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Inhibitory activity of *S*-adenosylhomocysteine hydrolase inhibitors against human cytomegalovirus replication

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Summary

Various acyclic and carbocyclic adenosine analogues, which are apparently targeted at the *S*-adenosylhomocysteine (AdoHcy) hydrolase have been reported to inhibit the replication of a number of pox-, rhabdo-, paramyxo-, arena-, and reoviruses. Here we show that this activity spectrum extends to human cytomegalovirus (HCMV). Of the compounds tested, neplanocin A, 3-deazaneplanocin A, 6′-*C*-methylneplanocin A and 5′-noraristeromycin were found to be the most potent inhibitors of HCMV replication in vitro. Their 50% inhibitory concentration ranged from 0.05 to 1.35 μg/ml. In general, the anti-HCMV activity of the adenosine analogues correlated well with their affinity (K_i) for AdoHcy hydrolase, suggesting that AdoHcy hydrolase may be considered as a target enzyme for anti-HCMV agents. For four compounds (3-deazaneplanocin A, 6′-*C*-methylneplanocin A (isomers I and II) and 3-deazaadenosine), anti-HCMV potency was greater than could be expected solely from their interaction with AdoHcy hydrolase, suggesting that these compounds may be functioning by an additional mechanism.

Human cytomegalovirus; Antivirals; AdoHcy hydrolase inhibitors

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Introduction

Human cytomegalovirus (HCMV) has been recognized as a life-threatening pathogen in immunocompromised patients, particularly those who received an organ (liver, lung, heart, kidney) or bone marrow transplant (BMT) (Schmidt, 1991), and in patients infected with human immunodeficiency virus type 1 (HIV-1), particularly during the advanced stage of AIDS (Tyms et al., 1989). HCMV mainly causes retinitis and pneumonitis in such patients, but can also lead to adrenalitis, colitis, hepatitis and encephalitis. HCMV infection gives little, if any, complications in immunocompetent individuals, but can cause serious malformations in infants infected before birth.

Two compounds have been licensed for the treatment of HCMV infections: 9-(1,3-dihydroxy-2-propoxymethyl)guanine (DHPG, ganciclovir, Cytovene^{R}) and the trisodium salt of phosphonoformic acid (PFA, foscarnet, Foscavir^{R}). Although ganciclovir and foscarnet effectively inhibit HCMV replication in vitro, they may fail to offer benefits in vivo, i.e., when given as single therapy in the treatment of such severe HCMV infections as pneumonitis in BMT patients (Klintmalm et al., 1985; Erice et al., 1987). Combination of ganciclovir with specific immune globulins has improved the outcome of these severe infections, in which case the response rate is increased to about 50% (Emanuel et al., 1988; Reed et al., 1988).

Concomitant with the increased use of antiviral drugs, there has been an increased emergence of drug-resistant virus strains. Thus, drug-resistant variants have been described for herpes simplex virus (HSV) (Coen, 1991), HIV-1 (Larder et al., 1989; Larder and Kemp, 1989; St. Clair et al., 1991; Nunberg et al., 1991; Mellors et al., 1992), varicella-zoster virus (VZV) (Jacobson et al., 1990), influenza A virus (Hayden et al., 1989) and HCMV (Erice et al., 1989; Stanat et al., 1991). HCMV may become resistant to both ganciclovir and foscarnet (Knox et al., 1991).

We have recently described a new class of compounds, the acyclic nucleoside phosphonate derivatives which show a broad spectrum of activity against retroviruses (De Clercq, 1991) and DNA viruses (De Clercq, 1991; De Clercq et al., 1986, 1987), including HSV and VZV mutants that are thymidine kinase deficient (TK^{-}). Foremost among the acyclic nucleoside phosphonates is (*S*)-1-(3-hydroxy-2-phosphonylmethoxypropyl)cytosine (HPMPC), a potent inhibitor of HCMV replication in vitro (Snoeck et al., 1988; Neyts et al., 1990; Andrei et al., 1991) and in vivo (Neyts et al., 1992). The compound elicits a long-lasting antiviral effect following its addition to cell cultures (Neyts et al., 1990) or administration in vivo (Neyts et al., 1992). As for ganciclovir and foscarnet, the target of anti-HCMV action of HPMPC is assumed to be the viral DNA polymerase.

Here we present anti-HCMV data obtained with a class of compounds which have proved to be potent inhibitors of the *S*-adenosylhomocysteine (AdoHcy) hydrolase (De Clercq and Cools, 1985; Hasobe et al., 1988, 1989a; Narayanan et al., 1988; Cools and De Clercq, 1989, 1990; McCarthy et al., 1989; Cools et

al., 1991; Patil et al., 1992; Shuto et al., 1992) and exert a broad-spectrum activity against a wide variety of pox-, rhabdo-, paramyxo-, arena-, and reoviruses (De Clercq and Montgomery, 1983; De Clercq, 1985; Holy et al., 1985, 1986; Hasobe et al., 1987; De Clercq et al., 1989a; Andrei and De Clercq, 1990; De Fazio et al., 1990; Cools et al., 1991; Wolfe and Borchardt, 1991; Shuto et al., 1992). AdoHcy hydrolase inhibitors may be expected to lead to the intracellular accumulation of AdoHcy, followed by inhibition of transmethylations, viral mRNA capping and viral progeny production.

Several adenosine analogues, e.g., neplanocin A, 3-deazaneplanocin A, 5′-noraristeromycin and 6′-*C*-methylneplanocin A were found to be potent inhibitors of HCMV replication in vitro. As a rule, a close correlation was found between the anti-HCMV activity of the adenosine analogues and their affinity (K_i) for AdoHcy hydrolase. However, four compounds (i.e., 6′-*C*-methylneplanocin A (isomers I and II), 3-deazaneplanocin A and 3-deazaadenosine) proved more effective against HCMV than could be expected from an action solely targeted at AdoHcy hydrolase.

Materials and Methods

Cells

All assays were carried out in human embryonic lung (HEL) fibroblasts (ATCC CCL-137) cultures (used between passages 10 and 20).

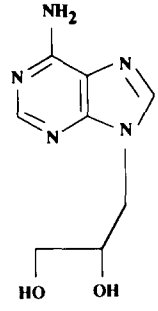
Viruses

The reference HCMV strains AD-169 (ATCC VR-538) and Davis (ATCC VR-807) were used as free viruses in all assays. Virus inoculum (20 or 100 PFU) was standardized according to titration of the virus in the same conditions as those used in the antiviral assays.

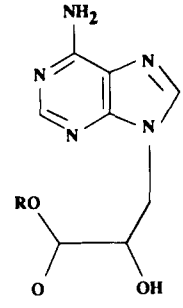
Compounds

The source of the test compounds was as follows: neplanocin A and 6′-*C*-methylneplanocin A (isomers I and II) (9-[(1*R*,2*S*,3*R*)-1,3-dihydroxy-4-(1-hydroxyethyl)-4-cyclopenten-1-yl]adenine), S. Shuto (Toyo Jozo Co., Tagata-Gun, Shizuoka-Ken, Japan); C-c^{3}Ado (carbocyclic 3-deazaadenosine), J.A. Montgomery (Kettering-Meyer Laboratory, Southern Research Institute, Birmingham, AL); AHPA ((*RS*)-3-adenin-9-yl hydroxypropanoic acid isobutyl ester), DHPA [(S)-(2,3-dihydroxypropyl)adenine) and HPMPC ((*S*)-1-(3-hydroxy-2-phosphonylmethoxypropyl)cytosine), A. Holy (Institute of Organic Chemistry and Biochemistry, Czechoslovak Academy of Sciences, Prague, Czechoslovakia); 3-deazaneplanocin A, V.E. Marquez (Laboratory of Pharmacology and Experimental Therapeutics, National Cancer Institute, Bethesda, MD); DHCeA (9-(*trans*-2′,*trans*-3′-dihydroxycyclopent-4′-enyl)adenine), c^{3}DHCeA (9-(*trans*-2′,*trans*-3′-dihydroxycyclopent-4′-enyl)-3-deazaadenine), DHCaA (9-(*trans*-2′,*trans*-3′-dihydroxycyclopentyl)adenine),

