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Anionic compounds as inhibitors of African swine fever virus replication in Vero cells

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Summary

The anionic compounds, aurintricarboxylic acid, Evans blue, glycyrrhizic acid, taurolithocholic acid, and fuchsin acid, have been tested for their capacity to inhibit the replication of African swine fever virus (ASFV) in Vero cells. Aurintricarboxylic acid suppressed ASFV cytopathic effects at 5 μg ml^{-1} and emerged as the most efficacious inhibitor with a selectivity index (S.I.) of 400, followed by Evans blue with 40, Fuchsin acid with 20, taurolithocholic acid with 10, and glycyrrhizic acid with 5.

All the compounds inhibited the virus adsorption or penetration to the cells in some degree, but inhibition was also found when they were added after virus adsorption, indicating that other mechanism (or mechanisms) are involved in viral inhibition.

Introduction

African swine fever virus is the causative agent of a fatal and important disease of domestic swine. The virus is a large enveloped cytoplasmic DNA virus which has icosahedral morphology. No vaccine is available for ASF, (Hess, 1981; Viñuela, 1985; Kihm *et al.*, 1987) because the sera from chronically infected animals do not neutralize the virus (De Boer, 1967) although some resistance to reinfection is acquired (Ruiz Gonzalvo *et al.*, 1986).

For this reason the development of a chemotherapeutic approach may combat the illness. Several compounds have been shown to inhibit African swine fever virus (ASFV) replication: 5-iodo-2′-deoxiuridine (Haag *et al.*, 1965; Gil-Fernández *et al.*, 1979), rifampicin (Dardiri *et al.*, 1971) and phosphonoacetic acid (PAA) (Moreno *et al.*, 1978; Gil-Fernández *et al.*, 1979) are also inhibitors of ASFV replication. Recently it has been established that PAA and phosphonoformic acid inhibit ASFV in cultured swine monocytes (Villinger *et al.*, 1990). Among other inhibitors of ASFV replication are: lysosomotropic substances (Geraldes and Valdeira, 1985; Alcamí *et al.*, 1989), several uridine 5-diphosphate glucose analogues (Gil-Fernández *et al.*, 1987b) and the fatty acids, monoolein, monolinolein and γ-linolenyl alcohol (Sola *et al.*, 1986a), iota carrageenan and suramin (Sola *et al.*, 1986b) also inhibit ASFV replication. Several nucleosides analogues, among them (S)-9-(3-hydroxy-2-phosphonylmethoxypropyl)adenine [(S)-HPMPA] proved to be the most potent and selective (Gil-Fernández and De Clercq, 1987a), and has been shown to inhibit ASFV DNA synthesis and viral late proteins (Arzuza *et al.*, 1988). Several phosphonylmethoxy alkylpurine and -pyrimidine derivatives related to (S)-HPMPA and 9-(2-phosphonylmethoxyethyl)adenine (PMEA) have also reportedly been selective inhibitors of ASFV (Gil-Fernández *et al.*, 1987a).

In our laboratory we have assayed a number of polysaccharides against ASFV (García-Villalón and Gil-Fernández, 1991) among which lambda carrageenan has shown the strongest inhibitory effect. These polysaccharides inhibit virus adsorption which may be due to the interaction with non saturable binding sites. In the present study we have extended the research to several anionic substances, namely; Aurintricarboxylic acid (ATA), Evans blue, fuchsin acid, glycyrrhizic acid, and taurolithocholic acid, with the aim of studying their inhibitory effect on replication of ASFV growing on Vero cells, and especially on virus adsorption.

