

Inhibition of measles virus replication by 5'-nor carbocyclic adenosine analogues

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Despite intense efforts to increase vaccine coverage, measles virus (MV) still causes significant morbidity and mortality in the world, sometimes as the result of severe, chronic, lethal disease. In an effort to develop therapies to supplement immunization strategies, a number of 5'-nor carbocyclic adenosine analogues were evaluated for anti-MV activity in CV-1 monkey kidney cells. Of those compounds tested, those either unsubstituted at C4 or possessing a hydroxyl, azido or amino substituent at that position were the most active, with particularly significant inhibition of MV, strain Chicago-1. The EC₅₀ values against this strain ranged from <0.1 to 1 mg/ml, as determined by cytopathic effect reduction assay, and confirmed by neutral red uptake. By virus yield reduction assay (+)-(1S,2S,3R,4S)-4-(6'-amino-9'H-purin-9'-yl)cyclopentane-1,2,3-triol (2) (-)-(1R,2S,3R)-1-(6'-amino-9'H-purin-9'-yl)-2,3-dihydroxycyclopent-4-ene (3) (-)-(1R,2S,3R)-1-(6'-amino-9'H-purin-9'-yl)cyclopentane-2,3-dihydroxycyclopentane (5) and (-)-(1R,2R,3R,4S)-4-amino-1-(6'-amino-9'H-purin-9'-yl)cyclopentane-2,3-diol (8) were the most potent

compounds tested, all with EC₉₀ values of ≤0.4 mg/ml. Compounds 3 and 5 were also tested against other MV strains, and similarly inhibited those strains except for four designated as Bil, Edmonston, SA and X-1108. Compound 8 did not potently inhibit these other MV strains. In addition, 3, 5 and 8 demonstrated synergistic (additive) inhibition of MV replication in combination with ribavirin at several concentrations. Compounds 3, 5 and 8 were also potent MV inhibitors even when added to infected cells 24 h after virus exposure. None of these three compounds was virucidal at concentrations that inhibited viral replication as determined by virus yield reduction assay. Most compounds tested were also not toxic at concentrations >100 mg/ml in actively growing and stationary-phase cells. Results suggest that these compounds may be clinically useful anti-MV virus agents.

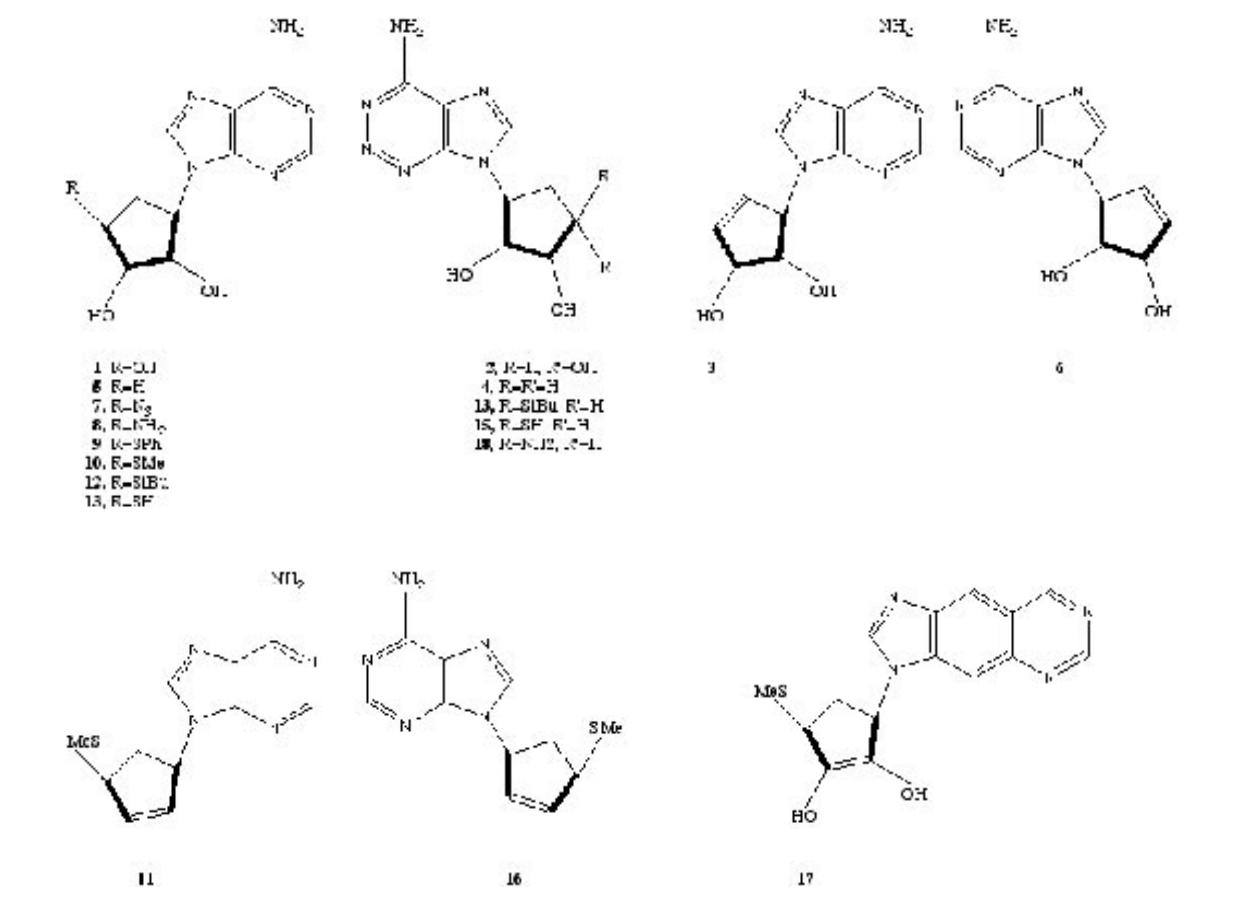
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Introduction

