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BROAD-SPECTRUM ANTIVIRAL ACTIVITY OF THE CARBOCYCLIC ANALOG OF 3-DEAZAADENOSINE

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The carbocyclic analog of 3-deazaadenosine (C-c^{3}Ado) was found to inhibit in vitro the replication of several DNA and RNA viruses, including vaccinia, reo, measles, parainfluenza and vesicular stomatitis, at a concentration of 0.2–1 μg/ml, while not being toxic for the host (primary rabbit kidney, HeLa, Vero) cells at a concentration of 400 μg/ml. In its activity against vesicular stomatitis virus, parainfluenza virus, measles and reo virus, C-c^{3}Ado proved about 100 times more potent than other established broad-spectrum antiviral agents such as ribavirin (virazole) and (*S*)-DHPA ((*S*)-9-(2,3-dihydroxypropyl)adenine). In vivo, C-c^{3}Ado protected newborn mice against a lethal infection of vesicular stomatitis virus when administered as a single dose of 20, 100 or 500 μg per mouse 1 h after virus infection.

carbocyclic 3-deazaadenosine; ribavirin; (*S*)-DHPA; 3-deazaadenosine; *S*-adenosyl-L-homocysteine hydrolase

INTRODUCTION

S-Adenosyl-L-homocysteine (AdoHcy) hydrolase can be considered as an important target for the antiviral action of adenosine analogs such as 9-β-D-arabinofuranosyladenine (Ara-A), (*S*)-9-(2,3-dihydroxypropyl)adenine ((*S*)-DHPA) and 3-deazaadenosine (c^{3}Ado) [6]. AdoHcy hydrolase hydrolyzes AdoHcy to homocysteine and adenosine. AdoHcy itself is both the product and a feedback inhibitor of *S*-adenosylmethionine (AdoMet)-dependent methylation reactions. When AdoHcy hydrolase is inhibited, AdoHcy accumulates, and, consequently, methylation reactions utilizing AdoMet as methyl donor are impaired. Such methylations are required for the maturation of viral mRNA, i.e. 5'cap formation, so that inhibitors of AdoHcy hydrolase may be expected to block virus replication by interference with the methylation of viral mRNA.

A typical example of an AdoHcy hydrolase inhibitor endowed with antiviral activity is c^{3}Ado (Fig. 1). Chiang and colleagues [5] have established that c^{3}Ado inhibits beef liver

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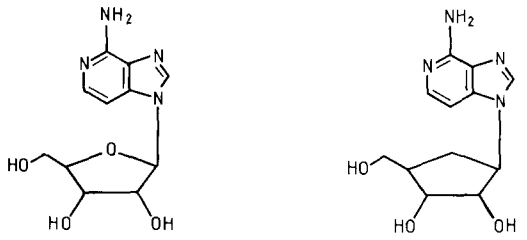

Fig. 1. Structural formulae of 3-deazaadenosine (c^3Ado) and carbocyclic 3-deazaadenosine (C-c^3Ado).

AdoHcy hydrolase with a K_i of 8 μM and that the compound affects the growth of a number of viruses including Rous sarcoma, vesicular stomatitis, Sindbis and Newcastle disease virus [1–3]. However, c^3Ado is neither potent nor very specific in its antiviral activity. As shown here, c^3Ado is toxic for the host cells at a concentration of 40 μg/ml, that is equal to or only 2–20-fold higher than its minimal antiviral concentration.

A dramatic improvement in both potency and selectivity has been achieved by the carbocyclic analog of c^3Ado (Fig. 1). Carbocyclic 3-deazaadenosine (C-c^3Ado) is, like c^3Ado itself, a potent inhibitor of AdoHcy hydrolase [10]. We have now found that C-c^3Ado inhibits the replication of several viruses, i.e. vaccinia, reo, measles, parainfluenza and vesicular stomatitis virus at a concentration of 0.2–1 μg/ml, while not being toxic for the host cells at a concentration of 400 μg/ml. C-c^3Ado also proved more potent in its antiviral activity than the well-known broad-spectrum antiviral agents (*S*)-DHPA [7] and ribavirin [11]. In vivo, it effected a significant decrease in the mortality rate of newborn mice infected with vesicular stomatitis virus, when administered as a single dose of 20, 100 or 500 μg per mouse 1 h after virus infection.