Results

Cytotoxicity was measured by three parameters, namely alteration of normal morphology evaluated by microscopic evaluation, staining of the cells with 1% crystal violet in ethanol as described previously (Gil-Fernández and De Clercq, 1987), and inhibition of host cell macromolecular (protein) synthesis on the basis of the incorporation of (^{35}S)-methionine. When the cytotoxicity was assayed with the dye-uptake method, Evans blue showed a 50% cytotoxicity concentration (CC_{50}) of 200 μg ml^{-1} followed by ATA and taurolithocholic acid with a CC_{50} of 400 and 500 μg ml^{-1} respectively (Table 1). Fuchsin acid and glycyrrhizic acid are the less cytotoxic with a CC_{50} of 2000 and 2500 μg ml^{-1} respectively. The cytotoxicity of all the compounds was larger when measured by the dye-uptake method than when measured by incorporation of (^{35}S)-

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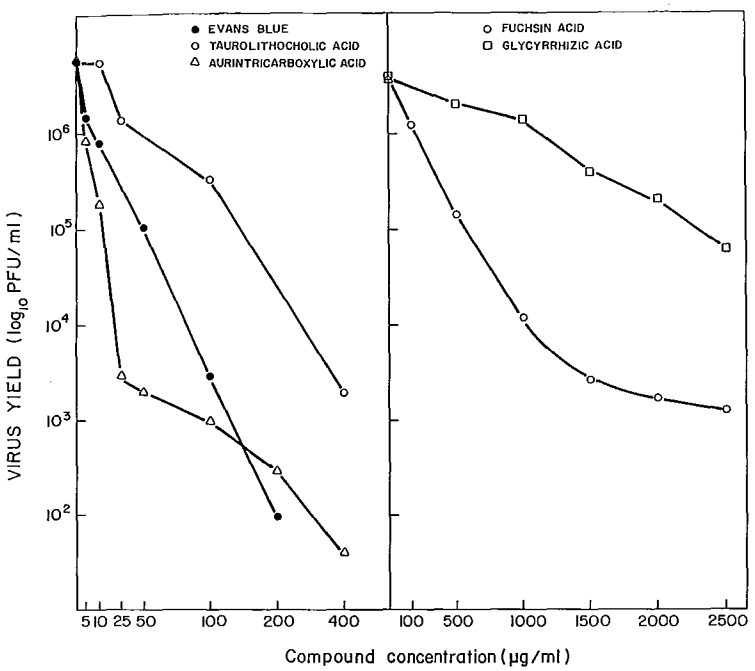

Fig. 1. Inhibitory effect of anionic compounds on the replication of ASFV in Vero cell cultures. The compounds were added immediately after virus adsorption (MOI 0.5). Virus yield was measured three days after infection by plaque formation in Vero cells.

methionine in the uninfected cells. The 50% antiviral effective concentration (EC_{50}) and the selectivity index (SI) are also shown. All the compounds were added after virus adsorption. From the selectivity indices, calculated as the ratio CC_{50}/EC_{50}, ATA could be considered to be the most specific inhibitor of ASFV (SI = 400), followed in order of decreasing specificity by Evans blue, fuchsin acid, taurolithocholic acid and glycyrrhizic acid.

Figure 1 shows the dose-response inhibition curves for the five compounds, when the drugs were added after virus adsorption. The most potent inhibitor was ATA followed by Evans blue, the EC_{50} values being 1 and 5 µg ml^{-1} respectively. The inhibition in virus yield exerted by all the compounds was large, amounting to 2–5 log_{10}s over the range of doses tested. Glycyrrhizic acid was found to be less effective with an inhibition of 2 log_{10}s that was obtained at high concentration (2500 µg ml^{-1}).

Figure 2 shows the results of the experiments examining the incorporation of (^{35}S)-methionine into protein after two days incubation of infected and uninfected cells with the different concentrations of the test compounds. Virus control and cell control without drug were also included. All percentages of (^{35}S)-methionine incorporated in infected and uninfected cells were made relative to untreated uninfected cells.

From the curves of incorporation of (^{35}S)-methionine into protein in uninfected cells (○) glycyrrhizic acid, followed by fuchsin acid, proved to be the least cytotoxic, while taurolithocholic acid was the most cytotoxic, with a quasi linear dose-dependent inhibition of incorporation of (^{35}S)-methionine. From the curves of incorporation of (^{35}S)-methionine into infected cells (●), at low drug doses the virus killed some of the cells. Thus little or no incorporation of radioactivity was detected. The uptake of (^{35}S)-methionine reached the maximum at the point where the greatest degree of viral cytopathic effect (CPE) inhibition occured, this being 10 µg ml^{-1} for ATA. This turned out to be the most inhibitory compound for ASFV at low concentrations, followed by Evans blue and then fuchsin acid. Taurolithocholic acid and glycyrrhizic acid were found to be the two compounds which showed the lowest degree of effectiveness of the drug. A definite correlation was observed between increasing uptake of (^{35}S)-methionine and increasing inhibition of ASFV found in the dose-response experiment. Eventually a concentration was reached, at which the dose of drug became toxic to cells which presumably do not support virus replication.