Measles virus (MV) is a potentially eradicable disease that still ranks as one of the leading causes of infectious disease-induced morbidity and mortality in childhood in the world. Currently, 30–40 million measles infections occur worldwide each year leading to 1–2 million deaths (Manchester *et al.*, 2000). In addition to causing an acute respiratory infection, measles is also associated with a serious, but transient suppression of cell-mediated immunity. This immunosuppression can lead to secondary infections, and major complications such as pneumonia and diarrhoea in children. In rare cases, it can also cause encephalitis and persistent central nervous system infections (Griffin &

Bellini, 1996). There are highly effective vaccines available, although their safety has occasionally been questioned because of tenuous links to Crohn disease and particularly to autism (Kawashima *et al.*, 2000). However, no studies to date have conclusively established a causal relationship with vaccine viruses and these two diseases (Afzal *et al.*, 2000).

Although the use of a live attenuated virus has nearly eradicated the endemic circulation of measles in the USA, reintroduction of the virus can cause repeated limited outbreaks (Gay, 2000). Therefore, campaigns need to be maintained that establish high routine coverage of the vaccine to prevent the reaccumulation of susceptible hosts that will

Figure 1. Structures of 5'-norcarbocyclic adenosine analogues

permit sustained measles transmission (Gay, 2000). Those who may be especially vulnerable are the non-immunized or immunized individuals with waning immunity, such as those living in extended care facilities (Clements & Cutts, 1995). In addition, with an increasing number of health care professionals having only received vaccine or never having been exposed to measles infection in infancy, nosocomially-acquired and -transmitted MV infections in health-care facilities may also contribute to a potential increase in MV infections (Steingart *et al.*, 1999). Thus, the potential for limited measles outbreaks always exists until indigenous virus transmission is eliminated in most populations of a given country (reviewed by De Serres *et al.*, 2000)

Of significance for the elimination of MV outbreaks is that virus isolates are not of single genotype or even serotype. Therefore, MV demonstrates considerable heterogeneity, as measured by monoclonal antibody reactivities and nucleotide sequencing (Rota *et al.*, 1998), as do other RNA viruses. Genotyping studies done in the last 10 years have indicated that the RNA sequences of the strains of

MV currently circulating have changed considerably from those isolated earlier. This suggests that measles virus may still be able to contribute significantly to worldwide morbidity and mortality caused by infectious diseases as it evolves under the pressure of elimination efforts.

Thus, there may be a need to supplement global immunization with non-toxic treatments that can be used to treat unexpected outbreak infections quickly and effectively. No chemotherapeutic agents have yet been approved for treatment of MV infections, although ribavirin has reportedly been efficacious when administered intravenously and orally (Gururangan *et al.*, 1990) alone or in combination with immune serum globulin in patients (Stogner *et al.*, 1993). To date, no other clinical trials have been done that support these findings of efficacy; on the contrary, a number of studies have also been done showing that ribavirin has no obvious clinical efficacy in MV infections when given as small-particle aerosol or intravenously (reviewed by Wyde *et al.*, 2000).

The recent reports of the broad-spectrum antiviral activity of 5'-noraristeromycin (1) and its enantiomer (Siddiqi *et*

Figure 2. Analysis of the antiviral and cytotoxic interactions of ribavirin with compounds **3**, **5** and **8**. (a) Antiviral interactions. (b) Cytotoxicity interactions. There was no cytotoxicity detected at any concentration of compound **8**.