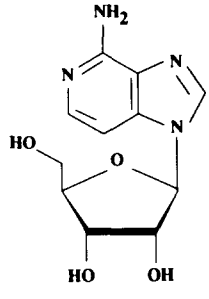
c^3DHCaA (9-(*trans*-2′,*trans*-3′-dihydroxycyclopentyl)-3-deazaadenine), and the 4′β-methyl, 4′α-methyl, 4′β-ethyl, 4′β-phenyl and 4′β-vinyl derivatives of DHCaA, R.T. Borchardt (Department of Biochemistry and Pharmaceutical Chemistry, University of Kansas, Lawrence, KA); adenosine dialdehyde, Sigma Chemical Co. (St. Louis, MO); ribavirin (1-(β-D-ribofuranosyl)-1,2,4-triazole-3-carboxamide), ICN Nutritional Biochemicals (Cleveland, OH); F-C-



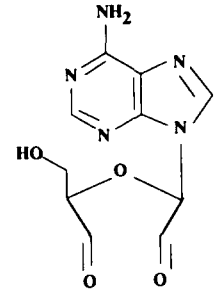
DHPA



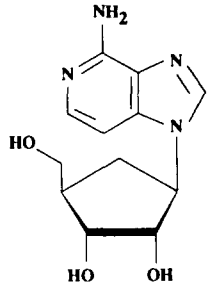
R = H : AHPA
R = $(CH_3)_2$ $CHCH_2$: AHPA isobutyl ester



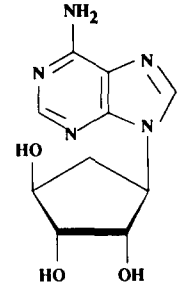
c^3 Ado



Ado dialdehyde

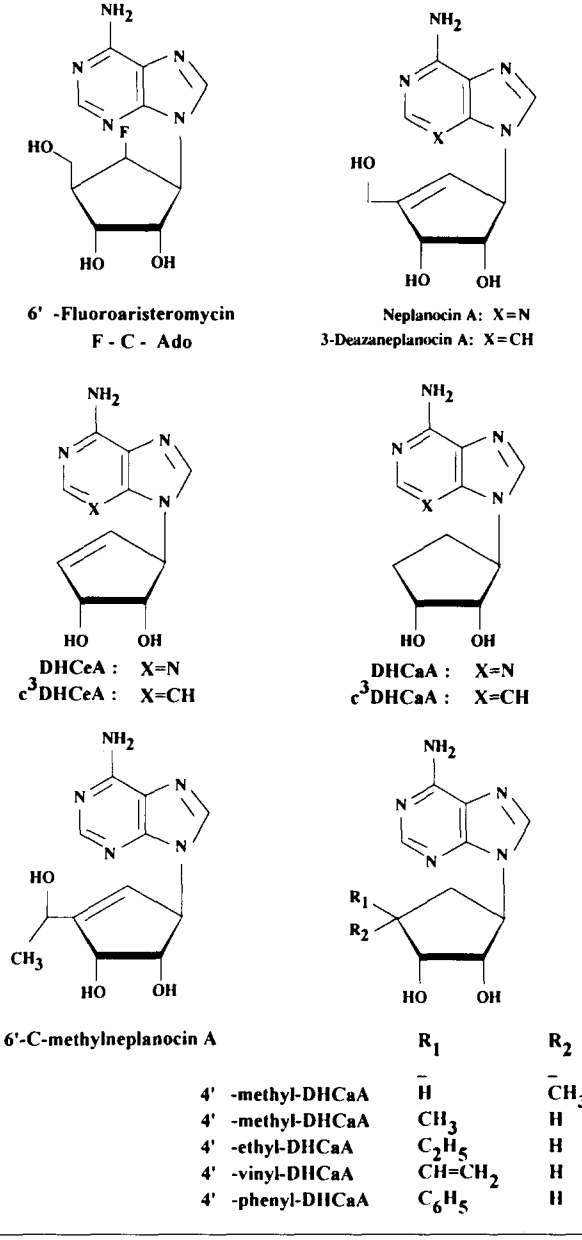


C - c^3 Ado



5'-Nor-aristeromycin

Ado ((±)-3-β-adenin-9-yl-4β-fluoro-5β-(hydroxymethyl)-1α,2α-cyclopentanediol, 6′β-fluoroaristeromycin), Syntex Research, Palo Alto, CA; c^3Ado (3-deazaadenosine), Drug Synthesis and Chemistry Branch of the National Cancer Institute (Bethesda, MD); 5′-noraristeromycin ((±)-(1α,2β,3β,4α)-4-(6-



← ↑

Fig. 1. Formulae of adenosine analogues which act as inhibitors of AdoHcy hydrolase.

amino-9*H*-purin-9-yl)-1,2,3-cyclopentanetriol), S.W. Schneller (Department of Chemistry, University of South Florida, Tampa, FL). The structural formulae of the compounds are presented in Fig. 1.

Antiviral assays

The assays were done in confluent HEL cells in 96-well microtiter plates (Becton-Dickinson, Belgium). The cells were inoculated with virus (at an input of 20 or 100 PFU, i.e., at a multiplicity of infection (MOI) of 0.004 or 0.02, respectively) for two hours, residual virus was removed and replaced by serial dilutions (in duplicate) of the test compounds in minimum essential medium (MEM) (Gibco, Paisley, Scotland) supplemented with 2% inactivated fetal calf serum (FCS) (Integro, Zaandam, The Netherlands). Microtiter trays were incubated at 37°C in 5% CO_2 atmosphere for 7 days. The microtiter plates were fixed with ethanol, stained with Giemsa, and plaque formation (cells infected with HCMV at 20 PFU) or cytopathicity (cells infected with HCMV at 100 PFU) was evaluated. The 50% inhibitory concentration (IC_{50}) was defined as the concentration required to reduce virus plaque formation or cytopathicity by 50%.

Cytotoxicity assays

The cytotoxicity of the compounds was estimated in growing HEL cells in 96-well microtiter plates. Per well, 5×10^3 cells were seeded and allowed to grow for 24 hours in MEM supplemented with 20% inactivated FCS. The different compound dilutions (in MEM supplemented with 2% inactivated FCS) were added in duplicate to the growing cells. After 3 more days under these conditions, the cells were trypsinized (Trypsin Rega 5, Gibco, Paisley, Scotland), resuspended in Diluid (J.T. Backer, Deventer, The Netherlands) and counted in a Coulter counter (Analis, Belgium). The 50% cytotoxic concentration (CC_{50}) was defined as the compound concentration required to reduce cell growth by 50% compared to the control.

Selectivity index (S.I.) was defined as the CC_{50}/IC_{50} ratio.

[^{3}H]Leucine incorporation assays

Toxicity of the different compounds was evaluated by a [^{3}H]leucine incorporation assay. The conditions were similar to those described above for the cytotoxicity. Eighteen hours before harvest, 10 μl of MEM containing 0.25 μCi of [^{3}H]leucine were added to each well. On day 4, the cells were washed and fixed with ethanol and analyzed in a scintillation counter after cell lysis with NaOH, 1 N.

Virus yield assays

Confluent HEL cells in 6-well microtiter plates (Nunc, Denmark) were infected with HCMV (reference strain AD-169) at a multiplicity of infection (MOI) of approximately 0.1. After 2 hours, residual virus was removed and replaced by 4 ml of MEM supplemented with 2% FCS containing different

dilutions of the test compounds. At days 4, 5, 6 and 7 post infection, the cell supernatants were harvested, cell debris was removed by centrifugation at 1800 rpm for 10 min, and the clarified supernatants were frozen at −70°C until titration. Serial ten-fold dilutions were assayed in quadruplicate in 96-well microtiter plates incubated for 7 days at 37°C in 5% CO_2 atmosphere. The mean number of plaques was determined for each dilution, and the virus titer expressed in PFU/ml.

