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Activity of several S-adenosylhomocysteine hydrolase inhibitors against African swine fever virus replication in Vero cells

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Summary

Several inhibitors of S-adenosylhomocysteine (AdoHcy) hydrolase have been found to selectively suppress the replication of African swine fever virus (ASFV) in Vero cells. Of the compounds tested, 3-deazaneplanocin A proved to be the most potent and selective inhibitor of ASFV replication. Its selectivity index (SI) was 3000. Then followed 9-(*trans*-2′,*trans*-3′-dihydroxycyclopentyl)-3-deazaadenine (SI = 2500), the 4′β-vinyl derivative of 9-(*trans*-2′,*trans*-3′-dihydroxycyclopentyl)adenine (SI = 2000), 6′β-fluoroaristeromycin (SI = 1250), 4′,5′-unsaturated 5′-fluoroadenosine (MDL 28842) and 9-(*trans*-2′,*trans*-3′-dihydroxycyclopentyl)adenine (SI = 667), 9-(*trans*-2′,*trans*-3′-dihydroxycyclopent-4′-enyl)adenine and the 4β-methyl derivative of 9-(*trans*-2′,*trans*-3′-dihydroxycyclopentyl)adenine (SI = 400), 9-(*trans*-2′,*trans*-3′-dihydroxycyclopent-4′-enyl)-3-deazaadenine (SI = 200). We postulate that the mechanism of anti-ASFV action of these compounds is based on the inhibition of AdoHcy hydrolase, thus resulting in the accumulation of AdoHcy and suppression of methylation reactions needed for viral mRNA maturation.

S-Adenosylhomocysteine hydrolase inhibitor; African swine fever virus

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Introduction

African swine fever virus (ASFV) is a large enveloped cytoplasmic DNA virus which produces an important disease of domestic pigs. Since several attempts to develop vaccines for prophylaxis of ASFV infection have proved unsuccessful (Kihm et al., 1987), the search for antiviral compounds able to inhibit virus replication appears as a reasonable alternative approach to combat the illness.

The replication of ASFV is inhibited by 5-iodo-2′-deoxyuridine (Haag et al., 1965; Gil-Fernández et al., 1979), rifampicin (Dardiri et al., 1971), phosphonoacetic acid (Moreno et al., 1978; Gil-Fernández et al., 1979). In swine monocyte cultures ASFV is inhibited by phosphonoacetic acid and phosphonoformic acid (Villinger et al., 1990). Lysosomotropic substances inhibit the production of ASFV in Vero cells (Geraldes and Valdeira, 1985; Alcamí et al., 1989). The fatty acids monoolein, monolinolein and γ-linolenyl alcohol (Sola et al., 1986a), ι carrageenan, suramin (Sola et al., 1986b) and several uridine 5′-diphosphate glucose analogues (Gil-Fernández et al., 1987b) are also inhibitory to ASFV replication. Human interferon alpha (IFN-α) and interferon gamma (IFN-γ) inhibit ASFV replication in Vero cells and act synergistically against the infection. Continuous treatment with IFN-α cures Vero cells from lytic and persistent infections with ASFV (Páez et al., 1990).

Among the nucleoside analogues, (*S*)-9-(3-hydroxy-2-phosphonylmethoxypropyl)adenine (HPMPA) inhibits ASFV replication with a selectivity index of 15 000 (Gil-Fernández and De Clercq, 1987a). This compound has also been shown to inhibit ASFV DNA synthesis (Arzuza et al., 1988). Several phosphonylmethoxyalkylpurine and -pyrimidine derivatives related to HPMPA and 9-(2-phosphonylmethoxyethyl)adenine (PMEA) have also been reported to be selective inhibitors of ASFV (Gil-Fernández et al., 1987b).

These studies have recently been extended to a number of sulfated polysaccharides (García-Villalón and Gil-Fernández, 1991) and several other anionic substances, i.e. aurintricarboxylic acid, Evans blue, fuchsin acid, glycyrrhizic acid and taurolithocholic acid (García-Villalón and Gil-Fernández, 1992). The sulfated polysaccharides as well as the anionic compounds in general are assumed to interfere with the virus adsorption step. Since κ- and λ-carrageenan and the anionic compounds also inhibit virus replication when added after virus adsorption, they may, in addition to their inhibitory effect on virus adsorption, also interfere with other steps in the virus replicative cycle.