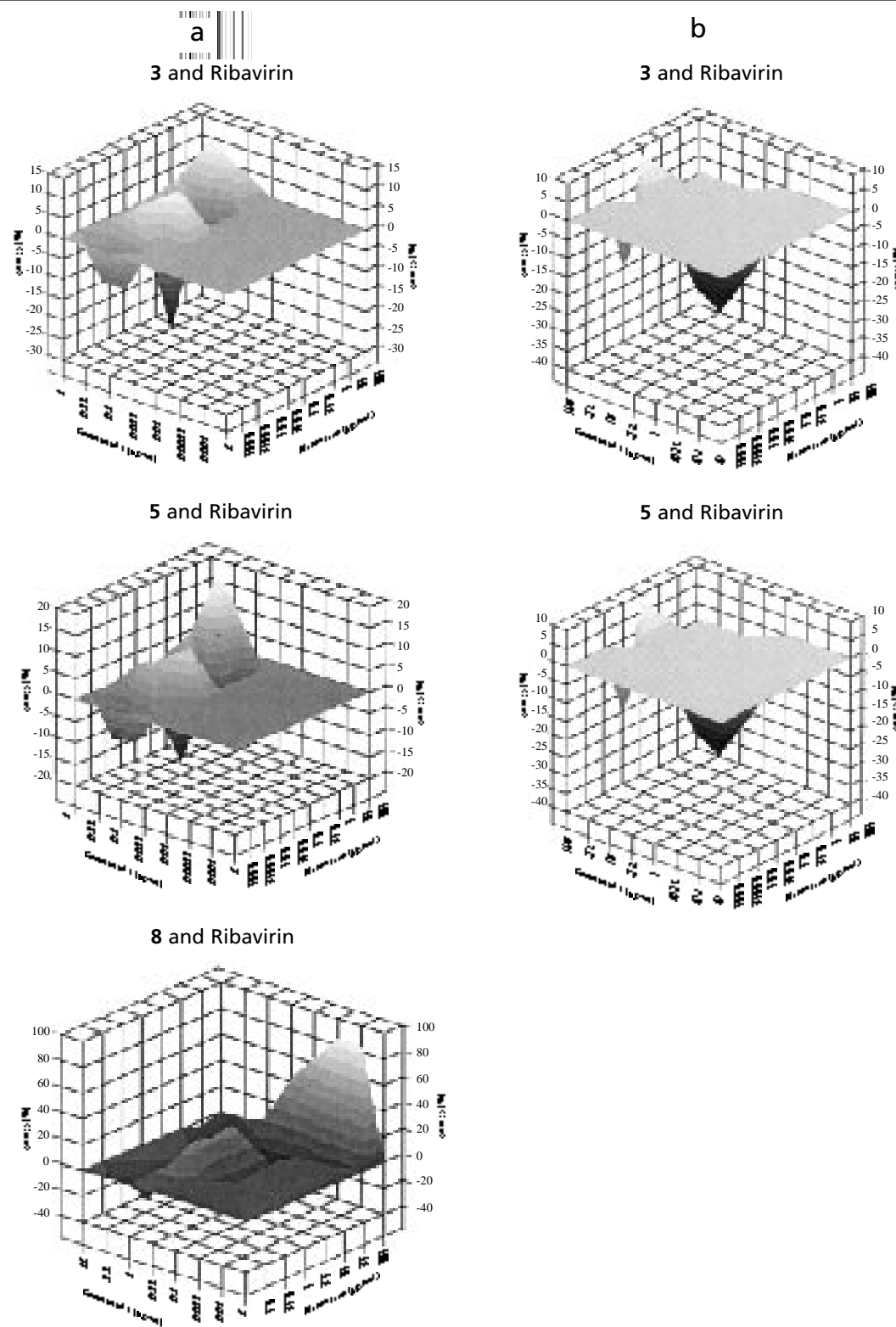


Table 1. Inhibition of strain Chicago-1 measles virus replication in African green monkey kidney (CV-1) cells by 5'-nor carbocyclic adenosine analogues

Compound	Visual assay			Neutral red assay			Virus yield reduction assay	Virucidal assay	Cell cytotoxicity*
	EC ₅₀ (µg/ml)	IC ₅₀ (µg/ml)	SI	EC ₅₀ (µg/ml)	IC ₅₀ (µg/ml)	SI	EC ₉₀ (µg/ml)	Lowest virucidal concentration (µg/ml)	EC ₅₀ (µg/ml)
1	1.0	>1000†	>1000	<1.0‡	550	>550	0.5	ND	>100
2	1.0	>100	>100	1.0	75	75	0.2	ND	>100
3	1.0	95	95	1.0	70	70	<0.1	≥0.64	100
4	<0.1	60	>600	<0.1	61	>610	3.0	ND	100
5	<0.1	>100	>1000	<0.1	>100	>1000	0.4	≥1	100
6	1.0	>100	>100	<0.1	100	>1000	>100	ND	100
7	0.1	>100	>1000	0.5	>100	>200	11.0	ND	>100
8	<0.1	>100	>1000	<0.1	>100	>1000	0.1	>100	>100
Ribavirin	25.0	270	11	20.0	196	10	2.0	>1000	33

*Cytotoxicity was determined in actively growing cells by neutral red assay.

†Concentrations higher than those shown were not tested.

‡Concentrations lower than those shown were not tested.

EC₅₀, 50% effective concentration; IC₅₀, 50% cytotoxic dose; EC₉₀, 90% effective concentration; SI, selectivity index; ND, not determined.

al., 1993, 1994; Seley *et al.*, 1997) prompted us to explore related adenosine analogues (Figure 1) as a potential source of new agents for treating MV. The results of this study are reported here.

