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INHIBITION OF AFRICAN SWINE FEVER VIRUS DNA SYNTHESIS BY (S)-9-(3-HYDROXY-2-PHOSPHONYLMETHOXYPROPYL)ADENINE

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The acyclic nucleotide analogue (S)-9-(3-hydroxy-2-phosphonylmethoxypropyl)adenine [(S)-HPMPA] is a potent and selective inhibitor of African swine fever virus (ASFV) replication. Using the DNA-DNA hybridization technique with plasmid pRPEL-2 as probe, we have shown that (S)-HPMPA exerts a specific, dose-dependent, inhibitory effect on viral DNA synthesis. Also, (S)-HPMPA inhibits the production of late viral proteins, especially IP-73, in ASFV-infected MS and Vero cells. When evaluated under the same experimental conditions, phosphonoacetic acid (PAA) also caused an inhibition of viral DNA and late viral protein synthesis but only so at a concentration which was 10- to 20-fold higher than that required for (S)-HPMPA.

African swine fever can occur as an acute, subacute, chronic or inapparent disease of domestic swine (1,2). It is the cause of enormous economic losses in the affected countries. The etiologic agent, African swine fever virus (ASFV), is a double-stranded DNA virus which replicates in the cytoplasm of infected cells. Although the virus has an icosahedral morphology similar to that of the Iridoviridae, it has recently been excluded from the Iridoviridae family (3). The viral proteins have been resolved by polyacrylamide gel electrophoresis (4-8). Using immunoprecipitation, Letchworth and Whyard (9) were able to identify at least 37 viral proteins participating in antigen-antibody reactions. Tabarés et al. (10) found that at least eight of the viral polypeptides were phosphoproteins and at least three glycoproteins. Escribano and Tabarés (11) identified 44 polypeptides as either immediate early, early or late proteins. More recently, two dimensional analysis of the proteins synthesized in virus-infected cells revealed from 95 to 116 virus-specific proteins (12-14).

Studies of the viral proteins are contributed to the knowledge of the antigenic structure of the virus. Although some types of antibodies have been found there is no evidence for the presence of virus-neutralizing antibodies

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(1,15). Other authors have found that sera taken from infected animals challenged after infection to an homologous or heterologous virus were able to inhibit virus replication in vitro and in vivo (16). From virus-neutralization assays it was clear, however, that a 10 % fraction of the virus was not neutralized (17). These facts, and also the antigenic variability of the virus, make the development of a vaccine elusive. Therefore, other means such as a chemotherapeutic approach should be envisaged.

Quite a variety of drugs have been examined for their inhibitory effect on ASFV in cell culture. Inhibition of ASFV replication has been observed with 5-iodo-2'-deoxyuridine (18,19), rifampicin (20), phosphonoacetic acid (19,21), chloroquine (22), suramine, megalomycin C, atropine and ι-carrageenan (23). Monoolein, monolinolein and γ-linonenyl alcohol have been shown to inactivate or inhibit the virus (24). Recently, several uridine 5'-diphosphate-glucose analogues were evaluated for their activity against ASFV (25), and these studies were extended to nucleoside analogues with broad-spectrum antiviral potential (26,27). From all the compounds that were evaluated (S)-9-(3-hydroxy-2-phosphonylmethoxypropyl)adenine [(S)-HPMPA], emerged as the most potent and selective inhibitor of ASFV [MIC (minimum inhibitory concentration) : 0.01 μg/ml; selectivity index of 15,000]. The present investigation was aimed at gaining better insight in the mode of action of (S)-HPMPA against ASFV. In these studies phosphonoacetic acid (PAA) was incluced for comparative reasons. PAA is known to be a selective inhibitor of the synthesis of ASFV DNA (21) and late viral proteins (6,7).

MATERIALS AND METHODS

Cells and Viruses. The Spain-70 strain of ASFV was used after 45 passages in the MS (monkey kidney) cell line (28). This strain is referred to as E70MS45 in the text. The Badajoz-71 (BA-71) isolate of ASFV (29) was used after 21 passages in Vero (green monkey kidney) cells. Vero and MS cells were grown in Dulbecco's modified Eagle's minimum essential medium supplemented with 10 % newborn calf serum (growth medium). Maintenance medium contained only 2 % newborn calf serum.