Several adenosine analogues have been found to exert their antiviral activity through inhibition of S-adenosylhomocysteine (AdoHcy) hydrolase. This is a key enzyme in transmethylation reactions, using S-adenosylmethionine (AdoMet) as the methyl donor. Viruses with a methylated cap structure, i.e., $m^7G(5')ppp(5')NmpN$, at the 5′-end of their mRNAs are particularly sensitive to inhibition by the adenosine analogues, as this 5′ cap is required for the efficient translation of the viral mRNAs.

Since the mRNAs synthesized by ASFV contain a methylated cap structure

[mainly $m^7G(5')ppp(5')AmpN$ (Salas et al., 1981)], one may expect its synthesis to be inhibited by the adenosine analogues targeted at the AdoHcy hydrolase. The AdoHcy hydrolase inhibitors (*S*)-9-(2,3-dihydroxypropyl)adenine (DHPA), (*RS*)-3-adenin-9-yl-2-hydroxypropanoic acid (AHPA) alkyl esters, carbocyclic 3-deazaadenosine (C-c^3Ado) have been previously shown to inhibit ASFV replication (Gil-Fernández and De Clercq, 1987a). We have now extended these studies to several adenosine analogues which have recently been identified as potent AdoHcy hydrolase inhibitors, namely: neplanocin A (De Clercq, 1985), 3-deazaneplanocin A (Tseng et al., 1989; De Clercq et al., 1989), 9-(*trans*-2′,*trans*-3′-dihydroxycyclopent-4′-enyl)adenine (DHCeA) and 9-(*trans*-2′,*trans*-3′-dihydroxycyclopent-4′-enyl)-3-deazaadenine (c^3DHCeA) (Hasobe et al., 1987, 1988; De Clercq et al., 1989), 9-(*trans*-2′,*trans*-3′-dihydroxycyclopentyl)adenine (DHCaA), 9-(*trans*-2′,*trans*-3′-dihydroxycyclopentyl)-3-deazaadenine (c^3DHCaA), 4′β-vinyl, 4′β-phenyl, 4′α-methyl, 4′β-methyl and 4′β-ethyl derivatives of DHCaA (Wolfe et al., 1991), 6′β-fluoroaristeromycin (F-C-Ado) (Madhavan et al., 1988; Cools et al., 1991) and the 4′,5′-unsaturated 5′-fluoroadenosine MDL 28842 (McCarthy et al., 1989).

Materials and Methods

Cells and viruses

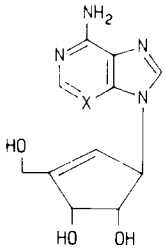
Vero cells (CCL 81) were grown at 37°C in Dulbecco's modified Eagle's medium (DMEM) with 10% newborn calf serum and antibiotics. Maintenance medium contained only 2% newborn calf serum. ASFV (BA71V strain) was adapted to grow in Vero cells, as described previously (Enjuanes et al., 1976).

Compounds

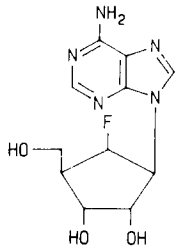
The source of the test compounds was as follows: neplanocin A, T. Saito (Toyo Jozo Co., Tokyo, Japan); 3-deazaneplanocin A, V.E. Marquez (National Cancer Institute, NIH, Bethesda, MD); DHCeA, c^3DHCeA, DHCaA, c^3DHCaA and the 4′-substituted derivatives of DHCaA, R.T. Borchardt (Departments of Medicinal Chemistry and Biochemistry, University of Kansas, Lawrence, KS); 6′β-fluoroaristeromycin, E.J. Prisbe (Syntex Research, Palo Alto, CA); and MDL 28842, J.R. McCarthy (Merrell Dow Research Institute, Cincinnati, OH). The formulae of the test compounds are presented in Fig. 1.

Determination of virus-inhibitory compound concentrations

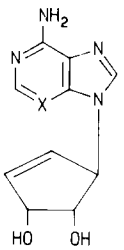
The procedure for measuring anti-ASFV activity in Vero cells has been described previously (Gil-Fernández and De Clercq, 1987a). Briefly, monolayers of Vero cells, growing in 24-well plates were infected with ASFV at a multiplicity of infection (moi) of 0.5 pfu/cell. After virus adsorption, medium



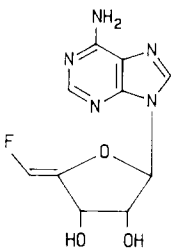
Neplanocin A: X=N
3-Deazaneplanocin A: X=CH



