T. Watanabe, and J. Abe.** 1980. Neplanocins, new antitumor agents: biological activities, p. 1559–1561. *In* J. D. Nelson and C. Grassi (ed.), Current chemotherapy and infectious disease, vol. 2. American Society for Microbiology, Washington, D.C.
22. **Votruba, I., and A. Holý.** 1980. Inhibition of *S*-adenosyl-L-homocysteine hydrolase by the aliphatic nucleoside analogue -9-(*S*)-(2,3-dihydroxypropyl)adenine. Coll. Czech. Chem. Commun. **45**:3039–3044.
23. **Yaginuma, S., N. Muto, N. Tsujino, Y. Sudate, M. Hayashi, and M. Otani.** 1981. Studies on neplanocin A, new antitumor antibiotic. I. Producing organism, isolation and characterization. J. Antibiot. **34**:359–366.
24. **Yaginuma, S., M. Tsujino, N. Muto, M. Otani, M. Hayashi, F. Ishimura, T. Fujii, S. Watanabe, T. Matsuda, T. Watanabe, and J. Abe.** 1980. Neplanocins, new antitumor agents: discovery, isolation, and structure, p. 1558–1559. *In* J. D. Nelson and C. Grassi (ed.), Current chemotherapy and infectious disease, vol. 2. American Society for Microbiology, Washington, D.C.