Table 1. Comparative potency, cytotoxicity, and selectivity of several anionic substances as inhibitors of ASFV *in vitro*.

Compound	CC_{50} (µg ml^{-1}) (A)	EC_{50} (µg ml^{-1}) (B)	Selectivity index A/B
Aurintricarboxylic acid	400	1	400
Evans blue	200	5	40
Fuchsin acid	2000	100	20
Taurolithocholic acid	500	50	10
Glycyrrhizic acid	2500	500	5

A: Cytotoxic concentration affecting 50% of the cells, assessed colorimetrically by the dye uptake method. These determinations were made in confluent cell monolayers.
B: Antivirally effective concentration required to effect a 50% reduction in virus yield.

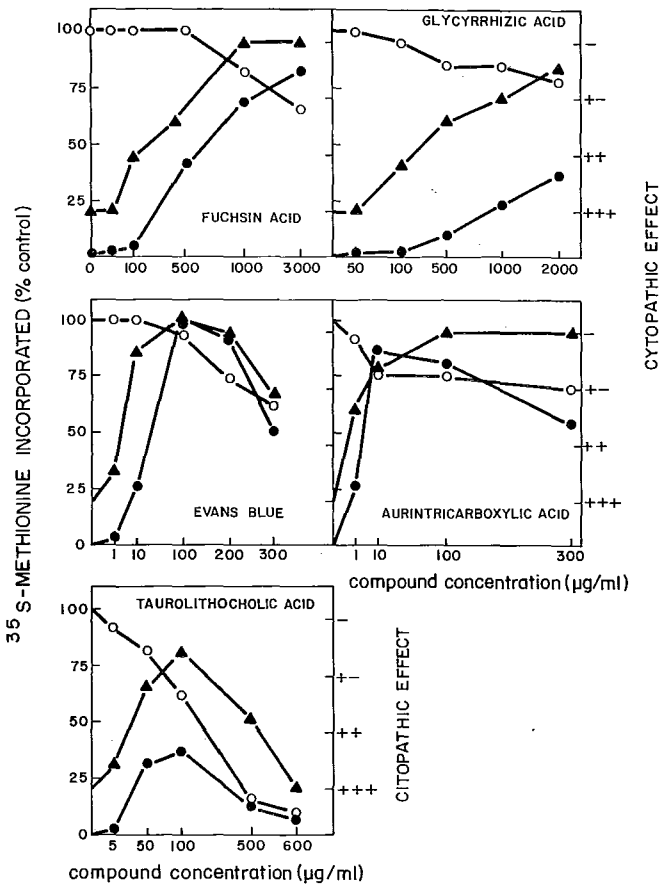

Fig. 2. Protein synthesis in infected and uninfected cells and cytopathic effect (CPE) of ASFV in Vero cells (MOI: 1). CPE was recorded 48 h.p.i. (▲). −, +−, ++ and +++ represent CPE on a linear scale; (−) no CPE; (+++) maximum CPE. Protein synthesis was measured for ASFV-infected cells (●) and uninfected control cells (○) in the presence of anionic compounds.

Then the curve of incorporation of (^{35}S)-methionine in infected cells parallels the toxicity curve of the uninfected cultures and the CPE curve. This technique indicates the maximum effective concentration of each inhibitor and thus indicates cell viability and indirectly indicates viral inhibition. The sensitivity of the technique is demonstrated in these experiments.