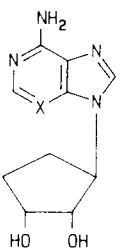
6'β-Fluoroaristeromycin



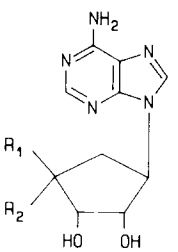
DHCeA: X=N
c^{3}DHCeA: X=CH



MDL 28842



DHCaA: X=N
c^{3}DHCaA: X=CH



	R_1	R_2
4'α-methyl-DHCaA	H	CH_3
4'β-methyl-DHCaA	CH_3	H
4'β-ethyl-DHCaA	C_2H_5	H
4'β-vinyl-DHCaA	$CH{=}CH_2$	H
4'β-phenyl-DHCaA	C_6H_5	H

Fig. 1. Formulae of test compounds.

with compounds at various concentrations was added. When the virus controls without drug showed complete destruction, the cells were removed from the wells and total virus yield was determined by plaque formation.

Having determined virus inhibition by the different compounds, further experiments were carried out to determine the EC_{50}, defined as the concentration of the test compound required to reduce plaque formation by 50%. To this end, the effect of different drug concentrations, measured by the

reduction in plaque formation, was studied as follows: monolayers of cells were infected with ASFV at 50 to 10 pfu per well and after an adsorption period of 1.5 h the monolayers were washed with phosphate-buffered saline to remove unadsorbed virus and then they were covered with 1.8% agar and doubly concentrated Dulbecco's medium containing different concentrations of the test compounds. Four wells were used for each compound concentration. After 7 days the overlayer was removed, the cell monolayer was stained with 1% crystal violet diluted in ethanol and the number of pfu was counted. Every single experiment was repeated at least three times.

Estimation of the cytopathic effect and cytotoxicity

Cells were seeded in 96-well trays (10^4 cells/well). When the cell monolayers were confluent, wells were inoculated with the virus at an moi equal to 1 pfu/cell. After virus adsorption, medium with different dilutions of the compounds was added. Between the rows of infected cells, rows of uninfected cells with the same final concentration of the inhibitors were included to assess the cytotoxicity of the compounds. When the virus controls without drug were completely destroyed, cytotoxicity and cytopathic effect were evaluated by (a) microscopic evaluation of cell morphology, (b) staining of the cells with 1% crystal violet in ethanol as described by Gil-Fernández and De Clercq (1987a), and (c) inhibition of protein synthesis as described by García-Villalón and Gil-Fernández (1991). We define the minimum cytotoxic concentration (CC_{50}) as the concentration of drug required to produce 50% cytotoxicity, as assessed colorimetrically (dye uptake method). The selectivity index (SI) was defined as the ratio CC_{50}/EC_{50}.

TABLE 1

Comparative potency, cytotoxicity and selectivity of several *S*-adenosylhomocysteine hydrolase inhibitors against ASFV replication in vitro

Compound	CC_{50}[a] (μg/ml)	EC_{50}[b] (μg/ml)	Selectivity index
6′β-Fluoroaristeromycin	10	0.008	1,250
Neplanocin A	10	0.15	66
3-Deazaneplanocin A	30	0.01	3,000
MDL 28842	20	0.03	667
DHCeA	200	0.5	400
c^3DHCeA	200	1	200
DHCaA	200	0.3	667
c^3DHCaA	200	0.08	2,500
4′β-vinyl-DHCaA	200	0.1	2,000
4′β-phenyl-DHCaA	150	10	15
4′α-methyl-DHCaA	200	10	20
4′β-methyl-DHCaA	200	0.5	400
4′β-ethyl-DHCaA	200	5	40

[a]Cytotoxic concentration affecting 50% of the cells, as assessed colorimetrically (dye uptake).
[b]Antivirally effective concentration required to effect a 50% reduction in plaque formation.

Results

We have evaluated a series of AdoHcy hydrolase inhibitors for their activity against the replication of ASFV in Vero cells. For each compound were determined the antivirally effective concentration to effect a 50% reduction in plaque formation (EC_{50}) and the cytotoxic concentration affecting 50% of the cells (CC_{50}). In general all the compounds are inhibitors of ASFV replication at very low concentrations. The most potent inhibitor proved to be 6′β-fluoroaristeromycin, with an EC_{50} of 0.008 μg/ml. This is followed by 3-deazaneplanocin A (EC_{50}: 0.01 μg/ml), MDL 28842 (EC_{50}: 0.03 μg/ml), c^3DHCaA (EC_{50}: 0.08 μg/ml), 4′β-vinyl DHCaA (EC_{50}: 0.1 μg/ml), neplanocin