Materials and methods: chemistry

Synthesis of 5'-nor carbocyclic nucleoside analogues

The syntheses of the compounds described herein yielded enantiomerically pure products and were done following the procedures developed in the Auburn university laboratory, Ala., USA: Siddiqi *et al.* (1993) for (-)-(1S,2R,3S,4R)-4-(6'-amino-9H-purin-9'-yl)cyclopentane-1,2,3-triol (**1**); Seley *et al.* (1998) for (+)-(1S,2S,3R,4S)-4-(6'-amino-9H-purin-9'-yl)cyclopentane-1,2,3-triol (**2**); (-)-(1R,2S,3R)-1-(6'-amino-9'-H-purin-9'-yl)-2,3-dihydroxycyclopent-4-ene (**3**); (+)-(1S,2R,3S)-1-(6'-amino-9'-H-purin-9'-yl)cyclopentane-2,3-dihydroxycyclopentane (**4**); (-)-(1R,2S,3R)-1-(6'-amino-9'-H-purin-9'-yl)cyclopentane-2,3-dihydroxycyclopentane (**5**); (+)-(1S,2R,3S)-1-(6'-amino-9'-H-purin-9'-yl)-2,3-dihydroxycyclopent-4-ene (**6**); Hegde *et al.* (1998) for (-)-(1R,2R,3R,4S)-4-azido-1-(6'-amino-9'-H-purin-9'-yl)cyclopentane-2,3-diol (**7**); (-)-(1R,2R,3R,4S)-4-amino-1-(6'-amino-9'-H-purin-9'-yl)cyclopentane-2,3-diol (**8**); (+)-(1S,2S,3S,4R)-4-amino-1-(6'-amino-9'-H-purin-9'-yl)cyclopentane-2,3-diol (**18**); (-)-(1R,2S,3S,4S)-4-phenylthio-1-(6'-amino-9'-H-purin-9'-yl)cyclopentane-2,3-diol (**9**) (SR Das personal communication); (-)-(1R,2S,3S,4S)-4-methylthio-1-(6'-amino-

9'-H-purin-9'-yl)cyclopentane-2,3-diol (**10**); (-)-(1R,4S)-4-methylthio-1-(6'-amino-9'-H-purin-9'-yl)cyclopent-2-ene (**11**); (+)-(1S,4R)-4-methylthio-1-(6'-amino-9'-H-purin-9'-yl)cyclopent-2-ene (**16**); (-)-(1R,2S,3S,4S)-4-mercapto-1-(6'-amino-9'-H-purin-9'-yl)cyclopentane-2,3-diol (**14**), as developed by SR Das (personal communication); (+)-(1S,2R,3R,4R)-4-mercapto-1-(6'-amino-9'-H-purin-9'-yl)cyclopentane-2,3-diol (**15**); (-)-(1R,2S,3S,4S)-4-(isobutylthio)-1-(6'-amino-9'-H-purin-9'-yl)cyclopentane-2,3-diol (**12**), as developed by SR Das (personal communication); (+)-(1S,2R,3R,4R)-4-(isobutylthio)-1-(6'-amino-9'-H-purin-9'-yl)cyclopentane-2,3-diol (**13**); (-)-8-amino-3-[[1'R,2'S,3'R,4'S)-2',3',4'-trihydroxycyclopent-1'-yl]imidazo[4,5-g]-quinazoline (**17**), as developed by VP Rajappan (personal communication).

Ribavirin

Ribavirin, used as a positive control, was obtained from ICN Pharmaceuticals, Costa Mesa, Calif., USA.

Solubilization of Compounds

The 5'-nor-carbocyclic analogues were made soluble in mixture of methanol:DMSO (50:50) or in DMSO, prior to dilution in culture medium for antiviral assays. Ribavirin was soluble in aqueous media.

Materials and methods: virology

Cells and virus

African green monkey kidney cells (CV-1 cells) were

Table 2. 5'-Nor carbocyclic adenosine analogue not inhibiting strain Chicago-1 measles virus replication in African green monkey cells (CV-1) kidney

Compound	Visual assay			Neutral red assay		
	EC ₅₀ (µg/ml)	IC ₅₀ (µg/ml)	SI	EC ₅₀ (µg/ml)	IC ₅₀ (µg/ml)	SI
9	>100*	>100	0	>100	>100	0
10	>100	>100	0	>100	>100	0
11	>100	20	0	>100	15	0
12	100	>100	>1	>100	>100	0
13	>100	>100	0	>100	>100	0
14	>100	100	0	>100	100	0
15	>100	30	0	>100	100	0
16	>100	60	0	>100	70	0
17	100	>100	>1	100	>100	>1
18	>100	40	0	>100	20	0
Ribavirin	25	270	11	20	196	10

*Concentrations higher than those values shown were not tested. EC₅₀, 50% effective concentration; IC₅₀, 50% cytotoxic dose; SI, selectivity index.

obtained from American Type Culture Collection (ATCC, Manassas, Va., USA). The cells were grown in minimal essential medium (MEM, Gibco-BRL, Gaithersburg, Md., USA) supplemented with 0.1% NaHCO₃ and 10% fetal bovine serum (FBS, Hyclone Laboratories, Logan, Utah, USA). When performing antiviral assays, serum was reduced to 2%, and 50 mg/ml gentamicin (Sigma) was added to the medium.