Figure 3 shows the inhibition of the adsorption of ASFV at the different concentrations of the anionic compounds. In order to avoid possible non-specific effects on virus adsorption, cells were pretreated for 15 min with PBS containing foetal bovine serum, glucose, and bovine serum albumin, in order to block non specific adsorption. In the experiments concerning inhibition of adsorption of ASFV by the anionic compounds we also tested chloroquine, a lysosomotropic drug, which has been reported to have no effect on virus adsorption and penetration (Geraldes and Valdeira, 1985; Alcamí *et al.*, 1989). We confirmed the above results and so used chloroquine as our control. This had no effect on adsorption and penetration (not shown). At 5 µg ml^{-1}, and with increasing cocnentration Evans blue inhibited by 30% and taurolithocholic acid by 25%. The highest adsorption inhibition was found with ATA which reaches the value 68% at 300 µg ml^{-1}. Inhibitions of 50% are shown at 3000 µg ml^{-1} with fuchsin acid and glycyrrhizic acid.

Discussion

Alcamí *et al.* (1989) reported the attachment of African swine fever virus to saturable binding sites on the plasma membrane of Vero cells. The attachment step is an ideal target for compounds which prevent virus infection by inhibition of virus adsorption to the cell receptor. We have recently investigated the action of several polysaccharides on the adsorption inhibition of ASFV to Vero cells (García-Villalón and Gil-Fernández, 1991). Among the polysaccharides studied, gamma and kappa carrageenans, together with pentosan polysulphate, showed the largest inhibitory activity measured by adsorption inhibition of radiolabelled virus onto the cells. In previous studies we have shown that suramin, an anionic substance, inhibits ASFV replication (Sola *et al.*, 1986b). We have extended the present study to several anionic compounds which were investigated for their inhibitory effect on ASFV replication and particularly virus adsorption. The mode of action of these anionic compounds against the human immunodeficiency virus (HIV),

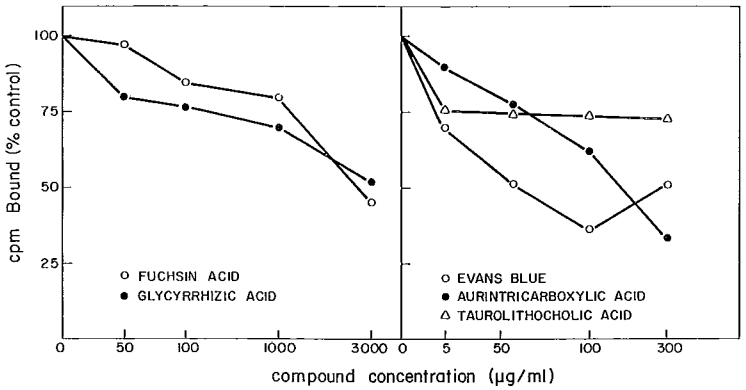

Fig. 3. Effect of the test compounds on ASFV adsorption. Monolayers of Vero cells were inoculated with purified radiolabelled virions (2.7×10^5 pfu ml^{-1} or 3×10^5 cpm ml^{-1}). The virus was adsorbed for 1 h prior to determining % bound.

reviewed by De Clercq (1989), is partly by inhibition of virus adsorption.

Among the anionic compounds assayed as inhibitors on the adsorption of ASFV to Vero cells, ATA and Evans blue were the most effective. At 5 μg ml^{-1} a 10–30% inhibition was detected. It reaches 65–70% at 100–300 μg ml^{-1} respectively. The potency and selectivity of these compounds was considerably higher than those reported previously for suramin (EC_{50}: 25 μg ml^{-1}; selectivity index 18; Sola *et al.*, 1986b). Fuchsin acid and glycyrrhizic acid showed an inhibition of 50% when high concentrations of the compounds were assayed (3000 μg ml^{-1}).

Together with their inhibitory action on ASFV adsorption, all the compounds inhibit virus replication when added after virus adsorption. ATA and Evans blue were found to reduce virus yield by 5 logs when measuring plaque formation. Evans blue has been reported to inhibit the cytopathogenicity of human T-cell lymphotropic virus type III/lymphadenopathy-associated virus (Balzarini *et al.*, 1986).