MATERIALS AND METHODS

Test compounds

C-c^3Ado was synthesized as described previously [10]. (*S*)-DHPA was provided by A. Holy (Czechoslovak Academy of Sciences, Prague, Czechoslovakia). Ribavirin was obtained from the American Cyanamid Company, Pear River, New York, whereas c^3Ado was provided by the Drug Synthesis and Chemistry Branch of the National Cancer Institute, Bethesda, Maryland.

Viruses

The origin of the viruses was as follows: herpes simplex virus type 1 (strains KOS, F and McIntyre), herpes simplex virus type 2 (strains Lyons, G and 196), see Ref. [8]; vaccinia virus, vesicular stomatitis virus, measles virus, Sindbis virus, coxsackie virus type B4 and polio virus type 1, see Ref. [9]; reo virus type 1 (ATCC VR-230) and parainfluenza virus type 3 (ATCC VR-93) were obtained from the American Type Culture Collection (Rockville, Maryland). The virus stocks were grown in primary rabbit kidney

cells (herpes simplex types 1 and 2, and vesicular stomatitis virus), Vero cells (measles, reo, parainfluenza and coxsackie virus), HeLa cells (polio virus), chick embryo cells (Sindbis virus) or chorioallantoic membrane cells (vaccinia virus).

Inhibition of virus-induced cytopathogenicity in vitro

Confluent primary rabbit kidney (PRK), human embryonic skin-muscle (ESM) fibroblasts, human epithelial (HeLa) cells, mouse (L-929) fibroblasts or green monkey kidney (Vero) cell cultures in microtiter trays were inoculated with 100 $CCID_{50}$ of virus, that is 100 times the virus dose needed to infect 50% of the cells. After 1 h virus adsorption to the cells, residual virus was removed and replaced by cell culture medium (Eagle's minimal essential medium (EMEM)) containing 3% fetal calf serum (FCS) and varying concentrations of the test compounds (C-c^3Ado, c^3Ado, (*S*)-DHPA or ribavirin). Viral cytopathogenicity (CPE) was recorded as soon as it reached completion in the untreated virus-infected cell cultures. The antiviral activity of C-c^3Ado, c^3Ado, (*S*)-DHPA and ribavirin is expressed as the minimal inhibitory concentration required to inhibit viral CPE by 50%.

Inhibition of virus multiplication in vitro

Confluent PRK or human skin fibroblast (VGS) cells in plastic Petri dishes were inoculated with 4.5 $\log_{10}$ plaque forming units (pfu) of vaccinia virus or 4.5 $\log_{10}$ $CCID_{50}$ of vesicular stomatitis virus, respectively. After 1 h virus adsorption to the cells, residual virus was removed and replaced by EMEM containing 3% FCS and varying concentrations of C-c^3Ado as indicated in the legend to Fig. 2. The cell cultures were frozen at -70°C at either 1, 24, 48 or 72 h after infection and the cell homogenates were assayed for virus content by plaque formation in mouse L-929 cells (vesicular stomatitis virus) or PPK cells (vaccinia virus).

Antiviral activity in vivo

Two-day-old NMRI mice were inoculated subcutaneously with 10 LD_{50} of vesicular stomatitis virus, that is 10 times the virus dose needed to kill 50% of the mice. 1 h after virus infection C-c^3Ado was injected intraperitoneally in a volume of 0.1 ml per mouse at varying doses as indicated in the legend to Fig. 3. Deaths were recorded daily up to 20 days after virus infection. There were 30 mice per group. Statistical significance for the differences in the final mortality rate was assessed by the χ^2-test with Yates' correction.

