Antiviral Research, 8 (1987) 273–282
Elsevier

AVR 00273

Phosphonylmethoxyalkylpurines and -pyrimidines as inhibitors of African swine fever virus replication in vitro

Carmen Gil-Fernández[1], Dolores García-Villalón[1], Erik De Clercq[2], Ivan Rosenberg[3] and Antonin Holý[3]

[1]*Centro de Investigaciones Biológicas, Consejo Superior de Investigaciones Científicas, Madrid, Spain;* [2]*Department of Human Biology, Division of Microbiology, Rega Institute for Medical Research, Katholieke Universiteit Leuven, Leuven, Belgium;* [3]*Institute of Organic Chemistry and Biochemistry, Czechoslovak Academy of Sciences, Prague, Czechoslovakia*

(Received 7 October 1987; accepted 10 November 1987)

Summary

Several phosphonylmethoxyalkylpurine and -pyrimidine derivatives related to (*S*)-9-(3-hydroxy-2-phosphonylmethoxypropyl)adenine [(*S*)-HPMPA] and 9-(2-phosphonylmethoxyethyl)adenine (PMEA) were evaluated as inhibitors of African swine fever virus (ASFV) replication in Vero cells. (*S*)-HPMPA has previously been shown to inhibit ASFV replication at a minimum inhibitory concentration (MIC) of 0.01 μg/ml with a selectivity index of 15000. Of the new compounds tested, the following emerged as the most potent and selective inhibitors of ASFV replication: the cyclic phosphonate of (*S*)-HPMPA [(*S*)-cHPMPA] with an MIC of 0.2 μg/ml and a selectivity index of 2500, the 2,6-diaminopurine analogue of (*S*)-HPMPA [(*S*)-HPMPDAP] with an MIC of 0.5 μg/ml and a selectivity index of 1400, and the cytosine [(*S*)-HPMPC] and guanine [(*RS*)-HPMPG] analogues with an MIC of 1 μg/ml and a selectivity index of 600–700.

African swine fever virus; Phosphonylmethoxyalkylpurines and -pyrimidines; (*S*)-HPMPA; PMEA; (*S*)-cHPMPA; (*S*)-HPMPC; (*RS*)-HPMPG; (*S*)-HPMPDAP; PMEDAP; PMEMAP.

Correspondence to: Dr. Carmen Gil-Fernández, Centro de Investigaciones Biológicas, Consejo Superior de Investigaciones Científicas, Velazques, 144, Madrid 28006, Spain.

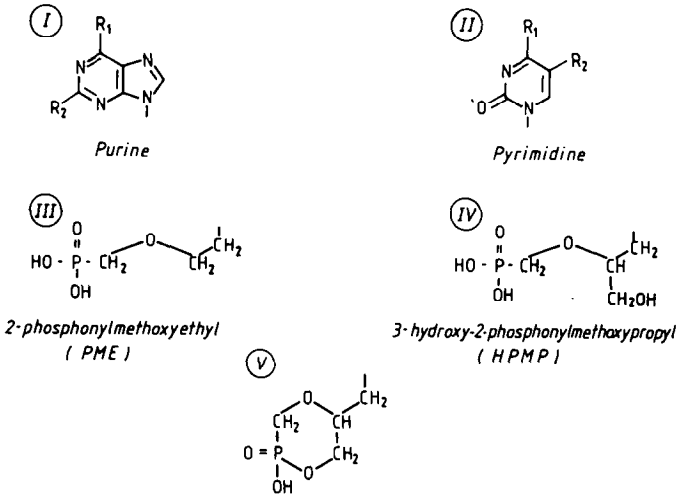
Introduction

African swine fever virus (ASFV) can cause an acute infection followed by persistent disease in several animal species. Virus can be isolated from the blood and organs of infected animals for long time periods and sometimes lifelong. Thus, animals which have survived the infection are able to further transmit the disease. These animals develop antibodies which can be detected by agar-gel precipitation, indirect immunofluorescence, immunoelectrophoresis, radial immunodiffusion, complement fixation, enzyme immunoassay and radioimmunoassay (Hess, 1981). However, it has proven impossible to demonstrate the presence of neutralizing antibodies, although some resistance to reinfection is acquired by animals which have survived the disease (Ruiz Gonzalvo et al., 1986). Immune sera, when added to cell cultures, inhibit the formation of virus plaques (Parker and Plowright, 1968). Inhibition of viral cytopathogenicity has also been observed in ASFV-infected buffy coat cultures in the presence of homologous serum (Malmquist, 1963). Also, sera taken from animals which had survived the disease and were challenged by an heterologous virus inhibit infection by homologous and several heterologous virulent viruses (Ruiz Gonzalvo et al., 1986). However, it is not known why immune serum has some inhibitory effect on the virus.