Compounds. (S)-HPMPA was provided by Dr. A. Holy (Institute of Organic Chemistry and Biochemistry, Czechoslovak Academy of Sciences, Prague). Phosphonoacetic acid was supplied by Abbott Laboratories, Chicago, Ill.

Radiolabelling of cells. Confluent MS or Vero cell monolayers were mock-infected or infected with ASFV at a multiplicity of about 10 $CCID_{50}$ per cell (1 $CCID_{50}$ being the infective dose for 50 % of the cell cultures). After a 2-hour virus adsorption period at 37°C, the cells were washed three times with PBS to remove unadsorbed virus. Medium with or without the appropriate concentrations of PAA or (S)-HPMPA was added and the virus was allowed to replicate for another 30 hours. Then the medium was discarded and replaced by methionine-free minimum essential medium (MEM) supplemented with 1 % calf serum and [^{35}S]methionine [specific radioactivity : 1140 Ci/mmol (New England Nuclear, Boston, U.S.A.)], 20 μCi/ml. After a 2-hour labelling period, the medium was removed, the monolayer washed 3 times with PBS and the cells were harvested and processed for electrophoresis. To this end, the cells were solubilized with 2 % sodium dodecyl sulphate (SDS) and 5 % β-mercaptoethanol and then heated at 100°C for two minutes. The SDS-solubilized proteins were subjected to electrophoresis

on polyacrylamide gel slabs, then dried under vacuum, and exposed to Valca rapida 90" medical X-ray film.

Dot hybridization. DNA was extracted from infected or non-infected cells by treatment with 2 % SDS and 0.2 N NaOH. Samples were neutralized and then deproteinized with a mixture of phenol, chloroform and isoamyl alcohol at 25:24:1. The DNAs were applied onto nitrocellulose paper and hybridized with P-pRPEL-2, essentially as described previously (28,30).

RESULTS

Inhibition of viral DNA synthesis by (S)-HPMPA. The inhibition of viral DNA synthesis was assessed by dot hybridization technique. The probe used for ASFV DNA detection was the plasmid pRPEL-2 which contains the ASFV (Spain-70 strain) H-ClaI DNA fragment, corresponding to the 0.53-0.565 mn region of the viral genome (31) and encoding for the late viral proteins (32). ASFV DNA from MS cells infected with the E70MS44 strain and ASFV DNA from Vero cells infected with the Ba-70 strain were hybridized with the pRPEL-2 plasmid. The infected cells had been treated with (S)-HPMPA at 1, 2.5, 5, 10 or 25 μg/ml or PAA at 10, 25, 10, 100 and 200 μg/ml. Results are shown in Fig. 1. Virtually complete inhibition of viral DNA synthesis was achieved by (S)-HPMPA at 5 μg/ml, that is a 10-fold lower concentration than that required for PAA (Fig. 1, panel M). (S)-HPMPA specifically inhibited viral DNA synthesis [in Vero cells, even at a concentration of 1 μg/ml (Fig. 1, panel V)]; it had no effect on cellular DNA synthesis at concentrations up to 100 μg/ml (Fig. 1, panel V).

Effect of (S)-HPMPA on viral protein synthesis. Analysis of the polypeptides produced in MS or Vero cells following ASFV infection revealed the presence of at least 44 polypeptides ranking in molecular weight from 9.5 K to 243 K (11). The synthesis of the following (all late viral proteins) appeared to be blocked in (S)-HPMPA- or PAA-treated cells: IP243, IP172, IP73, IP19, IP15, IP14.5, IP11.5, IP10 (Fig. 2). Some polypeptides, which may be identified as late viral proteins but band at positions corresponding to the early proteins IP16, IP16.5 and IP12, also showed a reduced synthesis. No differences were noted in the suppressive effects of (S)-HPMA and PAA on late viral protein synthesis, except that (S)-HPMA was effective at a much lower concentration (5 ug/ml) than PAA (100 ug/ml).