Glycyrrhizic acid inhibits virus growth and cytopathic effect of vaccinia, herpes simplex type I, Newcastle disease and vesicular stomatitis virus (Pompei *et al.*, 1979), and also the CPE of human immunodeficiency virus with inhibition of specific antigen expression (Ito *et al.*, 1987). The mechanism of the inhibitory effect of glycyrrhizic acid on human immunodeficiency virus is in part due to a reduction in protein kinase C which interferes with virus-cell binding (Ito *et al.*, 1989). Antiviral activity of glycyrrhizic acid was also found with hepatitis A virus (Crance *et al.*, 1990) and varicella-zoster (Baba and Shigeta, 1987).

The technique of incorporation of (^{35}S)-methionine in infected and uninfected cells (Fig. 2) proved to be a reliable method for measuring cell viability and, indirectly, inhibition of viral replication and the maximum effective concentration of each inhibitor.

The selectivity indices calculated from the CC_{50}, colorimetrically by the dye uptake method or based on the inhibition of (^{35}S)-methionine incorporation into cellular proteins (both determinations were made in confluent cell monolayers), were very similar: for ATA the former equalled 400 and the latter >300, for Evans blue the former equalled 20 and the latter >30, for taurolithocholic acid the former equalled 10 and the latter 6 and for glycyrrhizic acid the former equalled 5 and the latter >4.

We can correlate the results of (^{35}S)-methionine uptake with the results obtained in the virus yield reduction experiments. When the uptake of (^{35}S)-methionine increased at higher concentrations of the compounds, inhibition on viral replication also increased, as observed in viral yield reduction experiments until the point when the drug concentration became cytotoxic. Then the curve falls in parallel with the curve of the toxicity curve for uninfected cells.

Of all the anionic substances assayed glycyrrhizic acid and fuchsin acid were the less cytotoxic for Vero cells, with CC_{50} values of 2000 and 2500 respectively. At these concentrations adsorption of ASFV was inhibited. Fuchsin acid does not interfere with HIV adsorption at a 125 μM concentration that completely blocked HIV-1 replication (Baba *et al.*, 1988). We have not found such a strong inhibitory effect on ASFV replication but we have found inhibition of virus adsorption when the compounds were tested at high concentration.

Among all the compounds assayed against ASFV, ATA is the most efficacious inhibitor of virus replication. Several modes of action have been reported for ATA. It is reported to inhibit the attachment of messenger ribonucleic acid to ribosomes (Grollman and Stewart, 1968) thus preventing initiation of protein synthesis (Blumenthal and Landers, 1973; Huang and Grollman, 1972; Stewart *et al.*, 1971; Siegelman and Apirion, 1971). ATA has also been reported to inhibit RNA-directed DNA polymerase associated with Raucher murine leukaemia virus (Liao *et al.*, 1975) and avian myeloblastosis virus (Leis and Hurwitz, 1972) and to interfere with the ability of Moloney leukaemia RNA-directed DNA polymerase to bind a DNA primer molecule (Givens and Manly, 1976). ATA inhibits HIV-1 by preventing the attachment of the virus by a specific interaction

with the CD4 cellular receptors (Schols *et al.*, 1989). We have seen in the experiment with radiolabelled ASFV that ATA inhibits the adsorption but not as specifically as with HIV-1.

In the experiments with ASFV, ATA has been shown to inhibit different steps of virus replication. ATA inhibited at virus adsorption. However, when the compound was added after virus adsorption, the egress of ASFV from the cells was also inhibited by ATA. This possibility was tested by separate titrations of the virus remaining inside the cells, and of that liberated in the medium. The ratio of intracellular to released virus increased when ATA was present in the culture medium (results not shown).

Further research is in progress to elucidate the mechanism by which ASFV can be inhibited by ATA.

Materials and Experimental procedures

Virus and cells

ASFV, adapted to grow in Vero cells, was kindly provided by Professor E. Viñuela, Centro de Biología Molecular (Enjuanes *et al.*,1976). The virus was further propagated in Vero cells and the stock in the present study was that obtained after 16 passages. Vero cells (African green monkey kidney cells) were grown in Dulbecco's modified Eagle's medium, supplemented with 10% newborn calf serum for the growth and 2% for the maintenance medium.

