Antiviral Research, 7 (1987) 151–160
Elsevier

AVR 00228

Comparative efficacy of broad-spectrum antiviral agents as inhibitors of African swine fever virus replication in vitro

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(Received 5 August 1986; accepted 26 September 1986)

Summary

Various nucleoside analogues, selected on the basis of their previously established broad-spectrum antiviral properties, were evaluated for their potency and selectivity as inhibitors of the in vitro replication of the iridovirus, African swine fever virus (ASFV). The test compounds included (*S*)-9-(3-hydroxy-2-phosphonylmethoxypropyl)adenine [(*S*)-HPMPA], 9-(2-phosphonylmethoxyethyl)adenine, (*RS*)-3-adenin-9-yl-2-hydroxypropanoic acid isobutyl ester, (*S*)-9-(2,3-dihydroxypropyl)adenine, carbocyclic 3-deazaadenosine (C-c^{3}Ado), 3′-azido-2′,3′-dideoxythymidine, pyrazofurin and ribavirin. As the most efficacious inhibitors of ASFV replication emerged (*S*)-HPMPA followed by C-c^{3}Ado. The minimum inhibitory concentration of (*S*)-HPMPA for ASFV replication was 0.01 μg/ml, and its selectivity index was 15000. The corresponding values for C-c^{3}Ado were 0.025 μg/ml and 8000, respectively. It would seem justified to further pursue these compounds for their anti ASFV activity in vivo.

African swine fever virus; Adenosine analogues; (*S*)-HPMPA; PMEA; (*RS*)-AHPA; (*S*)-DHPA; C-c^{3}Ado; Azidothymidine; Pyrazofurin; Ribavirin

Introduction

African swine fever virus (ASFV) was first described by Montgomery in 1921 in Kenya as a disease which caused the death of swines within 48 h after infection. It appeared in 1957 in Portugal and spread through Spain to other European countries. More recent outbursts in Cuba, Haiti, Dominican Republic and Brazil have

led to considerable economic losses. In the Iberian peninsula the disease has become endemic, while the virus has undergone mutations to less virulent forms. Many swine are asymptomatic carriers, thus representing efficient means for transmitting the virus. The virus is borne by a tick of swine, *Ornithodorus erraticus* (Sanchez Botija, 1963), where it replicates and is transmitted sexually and transovarially (Plowright et al., 1970, 1974; Plowright, 1977). This property makes ASFV the only DNA virus capable of replicating in the arthropod vector.

Based on its icosahedral morphology, ASFV has been classified as a member of the *Iridoviridae* (Matthews, 1982), although it does have similarities with the *Poxviridae*. Thus, the enzymatic composition of ASFV is rather complex; associated with the virions are, among other enzymes, a specific viral DNA polymerase (Polatnick & Hess, 1972), a DNA topoisomerase (Salas *et al.*, 1983), two nucleoside triphosphate phosphohydrolases (Kuznar et al., 1981), a protein kinase (Polatnick et al., 1974) and a DNA-dependent RNA polymerase (Kuznar et al., 1980) which synthesizes in vitro at least four classes of polyadenylated, methylated and 5′-capped RNAs (Salas et al., 1981). Viral DNA synthesis and virion assembly take place in the cell nucleus and cytoplasm, respectively (Tabares and Sanchez Botija, 1979). The ASFV genome is cross-linked (Ortin et al., 1979), as is the poxvirus genome. These similarities suggest that ASFV, at least during the early phase of its replicative cycle, follows a strategy similar to that of the poxviruses.

Although the existence of some types of antibodies have been reported, there is no evidence that ASFV elicits neutralizing antibodies (Hess, 1971). This compounds the prospects for vaccine development and so does the genetic variability of the virus. Therefore, chemotherapeutic means should be envisaged to control ASFV infections. Preliminary studies have indicated that ASFV replication in vitro 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) and chloroquine (Geraldes and Valdeira, 1985). More recently, it has been shown that ASFV is also inhibited by megalomycin C, atropine, carrageenan and suramine, the latter being the most specific inhibitor of the four (Sola et al., 1986a). The virucidal and virustatic effects of monoolein, monolinolein and γ-linolenyl alcohol on ASFV have also been studied (Sola et al., 1986b).