Immunofluorescence assays

The immunofluorescence procedure has been described elsewhere (Schols et al., 1989). It was carried out in two chamber slides (Nunc, Denmark) containing confluent HEL cells infected with HCMV (AD-169 reference strain) at a MOI of approximately 0.1. After 2 hours incubation, the supernatant was removed and replaced by different concentrations of the test compounds. After 7 days, the cells were stained. The HCMV (AD-169)-infected cells were fixed for 20 minutes in acetone at 4°C. The cells were then incubated for 1 h at 37°C in the presence of the appropriate monoclonal antibody (mAb) (S. Michelson, Pasteur Institute, Paris, France) for the detection of late antigen expression (Amadei et al., 1983). The slides were rinsed with PBS and stained with fluorescein isothiocyanate-conjugated $F(ab')_2$ fragments of rabbit anti-murine immunoglobulin antibody (RaM-IG-$F(ab')_2$-FITC) (Dakopatts, Denmark) diluted 1/40. After 0.5 h incubation at 37°C, the slides were rinsed and dried. The samples were examined under a fluorescent microscope and the fluorescent cells of ten consecutive fields were counted for each dilution and compared to an untreated positive control.

Cesium chloride gradient analysis

HEL cells were seeded in 60-mm Petri dishes (Falcon, Becton Dickinson). Confluent HEL cells were infected with HCMV (reference strain AD-169) at an MOI of approximately 0.2. Unadsorbed virus was removed after 2 hours and the cell monolayers were overlayed with MEM containing 0.5% FCS and the appropriate concentrations of the test compounds (neplanocin A and 3-deazaneplanocin A). Mock-infected cells were prepared following a similar procedure. The cell cultures were incubated for 6 days, and 17 μCi of [*methyl*-^{3}H]dThd (specific radioactivity 45 Ci/mmol) were added to the medium 24 hours before harvesting the cells.

The cell monolayers were washed once with PBS and the cells were lysed with 200 μl of a solution containing 0.2% sodium dodecyl sulfate (SDS), 0.5% *N*-laurylsarcosylate, 1 mM sodium EDTA, 100 mM NaCl and 10 mM Tris-HCl, pH 7.4. Samples were frozen and stored at −70°C until used.

Each sample was layered on top of 8 ml of a cesium chloride (CsCl) solution (density 1.7067 g/ml) and centrifuged to equilibrium at 30 000 rpm in a Beckman L7-55 ultracentrifuge for 65 h at 20°C by using a TFT 65-13 rotor (Kontron, Beun de Ronde, Amsterdam, The Netherlands). Seven drop-fractions were collected from the bottom of the tubes, and the refractory

index was determined every fifth fraction. The acid-insoluble material of the fractions was precipitated on Whatman GF/C filters (Whatman International, Maidstone, UK) with 5% ice-cold trichloroacetic acid. Filters were dried with ethanol, and the radioactivity was determined in a toluene-based scintillant.

Correlation IC_{50} for HCMV/K_i for AdoHcy hydrolase

For the different test compounds, the correlation between the IC_{50} values for inhibition of HCMV replication and K_i values reported in the literature for different animal AdoHcy hydrolases (i.e., from murine L929 cells or beef liver extract) was determined on a Mackintosh computer (Apple) and plotted logarithmically (Stat. view 2, version 1.01).

Results

Antiviral assay

Activity of the different AdoHcy hydrolase inhibitors was evaluated against the two reference strains (AD-169 and Davis) of HCMV. As shown in Table 1, neplanocin A, 3-deazaneplanocin A, 6′-*C*-methylneplanocin A (isomer I) and F-C-Ado proved to be the most potent inhibitors of HCMV replication in vitro. The IC_{50} of neplanocin A ranged from 0.09 μg/ml (strain Davis, inoculum: 20 PFU) to 1.17 μg/ml (strain AD-169, inoculum: 100 PFU). IC_{50} values of 3-deazaneplanocin A and 6′-*C*-methylneplanocin A ranged from 0.05 to 0.30 μg/ml and from 0.053 to 1.35 μg/ml, respectively. Neplanocin A and 3-deazaneplanocin A were clearly more potent than their 5′-nor derivatives DHCeA and c^3DHCeA. DHCaA and c^3DHCaA were 5- to 10-fold more potent than DHCeA and c^3DHCeA. The β-aliphatic (ethyl-, phenyl-, vinyl- and methyl-) substituted derivatives of DHCaA were virtually inactive, as was the isomer II of 6′-*C*-methylneplanocin A, while the α-isomer of the methyl derivative was totally inactive and non-toxic.

The IC_{50} values of C-c^3Ado and adenosine dialdehyde were intermediate between those of neplanocin A and its 5′-nor derivatives, whereas 3-deazaadenosine and AHPA isobutyl ester were as potent as DHCeA and c^3DHCeA. Neither DHPA nor ribavirin or aristeromycin showed any activity against HCMV replication in vitro. The highest selectivity index (ratio CC_{50}/IC_{50}) was achieved by 3-deazaneplanocin A. The selectivity index of neplanocin A was slightly lower, as this compound was more toxic for both cell growth and [^{3}H]leucine incorporation (CC_{50}: 43 and 1.75 μg/ml, respectively). Of the 5′-nor derivatives of neplanocin A, the 3-deaza form was more toxic (CC_{50}: 13.5 μg/ml) than the 3-aza form (CC_{50}: 86 μg/ml), giving the latter a slight advantage in terms of selectivity index. Aristeromycin was the most toxic (CC_{50}: 1.25 and 1.5 μg/ml for cell growth and [^{3}H]leucine incorporation, respectively), followed by adenosine dialdehyde (CC_{50}: 16 μg/ml and 6.5 μg/ml, respectively).

TABLE 1

Anti-HCMV activity and cytotoxicity of AdoHcy hydrolase inhibitors in vitro

	Antiviral activity (IC_{50}) (μg/ml)[a]				Cytotoxicity (CC_{50}) (μg/ml)	
	AD-169 (20 PFU)	Davis (20 PFU)	AD-169 (100 PFU)	Davis (100 PFU)	Cell growth[b]	[^{3}H]Leucine incorporation[c]
Neplanocin A****	0.34 ± 0.12	0.09 ± 0.02	1.26 ± 0.36	0.30 ± 0.09	43	1.75
3-Deazaneplanocin A*	0.15 ± 0.035	0.05	0.27 ± 0.017	0.18 ± 0.03	125	20
6′-*C*-methylneplanocin A (isomer I)***	0.053 ± 0.005	0.35 ± 0.26	1.35 ± 0.78	0.05	>50	11
F-C-Ado*	0.15	ND	0.105 ± 0.031	0.035 ± 0.010	0.038	0.06
DHCeA***	2.5 ± 0.46	1.7 ± 0.38	19.25 ± 11.8	25.75 ± 12.63	86	>50
c^{3}DHCeA*	2.5 ± 1.06	3.5 ± 0.35	9.5 ± 3.18	13.5 ± 2.47	13.5	ND[d]
DHCaA	1.15	0.41	0.85	4	20	ND
c^{3}DHCaA	0.27	0.1	3	1	50	ND
C-c^{3}Ado***	1.63 ± 0.97	0.95 ± 0.43	4.72 ± 1.6	8.12 ± 4.88	85	>50
DHPA*	>100	>100	>100	>100	183	>50
Ribavirin**	>100	>100	>100	>100	27	15
Aristeromycin*	>100	>100	>100	>100	1.25	1.5
β-Vinyl-DHCaA	21.2	4	150	200	50	ND
β-Phenyl-DHCaA	18.1	18.1	60	90	150	ND
β-Ethyl-DHCaA	40	ND	>100	30	30	50
β-Methyl-DHCaA	>100	50	>100	>100	10	10
α-Methyl-DHCaA	175	25	250	250	200	ND
5′-Noraristeromycin	ND	0.4	ND	0.7	>50	>50
6′-*C*-methylneplanocin A (isomer II)	14.6	8.8	>100	ND	132	ND
AHPA isobutyl ester*	11.75 ± 5.83	1.5	22.5 ± 1.76	16	120	30
c^{3}Ado	2.5	3.5	10	10	>200	>50
Ado dialdehyde*	1.25 ± 0.53	0.25	6.25 ± 2.65	1.1 ± 0.63	16	6.5

[a]Concentration required to reduce viral cytopathicity (MOI: 100 PFU) or viral plaque formation (MOI: 20 PFU) by 50%.