MV strain CC was obtained from Pennsylvania State University. The Edmonston wild-type strain was purchased from ATCC and all other strains were acquired from PA Rota and JS Rota (Centers for Disease Control, Atlanta Ga., USA).

For all studies, except for the multiplicity of infection study, the viral m.o.i. ranged from 0.0005–0.01 for each virus tested.

Cytopathic effect inhibition assay

The protocol of Barnard *et al.* (1997a) was used. Test compounds were tested at varying concentrations (seven 1/2 log₁₀ dilutions). Virus was used at a m.o.i. of 0.001–0.010. Virus and compound were added in equal volumes to near-confluent cell monolayers in 96 well tissue culture plates. The m.o.i. used were virus-dependent and chosen for each strain, such that 100% of the cells in the virus controls showed cytopathic effects (CPE) within 5–7 days. The plates were incubated at 37°C until the cells in the virus control wells showed complete viral CPE as observed by light microscopy. Each concentration of drug was assayed for virus inhibition in quadruplicate and for cytotoxicity in duplicate. Four wells were set aside as uninfected, untreated cell controls per test and four wells per test compound

received virus in medium only and represented positive controls for virus replication. A positive control drug, ribavirin, was included for each set of compounds tested. For all CPE-based assays, the 50% effective concentrations (EC₅₀) were calculated by regression analysis of the means of the CPE ratings expressed as percentages of untreated, uninfected controls for each concentration. A neutral red assay of the same plate was done to verify the results from the CPE assay spectrophotometrically (see below). The usual correlation between the two assays has been greater than 95% (Barnard, 1997a).

Morphological changes resulting from cytotoxicity of a compound were graded on a scale of 0–5; 5 being defined as complete cytotoxicity. The 50% cytotoxic doses (IC₅₀) were calculated by regression analysis, and a selectivity index (SI) was calculated using the formula: SI=IC₅₀/EC₅₀. Neutral red uptake assay of CPE inhibition and compound cytotoxicity

This assay was done on the same CPE inhibition test plates described above to verify the inhibitory activity and the cytotoxicity observed by visual observation. The Neutral red (NR) assay was performed using a modified method of Cavanaugh *et al.* (1990) as described by Barnard *et al.* (1999). Briefly, medium was removed from each well of a plate scored for CPE from a CPE inhibition assay, 0.034% NR in Sörenson's citrate buffer (pH 4.0) was added to each well of the plate and the plate incubated for 2 h at 37°C in the dark. The NR solution was removed from the wells. After rinsing and aspirating to dryness, the remaining dye was extracted for 30 min, at room temperature in the dark, from the cells using absolute ethanol buffered with Sörenson's citrate buffer. Absorbances at 540 nm/450 nm

Table 3. Inhibition of the replication of various strains of measles virus in African green monkey kidney (CV-1) cells by 3, 5, 8 and ribavirin

Virus strain	3		5		8		Ribavirin	
	Visual assay	NR assay	Visual assay	NR assay	Visual assay	NR assay	Visual assay	NR assay
	EC ₅₀ (µg/ml)	EC ₅₀ (µg/ml)	EC ₅₀ (µg/ml)	EC ₅₀ (µg/ml)	EC ₅₀ (µg/ml)	EC ₅₀ (µg/ml)	EC ₅₀ (µg/ml)	EC ₅₀ (µg/ml)
Bil	>10*	>10	>10	>10	100	100	100	100
CC	0.05	0.05	0.05	0.05	30	10	20	20
Chicago	1	1	<0.1	<0.1	<0.1	<0.1	25	20
TN 1994	0.1	0.1	0.1	0.1	4	0.6	10	10
Edmonston	>64	>64	>64	>64	60	40	10	10
Halonen	<0.1†	<0.1	<0.1	<0.1	0.4	0.2	1	1
SA	50	>100	>100	>100	>100	>100	20	20
X-1108	>100	>100	>10	>10	10	15	20	20

*Concentrations higher than those shown were not tested.

† Concentrations lower than those shown were not tested.

EC₅₀, 50% effective concentration; NR, neutral red.

were read with a microplate reader (Bio-Tek EL 1309; Bio-Tek Instruments, Winooski, Vt., USA). Absorbance values were expressed as percentages of untreated controls and EC₅₀, IC₅₀ and SI values were calculated as described above.

Confirmatory virus yield reduction assay

All compounds with SI greater than 10 were evaluated in a more sensitive assay to confirm the results of the CPE inhibition/NR uptake assays. Infectious virus yields from each well from a second CPE inhibition assay were determined as previously described (Barnard *et al.*, 1997b). After CPE was scored as described above, each plate was frozen at -80°C and thawed. Sample wells at each compound concentration tested were pooled and titred for infectious virus by CPE assay. The wells were scored for CPE and virus titres calculated as described by Barnard *et al.* (1997b).