Since no neutralizing antibodies have been detected in ASFV-infected animals, and also because of the marked genetic variability of the virus, the development of a vaccine by conventional means has remained elusive for the time being. Therefore, particular attention has been given to chemotherapeutic approach. Now that efficient diagnostic techniques have been developed (Hess, 1981; Caballero and Tabares, 1986), compounds which have a potent and selective inhibitory effect on ASFV replication, may be of therapeutical usefulness in the treatment of the disease.

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), chloroquine (Geraldes and Valdeira, 1985), suramine, megalomycin C, atropine and i-carrageenan (Sola et al., 1986b). Monoolein, monolinolein and γ-linolenyl alcohol have also been shown to have a direct inactivating effect on the virus (Sola et al., 1986a).

Several nucleoside analogues with broad-spectrum antiviral potential have recently been evaluated against ASFV (De Clercq et al., 1986, 1987). Of these nucleoside analogues, (*S*)-9-(3-hydroxy-2-phosphonylmethoxypropyl)adenine [(*S*)-HPMPA] proved to be the most potent and selective inhibitor of ASFV replication. Its minimum inhibitory concentration (MIC) was 0.01 μg/ml, and its selectivity index 15000 (Gil-Fernández and De Clercq, 1987). These investigations have now been extended to a wide variety of phosphonylmethoxyalkylpurine and -pyrimidine derivatives related to (*S*)-HPMPA.



Abbreviation	Heterocyclic ring			Acyclic side chain
	I or II	R_1	R_2	III or IV
(S) - HPMPA	I	NH_2	H	IV
(S) - cHPMPA	I	NH_2	H	V
(S) - HPMPDAP	I	NH_2	NH_2	IV
(RS)- HPMPG	I	OH	NH_2	IV
(S) - HPMPC	II	NH_2	H	IV
(S) - HPMPT	II	OH	CH_3	IV
(RS)- HPMPU	II	OH	H	IV
(S) - HPMPMC	II	NH_2	CH_3	IV
PMEA	I	NH_2	H	III
PMEDAP	I	NH_2	NH_2	III
PMEMAP	I	H	NH_2	III
PMEHx	I	OH	H	III
PMEU	II	OH	H	III
PMEMC	II	NH_2	CH_3	III

Fig. 1. Structural formulae and abbreviations of the test compounds.

Materials and Methods

Virus

ASFV, adapted to grow in Vero cells, was kindly provided by E. Vinuela, Centro de Biologia Molecular (Enjuanes et al., 1976a,b). The virus was further propagated in Vero cells, and the stock in the present study was that obtained after the 21st passage.

Cells

Vero cells (green monkey kidney cells) were grown in Dulbecco's modified Ea-

gle's minimal essential medium supplemented with 10% newborn calf serum (growth medium). Maintenance medium contained only 2% newborn calf serum.

Compounds

The structural formulae and the abbreviations used for the test compounds are presented in Fig. 1. The compounds were prepared by the procedures described for (*S*)-HPMPA and PMEA: (*S*)-HPMPA and the derivatives of type IV according to Holý and Rosenberg (1987a,b); PMEA and the derivatives of type III according to Holý and Rosenberg (1987c); cHPMPA according to Rosenberg and Holý (1987). All compounds were purified by ion exchange chromatography to HPLC homogeneity and utilized as sodium salts.

Determination of virus-inhibitory compound concentrations

The effect of the different compounds on ASFV replication was determined by adding varying doses of the drugs to Vero cell cultures which had been grown to confluency 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, and infected with ASFV at a multiplicity of 1.0 plaque forming unit (PFU) per cell. After a 1.5 h adsorption period, unadsorbed virus was removed and the cultures were washed with phosphate-buffered saline. Then, maintenance medium containing 2% newborn calf serum and varying concentrations of the test compounds was added, and the cell cultures were further incubated at 37°C. Each culture was observed daily for viral cytopathic effects (CPE), and when the control cultures (inoculated with virus but not exposed to any of the compounds) showed complete destruction (usually 3 days after virus inoculation), the cells were removed from the wells with a rubber policeman and sonicated. Cell debris was removed by centrifugation and virus content in the supernatant was determined by plaque formation in Vero cells. 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 PFU per ml. All assays were repeated at least three times.