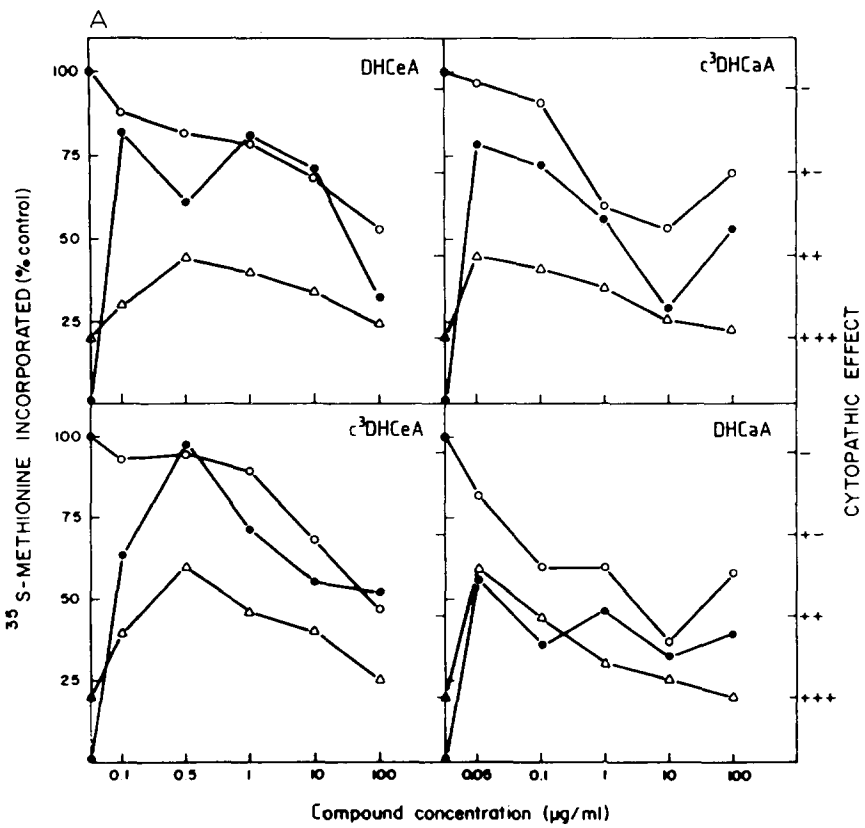


Fig. 2. Protein synthesis in infected and uninfected cells and cytopathic effect (CPE) of ASFV in Vero cells (MOI: 1). CPE was recorded at 48 h post infection (△). −, +−, + + and + + + represent CPE on a linear scale; (−) no CPE; (+ + +) maximum CPE. Protein synthesis was measured for ASFV-infected cells (●) and uninfected control cells (○) in the presence of S-adenosylhomocysteine hydrolase inhibitors. [^{35}S]methionine incorporation in infected and uninfected cells is expressed in percent of uninfected untreated cells. Note that scale on the *X*-axis is arbitrarily adjusted so as to avoid excessive expansion of the figure that would result from a linear scale. Numerical values for the compound concentration are explicitly indicated on the *X*-axis. This holds for Fig. 3 as well.

A (EC_{50}: 0.15 μg/ml), DHCaA (EC_{50}: 0.3 μg/ml), and DHCeA and 4′β-methyl-DHCaA (both with EC_{50}: 0.5 μg/ml). For the other compounds the EC_{50} ranged between 1 and 10 μg/ml. The cytotoxic concentration, required to reduce dye uptake by 50%, was 10 μg/ml for 6′β-fluoroaristeromycin and neplanocin A, 20 μg/ml for MDL 28842, 30 μg/ml for 3-deazaneplanocin A and >100 μg/ml for the other compounds (Table 1).

Fig. 2A, B and C show the results of the experiments carried out to determine the cytotoxicity by means of the [^{35}S]methionine incorporation in uninfected cells treated with the different drug concentrations. In parallel, inhibition of virus replication in infected and drug-treated cells was assessed. This inhibition was based upon the increase of [^{35}S]methionine incorporation. This incorporation is equal to that observed for uninfected untreated cells, or that achieved when the compound totally inhibits virus replication and moreover has no cytotoxicity. At the same time, cytopathicity determined by microscopic examination was recorded. [^{35}S]methionine incorporation is