DISCUSSION

(S)-HPMPA has been shown to be a potent and selective inhibitor of ASFV (26) as well as several other DNA viruses (33,34). The compound also proved effective in the treatment of herpetic keratitis caused by a thymidine kinase-deficient (TK^-) virus type 1 mutant (35). (S)-HPMPA is converted by cellular enzymes to the monophosphoryl and diphosphoryl derivative; the latter, termed (S)-HPMPApp, is assumed to be the active form of the molecule (36). (S)-HPMPA

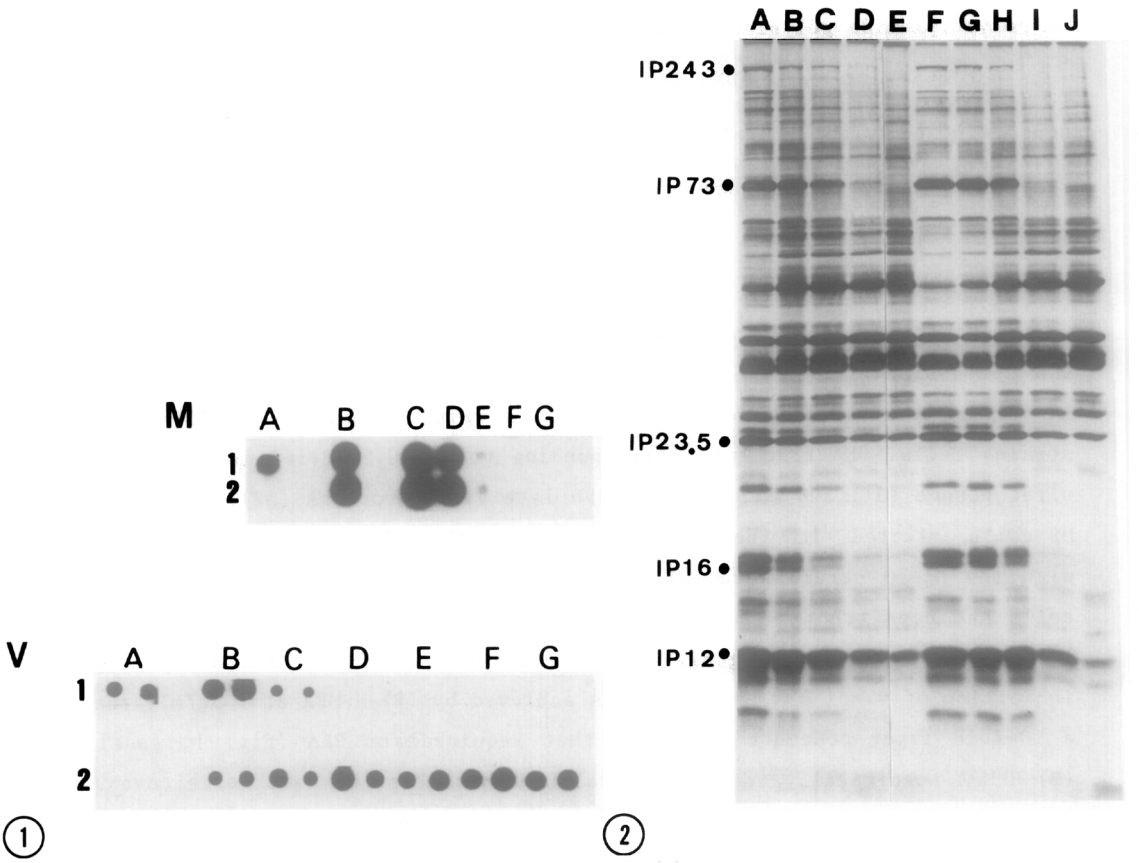

Figure 1. Inhibition of viral DNA synthesis by (S)-HPMA and PAA.
-Panel M : Hybridization of (^{32}P)-pRPEL2 DNA with viral DNA (A1), DNA from uninfected MS cells (A2), DNA from infected MS cells (B1,B2), DNA from infected MS cells treated with (S)-HPMA at 1 μg/ml (C1), 2.5 μg/ml (D1), 5 μg/ml (E1), 10 μg/ml (F1) or 25 μg/ml (G1), and from infected MS cells treated with PAA at 10 μg/ml (C2), 25 μg/ml (D2), 50 μg/ml (E2), 100 μg/ml (F2) or 200 μg/ml (G2).
-Panel V : Hybridization of (^{32}P)-pRPEL2 DNA with viral DNA (A1), DNA from infected Vero cells (B1), DNA from infected Vero cells treated with (S)-HPMA at 1 μg/ml (C1), 10 μg/ml (D1), 25 μg/ml (E1), 50 μg/ml (F1) or 100 μg/ml (G1). Hybridization of (^{32}P)-cellular DNA with viral DNA (A2), DNA from infected Vero cells (B2), DNA from infected Vero cells treated with (S)-HPMA at 1 μg/ml (C2), 10 μg/ml (D2), 25 μg/ml (E2), 50 μg/ml (F2) or 100 μg/ml (G2).Each assay was carried out by duplicate.

Figure 2. Fluorography showing inhibition of the synthesis of late viral proteins in ASFV-infected MS cells by (S)-HPMA and PAA. Control infected MS cells (A). Infected MS cells treated with (S)-HPMA at 1 μg/ml (B), 2.5 μg/ml (C), 5 μg/ml (D) or 25 μg/ml (E). Infected MS cells treated with PAA at 10 μg/ml (F), 25 μg/ml(G) 50 μg/ml (H), 100 μg/ml (I) or 200 μg/ml (J).

achieves a marked inhibitory effect on viral DNA synthesis at concentrations far below those that are required to inhibit cellular DNA synthesis. This phenomenon has been observed with cells infected by herpes simplex virus type 1 (36) or Epstein-Barr virus (37).

We have investigated whether (S)-HPMPA is inhibitory to viral DNA synthesis in ASFV-infected cells, using a molecular hybridization technique with as radioactive probe the plasmid pRPEL-2 as previously described (28,30). The sensitivity of the technique is such that it enables the detection of about 20 pg