[b]Concentration required to inhibit cell growth by 50%.

[c]Concentration required to inhibit [^{3}H]leucine incorporation by 50%.

[d]Not determined.

Results presented in this Table represent mean values for at least two (*), three (**), four (***) or seven (****) independent experiments ± standard error of the mean (SEM).

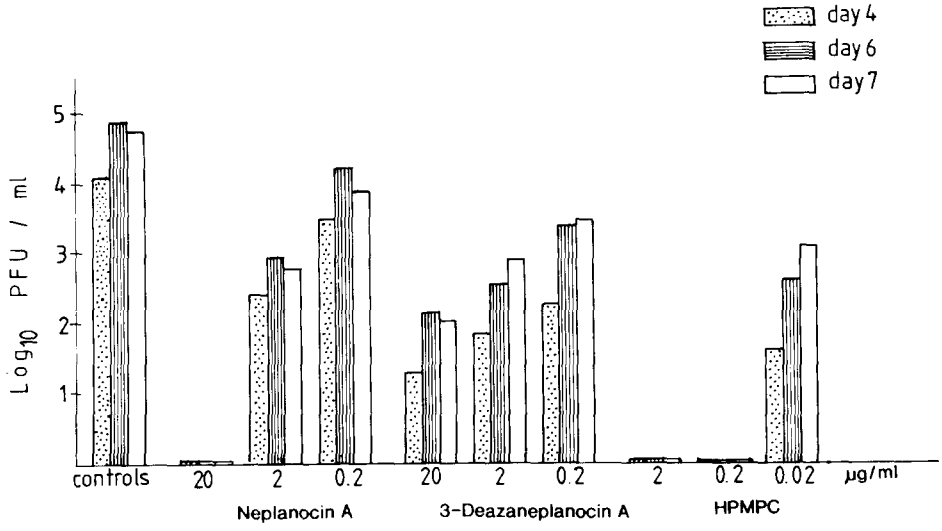


Fig. 2. HCMV free-virus production measured on days 4, 6 and 7 by titration on HEL cells, after treatment with 20, 2 and 0.2 µg/ml of neplanocin A and 3-deazaneplanocin A and HPMPC 2, 0.2 and 0.2 µg/ml.

Virus yield assay

Two of the most potent anti-HCMV compounds among the AdoHcy hydrolase inhibitors were compared with HPMPC for their ability to inhibit production of free virus. At a concentration of 0.2 µg/ml neither neplanocin A nor 3-deazaneplanocin A inhibited virus yield, while at a concentration of 2 µg/ml both molecules reduced virus production on days 6 and 7 by at least 2 logs and, at a concentration of 20 µg/ml, neplanocin A completely inhibited virus yield at days 4, 6 and 7 after infection. A similar degree of inhibition was observed with HPMPC at a concentration that was 10-fold lower than for the AdoHcy hydrolase inhibitors (Fig. 2).

TABLE 2

Immunofluorescent staining of CMV late antigen expression after exposure to different concentrations of selected AdoHcy hydrolase inhibitors

Compound	Concentrations (µg/ml)[a]						IC_{50}[b] (µg/ml)
	10	4	1	0.4	0.1	0.04	
Neplanocin A	3	12	60	92	113	97	2
3-Deazaneplanocin A	38	45	64	70	111	99	3
6′-*C*-methyl neplanocin A (isomer-I)	48	55	72	91	95	106	8

[a]Results are expressed as percentage of cells expressing fluorescence compared to infected untreated cells. Each concentration was assayed in duplicate.

[b]Concentration required to reduce viral late antigen expression by 50%.

Immunofluorescence assay

Inhibition of the viral late antigen production was quantified in an immunofluorescence assay. The results are given in Table 2 for neplanocin A, 3-deazaneplanocin A and 6′-C-methylneplanocin A (isomer I). The IC_{50} values obtained by this method were comparable to those obtained by the classic plaque assay. It should be noted that even at the highest concentration used (10 μg/ml) full inhibition of late antigen expression was not achieved. Neplanocin A and 3-deazaneplanocin A did not interfere with HCMV immediate early antigen expression (data not shown).

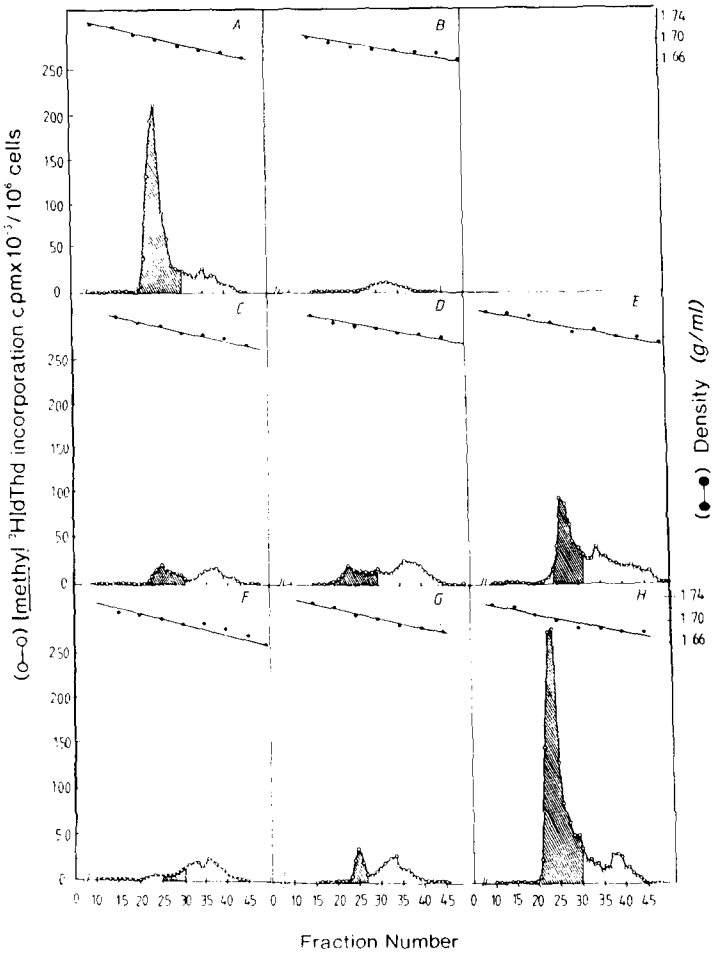


Fig. 3. Cesium chloride equilibrium gradient analysis of DNA from either mock-infected (Panel B) or HCMV-infected cells (Panels A and C–H). Panel A represents the control virus-infected cells. Panels C, D and E represent infected cells treated for 6 days with deazaneplanocin A at 10, 1 and 0.1 μg/ml, respectively. Panels F, G and H represent infected cells treated for 6 days with neplanocin A at 10, 1 and 0.1 μg/ml, respectively. DNA was labelled with [*methyl*-^{3}H]dThd 12 hours before harvesting (day 6). Shaded areas represent viral DNA.