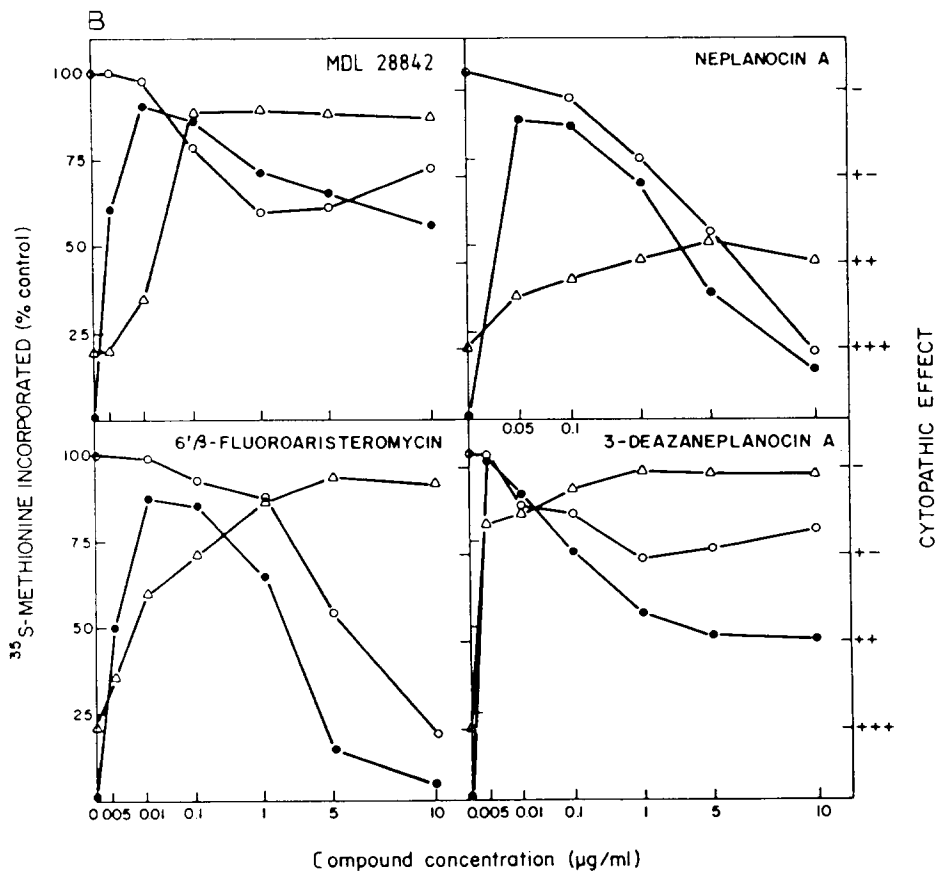


Fig. 2. Continued.