These investigations have now been extended to a series of nucleoside analogues, i.e. (*S*)-9-(3-hydroxy-2-phosphonylmethoxypropyl)adenine [(*S*)-HPMPA], 9-(2-phosphonylmethoxyethyl)adenine (PMEA), (*RS*)-3-adenin-9-yl-2-hydroxypropanoic acid [(*RS*)-AHPA] isobutyl ester, (*S*)-9-(2,3-dihydroxypropyl)adenine [(*S*)-DHPA], carbocyclic 3-deazaadenosine (C-c^{3}Ado), 3′-azido-2′,3′-dideoxythymidine (azidothymidine), pyrazofurin [3-(β-D-ribofuranosyl)-4-hydroxypyrazole-5-carboxamide] and ribavirin [1-(β-D-ribofuranosyl)-1,2,4-triazole-3-carboxamide]. The compounds were selected for the following reasons: (*S*)-HPMPA and PMEA because of their selective broad-spectrum anti-DNA virus activity encompassing all DNA viruses, i.e. herpes simplex, varicella-zoster, cytomegalo, adeno, vaccinia, which have so far been looked at (De Clercq et al., 1986); (*RS*)-AHPA isobutyl ester (De Clercq and Holý, 1985), (*S*)-DHPA (De Clercq et al., 1978) and

C-c^{3}Ado (De Clercq and Montgomery, 1983) for their broad-spectrum antiviral activity including (–)RNA viruses (i.e. vesicular stomatitis virus), (±)RNA viruses (i.e. rotavirus) as well as DNA viruses (i.e. poxviruses) (De Clercq et al., 1978; De Clercq and Montgomery, 1983; De Clercq and Cools, 1985; De Clercq and Holý, 1985; Kitaoka et al., 1986); azidothymidine (AZT) (Mitsuya et al., 1985), which is specifically active against human T-lymphotropic virus type III/lymphadenopathy-associated virus (HTLV-III/LAV), the virus that is etiologically linked to AIDS (acquired immune deficiency syndrome) but may also share some common properties with ASFV (Beldekas et al., 1986); and pyrazofurin (Descamps and De Clercq, 1978) and ribavirin (Sidwell et al., 1972), which have been found effective against a wide variety of RNA and DNA viruses and of which the latter is currently being evaluated for clinical use in the treatment of respiratory tract (i.e., respiratory syncytial) virus and hemorrhagic (i.e. Lassa) fever virus infections.

Materials and Methods

Virus

ASFV, adapted to grow in Vero cells, was kindly provided by E. Viñuela, Centro de Biologia Molecular (Enjuanes et al., 1976a,b). The virus was further propagated in Vero cells, and the stock used in the present study was that obtained after the 20th passage.

Cells

Vero cells (green monkey kidney cells) were grown in Dulbecco's modified Eagle's minimal essential medium supplemented with 10% newborn calf serum (growth medium). Maintenance medium contained only 2% newborn calf serum.

Compounds

(*S*)-HPMPA, PMEA, (*RS*)-AHPA isobutyl ester and (*S*)-DHPA were kindly provided by A. Holý (Institute of Organic Chemistry and Biochemistry, Czechoslovakian Academy of Sciences, Prague, Czechoslovakia). C-c^{3}Ado was kindly provided by J.A. Montgomery (Kettering-Meyer Laboratory, Southern Research Institute, Birmingham, AL, U.S.A.). Azidothymidine was synthesized by P. Herdewijn (Rega Institute for Medical Research, Katholieke Universiteit Leuven, Leuven, Belgium). Pyrazofurin was purchased from Calbiochem Behring Corporation (Lucerne, Switzerland) and ribavirin was obtained from ICN Nutritional Biochemicals (Cleveland, OH, U.S.A.).