Cesium chloride gradient assay

The inhibitory effects of neplanocin A and 3-deazaneplanocin A on viral DNA synthesis were monitored by equilibrium cesium chloride gradient centrifugation. The results are depicted in Fig. 3. Both compounds achieved a concentration-dependent inhibition of viral DNA synthesis in HCMV-infected cells. At a concentration of 10 or 1 μg/ml, neplanocin A and 3-deazaneplanocin A almost completely suppressed viral DNA synthesis, without affecting cellular DNA synthesis (Fig. 3, panels F,G and C,D, respectively). At 0.1 μg/ml, neplanocin A did not inhibit viral DNA synthesis, while at this concentration 3-deazaneplanocin A reduced the amount of [*methyl*-^{3}H]thymidine incorporated into viral DNA by about 50% (Fig. 3, panels H and E, respectively).

IC_{50}/K_i correlation analysis

The K_i values of various AdoHcy hydrolase inhibitors for AdoHcy hydrolases from either murine L-929 cells or beef liver are listed in Table 3. For the compounds of Table 3, known to be targeted at AdoHcy hydrolase, and for which the K_i for AdoHcy hydrolase was determined with both the murine L929 cells and bovine liver enzymes, an excellent correlation (r = 0.990) between the K_i values obtained in both systems was observed (Fig. 4).

The correlation between the K_i values for the L929-cell AdoHcy hydrolase

TABLE 3

K_i values of different AdoHcy hydrolase inhibitors for AdoHcy hydrolase

Compound	AdoHcy hydrolase extracted from		References
	Murine L-929 cells (nM)	Beef liver (nM)	
Neplanocin A	2.6	2	De Clercq and Cools (1985) Cools and De Clercq (1989)
3-Deazaneplanocin A	22	-	Cools and De Clercq (1989)
C-c^{3}Ado	18	13	De Clercq and Cools (1985) Cools and De Clercq (1989)
DHPA	280	1400	De Clercq and Cools (1985) Cools and De Clercq (1989)
Ado dialdehyde	4.3	2.39	Cools and De Clercq (1989)
AHPA isobutyl ester	52	73	De Clercq and Cools (1985) Cools and De Clercq (1989)
DHCeA	-	41[a]	Narayanan et al. (1988)
c^{3}DHCeA	-	35[a]	Narayanan et al. (1988)
F-C-Ado	3.1	-	Cools et al. (1991)
Aristeromycin	-	5–110	Guranowski et al. (1981) Houston et al. (1985)
c^3-Ado	-	4000	Guranowski et al. (1981)
6′-*C*-Methylneplanocin A (isomer I)	86	-	our unpublished data
6′-*C*-Methylneplanocin A (isomer II)	55,660	-	our unpublished data
5′-Noraristeromycin	11.1	-	our unpublished data

[a]Inactivation constants. All the other data represent inhibition constants (K_i).

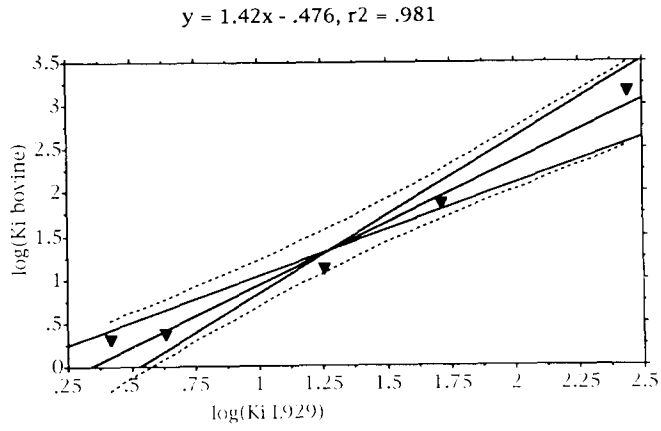


Fig. 4. Linear regression for K_i of neplanocin A, C-c[3]Ado, DHPA, Ado dialdehyde and AHPA isobutyl ester for the bovine enzyme as a function of the K_i obtained in L929 cells. Continuous lines represent the slopes and broken lines represent the 95% confident interval.

and the IC_{50} values for inhibition of HCMV replication was assessed with the IC_{50} values being determined for both AD-169 and Davis strains at an inoculum of 20 or 100 PFU. The correlation coefficient (r) was 0.594. Three compounds were responsible for this low correlation rate: 3-deazaneplanocin A and the two isomers of 6′-*C*-methylneplanocin A. The IC_{50} of these three compounds was significantly lower than could be expected from their K_i for the L929-cell AdoHcy hydrolase. When these three compounds were omitted from the correlation analysis, r was 0.908 (Fig. 5). When the IC_{50}/K_i correlation analysis was done with the K_i values obtained from the bovine enzyme, the

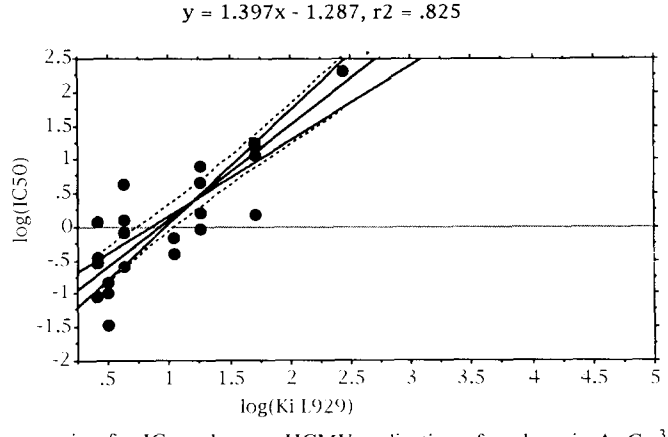


Fig. 5. Linear regression for IC_{50} values on HCMV replication of neplanocin A, C-c[3]Ado, DHPA, Ado dialdehyde, AHPA isobutyl ester, F-C-Ado and 5′-noraristeromycin as a function of their K_i for L-929 cell AdoHcy hydrolase. Explanation of the lines is given in the legend of Fig. 4.

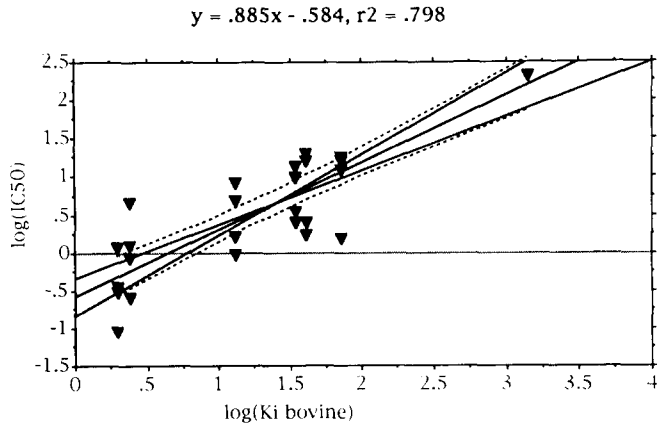


Fig. 6. Linear regression for IC_{50} values on HCMV replication of neplanocin A, C-c^{3}Ado, DHPA, Ado dialdehyde, AHPA isobutyl ester, DHCeA and c^{3}DHCeA as a function of their K_i for bovine liver AdoHcy hydrolase. Explanation of the lines is given in the legend of Fig. 4.

correlation coefficient was 0.633. Again, this relatively low r value was due to the behavior of one compound, 3-deazaadenosine. When c^{3}Ado was excluded from the analysis, r was 0.857 for the K_i and the IC_{50} of the remaining 8 compounds. If aristeromycin was removed from the analysis (since the literature (Guranowski et al., 1981; Houston et al., 1985) gives two divergent values for its K_i (5 and 110 nM)), an even better correlation was found (r = 0.893) (Fig. 6).