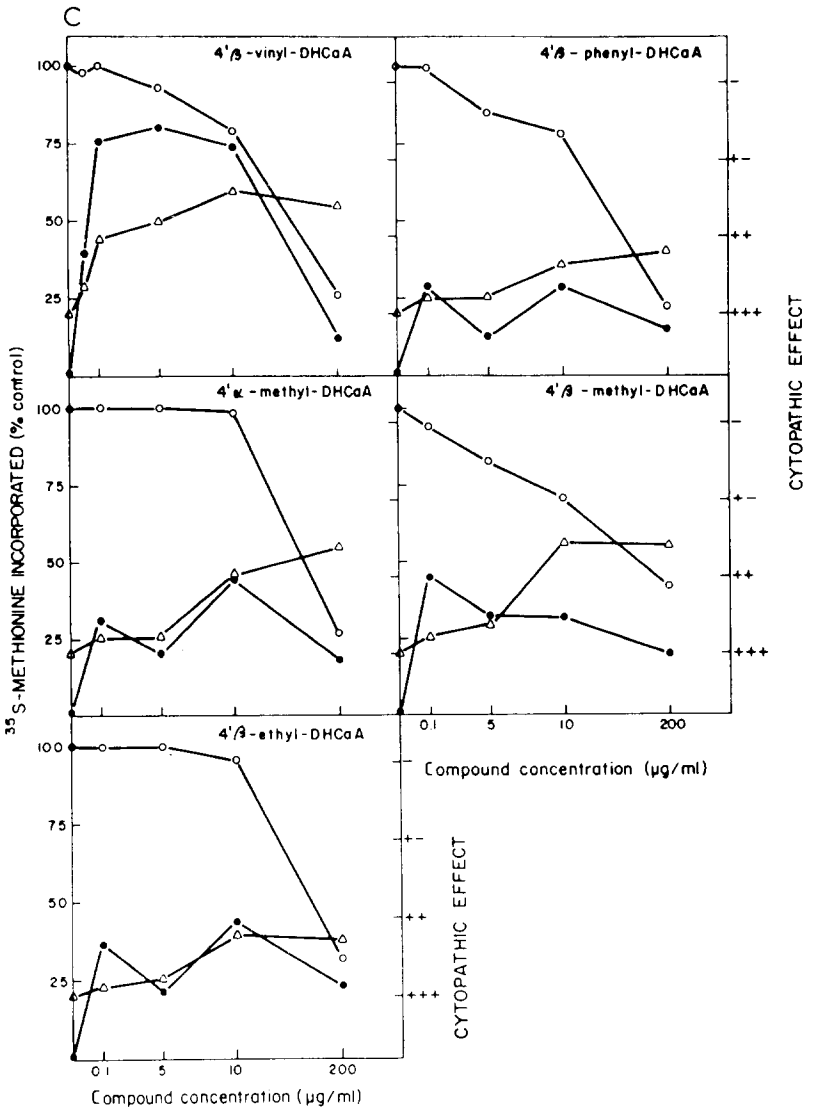


Fig. 2. Continued.

indicated at percent relative to the uninfected untreated cells.

From the data on the incorporation of [^{35}S]methionine in uninfected cells, 4′α-methyl-DHCaA and 4′β-ethyl-DHCaA appeared to be the least cytotoxic, whereas 6′β-fluoroaristeromycin and neplanocin A were the most cytotoxic. If the drug inhibits virus replication, incorporation of [^{35}S]methionine in infected cells is close to that of the uninfected controls. When there is no inhibition, then the cell monolayers are destroyed and [^{35}S]methionine incorporation is

negligible. All the drugs tested inhibited virus replication at low concentrations, as demonstrated by [^{35}S]methionine incorporation. [^{35}S]methionine incorporation was 100% at a concentration of 0.005 μg/ml for 3-deazaneplanocin A and 95% at a concentration of 0.5 μg/ml for c^{3}DHCeA. For compounds MDL, neplanocin A, DHCeA and c^{3}DHCaA incorporation of [^{35}S]methionine was $>75\%$ at drug concentrations ranging from 0.005 to 0.5 μg/ml. When the drug became cytotoxic, incorporation of [^{35}S]methionine decreased in parallel in