Inhibition of ASFV replication

Cells were seeded in 24-well plates (10^5 cells/well) in growth medium containing 3.7% sodium bicarbonate, and incubated at 37°C in 5 % CO_2 and 95% humidity. When confluent, the cell monolayers were infected with ASFV at a multiplicity of 0.5 PFU per cell. After a 1.5 h adsorption period, unadsorbed virus was removed,

and the monolayers were washed with phosphate-buffered saline. Then, culture medium containing different concentrations of the test compounds was added (4 wells per compound concentration). The cell cultures were incubated at 37°C. When the control cultures (inoculated with virus but not exposed to any of the compounds) showed complete destruction (usually three days after virus inoculation), the cells were removed from the wells with a rubber policeman and sonicated. Cellular debris was then removed by centrifugation and the virus content in the supernatant was determined by plaque formation in Vero cells. To this end, the cells were exposed to serial dilutions of the supernatants (4 wells per dilution) and overlaid with agar. Virus plaques were counted on days 7 or 8 after inoculation, after removing the agar coat and staining the cell monolayers with 1% crystal violet in alcohol. The resulting virus titers are expressed in plaque forming units (PFU) per ml. All assays were repeated at least three times.

Cytotoxicity

Cells (in growth medium) were seeded in 96-well plates (10^5 cells/well). When the cell monolayers were almost confluent, growth medium was replaced by maintenance medium containing different concentrations of the test compounds. Three days later, cytotoxicity was evaluated by two different procedures: by (*i*) microscopic observation of cell morphology, and (*ii*) staining of the cells with 1% crystal violet in ethanol. This dye uptake method was essentially similar to that used by Finter (1969) for measuring viral cytopathogenicity in virus-infected cells treated with interferon, with the only difference that in the present investigations crystal violet was used instead of neutral red. The former has the advantage that it stains the cells in 2–3 min with an intense violet color displaying a wide variety of intensities depending on the health and number of the cells. Following the 2–3 min staining period, excess dye was removed by washing the cell monolayer 3 times with saline. The plates were then let to dry upside down and the amount of dye bound was determined directly by inspection of the cell sheets against a white background. Depending on the color intensity, a numerical index from 0 to 4 was established, whereby 2 corresponds to 50% cell destruction. Each experiment was repeated 3 times, using two wells for each drug concentration.

Results

Cytotoxicity measurements were based on the dye (crystal violet) uptake method (see Materials and Methods) and carried out after the Vero cells had been exposed to the test compounds for 3 days. This time interval was chosen because virus yield reductions were also measured after a 3-day incubation period. The minimum toxic concentration (MTC_{50}) expressed as the concentration required to reduce cellular uptake of the dye by 50%, was well above 100 μg/ml for all test compounds: it varied from 150 μg/ml for (*S*)-HPMPA to 400 μg/ml for (*S*)-DHPA (Table 1).

All compounds were evaluated at several concentrations for their inhibitory ef-

TABLE 1

Comparative potency, cytotoxicity and selectivity of antiviral compounds as inhibitors of ASFV in vitro

Compound	MTC_{50}[a] (μg/ml)	MIC_{50}[b] (μg/ml)	Selectivity index[c]
(*S*)-HPMPA	150	0.01	15 000
C-c^{3}Ado	200	0.025	8 000
Pyrazofurin	200	1	200
PMEA	200	5	40
(*RS*)-AHPA isobutyl ester	200	8	25
(*S*)-DHPA	400	10	40
Ribavirin	300	25	25
Azidothymidine	200	25	8

[a] Minimum toxic concentration, affecting 50% of the cells, as assessed colorimetrically by the dye uptake method.

[b] Minimum inhibitory concentration, required to effect a 50% reduction in virus yield.

[c] Ratio of MTC_{50} to MIC_{50}.

fects on ASFV replication. The dose–response curves for the eight compounds are presented in Figs. 1–8. Virus yield measurements, which were, as mentioned above, usually done at 3 days after virus infection, pointed to (*S*)-HPMPA as the most potent inhibitor. At 50 μg/ml (*S*)-HPMPA completely suppressed ASFV replication (Fig. 1). Its minimum inhibitory concentration (MIC_{50}) required to effect a 50% reduction in virus yield was 0.01 μg/ml.