A 90% reduction in virus yield was then calculated by regression analysis. This represented a 1 log₁₀ inhibition in titre compared with untreated virus controls (Barnard *et al.*, 1997b). SI was calculated by the formula $SI = IC_{50}/EC_{90}$. The IC₅₀ was derived from visual observation of cytotoxicity of the same CPE inhibition assay prior to freezing and harvesting the virus.

Virucidal assay

For compounds showing good antiviral inhibitory activity, a virucidal test was done to exclude the possibility that the compounds inhibited the virus by physically inactivating or disrupting the virion. The method of Barnard *et al.* (1997b) was used. Concentrations tested bracketed the concentration determined to represent the EC₅₀; each concentration of test compound was assayed in quadruplicate. Surviving virus was quantified by CPE assay and titres calculated as described by Barnard *et al.* (1997b).

Cytotoxicity testing

Cytotoxicity in rapidly dividing cells was evaluated by determining the total number of cells as reflected by a NR uptake assay after a 3-day exposure to several concentrations of compound. The method of Barnard *et al.* (1997b) was used. To quantitate cell growth after 72 h in the presence or absence of drug, the plates were then treated as described above for the NR assay. Absorbance values were expressed as percentages of untreated controls, and IC₅₀ values were calculated by regression analysis.

Multiplicity of infection assay

This assay was done as described by Barnard *et al.* (1997a). Virus (strain Chicago-1) was used to study the inhibition by selected compounds at different m.o.i. (Table 2). Compounds at varying concentrations were evaluated as described above for the CPE inhibition and NR assays in the presence of virus at a selected m.o.i. EC₅₀ and IC₅₀ values were determined as previously described.

Cytopathic effect inhibition timing assay

The CPE inhibition assay, used in this part of the study, was performed as previously described, although the time that the compound was added to the plates was varied relative to the time of virus exposure. At the appropriate time after virus exposure (0, 1, 2, 4, 6, 8, 24 h), varying dilutions of test compounds in a small volume were added to the cells without removal of the virus. When a pretreatment was done (-1 h), compound was added to the wells and incubated for 1 h at 37°C, after which, virus at an appropriate m.o.i. was added to each well in a small volume. At '0' time, the virus were added within 5 min of drug addition as described previously for the CPE inhibition assay (Barnard *et al.*, 1997a). The EC₅₀, IC₅₀ and SI values were derived

Table 4. Effects of the multiplicity of infection on the inhibition of measles virus Chicago replication in African green monkey kidney cells (CV-1) by **8** and ribavirin

m.o.i.*	Compound 8						Ribavirin					
	Visual assay			Neutral red assay			Visual assay			Neutral red assay		
	EC ₅₀ (µg/ml)	IC ₅₀ (µg/ml)	SI	EC ₅₀ (µg/ml)	IC ₅₀ (µg/ml)	SI	EC ₅₀ (µg/ml)	IC ₅₀ (µg/ml)	SI	EC ₅₀ (µg/ml)	IC ₅₀ (µg/ml)	SI
0.01	>100†	>100	0	>100	>100	0	32	190	6	10	80	8
0.005	100	>100	>1	100	>100	>1	11	190	17	10	210	21
0.002	20	>100	>5	20	>100	>5	10	190	19	10	230	23
0.001†	4.8	>100	>21	3	>100	>33	10	180	18	4.9	100	20
0.0004	<0.1‡	>100	>1000	100	>1000	6	190	32	4	102	51	

*m.o.i. for antiviral tests was 0.0005–0.001.

†Concentrations higher than those shown were not tested.

‡Concentrations lower than those shown were not tested.

EC₅₀, 50% effective concentration; IC₅₀, 50% cytotoxic dose; SI, selectivity index.

from visual and NR assays as described above.

Drug combination assays

Concentrations of test compounds were evaluated alone and in combination with varying concentrations of ribavirin. The following drug concentrations were used when antiviral interactions were analysed: **3**=0.1–100 mg/ml, **5**=0.01–100 mg/ml, **8**=0.01–100 g/ml, ribavirin=0.1–100 mg/ml. The following drug concentrations were used when cytotoxic interactions were analysed: **3**=0.1–100 mg/ml, **5**=0.1–100 mg/ml, **8**=0.1–100 mg/ml, ribavirin=1–1000 mg/ml. All combinations, as well as each drug concentration used alone, were done in quadruplicate in 96-well microplates. Toxicity controls for each combination were also run in parallel on the same plate; these consisted of uninfected cells similarly treated with each drug combination, as well as each concentration of either drug used alone. The data from the visual assays were verified by NR assays, although only the data from the visual assays are shown in Figure 2.

The evaluation of synergism was done using the 3-dimensional computerized system of Prichard and Shipman (1990) with the MacSynergy™ computer program. The difference between the observed effects and those expected if the interactions had occurred independently, are expressed as synergy volume above and below the plane that represents no interactive effects. Any peaks above the plane are indicative of synergy, while any peaks below the plane are indicative of metabolism.