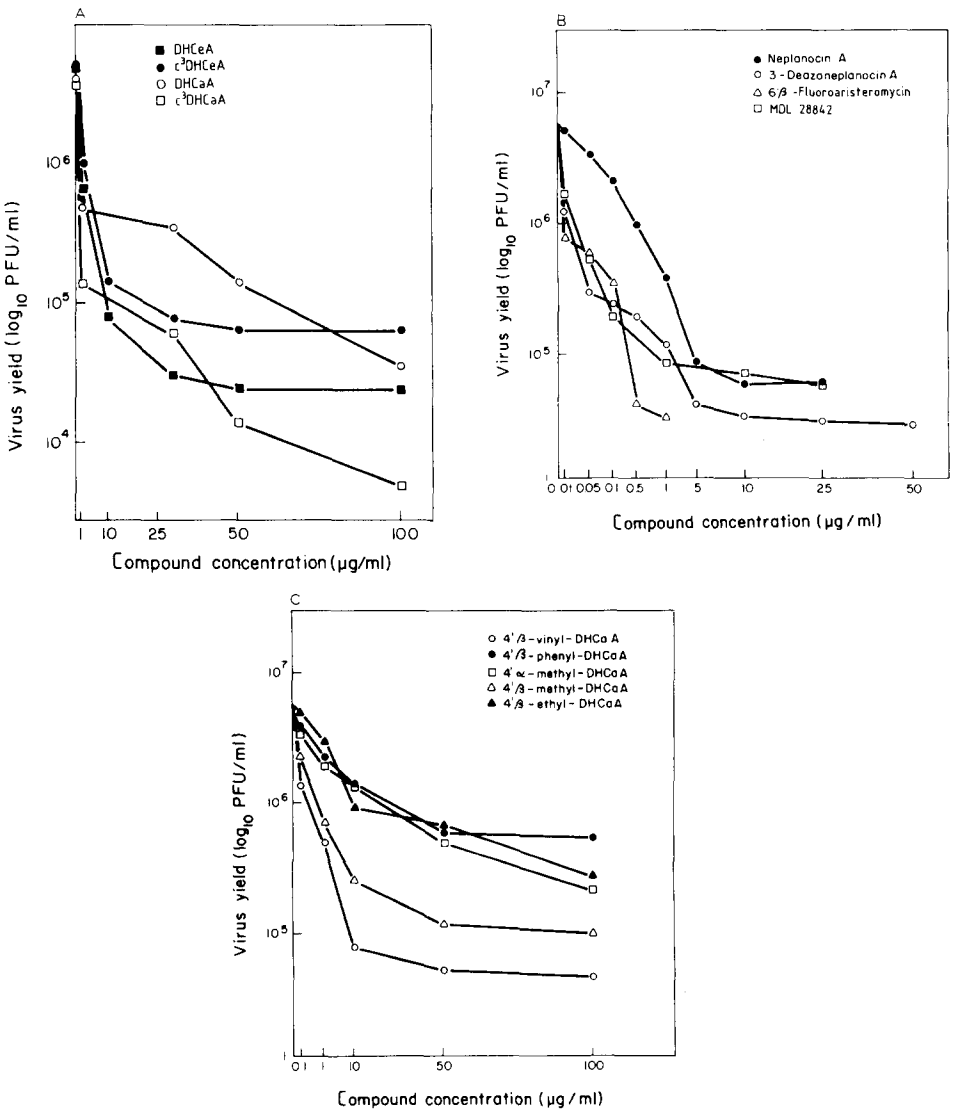


Fig. 3. Inhibitory effects of *S*-adenosylhomocysteine hydrolase inhibitors on the replication of ASFV in Vero cell cultures. The compounds were added immediately after virus adsorption (MOI: 0.5). Virus yield was measured three days after infection by plaque formation in Vero cells.

both infected and uninfected cell cultures. This could be clearly seen for neplanocin A and 6′β-fluoroaristeromycin, which were in fact the two most cytotoxic compounds, inhibiting [^{35}S]methionine incorporation in uninfected cells at a concentration of 5 μg/ml. Viral cytopathicity was most efficiently blocked by deazaneplanocin A, 6′β-fluoroaristeromycin and MDL 28842.

Fig. 3A, B and C shows the concentration–response curves for the compounds when evaluated for their inhibitory effect on virus yield. The slopes of the curves are very steep at concentrations below 1 μg/ml, showing at this low concentration range a decrease in virus yield of 2 logs. At 1 to 10 μg/ml the curves levelled of for all compounds except for c^{3}DHCaA for which a linear correlation was noted up to a concentration of 100 μg/ml.

As the most selective inhibitor of ASFV replication emerged 3-deazaneplanocin A with a selectivity index (SI) of 3000, followed by c^{3}DHCaA (SI = 2500); 4′β-vinyl-DHCaA (SI = 2000); 6′β-fluoroaristeromycin (SI = 1250); MDL 28842 and DHCaA (SI = 667); DHCeA, 4′β-methyl-DHCaA (SI = 400); c^{3}DHCeA (SI = 200); and the other compounds (including neplanocin A) with a SI < 100.

Discussion

All the compounds which were studied for their activity against ASFV replication are inhibitors of *S*-adenosylhomocysteine hydrolase. Hydrolysis of AdoHcy by this enzyme is a reversible reaction. When AdoHcy hydrolase is inhibited, AdoHcy accumulates and this leads to a shut-off of the methylation utilizing AdoMet as methyl donor. These methylations are required for the 5′-cap formation of viral mRNAs.

Of the adenosine analogues that are targeted at AdoHcy hydrolase we found previously DHPA, AHPA and C-c^{3}Ado to be selective inhibitors of ASFV replication (Gil-Fernández and De Clercq, 1987a). Of the new compounds that were studied against ASFV, neplanocin A has been shown previously to be a potent inhibitor of vaccinia virus replication in L-cell cultures (Borchardt et al., 1984; Cools and De Clercq, 1989). Neplanocin A also is a potent inhibitor of ASFV (EC_{50}: 0.15 μg/ml), but it is rather cytotoxic and thus its selectivity index is not higher than 66. To minimize the toxicity, the 9-(*trans*-2′, *trans*-3′-dihydroxycyclopent-4′-enyl) derivatives of adenine (DHCeA) and 3-deazaadenine (c^{3}DHCeA), which lack the 4′-hydroxymethyl group, were synthesized (Borcherding et al., 1987). They were found to exhibit selective antiviral activity in vaccinia virus-infected L cells (Hasobe et al., 1987). As compared to neplanocin A, DHCeA also is a more selective inhibitor of ASFV replication (SI = 400). As the most selective ASFV inhibitor, however, emerged 3-deazaneplanocin A (SI = 3000), followed by c^{3}DHCaA, 4′β-vinyl DHCaA, 6′β-fluoroaristeromycin, DHCaA and MDL 28842 (Table 1). All these compounds have been previously shown to exhibit an antiviral activity spectrum that is characteristic of AdoHcy hydrolase inhibitors (De Clercq,

1987, 1991; De Clercq et al., 1989; Cools et al., 1991; and De Clercq and Borchardt, unpublished observations).