The second most potent anti-ASFV agent was C-c^{3}Ado, which effected a 3.5 $\log_{10}$

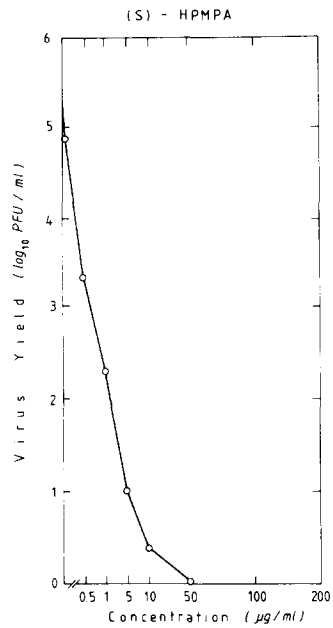


Fig. 1.

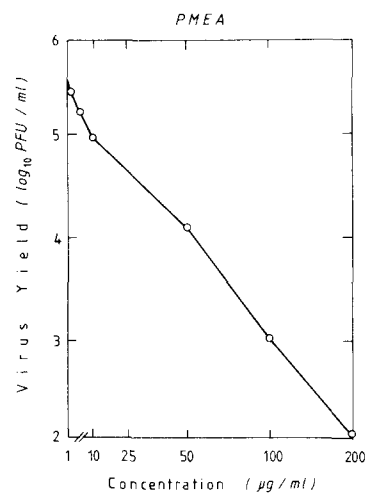
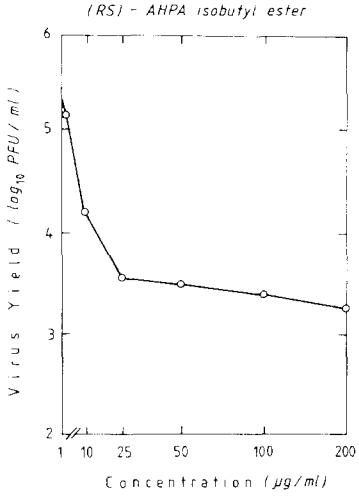


Fig. 2.

For figure legends see p. 157.

Fig. 3.



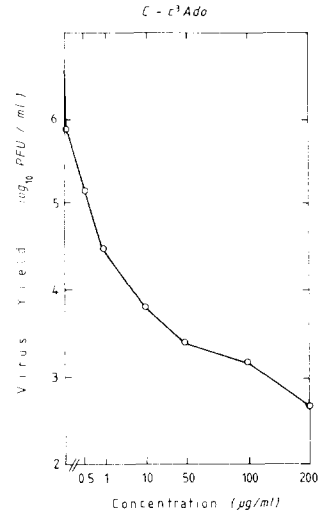
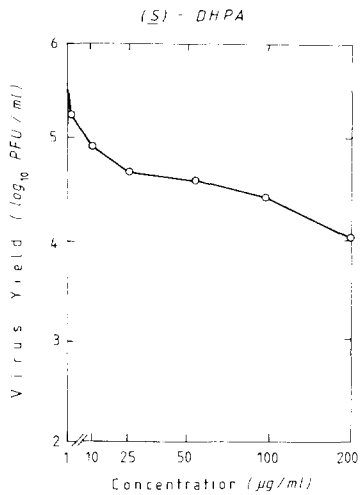


Fig. 5.

Fig. 4.



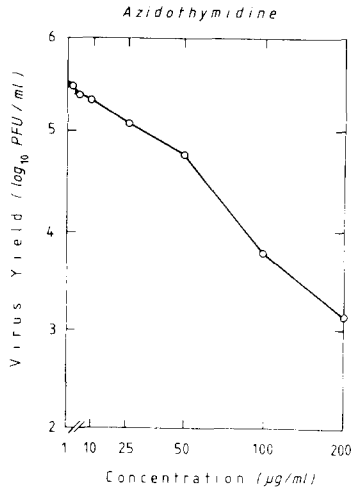


Fig. 6.