Compounds

Aurintricarboxylic acid, free acid (ATA), Evans blue, fuchsin acid, glycyrrhizic acid (ammonium salt), and taurolithocholic acid (sodium salt) were purchased from Sigma Chemical Co. (St Louis, MO).

Inhibition of ASFV replication

Experiments were done as described previously (Gil-Fernández and De Clercq, 1987). Briefly, monolayers of Vero cells growing in 24-well plates were infected with ASFV at a multiplicity of infection (MOI) 0.5 PFU per cell.

After 1.5 h of adsorption period unadsorbed virus was removed and the monolayers were washed off with phosphate-buffered saline (PBS). Then culture medium containing different concentrations of the compounds was added. When the controls, consisting of infected cultures without drugs, showed complete destruction, the cells were removed from the wells and sonicated. Total virus yield was determined by plaque formation in Vero cells. All assays were repeated at least three times. Three wells were used for each compound concentration.

Virus purification and binding of radiolabelled virus to cells

The process of virus purification has been described elsewhere (García-Villalón and Gil-Fernández, 1991). In short, 100mm plates were inoculated with ASFV at 2 pfu cell^{-1}. After the adsorption period and removal of the inoculum, medium supplemented with 15 μg ml^{-1} (^{35}S)-methionine was added and incubated at 37°C for 48h. Cells were harvested and disrupted by sonication, cellular debris was centrifuged a 2000 rpm for 5 min. The supernatants were layered on top of a cushion of 35% sucrose and centrifuged at 20K rpm for 1 h at 4°C. Pellets were suspended in 1 ml of PBS. The specific activity of (^{35}S)-methionine labelled virus was 3×10^5 cpm ml^{-1} or 2.7×10^5 pfu ml^{-1}. Binding of radiolabelled virus to the cells was done in 96 well plates pretreated during 15 min with PBS containing 1% foetal bovine serum, 0.1% glucose and bovine serum albumin (5 mg ml^{-1}) in order to block nonspecific adsorption. Then the cells were inoculated with purified radiolabelled virus in 50 μl of PBS supplemented with serum albumin (1 mg ml^{-1}) and with or without the compounds to be tested. After 1 h adsorption at 37°C unadsorbed virus was removed and cells were rinsed three times with PBS. Cells with bound or penetrated virus were lysed and radioactivity was counted in a liquid scintillation spectrometer.

Estimation of the cytopathic effect and cytotoxicity

Cytopathic effects and cytotoxicity were measured as explained elsewhere (García-Villalón and Gil-Fernández, 1991). Monolayers of Vero cells growing in 96-well plates were inoculated at MOI equal to 1 in the presence of inhibitors, with the exception of the virus control. Uninfected cells with and without the drug were run in parallel to study the cytotoxicity of the compounds. All assays were repeated at least three times. Each point in the figures represents the mean value of three experiments.

In addition, inhibition of protein synthesis in infected and uninfected cells, and CPE, was estimated as described previously (Sola *et al.*, 1986b), with small variations. Controls of both, cells and virus without drug were also included. Monolayers of Vero cells growing in 96-well plates were inoculated at MOI equal to 1. After 1 h, of 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. When the control cultures (inoculated with virus but not exposed to any of the compounds) showed 50% CPE, the culture medium was removed and 1 μCi of (^{35}S)-methionine (1153 Ci mmol^{-1}; Radiochemical Centre, Amersham, England) per well was added in Dulbecco modified Eagle medium without methionine and incubated for 1 h at 37°C. Afterwards, the radioactive medium was removed and the cell monolayers were precipitated with 0.1 ml of 5% trichloroacetic acid. Nonspecific material was removed and the cell monolayers were washed twice with ethanol, dried and dissolved with 50 μl of 0.1 N NaOH–1% sodium dodecyl sulphate. A total of 100 μl (two wells per concentration) was counted in a liquid scintillation spectrometer.

This method measured the inhibition of host cell macromolecule (protein) synthesis on the basis of the incorporation of (^{35}S)-methionine. To differentiate between viral and cellular protein synthesis a different technique SDS-PAGE/fluorography will be necessary.

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