RESULTS

C-c^3Ado was evaluated in vitro against a wide variety of viruses belonging to either the poxviridae (vaccinia), herpetoviridae (herpes simplex), rhabdoviridae (vesicular stomatitis), picornaviridae (polio, coxsackie), togaviridae (Sindbis), paramyxoviridae (measles, parainfluenza) or reoviridae (reo). For each virus we determined the minimal inhibitory concentration required to suppress viral cytopathogenicity (CPE) by 50%. For

TABLE 1

Inhibitory effects of C-c^{3}Ado on virus-induced cytopathogenicity in vitro

Virus[a]	Cell culture[b]	Minimal inhibitory concentration (μg/ml)			
		C-c^{3}Ado	c^{3}Ado	(*S*)-DHPA	Ribavirin
DNA viruses					
Vaccinia	PRK	0.8	7 (40)[c]	40	15
Herpes simplex type 1	PRK	⩾200	⩾40 (40)	⩾300	>400
Herpes simplex type 2	PRK	⩾300	⩾40 (40)	⩾400	>400
RNA viruses					
Vesicular stomatitis	PRK	0.2	7 (40)	10	25
Vesicular stomatitis	ESM	0.2	nd	15	20
Vesicular stomatitis	HeLa	0.3	20 (40)	50	10
Vesicular stomatitis	L-929	1	nd	70	70
Polio type 1	HeLa	>400	⩾40 (40)	>400	15
Coxsackie type B4	HeLa	>400	⩾40 (40)	>400	20
Coxsackie type B4	Vero	1	10 (40)	40	200
Sindbis	Vero	20	⩾40 (40)	>400	40
Measles	Vero	0.4	⩾40 (40)	40	20
Parainfluenza type 3	Vero	0.2	2 (40)	20	20
Reo type 1	Vero	1	10 (40)	50	80

[a] One strain per virus except for herpes simplex types 1 and 2, where the antiviral data represent average values for three herpes simplex type 1 (KOS, F, McIntyre) strains and three herpes simplex type 2 (Lyons, G, 196) strains.

[b] The abbreviations for the cell cultures are indicated in the Materials and Methods section.

[c] The data indicated in parentheses refer to the minimal cytotoxic concentration (μg/ml) of compound causing a microscopically detectable alteration of normal cell morphology (in uninfected cell cultures). C-c^{3}Ado, (*S*)-DHPA and ribavirin did not cause such cytotoxicity at the highest concentration tested (400 μg/ml).

nd, not determined.

vesicular stomatitis and coxsackie B4 virus these CPE-inhibition assays were extended to more than one cell system.

As shown in Table 1, C-c^{3}Ado inhibited the CPE of vaccinia, vesicular stomatitis, measles, parainfluenza and reo virus at a concentration ranging from 0.2–1 μg/ml. Sindbis was inhibited at 20 μg/ml, herpes simplex at 200–300 μg/ml and polio not at all. A rather surprising result was noted for coxsackie B4: this virus was inhibited at 1 μg/ml in Vero cells but did not prove sensitive to C-c^{3}Ado in HeLa cells. The spectrum of activity of C-c^{3}Ado was remarkably similar to that of (*S*)-DHPA. However, C-c^{3}Ado was 50–100-fold more potent in its antiviral action than (*S*)-DHPA. Also, C-c^{3}Ado proved about 100 times more potent than ribavirin in its activity against vesicular stomatitis, measles, parainfluenza and reo virus. C-c^{3}Ado and ribavirin showed a marked difference in their activity spectrum, in that the latter was active against polio and coxsackie in HeLa cells, whereas the former was not (Table 1).