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reduction in virus yield at a concentration of 200 µg/ml (Fig. 5). The MIC_{50} of C-c^3Ado was 0.025 µg/ml. Within the concentration range of 10–200 µg/ml, pyrazofurin caused a similar reduction in virus yield as did C-c^3Ado (Fig. 7). However, the MIC_{50} of pyrazofurin (1 µg/ml) was considerably higher than that of C-c^3Ado.

Next in potency was PMEA with an MIC_{50} of 5 µg/ml. At 200 µg/ml PMEA effected a similar (3.5 $\log_{10}$) reduction in ASFV yield as did C-c^3Ado and pyrazofurin, but at lower concentrations (5–100 µg/ml) it was definitely less effective (Fig. 2). PMEA (Fig. 2), like (*S*)-HPMPA (Fig. 1), exhibited a linear dose– response relationship within the concentration ranges at which they were evaluated.

Fig. 7.

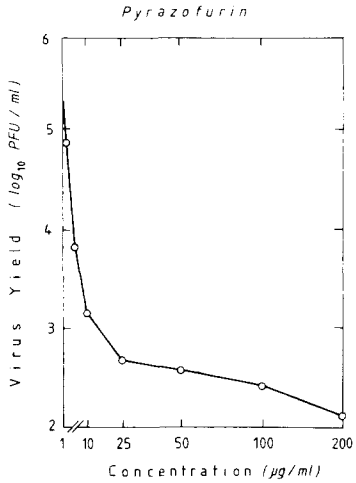
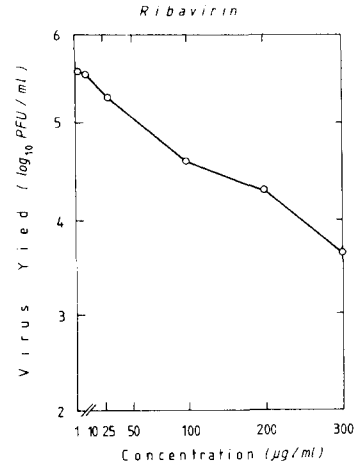


Fig. 8.



Figs. 1–8.
Inhibitory effects of antiviral compounds on the replication of ASFV in Vero cell cultures. The compounds were added following virus adsorption (multiplicity of infection: 0.5). Virus yield was measured 3 days after infection by plaque formation in Vero cells (see Materials and Methods). Antiviral compounds: (*S*)-HPMPA (Fig. 1), PMEA (Fig. 2), (*RS*)-AHPA isobutyl ester (Fig. 3), (*S*)-DHPA (Fig. 4), C-c^{3}Ado (Fig. 5), azidothymidine (Fig. 6), pyrazofurin (Fig. 7), and ribavirin (Fig. 8).

(*RS*)-AHPA isobutyl ester (Fig. 3) and (*S*)-DHPA (Fig. 4) were about equally active in terms of MIC_{50} (8 and 10 µg/ml, respectively), but at higher concentrations (25–200 µg/ml) (*RS*)-AHPA isobutyl ester was clearly more effective than (*S*)-DHPA. For both compounds, and in particular (*RS*)-AHPA isobutyl ester, the antiviral activity seemed to level off from a concentration of 25 µg/ml.

With a MIC_{50} of 25 µg/ml, azidothymidine and ribavirin could be considered as the least potent anti-ASFV agents. However, in contrast with (*S*)-DHPA and (*RS*)-AHPA isobutyl ester, azidothymidine (Fig. 6) and ribavirin (Fig. 8) showed a quasi linear dose-dependence in that their antiviral potency increased proportionally with increasing concentrations.

From the selectivity indexes, calculated as the ratios of MTC_{50} to MIC_{50} (Table 1), (*S*)-HPMPA could be considered as the most specific inhibitor of ASFV, followed, in order of decreasing specificity, by C-c^{3}Ado > pyrazofurin > PMEA = (*S*)-DHPA > (*RS*)-AHPA isobutyl ester > ribavirin > azidothymidine.