Results

Primary characterization of antiviral activity

A series of 5'-norcarbocyclic nucleoside analogues were evaluated for anti-measles virus activity using strain Chicago-1. Of the compounds tested, eight (compounds **1–8**) were found to be active, with selective indices greater

than 10 (Table 1). The EC₅₀ values ranged from <0.1 to 1 mg/ml by CPE inhibition assay, as verified by neutral red assay. Compounds **4**, **5**, **7** and **8** were the most potent (see EC₅₀ values). Most of the active compounds were not toxic at the concentrations used (Table 1). Compounds **9–17** were considered inactive (Table 2). Using a virus yield reduction assay, compounds **2**, **3**, **5** and **8** were still found to potently inhibit MV replication; EC₉₀ values were ≤0.4 mg/ml. Further characterization of the antiviral activity of **3**, **5** and **8**, revealed that the antiviral activity of two of the three analogues could not be attributed to the physical disruption of the virus (Table 1). For example, compound **5**, with an EC₅₀ <0.1 mg/ml and an EC₉₀=0.4 mg/ml, was not virucidal at concentrations lower than 1 mg/ml and **8** was not virucidal at any concentration tested. However, compound **3** probably inhibited virus by both a virucidal mechanism as well as an antiviral mechanism, since it inhibited 90% of the virus even at 1 mg/ml. The antiviral concentrations were EC₅₀=1 mg/ml and EC₉₀ <0.1 mg/ml (Table 1). In addition, none of the active compounds tested were cytotoxic to actively growing CV-1 cells (Table 1).

Other MV strains

Compound **3** inhibited MV strains CC, TN 1994 and Halonen (Table 3), but did not inhibit the Bil, and X-1108 strains, and only slightly inhibited Edmonston and SA strains. However, the three sensitive strains were inhibited at even lower concentrations of **3** (<0.1 µg/ml compared with 1 mg/ml versus Chicago-1, Table 1) than strain Chicago-1, the initial virus used to evaluate antiviral activity. The inhibition spectrum of compound **5** was nearly identical to that of compound **3** (Table 3). However, the 5m-nor carbocyclic analogue **8** only potently inhibited strains Halonen and TN 1994 (Table 3); strain X-1108 was 2–20-fold less sensitive to inhibition compared with strains Halonen and TN 1994. Strains CC and Edmonston were

Table 5. The effects of the time of addition of **3**, **5** and **8** on measles virus replication in African green monkey kidney cells (CV-1)

Time of addition of compound	EC ₅₀							
	3		5		8		Ribavirin	
	Visual assay	Neutral red assay	Visual assay	Neutral red assay	Visual assay	Neutral red assay	Visual assay	Neutral red assay
-1*	0.5	3	0.01	0.01	0.1	0.2	6	0.6
0†	0.1	0.1	0.01	0.01	0.2	0.4	3	0.3
1‡	0.2	<0.1§	0.02	0.01	0.06	0.1	13	5
2	0.5	1	0.02	0.02	0.1	0.18	15	12
4	0.5	3	<0.01	<0.01	0.1	0.1	18	12
6	0.5	3	<0.01	<0.01	0.1	0.09	15	12
8	0.5	1	<0.01	<0.01	1	0.5	18	12
24	0.5	3	0.05	0.03	1	2.6	15	12

*Compound was added to the cells 1 h before exposure to virus. At 0 time, virus was added, at the appropriate m.o.i., to the compound-treated cells and incubated with the cells until the assay was ended.

†An equal volume of virus was added to dilutions of compound present on cells within 5 min of compound addition to cells.

‡After exposure of cells to virus for one the time periods (1–24 h) indicated, an appropriate concentration was added to the virus-infected cells and incubated with those cells until the assay ended.

§Concentrations lower than shown were not tested. EC₅₀, 50% effective concentration.

slightly inhibited by compound **8** and strain SA was not inhibited at all. The potency of inhibition (EC₅₀) of strain Halonen by **8** was 0.4 mg/ml by visual CPE inhibition assay, which was verified by NR assay (EC₅₀=0.2 mg/ml); for strain TN 1994 the EC₅₀ values were 4 mg/ml and 0.6mg/ml by visual CPE inhibition and NR assay, respectively.

Effects of multiplicity of infection

Analogue **8** was evaluated for the effects of virus concentration on the potency of inhibition. At higher m.o.i., the compound was not inhibitory to strain Chicago-1 (Table 4). Only at virus/cell ratios below 0.002 did **8** inhibit the virus strikingly. The positive control drug, ribavirin, also showed this 'm.o.i. dependency'.