C-c^{3}Ado was effective against the same viruses that proved susceptible to c^{3}Ado, but it was 10 times more potent in its antiviral action than c^{3}Ado (Table 1). Whereas c^{3}Ado was uniformly cytotoxic at 40 μg/ml, regardless of the nature of the host (PRK, HeLa or Vero) cells, C-c^{3}Ado did not cause a morphological alteration of the host cells even at 400 μg/ml, the highest concentration tested. Neither did C-c^{3}Ado inhibit cellular DNA, RNA or protein synthesis (as monitored by 2′-[*methyl*-^{3}H]deoxythymidine, [5-^{3}H]-uridine or [4,5-^{3}H]leucine incorporation, respectively), when added to uninfected PRK (primary rabbit kidney) or HeLa cells for 1 day at a concentration up to 400 μg/ml.

That the inhibitory effects of C-c^{3}Ado on viral CPE truly reflected an inhibition of virus replication was ascertained by virus growth experiments performed with two viruses, vesicular stomatitis and vaccinia, which proved particularly sensitive to C-c^{3}Ado in the CPE inhibition assay. At a concentration of 1 μg/ml C-c^{3}Ado caused a greater than 2 $\log_{10}$ reduction in the 24-h-yield of vesicular stomatitis virus (Fig. 2A) and at 10 μg/ml it effected a 3 $\log_{10}$ reduction in the 48-h-yield of vaccinia virus (Fig. 2B). Similar reductions in vaccinia and vesicular stomatitis virus yield were achieved by ribavirin and (*S*)-DHPA at a concentration 100-fold greater than that required for C-c^{3}Ado (data not shown).

C-c^{3}Ado was further explored for its potential to protect newborn mice against a lethal infection with vesicular stomatitis virus. Two-day-old mice were infected subcutaneously with vesicular stomatitis virus at 10 LD_{50} per mouse and treated intraperitoneally with a single dose of C-c^{3}Ado 1 h after virus infection. When administered at 1 or 5 μg per mouse, C-c^{3}Ado did not affect the mortality rate of the virus-infected mice, but at 20, 100 and 500 μg per mouse it brought about a significant increase in the final number of surviving mice ($P < 0.001$) (Fig. 3). This protective effect was dose-dependent. With a dose of 500 μg C-c^{3}Ado per mouse, the final number of survivors reached 80%, as compared to 10% in the control group. When tested under similar conditions (data not

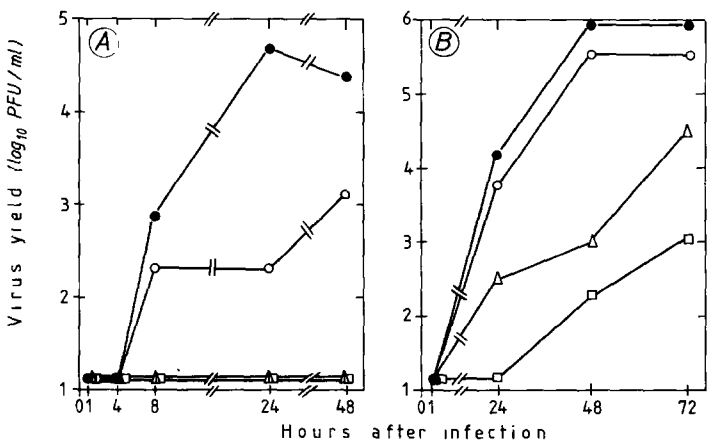

Fig. 2. Effects of C-c^{3}Ado on the multiplication of vesicular stomatitis virus in VGS cell cultures (A) and the multiplication of vaccinia virus in PRK cell cultures (B). Concentrations of C-c^{3}Ado : 100 μg/ml (□), 10 μg/ml (△), 1 μg/ml (○) or 0 μg/ml (●).