Discussion

From the present series of compounds which were evaluated for their inhibitory effects on ASFV replication, (*S*)-HPMPA exhibited the highest potency (MIC_{50}: 0.01 µg/ml) and greatest specificity (selectivity index: 15000), followed by C-c^{3}Ado

as the second most potent and selective anti-ASFV agent (MIC_{50}: 0.025 μg/ml, selectivity index: 8000). The potency and selectivity of these compounds was considerably higher than those reported previously for suramin (MIC_{50}: 25μg/ml; selectivity index: 18), megalomycin C, atropine and carrageenan (Sola et al., 1986a).

(*S*)-HPMPA and C-c^{3}Ado may achieve their anti-ASFV activity through different action mechanisms. The antiviral action of C-c^{3}Ado, as well as that of (*RS*)-AHPA isobutyl ester and (*S*)-DHPA, may be mediated by an inhibition of *S*-adenosylhomocysteine (SAH) hydrolase, and, concomitantly, transmethylation reactions. Indeed, C-c^{3}Ado, (*RS*)-AHPA and (*S*)-DHPA have been identified as rather potent inhibitors of SAH hydrolase (De Clercq and Cools, 1985; and references cited therein).

(*S*)-HPMPA and PMEA, however, are not inhibitory to SAH hydrolase (A. Holý, personal communication). The exact mechanism of action of these compounds remains to be elucidated. Recent observations (E. De Clercq, T. Sakuma and R. Bernaerts, unpublished data) indicate that (*S*)-HPMPA preferentially inhibits viral DNA synthesis, as compared to cellular DNA synthesis, within herpes simplex virus type 1 (strain KOS)-infected Vero cells. Being phosphonyl derivatives, (*S*)-HPMPA and PMEA cannot be dephosphorylated and are probably taken up as such by the cells. Whether they are phosphorylated intracellularly to their diphosphoryl derivatives, as has been recently demonstrated for the homophosphonate of DHPG [9-(1,3-dihydroxy-2-propoxymethyl)guanine] (Duke et al., 1986; Prisbe et al., 1986), is an interesting possibility that would be worth exploring.

For azidothymidine intracellular phosphorylation to the 5′-triphosphate would seem mandatory if the compound were to interact, as a chain terminator, with the viral DNA polymerase. Such a mechanism of action has been proposed for the inhibitory effect of azidothymidine on HTLV-III/LAV (Mitsuya et al., 1985) and may also account for its, albeit modest, activity against ASFV.

For pyrazofurin, an action at both AICAR (5-aminoimidazole-4-carboxamide-1-β-D-ribofuranosyl 5′-monophosphate) formyltransferase and OMP (orotidine 5′-monophosphate) decarboxylase has been proposed (Worzalla and Sweeney, 1980). It is difficult to visualize, however, how an interference at these enzyme levels could confer any specificity towards virus (i.e. ASFV)-infected cells.

In the antiviral action of ribavirin several factors may interplay (Smith, 1980), such as a reduction in purine nucleotide pool size and/or inhibition of the initiation of viral mRNA transcription (as demonstrated particularly for influenza virus (Wray et al., 1985a,b)). Whether any of these factors are involved in the mechanisms of anti-ASFV action of ribavirin remains subject of further study.

In summary, the present findings, taken together with previous studies of anti-ASFV agents (Haag et al., 1965; Dardiri et al., 1971; Moreno et al., 1978; Gil-Fernández et al., 1979; Geraldes and Valdeira, 1985; Sola et al., 1986a,b), point to (*S*)-HPMPA and C-c^{3}Ado as the most selective and most potent inhibitors of ASFV which have been reported so far. It would now seem imperative to further examine (*S*)-HPMPA and C-c^{3}Ado for their potential in the treatment of ASFV infections in vivo.

Acknowledgments

This work was supported by the Spanish Comisión Asesora de Investigación Cientifica Y Técnica (CAICYT), the Belgian Fonds voor Geneeskundig Wetenschappelijk Onderzoek (Project no. 3.0040.83) and the Belgian Geconcerteerde Onderzoeksacties (Project 85/90-79). The authors are grateful to Christiane Callebaut for her excellent assistance in the preparation of the manuscript.

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