Discussion

Despite the availability of two anti-HCMV drugs (ganciclovir and foscarnet), the difficulties encountered in the treatment of immunosuppressed patients with HCMV disease have stimulated the search for new drugs and new target sites in the viral replicative cycle. Compounds acting at such new targets appear desirable because of increasing evidence that the lack of clinical response is linked to the emergence of drug-resistant viruses (Erice et al., 1989; Knox et al., 1991; Stanat et al., 1991). We have previously shown that acyclic nucleoside phosphonate derivatives such as HPMPC and HPMPA achieve a potent inhibitory effect on HCMV replication through an action targeted at the viral DNA synthesis step (Snoeck et al., 1988; Neyts et al., 1990; De Clercq, 1991). Combination therapy for the treatment of HCMV infections may also be considered as a feasible approach (Snoeck et al., 1993).

The AdoHcy hydrolase has been previously established as a target for antiviral chemotherapy (De Clercq, 1987). Inhibitors of AdoHcy hydrolase have been shown to be broad-spectrum antiviral agents interfering with the replication of (−)RNA viruses, including measles, respiratory syncytial virus,

influenza A and B, rabies and arenaviruses (Tacaribe, Junin), (+)RNA viruses (reo, rota) and some DNA viruses (e.g. vaccinia and African swine fever) (Andrei and De Clercq, 1990; De Clercq, 1987). The similarity in the antiviral activity spectrum of all the AdoHcy inhibitors points to a common mechanism and target of action. The viruses which are sensitive to the AdoHcy hydrolase inhibitors are known to multiply in the cytoplasm of the infected cells and to depend on their own methyltransferases for the methylation (5′-capping) of their mRNA. This suggests that the mechanism of action of the AdoHcy hydrolase inhibitors may involve these methyltransferases (De Clercq, 1987).

Here, we demonstrate that the AdoHcy inhibitors are also potent inhibitors of HCMV replication in vitro, as monitored by the classic plaque assay, virus yield or viral antigen detection, and further confirmed by analysis of viral DNA synthesis.

As the most active anti-HCMV agents emerged neplanocin A, 3-deazaneplanocin A, 6′-*C*-methylneplanocin A (isomer I) and 5′-noraristeromycin. The 5′-nor derivatives of neplanocin A and 3-deazaneplanocin A were clearly less active than their parent counterparts. This difference was already noted by De Clercq et al. (1989a) when the two pairs of drugs were tested against vaccinia virus, vesicular stomatitis virus, parainfluenza virus and reovirus. Whereas 5′-noraristeromycin showed selective anti-HCMV activity, aristeromycin was inactive and cytotoxic. Of the two diastereoisomers of 6′-*C*-methylneplanocin A, isomer I proved particularly active against HCMV, whereas isomer II was much less active, which is in keeping with previously reported antiviral data for this compound (Shuto et al., 1992).

F-C-Ado was as active as 3-deazaneplanocin A against HCMV replication but its high cytotoxicity as monitored by inhibition of both cell growth and [^{3}H]leucine incorporation makes this compound's selectivity index almost equal to 1, compared to more than 800 for 3-deazaneplanocin A.

Although the AdoHcy hydrolase inhibitors caused a marked inhibition of HCMV replication, whatever assay was used to measure virus replication, total inhibition of virus replication could not be achieved, even at the highest concentration used. As shown by the cesium chloride gradient results (Fig. 3), at a concentration of 10 μg/ml (100 times the IC_{50}) both neplanocin A and 3-deazaneplanocin A not completely suppressed viral DNA synthesis: there was still some viral DNA detectable. This was not the case when Neyts et al. (1990) applied a similar technique to evaluate the effects of HPMPC on HCMV DNA synthesis. At 100 times the IC_{50} HPMPC made viral DNA undetectable. Also, immunofluorescence assays carried out at 7 days postinfection indicated the lack of complete inhibition of late antigen production at the highest concentration used (10 μg/ml) for the test compounds (Table 2). Under the same conditions HPMPC achieved a complete inhibition of viral antigen expression, as demonstrated previously (Andrei et al., 1991; Snoeck et al., 1991). Similarly, the AdoHcy hydrolase inhibitors did not totally shut off free virus production (Fig. 2), except for neplanocin A at 20 μg/ml (which is close to the CC_{50} for cell growth). Since AdoHcy hydrolase inhibitors are exerting their

antiviral activity through the inhibition of a cellular enzyme, they may be expected to achieve full viral inhibition only at concentrations impairing cell survival as well.

It is unlikely that the lack of complete inhibition of HCMV at the highest concentrations of the compounds may be due to the emergence of drug-resistant virus strains. There is no evidence that compounds acting on cellular enzymes induce resistance (De Clercq, 1992). AdoHcy hydrolase inhibitors lead to an accumulation of AdoHcy which is a product inhibitor of methylation reaction catalyzed by methyltransferases with *S*-adenosylmethionine (AdoMet) as methyl donor (De Clercq, 1987). To relieve the methyltransferases from the restrains of AdoHcy, the latter has to be removed continuously by AdoHcy hydrolase. If this enzyme is blocked, AdoHcy accumulates, and thus methylations, particularly those required for viral mRNA capping, are shut off (De Clercq, 1987).

HCMV is known to stimulate cellular metabolism, particularly the synthesis of DNA polymerases, thymidine kinase and ornithine decarboxylase (Ho, 1991). This makes a direct comparison of the mechanism of anti-HCMV action with the mechanism of action of AdoHcy hydrolase inhibitors against other viruses more difficult. In addition, among those viruses that are sensitive to AdoHcy hydrolase inhibitors, HCMV is the only one that replicates intranuclearly.

A close correlation has been found between the antiviral activity of the AdoHcy inhibitors and their inhibitory effect on AdoHcy hydrolase (De Clercq and Cools, 1985; Borcherding et al., 1988; Cools and De Clercq, 1989). The increase in intracellular AdoHcy pool levels and the concomitant elevation in the ratio AdoHcy/AdoMet correlated closely with the antiviral effects (Hasobe et al., 1988, 1989a; Cools and De Clercq, 1990; Votruba et al., 1990).

We analyzed the correlation between the IC_{50} of the AdoHcy hydrolase inhibitors with regard to anti-HCMV activity and their K_i values for AdoHcy hydrolase as described in the literature (see Table 3 for references). For four compounds (3-deazaneplanocin A, 6′-*C*-methylneplanocin A (isomers I and II) and 3-deazaadenosine) the anti-HCMV activity was higher than could be expected from their K_i for inhibition of AdoHcy hydrolase. When these compounds were excluded from the analysis, a close correlation was found between the IC_{50} for antiviral activity and the K_i for the L929 and bovine AdoHcy hydrolase ($r = 0.908$ and 0.893, respectively). Cools and De Clercq (1989) found an r value of 0.993 and 0.998 when the DHPA, AHPA, C-c^{3}Ado, 3-deazaneplanocin A, adenosine dialdehyde and neplanocin A on the replication of vaccinia virus and vesicular stomatitis virus were compared with the K_i values for the AdoHcy hydrolase of L929 cells. The close correlation now found between the IC_{50} for HCMV and the K_i for AdoHcy hydrolase indicates that as for vaccinia and vesicular stomatitis virus, AdoHcy hydrolase may also function as a target for the inhibitory effects of these compounds on HCMV.

For the compounds (i.e., 3-deazaneplanocin A, 6′-*C*-methylneplanocin A, 3-

deazaadenosine) excluded from the analysis, an additional mechanism of antiviral action can be postulated. It is plausible, therefore, that, in addition to their inhibitory effect on AdoHcy hydrolase, they also interfere with other enzymatic processes (e.g., viral DNA polymerase following phosphorylation to their triphosphate form) involved in HCMV replication.

It would also seem necessary to further investigate whether the anti-HCMV activity of AdoHcy hydrolase inhibitors can be enhanced by homocysteine, as has been shown for the other antiviral effects of these compounds (De Clercq et al., 1989b; Hasobe et al., 1989b; Cools and De Clercq, 1990; Cools et al., 1990). Also, these adenosine analogues should serve as the model compounds for the design of new congeners with improved anti-HCMV selectivity (increased potency, decreased cytotoxicity).