Determination of cytotoxic compound concentrations

Vero cells grown to confluency in 96-well plates were exposed to different concentrations of the test compounds (four wells per compound concentration) in maintenance medium. After two or three days of incubation, cytotoxicity was evaluated by two different procedures: (*i*) staining of the cells with 1% crystal violet in ethanol, and (*ii*) inhibition of [^{35}S]methionine incorporation.

The dye uptake method (*i*) was essentially similar to that used by Finter (1969) for measuring viral CPE in virus-infected cells treated with interferon, the only technical difference being that crystal violet was used instead of neutral red. After a staining period of 2–3 min, excess dye was removed by washing the cell monolayer 3 times with saline. Readings were done directly by visualizing the cell monolayers against a white background. Depending on the color intensity, a numeral index from 0–4 was established, whereby 2 corresponds to 50% cell destruction.

To monitor protein synthesis (*ii*), the cell culture medium was removed and 5 μCi [^{35}S]methionine [specific radioactivity: 1095 Ci/mmol (The Radiochemical Centre, Amersham, U.K.)] per ml of Dubecco's modified Eagle's medium free methionine were added, and the cells were incubated for 1 h at 37°C. The radioactive medium was then discarded and the cell monolayer was precipitated with 1 ml of 5% trichloroacetic acid. Non-precipitable material was removed and the cell monolayer was washed twice with phosphate-buffered saline and twice with ethanol. The monolayer was then dried at 37°C and dissolved with 250 μl of 0.1 N NaOH and 1% sodium dodecyl sulfate. The samples (100 μl) were then analyzed for radioactivity in a liquid scintillation spectrometer LKB Wallac 1219 Rackbeta.

Results and Discussion

Only Vero cells were used as substrate for the evaluation of the anti-ASFV activity of the compounds, because it appeared to be the sole cell line in which plaques

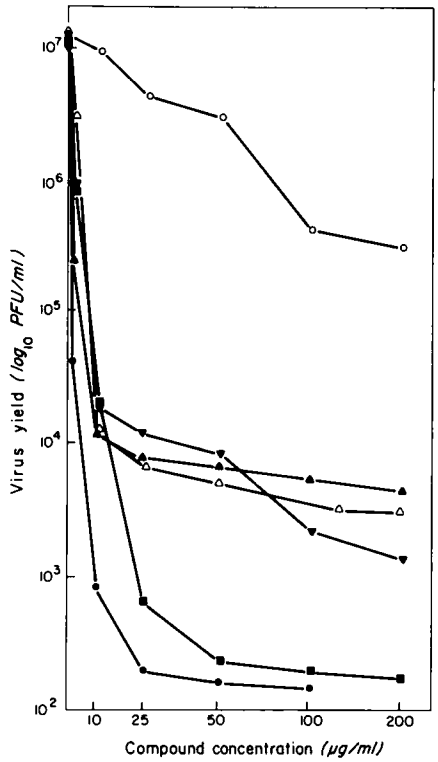


Fig. 2. Inhibitory effect of the test compounds on the replication of ASFV in Vero cell cultures. The compounds were added immediately after virus adsorption (multiplicity of infection: 1.0). Virus yield was measured three days after infection by plaque formation in Vero cells (see Materials and Methods). Test compounds: (*S*)-cHPMPA (-●-), (*S*)-HPMPDAP (-▲-), (*S*)-HPMPC (-■-), (*RS*)-HPMPG (-▼-), PMEDAP (-△-) and PMEMAP (-○-).

were developed upon ASFV infection, and, in addition the virus had been adapted to grow in these cells.

The two prototype compounds (*S*)-HPMPA and PMEA have been shown previously to inhibit ASFV replication in Vero cells (Gil-Fernández and De Clercq, 1987). Their minimum inhibitory concentration (MIC_{50}) required to effect a 50% reduction in virus yield were 0.01 and 5 μg/ml, respectively. Virus yield reduction experiments have now been carried out with various other phosphonylmethoxyalkylpurine and -pyrimidine derivatives, i.e. (*S*)-cHPMPA, (*S*)-HPMPDAP, (*S*)-HPMPC, (*RS*)-HPMPG, PMEDAP and PMEMAP, and dose-response curves were constructed for these compounds (Fig. 2), as was done previously for (*S*)-HPMPA and PMEA (Gil-Fernández and De Clercq, 1987).