A precise comparison of the results obtained here with those obtained by other authors for the potency of the adenosine analogues as inhibitors of AdoHcy hydrolase is difficult, due to the diversity of sources from which the enzyme was obtained and the assay conditions used to assess its activity. For example, Borchardt et al. (1984) reported for neplanocin A a K_i of 8.39 nM for the beef liver enzyme, whereas for the same compound assayed against the same enzyme a K_i of 2 nM was found by De Clercq and Cools (1985).

When the different adenosine analogues were evaluated in parallel against AdoHcy hydrolase from murine L929 cells the most potent AdoHcy hydrolase inhibitor was neplanocin A (K_i: 2.6 nM), followed 3-deazaneplanocin A (K_i: 22 nM), C-c^{3}Ado (K_i: 18 nM), (*RS*)-AHPA isobutyl ester (K_i: 52 nM) and (*S*)-DHPA (K_i: 280 nM) (Cools and De Clercq, 1985). Cools et al. (1991), using purified AdoHcy hydrolase from the murine L929 cells, found a K_i of 3.1 nM for 6′β-fluoroaristeromycin.

For neplanocin A, 3-deazaneplanocin A, 6′β-fluoroaristeromycin and the previously studied C-c^{3}Ado, (*S*)-DHPA and (*RS*)-AHPA isobutyl ester (Gil-Fernández and De Clercq, 1987) there is a close correlation between the inhibition of the enzyme and the inhibition of virus replication.

(*S*)-DHPA and (*RS*)-AHPA are the least potent inhibitors of ASFV replication and of AdoHcy hydrolase (EC_{50}: 8 μg/ml and 10 μg/ml, respectively). 6′β-fluoroaristeromycin, which was found by Cools et al. (1991) to be a highly potent inhibitor of purified AdoHcy hydrolase, is the most potent inhibitor of ASFV replication (EC_{50}: 0.008 μg/ml). 3-Deazaneplanocin A is a more potent inhibitor of ASFV replication than neplanocin A (EC_{50}: 0.01 μg/ml and 0.15 μg/ml, respectively). 3-Deazaneplanocin A was found to be a highly potent inhibitor of hamster liver AdoHcy hydrolase with a K_i value of 0.05 nM (Glazer et al., 1986).

C-c^{3}Ado also is a potent inhibitor of ASFV (EC_{50}: 0.025 μg/ml). Different K_i values of purified AdoHcy hydrolase have been reported for C-c^{3}Ado by various authors depending on the origin of the enzyme: 1 nM for the enzyme from hamster liver (Montgomery et al., 1982), 4 nM and 13 nM for the enzyme from bovine liver (Houston et al., 1985; De Clercq and Cools, 1985) and 18 nM for the enzyme from murine L929 cells (Cools and De Clercq, 1988).

From the apparent correlation between the inhibitory effect of these compounds on the replication of ASFV and their inhibitory action on AdoHcy hydrolase, we postulate that the inhibition of AdoHcy hydrolase by the compounds accounts for their antiviral activity.

As has been proposed for the activity of the AdoHcy hydrolase inhibitors against vaccinia virus (Borchardt et al., 1984; De Clercq, 1985), reovirus, vesicular stomatitis virus and some other viruses (reviewed by De Clercq, 1987), their activity against ASFV could also be attributed to inhibition of the methyltransferases involved in the 5′-capping of viral mRNA. ASFV infection leads to the synthesis of four classes of capped and polyadenylated RNAs

(Salas et al., 1981).

Although this finding implies that ASFV may encode for specific enzymes catalyzing these reactions, no purification of virus-specific methyltransferases (i.e. guanine 7-methyltransferase and 2'-O-nucleoside methyltransferase), as in the case of vaccinia virus (Martin et al., 1975), has not yet been described for ASFV.

Together with the previously described AdoHcy hydrolase inhibitors (i.e. C-c^3Ado) (Gil-Fernández and De Clercq, 1987a), some of the compounds described here (i.e. 3-deazaneplanocin A) may be considered as potential candidates for the treatment of African swine fever infection in vivo.

Acknowledgements

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