viral DNA (30). (S)-HPMPA inhibit viral DNA synthesis at a concentration of 1 µg/ml, while not being inhibitory to cellular DNA synthesis at a concentration up to 100 µg/ml. Similar results were obtained with the Ba-71 and E70MS45 strains in Vero and MS cells, respectively. These results thus confirm and extend the observations of Votruba et al. (36) and Lin et al. (37) in that (S)-HPMPA is specifically inhibitory to viral DNA synthesis, including ASFV DNA synthesis.

The inhibitory effect of (S)-HPMPA on ASFV DNA synthesis has been compared to that of a well-known DNA synthesis inhibitor, namely PAA. PAA is thought to suppress ASFV replication (19,21) through inhibition of the viral DNA polymerase (21). (S)-HPMPA inhibited ASFV DNA synthesis at a concentration (5 µg/ml) which was at least 10-fold lower than that required for PAA to inhibit ASFV DNA synthesis.

To assess whether inhibition of viral DNA synthesis was followed by an inhibition of viral protein synthesis, polyacrylamide gels were run with extracts from both ASFV (Ba-71 strain)-infected Vero cells and ASFV (E70MS45 strain)-infected MS cells, respectively. (S)-HPMPA or PAA treatment of these cells resulted in a marked inhibition in the synthesis of late viral proteins, (S)-HPMPA being effective at a 20-fold lower concentration than PAA. Also, the synthesis of IP16 and IP16.5, two polypeptides that have been classified as early proteins (11), was apparently inhibited by (S)-HPMPA and PAA. This may indicate the presence of late proteins in the corresponding bands. In fact, Urzainqui et al. (14), using two-dimensional gel electrophoresis, found two late polypeptides with a molecular weight of approximately 16 K. The synthesis of another so-called early protein, IP12, was partially inhibited by PAA and (S)-HPMPA. The IP12 band actually consists of eight polypeptides with a similar molecular weight. They can be individualized by two-dimensional gel electrophoresis. Out of these 8 polypeptides, two have been classified as immediate early, two as early and four as late proteins (14).

From the findings presented here we can conclude that (S)-HPMPA inhibits ASFV replication through a specific inhibition of viral DNA synthesis; whether this effect results from the inhibitory effect of (S)-HPMPApp on the viral DNA polymerase is an interesting possibility worthy of further study.

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