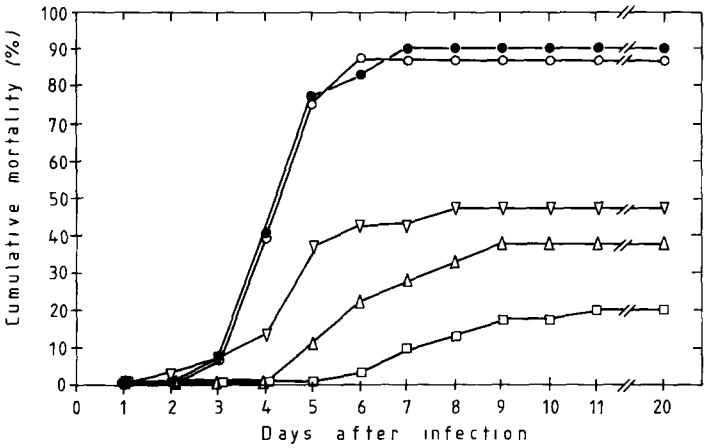

Fig. 3. Effects of C-c^{3}Ado on mortality of newborn mice infected with vesicular stomatitis virus. Doses of C-c^{3}Ado (per mouse) : 500 μg (□), 100 μg (Δ), 20 μg (▽), 5 μg (○) or 0 μg (●). The mortality rate of the groups treated with 20, 100 or 500 μg C-c^{3}Ado per mouse was significantly different ($P < 0.001$) from the mortality rate of the control group.

shown) (*S*)-DHPA at 500 μg per mouse increased the final survival rate from 10% to 50%, as did C-c^{3}Ado at 20 μg per mouse (Fig. 3). No signs of toxicity were noted when uninfected 2-day-old mice were given a single intraperitoneal dose of 500 μg C-c^{3}Ado per mouse. While preliminary data suggest that single intraperitoneal doses of 5 mg C-c^{3}Ado administered 1 h after infection may reduce the severity of vesicular stomatitis and vaccinia virus infections in older mice (11–13 g), no beneficial effects were obtained with C-c^{3}Ado at doses up to 500 μg per mouse in 2-day-old mice infected with coxsackie B4 virus (data not shown). The latter results are consistent with the lack of activity of C-c^{3}Ado against coxsackie B4 in HeLa cells (Table 1).

DISCUSSION

C-c^{3}Ado has already been the subject of some previous antiviral studies [10]. From these studies C-c^{3}Ado appeared to be inferior to ara-A in its activity against herpes simplex virus type 1 but superior to ara-A in its activity against vaccinia virus. However, no attempts were made to determine the activity of C-c^{3}Ado against (-)RNA viruses, and, as demonstrated by the present study, these are the viruses that are most sensitive to the antiviral action of C-c^{3}Ado.

The precise mechanism of antiviral action of C-c^{3}Ado remains to be determined. C-c^{3}Ado is not subject to deamination or phosphorylation [10]. Hence it is probably not incorporated into DNA or RNA. However, C-c^{3}Ado is a competitive inhibitor of AdoHcy hydrolase with a K_i of 3 μM and 1 nM for the enzyme from beef and hamster liver, respectively [10]. Within the cell, the inhibition of AdoHcy hydrolase would lead to an accumulation of AdoHcy and this would, in turn, lead to an inhibition of methylation

reactions including those required for viral mRNA maturation. Alternatively or additionally, C-c^{3}Ado may also perturb the biosynthesis of polyamines (spermine, spermidine) [4], but it is not yet clear to what extent this perturbation may account for the antiviral activity of the compound.

It is noteworthy that the activity spectrum of C-c^{3}Ado, like that of (*S*)-DHPA (Table 1), is particularly directed towards pox- and (-)RNA viruses such as vesicular stomatitis, parainfluenza, whereas herpes- and (+)RNA viruses such as polio are only minimally affected or not inhibited at all. This activity spectrum seems compatible with an impairment of viral mRNA transcription and/or processing.

Our results indicate that C-c^{3}Ado should be further pursued for its therapeutic potentials in the treatment of infections caused by rhabdoviruses (i.e., rabies), reoviruses (i.e., rota), paramyxoviruses (i.e., parainfluenza and measles) and poxviruses (i.e., vaccinia). C-c^{3}Ado should also be explored for its inhibitory effects on a number of (-)RNA viruses such as orthomyxo-, arena- and bunyaviruses, which were not included in the present study.

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