As the most potent inhibitor among the new (*S*)-HPMPA analogues that were evaluated for their inhibitory effect on ASFV replication emerged (*S*)-cHPMPA, with a MIC_{50} of 0.2 μg/ml. The second most potent compound was (*S*)-HPMPDAP (MIC_{50} : 0.5 μg/ml). Then followed (*RS*)-HPMPG and (*S*)-HPMPC with an MIC_{50} of 1 μg/ml and PMEDAP with an MIC_{50} of 2 μg/ml. PMEMAP was only weakly inhibitory (MIC_{50}: 100 μg/ml), and the other compounds [(*S*)-HPMPT, (*RS*)-HPMPU, (*S*)-HPMPMC, PMEHx, PMEU and PMEMC] could be considered as inactive against ASFV replication. Of the active compounds, (*S*)-cHPMPA and (*S*)-HPMPC achieved the greatest inhibition in virus yield (> 4.5 $\log_{10}$), whereas the

TABLE 1

Comparative potency, cytotoxicity and selectivity of phosphonylmethoxyalkyl purine and -pyrimidine derivatives as inhibitors of ASFV in vitro.

Compound	MTC_{50}[a] (μg/ml)		MIC_{50}[b] (μg/ml)	Selectivity index	
	[A]	[B]	[C]	A/C	B/C
(*S*)-HPMPA	150[c]	200	0.01[c]	15000[c]	20000
(*S*)-cHPMPA	500	750	0.2	2500	3750
(*S*)-HPMPDAP	700	1000	0.5	1400	2000
(*RS*)-HPMPG	600	1000	1	600	1000
(*S*)-HPMPC	700	1000	1	700	1000
(*S*)-HPMPT	> 500	ND[e]	200[d]	≥ 2.5	...
(*RS*)-HPMPU	> 500	ND	300[d]	≥ 1.6	...
(*S*)-HPMPMC	> 500	ND	300[d]	≥ 1.6	...
PMEDAP	200	165	2	100	82.5
PMEA	200[c]	150	5[c]	40[c]	30
PMEMAP	600	ND	100	6	...
PMEHx	> 500	ND	200[d]	≥ 2.5	...
PMEU	> 500	ND	300[d]	≥ 1.6	...
PMEMC	> 500	ND	200[d]	≥ 1.6	...

[a]Minimum toxic concentration affecting 50% of the cells as either [A] assessed colorimetrically by the dye uptake method or [B] based on the inhibition of [^{35}S]methionine incorporation into cellular proteins.

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

[c]Data taken from Gil-Fernández and De Clercq (1987).

[d]These concentrations were those required to inhibit virus-induced cytopathogenicity by 50%.

[e]Not determined.

virus yield reduction effected by (*S*)-HPMPDAP and PMEDAP did not exceed 3.5 $\log_{10}$. For all compounds, except for (*S*)-cHPMPA, the virus yield reduction seemed to level off at the higher concentrations (Fig. 2).

Cytotoxicity measurements were based on the dye (crystal violet) uptake method and on the incorporation of [^{35}S]methionine into cellular proteins. The readings were done after the cells had been exposed to the compounds for 3 days (dye uptake method) or two days (methionine incorporation). These time intervals were chosen because virus yield reductions were also measured after an incubation period of 2–3 days. The minimum toxic concentration (MTC_{50}) was expressed as the concentration required to achieve a 50% reduction in the cellular uptake of the dye or the incorporation of [^{35}S]methionine.

The MTC_{50} values based on inhibition of cellular uptake of crystal violet corresponded closely with those that were based on the inhibition of [^{35}S]methionine incorporation (Table 1). None of the compounds proved cytotoxic at a concentration of $\leq$ 100 μg/ml. At concentrations higher than 100 μg/ml, cytotoxicity could best be assessed by monitoring the inhibition of [^{35}S]methionine incorporation (Fig. 3). From the dose-response curves for inhibition of [^{35}S]methionine incorporation, PMEA, PMEDAP and (*S*)-HPMPA appeared clearly more cytotoxic than the other four compounds, (*S*)-cHPMPA, (*RS*)-HPMPG, (*S*)-HPMPC and (*S*)-HPMPDAP: whereas the former exhibited an MTC_{50} of 150–200 μg/ml, the latter three were non-toxic at a concentration up to 500 μg/ml.