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References

Amadei, C., Tandy-Panit, M., Couillin, P., Coulon, M., Cabau, N., Boué, A. and Michelson, S. (1983) Kinetic study of the development and localization of human cytomegalovirus-induced antigens, using monoclonal antibodies. Ann. Virol. 134E, 165–180.

Andrei, G. and De Clercq, E. (1990) Inhibitory effect of selected antiviral compounds on arenavirus replication in vitro. Antiviral Res. 14, 287–300.

Andrei, G., Snoeck, R., Schols, D., Goubau, P., Desmyter, J. and De Clercq, E. (1991) Comparative activity of selected antiviral compounds against clinical isolates of human cytomegalovirus. Eur. J. Clin. Microbiol. Infect. Dis. 10, 1026–1033.

Borcherding, D.R., Narayama, S., Hasobe, M., McKee, J.G., Keller, B.T. and Borchardt, R.T. (1988) Potential inhibitors of (*S*)-adenosylmethionine-dependent methyltransferases. 11. Molecular dissection of neplanocin A as potential inhibitor of (*S*)-adenosylhomocysteine hydrolase. J. Med. Chem. 31, 1729–1738.

Coen, D.M. (1991) The implications of resistance to antiviral agents for herpesvirus drug targets and drug therapy. Antiviral Res. 15, 287–300.

Cools, M. and De Clercq, E. (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.

Cools, M. and De Clercq, E. (1990) Influence of *S*-adenosylhomocysteine hydrolase inhibitors on *S*-adenosylhomocysteine and *S*-adenosylmethionine pool levels in L929 cells. Biochem. Pharmacol. 40, 2259–2264.

Cools, M., Hasobe, M., De Clercq, E. and Borchardt, R.T. (1990) Mechanism of the synergistic antiviral and cytostatic activity of (*RS*)-3-(adenin-9-yl)-2-hydroxypropanoic acid isobutyl ester

and D,L-homocysteine. Biochem. Pharmacol. 39, 195–202.
Cools, M., Balzarini, J. and De Clercq, E. (1991) Mechanism of antiviral and cytotoxic action of (±)-6′β-fluoroaristeromycin, a potent inhibitor of *S*-adenosylhomocysteine hydrolase. Mol. Pharmacol. 39, 718–724.
De Clercq, E. (1985) Antiviral and antimetabolic activities of neplanocins. Antimicrob. Agents Chemother. 28, 84–89.
De Clercq, E. (1987) *S*-Adenosylhomocysteine hydrolase inhibitors as broad-spectrum antiviral agents. Biochem. Pharmacol. 36, 2567–2575.
De Clercq, E. (1991) Broad-spectrum anti-DNA virus and antiretrovirus activity of phosphonylmethoxyalkylpurines and -pyrimidines. Biochem. Pharmacol. 42, 963–972.
De Clercq, E. (1993) Antiviral agents: characteristic activity spectrum depending on the molecular target they interact with. In: K. Maramorosch, F.A. Murphy and A.J. Shatkin (Eds), Advances in Virus Research, Vol. 42, pp. 1–55. Academic Press, Orlando, FL.
De Clercq, E. and Montgomery, J.A. (1983) Broad-spectrum antiviral activity of the carbocyclic analog of 3-deazaadenosine. Antiviral Res. 3, 17–24.
De Clercq, E. and Cools, M. (1985) Antiviral potency of adenosine analogues: correlation with inhibition of *S*-adenosylhomocysteine hydrolase. Biochem. Biophys. Res. Commun. 129, 306–311.
De Clercq, E., Holy, A., Rosenberg, I., Sakuma, T., Balzarini, J. and Maudgal, P.C. (1986) A novel selective broad-spectrum anti-DNA virus agent. Nature 323, 464–467.
De Clercq, E., Sakuma, T., Baba, M., Pauwels, R., Balzarini, J., Rosenberg, I. and Holy, A. (1987) Antiviral activity of phosphonylmethoxyalkyl derivatives of purine and pyrimidines. Antiviral Res. 8, 261–272.
De Clercq, E., Cools, M., Balzarini, J., Marquez, V.E., Borcherding, D.R., Borchardt, R.T., Drach, J.C., Kitaoka, S. and Konno, T. (1989a) Broad-spectrum antiviral activities of neplanocin A, 3-deazaneplanocin A, and their 5′-nor derivatives. Antimicrob. Agents Chemother. 33, 1291–1297.
De Clercq, E., Cools, M. and Balzarini, J. (1989b) Homocysteine potentiates the antiviral and cytostatic activity of those nucleoside analogues that are targeted at *S*-adenosylhomocysteine hydrolase. Biochem. Pharmacol. 38, 1771–1778.
De Fazio, G., Alba, A.P.C., Vicente, M. and De Clercq, E. (1990) Antiviral activity of *S*-adenosylhomocysteine hydrolase inhibitors against plant viruses. Antiviral Res. 13, 219–226.
Emanuel, D., Cunningham, I., Jules-Elysee, K., Brochstein, J.A., Kernan, N.A., Laver, J., Stover, D., White, D.A., Fels, A., Polsky, B., Castro-Malaspina, H., Peppard, J.R., Bartus, P., Hammerling, U. and O'Reilly, R.J. (1988) Cytomegalovirus pneumonia after bone marrow transplantation successfully treated with the combination of ganciclovir and high-dose intravenous immune globulin. Ann. Intern. Med. 109, 777–782.
Erice, A., Jordan, M., Chace, B., Fletcher, C., Chinnock, B. and Balfour, H. (1987) Ganciclovir treatment of cytomegalovirus disease in transplant patients and other immunocompromised hosts. JAMA 257, 3082–3087.
Erice, A., Chou, S., Biron, K.K., Stanat, S.C., Balfour, H.H., Jr. and Jordan, M.C. (1989) Progressive disease due to ganciclovir-resistant cytomegalovirus in immunocompromised patients. N. Engl. J. Med. 320, 289–293.
Guranowski, A., Montgomery, J.A., Cantoni, G.L. and Chiang, P.K. (1981) Adenosine analogues as substrates and inhibitors of (*S*)-adenosylhomocysteine hydrolase. Biochemistry 20, 110–115.
Hasobe, M., McKee, J.G., Borcherding, D.R. and Borchardt, R.T. (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.
Hasobe, M., McKee, J.G., Borcherding, D.R., Keller, B.T. and Borchardt, R.T. (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.
Hasobe, M., McKee, J.G. and Borchardt, R.T. (1989a) Relationship between intracellular concentration of *S*-adenosylhomocysteine and inhibition of vaccinia virus replication and inhibition of murine L-929 cell growth. Antimicrob. Agents Chemother. 33, 828–834.

Hasobe, M., McKee, J.G., Ishii, H., Cools, M., Bordhardt, R.T. and De Clercq, E. (1989b) Elucidation of the mechanism by which homocysteine potentiates the anti-vaccinia virus effects of the *S*-adenosylhomocysteine hydrolase inhibitor 9-(*trans*-2′,*trans*-3′-dihydroxycyclopent-4′-enyl)-adenine. Mol. Pharmacol. 36, 490–496.

Hayden, F.G., Belshe, R.B., Clover, R.D., Hay, A.J., Oakes, M.G. and Soo, W. (1989) Emergence and apparent transmission of rimantadine-resistant influenza A virus in families. N. Engl. J. Med. 321, 1696.

Ho, M. (Ed) (1991) Virological diagnosis and infections in cells and tissues. In: Cytomegalovirus: Biology and Infection, 2nd Edn. Chapter IV, pp. 57–72. Plenum Medical, New York.

Holy, A., Votruba, I. and De Clercq, E. (1985) Structure-activity studies on open-chain analogues of nucleosides: inhibition of *S*-adenosyl-L-homocysteine hydrolase and antiviral activity. 2. Acid open-chain analogues. Collect. Czech. Chem. Commun. 50, 262–279.