Preliminary mode of action studies

To understand the potential viral targets of these compounds, times of addition studies were done. When the times that **3**, **5** and **8** were added to virus infected cells were analysed for potency of inhibition, all three compounds were very inhibitory for strain Chicago-1 replication, even up to 24 h post-virus exposure (Table 5). However, of the three analogues, **5** was the most inhibitory, and consistently remained so up to and including 24 h post-virus exposure. It could also be used prophylactically at similar concentrations to achieve inhibition of virus replication comparable to the times when it was added after virus exposure. Compound **3** was also consistently inhibitory, regardless of the time of addition. However, the inhibitory activity of **8** began to wane at 8 h post-virus exposure.

Drug combination studies

It was interesting to determine how **3**, **5** and **8** would inter-

act with ribavirin when used in combination with that compound. Therefore, each of these 5'-nor carbocyclic analogues were evaluated for efficacy in inhibiting MV strain Chicago-1 replication in combination with ribavirin. Varying concentrations of an analogue were evaluated alone and in combination with varying concentrations of ribavirin. The peaks of synergism or antagonism, as ascertained by the MacSynergy™ computer program, were used as measure of the effects of the drug combinations. Compounds **5** and **8** appeared to augment the anti-MV effects of ribavirin (Figure 2a) especially at lower concentrations. Compound **3** seemed to have less of an additive interaction with ribavirin than either **5** or **8**. At lower concentrations, compound **8** had a large additive effect on the antiviral effect of ribavirin. In addition, at higher concentrations, **3** and **5** seemed to slightly ameliorate (antagonize) the cytotoxicity of ribavirin (Figure 2b). Because **8** was not cytotoxic at the concentrations used when assaying the combination of **8** and ribavirin, interactions were not demonstrated in that particular assay.

Discussion

Various acyclic and carbocyclic adenosine analogue, which apparently target S-adenosylhomocysteine (AdoHcy) have been reported to inhibit the replication of a variety of viruses, including arena-, herpes-, irido-, filo- pox-, rhabdo-, paramyxo-, and reo- and retro-viruses (De Clercq, 1998). The recent reports of the broad spectrum antiviral activity of 5'-noraristeromycin (**1**), and the C4'-epimer of its enantiomer (**2**), stimulated interest to further explore the potential of 5'-nor carbocyclic nucleosides as potent, non-toxic antiviral agents (Siddiqi *et al.*, 1993, 1994; Seley *et al.*, 1997).

Some structure activity relationships could be determined from the data. For example, removal of the C4' hydroxyl from 5'-noraristeromycin (**1**) and from (**2**) to give **4** and **5** enhanced the MV inhibitory activity by 10-fold, as detected by CPE inhibition and NR assays as was likewise reported by Seley *et al.* (1998) for anti-HBV activity. Addition of an amine group at the C-5' position (**8**) improved the inhibitory activity as measured by all assays, but the L-enantiomer (**18**) was inactive. By contrast, an azido addition at the same position (**7**) did not substantially affect the potency relative to the other compounds. Additions of sulfur-containing groups at C4' resulted in inactive compounds (**9-18**); this was presumably due to electronic or steric effects from these constituents.

In our hands **1** showed an antiviral profile similar to that of Siddiqi *et al.* (1994). Unlike compound **1**, compounds **3-8** apparently are not broad-spectrum inhibitors of virus replication. Although compounds **3-8** inhibited a number of MV in this study, those same compounds did not inhibit an adenovirus, a flavivirus, an arena virus, a bunya virus, influenza A viruses, other paramyxoviruses and a rhinovirus (data not shown). However, compounds **3** and **5** did inhibit vaccinia virus and compounds **3-8** inhibited several influenza B viruses, although at concentrations 4-50-fold higher than those that inhibited MV replication (data not shown). Thus, unlike other carbocyclic adenosine analogues, the spectrum of viruses inhibited by these analogues of **1** is quite limited. This suggests that although the target of inhibition could be S-adenosylhomocysteine hydrolase activity, as has been theorized for other carbocyclic adenosine analogues (reviewed by De Clercq, 1998), other targets of inhibition may be possible. The timing studies show that compounds **3**, **5**, and **8** effectively inhibit some target up to 24 h post infection. That could imply that an event occurring late in infection such as assembly or egress could be inhibited. Alternatively, the compounds might be irreversible inhibitors of an earlier replication event.

When three of the active compounds (**3**, **5**, **8**) were evaluated against other MV strains, the inhibitory activity demonstrated strain specificity. Strains Bil, Edmonston, SA and X-1108 were not sensitive to inhibition by any of these compounds when tested at the concentrations used in the antiviral assays. The spectrum of MV strains inhibited by these carbocyclic analogues did not necessarily correlate with any particular clade system or genetic grouping system developed for MV (Bellini & Rota, 1998, Rota *et al.*, 1998; Takeda *et al.*, 1999;). For example, Halonen and Edmonston are in the group 1 genotype, but the replication of strain Halonen, a wild-type strain, was extremely sensitive to inhibition by these compounds; Edmonston, a wild-type strain, replication was not inhibited.

This series of 5'-nor carbocyclic nucleoside analogues are potent, relatively non-toxic, specific inhibitors of MV repli-

cation. Thus, they represent a new potent class of compounds that may become clinically relevant anti-MV agents

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DL Barnard *et al.*

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