Based on the MTC_{50}/MIC_{50} ratios (Table 1) selectivity indexes were calculated, and irrespective of the parameter (dye uptake or [^{35}S]methionine) that was used for monitoring host cell toxicity, the order of (decreasing) selectivity was as fol-

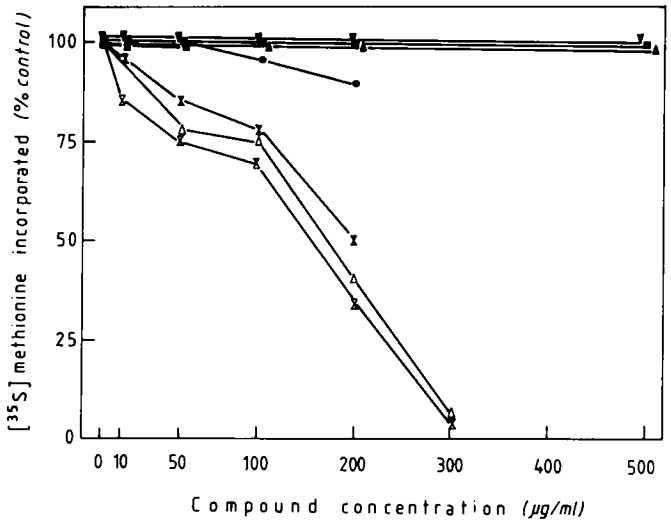


Fig. 3. Inhibitory effects of the test compounds on host cell protein synthesis as monitored by the incorporation of [^{35}S]methionine in uninfected Vero cell cultures (see Materials and Methods). Test compounds: (*S*)-HPMPA (-⧗-), (*S*)-cHPMPA (-●-), (*S*)-HPMPDAP (-▲-), (*S*)-HPMPC (-■-), (*RS*)-HPMPG (-▼-), PMEDAP (-△-) and PMEA (-⧖-).

lows: (*S*)-HPMPA > (*S*)-cHPMPA > (*S*)-HPMPDAP > (*S*)-HPMPC ~ (*RS*-HPMPG > PMEDAP > PMEA > PMEMAP. The same order was noted for the anti-ASFV potency of the compounds (Table 1), which thus makes (*S*)-HPMPA and (*S*)-cHPMPA as both the most selective and most potent ASFV inhibitors of the phosphonylmethoxyalkylpurines and -pyrimidines that have been investigated so far.

The mechanism of anti-ASFV action of (*S*)-HPMPA, (*S*)-cHPMPA and their congeners remains subject of further investigation. (*S*)-HPMPA can as such be taken up by the cells and would then be converted to its mono- and diphosphoryl derivatives by cellular enzymes (Votruba et al., 1987). (*S*)-HPMPA is much more inhibitory to viral DNA synthesis than cellular DNA synthesis, as has been demonstrated in Vero cells and human embryonic lung (HEL) cells infected with herpes simplex virus type 1 (HSV-1) (Votruba et al., 1987) as well as Raji cells infected with Epstein-Barr virus (EBV) (Lin et al., 1987). Also PMEA specifically inhibits EBV DNA synthesis in the superinfected Raji cells (Lin et al., 1987). We will examine whether (*S*)-HPMPA and PMEA also achieve a specific inhibition of ASFV DNA synthesis in ASFV-infected Vero cells.

From a structural-function viewpoint the present results indicate that as a group the 3-hydroxy-2-phosphonylmethoxypropyl ('HPMP') derivatives are more effective inhibitors of ASFV replication than are the 2-phosphonylmethoxyethyl ('PME') derivatives. This also holds for other viruses, such as vaccinia, adeno, cytomegalo and varicella-zoster virus (De Clercq et al., 1987). However, the 'PME' compounds are about equally active as the 'HPMP' congeners against HSV-1, HSV-2 and thymidine kinase-deficient (TK^-)mutants of HSV-1 (De Clercq et al., 1987).

It would now seem imperative to examine the most selective ASFV inhibitors of both the 'HPMP' and 'PME' series, i.e. (*S*)-HPMPA, (*S*)-cHPMPA, (*S*)-HPMPDAP, PMEDAP and PMEA, for their efficacy against African swine fever virus infection in an animal model system.

Acknowledgements

This work was supported by the Spanish Comisión Asesora de Investigación Ciéntífica y Técnica (CAICYT), the Belgian Fonds voor Geneeskundig Wetenschappelijk Onderzoek (Project no. 3.0040.83) and the Belgian Geconcerteerde Onderzoeksacties (Project no. 85/90-79). D.G.V. is the holder of a Consejo Superior de Investigaciones Científicas fellowship. The authors are indebted to Christiane Callebaut for her excellent editorial assistance.