Holy, A., Votruba, I., Merta, A., De Clercq, E., Jelínek, R., Sláma, K., Benes, K. and Melichar, O. (1986) Biological consequences of *S*-adenosyl-L-homocysteinase inhibition by acyclic adenosine analogs. In: R.T. Borchardt, C.R. Creveling and P.M. Ueland (Eds), Biological Methylation and Drug Design, pp. 397–408. Humana Press, Clifton, NJ.

Houston, D.M., Dolence, E.K., Keller, B.T., Patel-Thombre, U. and Borchardt, R.T. (1985) Potential inhibitors of *S*-adenosylmethionine dependent methyltransferases. 8. Molecular dissections of carbocyclic 3-deazaadenosine as inhibitors of *S*-adenosylhomocysteine hydrolase. J. Med. Chem. 28, 471–477.

Jacobson, M.A., Beger, T.G., Fikrig, S., Becherer, P., Moohr, J.W., Stanat, S.C. and Biron, K.K. (1990) Acyclovir-resistant varicella zoster virus infection after chronic oral acyclovir therapy in patients with the acquired immunodeficiency syndrome (AIDS) Ann. Intern. Med. 112, 187–191.

Klintmalm, G., Lonnqvist, B., Öberg, B., Gahrton, G., Lernestedt, J.O., Lundgren, G., Ringden, O., Robert, K.H., Wahren, B. and Groth, C.G. (1985) Intravenous foscarnet for the treatment of severe cytomegalovirus infection in allograft recipients. Scand. J. Infect. Dis. 17, 157–163.

Knox, K., Dobryski, W. and Carrigan, D. (1991) Cytomegalovirus isolate resistant to ganciclovir and foscarnet from a marrow transplant patient. Lancet 337, 1292–1293.

Larder, B.A. and Kemp, S.D. (1989) Multiple mutations in HIV-1 reverse transcriptase confer high-level resistance to zidovudine (AZT). Science 246, 1155–1158.

Larder, B.A., Darby, G. and Richman, D.D. (1989) HIV with reduced sensitivity to zidovudine (AZT) isolated during prolonged therapy. Science 243, 1731–1734.

McCarthy, J.R., Jarvi, E.T., Matthews, D.P., Edwards, J.L., Prakash, N.J., Bowlin, T.L., Mehdi, S., Sunkara, P.S. and Bey, P. (1989) 4,5′-Unsaturated 5′-fluoroadenosine nucleosides: potent mechanism-based inhibitors of *S*-adenosyl-L-homocysteine hydrolase. J. Am. Chem. Soc. 111, 1127–1128.

Mellors, J.W. Dutschman, G.E., Im, G.-J., Tramontano, E., Winkler, S.R. and Cheng, Y.-C. (1992) In vitro selection and molecular characterization of human immunodeficiency virus-1 resistant to non-nucleoside inhibitors of reverse transcriptase. Mol. Pharmacol. 41, 446–451.

Narayanan, S.R., Keller, B.T., Borcherding, D.A., Scholtz, S.A. and Borchardt, R.T. (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.

Neyts, J., Snoeck, R., Schols, D., Balzarini, J. and De Clercq, E. (1990) Selective inhibition of human cytomegalovirus DNA synthesis by (*S*)-1-(3-hydroxy-2-phosphonylmethoxypropyl)cytosine [(*S*)-HPMPC] and 9-(1,3-dihydroxy-2-propoxymethyl)guanine (DHPG). Virology 179, 41–50.

Neyts, J., Balzarini, J., Naesens, K. and De Clercq, E. (1992) Efficacy of (*S*)-1-(3-hydroxy-2-phosphonylmethoxypropyl)cytosine and 9-(1,3-dihydroxy-2-propoxymethyl)guanine for the treatment of murine cytomegalovirus infection in severe combined immunodeficiency mice. J. Med. Virol. 37, 67–71.

Nunberg, J.H., Schleif, W.A., Boots, E.J., O'Brien, J.A., Quintero, J.C., Hoffman, J.M., Emini, E.A. and Goldman, M.E. (1991) Viral resistance to human immunodeficiency virus type 1-specific pyridinone reverse transcriptase inhibitors. J. Virol. 65, 4887–4892.

Patil, S.D., Schneller, S.W., Hosoya, M., Snoeck, R., Andrei, G., Balzarini, J. and De Clercq, E.

(1992) Synthesis and antiviral properties of (±)-5′-noraristeromycin and related purine carbocyclic nucleosides. A new lead for anti-human cytomegalovirus agent design. J. Med. Chem. 35, 3372–3377.

Reed, E.C., Bowden, R.A., Dandliker, P.S., Lilleby, K.E. and Meyers, J.D. (1988) Treatment of cytomegalovirus pneumonia with ganciclovir and intravenous cytomegalovirus immunoglobulin in patients with bone marrow transplants. Ann. Intern. Med. 109, 783–788.

Schmidt, G. (1991) Treatment of CMV infections and disease in transplantation. Transplant. Proc. 23, Suppl. 3, 126–130.

Schols, D., Snoeck, R., Neyts, J. and De Clercq, E. (1989) Detection of immediate early, early and late antigens of human cytomegalovirus by flow cytometry. J Virol. Methods. 26, 247–254.

Shuto, S., Obara, T., Toriya, M., Hosoya, M., Snoeck, R., Andrei, G., Balzarini, J. and De Clercq, E. (1992) New neplanocin analogues 1. Synthesis of 6′-modified neplanocin A derivatives as broad-spectrum antiviral agents. J. Med. Chem. 35, 324–331.

Snoeck, R., Sakuma, T., De Clercq, E., Rosenberg, I. and Holy, A. (1988) (*S*)-1-(3-Hydroxy-2-phosphonylmethoxypropyl)cytosine, a potent and selective inhibitor of human cytomegalovirus replication. Antimicrob. Agents Chemother. 32, 1839–1844.

Snoeck, R., Schols, D., Andrei, G., Neyts, J. and De Clercq, E. (1991) Antiviral activity of anti-cytomegalovirus agents (HPMPC, HPMPA) assessed by a flow cytometric method and DNA hybridization technique. Antiviral Res. 16, 1–9.

Snoeck, R., Andrei, G., Schols, D. and De Clercq, E. (1993) Activity of different antiviral drug combinations against human cytomegalovirus replication in vitro. Eur. J. Clin. Microbiol. Infect. Dis. 11, 1144–1155.

St. Clair, M.H., Martin, J.L., Tudor-Williams, G., Bach, M.C., Vavro, C.L., King, D.M., Kellam, P., Kemp, S.D. and Larder, B.A. (1991) Resistance to ddI and sensitivity to AZT induced by a mutation in HIV-1 reverse transcriptase. Science 253, 1557–1559.

Stanat, S.C., Reardon, J.E., Erice, A., Jordan, M.C., Drew, W.L. and Biron, K.K. (1991) Ganciclovir-resistant cytomegalovirus clinical isolates: mode of resistance to ganciclovir. Antimicrob. Agents Chemother. 35, 2191–2197.

Tyms, A., Taylor, D. and Parkin, J. (1989) Cytomegalovirus and the acquired immunodeficiency syndrome. J. Antimicrob. Chemother. 23, Suppl A, 89–105.

Votruba, I., Hasobe, M., Holy, A. and Borchardt, R.T. (1990) 2-Methylpropyl ester of 3-(adenin-9-yl)-2-hydroxypropanoic acid. Mechanism of antiviral action in vaccinia-infected L929 cells. Biochem. Pharmacol. 39, 1573–1580.

Wolfe, M.S. and Borchardt, R.T. (1991) *S*-Adenosyl-L-homocysteine hydrolase as a target for antiviral chemotherapy. J. Med. Chem. 34, 1521–1530.