References

Caballero, R.G. and Tabares, E. (1986) Application of pRPEL 2 plasmid to detect African swine fever virus by DNA-DNA hybridization. Arch. Virol. 87, 119–125.

Dardiri, A.H., Bachrach, H.L. and Heller, E. (1971) Inhibition by rifampicin of African swine fever virus replication in tissue culture. Infect. Immun. 4, 34–36.

De Clercq, E., Holý, 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 Holý, A. (1987) Antiviral activity of phosphonylmethoxyalkyl derivatives of purines and pyrimidines. Antiviral Res. 8, 000–000.

Finter, N.B. (1969) Dye uptake methods for assessing viral cytopathogenicity and their application to interferon assays. J. Gen. Virol. 5, 419–427.

Geraldes, A. and Valdeira, M.L. (1985) Effect of chloroquine on African swine fever virus infection. J. Gen. Virol. 66, 1145–1148.

Gil-Fernández, C. and De Clercq, E. (1987) Comparative efficacy of broad-spectrum antiviral agents as inhibitors of African swine fever virus replication in vitro. Antiviral Res. 7, 151–160.

Gil-Fernández, C., Páez, E., Vilas, P. and García-Gancedo, A. (1979) Effect of disodium phosphonoacetate and iododeoxyuridine on the multiplication of African swine fever virus in vitro. Chemotherapy 25, 162–169.

Haag, J., Larenaudie, B. and Gonzalvo, F.R. (1965) Peste porcine Africaine, action de la 5-iodo-2'désoxyuridine sur la culture du virus in vitro. Bull. Off. Int. Epiz. 63, 717–722.

Hess, W.R. (1981) African swine fever: a reassessment. Adv. Vet. Sci. Comp. Med. 25, 39–69.

Holý, A. and Rosenberg, I. (1987a) Sterospecific syntheses of 9-(*S*)-(3-hydroxy-2-phosphonylmethoxypropyl)adenine (HPMPA). Nucleic Acids Res. Symp. Series 18, 33–36.

Holý, A. and Rosenberg, I. (1987b) Synthesis of isomeric and enantiomeric *O*-phosphonylmethyl derivatives of 9-(2,3-dihydroxypropyl)adenine. Collect. Czech. Chem. Commun. 52, in press.

Holý, A. and Rosenberg, I. (1987c) Synthesis of 9-(2-phosphonylmethoxyethyl)adenine and related compounds. Collect. Czech. Chem. Commun. 52, 2801–2809.

Lin, J.-C., De Clercq, E. and Pagano, J.S. (1987) Novel acyclic adenosine analogs inhibit Epstein-Barr virus replication. Antimicrob. Agents Chemother. 31, 1431–1433.

Malmquist, W.A. (1963) Serologic and immunologic studies with African swine fever virus. Am. J. Vet. Res. 24, 450–459.

Moreno, M.A., Carrascosa, A.L., Ortin, J. and Vinuela, E. (1978) Inhibition of African swine fever (ASF) virus replication by phosphonoacetic acid. J. Gen. Virol. 93, 253–258.

Parker, J. and Plowright, W. (1968) Plaque formation by African swine fever virus. Nature 219, 524–525.

Rosenberg, I. and Holý, A. (1987) Synthesis of potential prodrugs and metabolites of 9-(*S*)-(3-hydroxy-2-phosphonylmethoxypropyl)adenine. Collect Czech. Chem. Commun. 52, in press.

Ruiz Gonzalvo, F., Carnero, M.E., Caballero, C. and Martinez, J. (1986) Inhibition of African swine fever infection in the presence of immune sera in vivo and in vitro. Am. J. Vet. Res. 47, 1249–1252.

Sola, A., Rodriguez, S., García-Gancedo, A., Vilas, P. and Gil-Fernández, C. (1986a) Inactivation and inhibition of African swine fever virus by monoolein, monolinolein and γ-linolenyl alcohol. Arch. Virol. 88, 285–292.

Sola, A., Rodriguez, S., Gil-Fernández, C., Alarcon, B., Gonzalez, M.E. and Carrasco, L. (1986b) New agents active against African swine fever virus. Antimicrob. Agents Chemother. 29, 284–288.

Votruba, I., Bernaerts, R., Sakuma, T., De Clercq, E., Merta, A., Rosenberg, I. and Holý, A. (1987) Intracellular phosphorylation of broad-spectrum anti-DNA virus agent (*S*)-9-(3-hydroxy-2-phosphonylmethoxypropyl)adenine and inhibition of viral DNA synthesis. Mol. Pharmacol., 32, 524–529.