Antiviral activity of lauryl gallate against animal viruses

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Short Title: Antiviral activity of LG against animal viruses

Abstract

The effect of the anti-tumoral drug lauryl gallate on the infectivity of the African swine fever virus among other DNA (Herpes simplex and Vaccinia) and RNA (Influenza, Porcine transmissible gastroenteritis and Sindbis) viruses, involved in animal and human diseases, is analyzed. Viral production was strongly inhibited in different cell lines at non-toxic concentrations of the drug (1-10 μM), reducing the titres from 3 to more than 5 log. units depending on the multiplicity of infection. In our model system (African swine fever virus in Vero cells), the addition of the drug 1 h before virus adsorption, completely abolished virus productivity in a one-step growth virus cycle. Interestingly, no inhibitory effect was observed when lauryl gallate was added after 5 to 8 hpi. Both cellular and viral DNA synthesis and late viral transcription were inhibited by the drug, but, however, the early viral protein synthesis and the virus-mediated increasing of p53 remained unaffected. Activation of the apoptotic effector caspase-3 was not detected after lauryl gallate treatment of Vero cells, and, furthermore, the presence of the drug abrogated the activation of this protease induced by the virus infection. The overall results likely indicate that a cellular factor/function might be the target of the antiviral action of alkyl gallates.

Keywords: Lauryl gallate, ASFV, Herpesvirus, Influenza, Antiviral therapy

1. Introduction

Conventional antiviral agents can interfere successfully with viral proteins or functions [6], but exhibit a relatively narrow spectrum of action and often provoke the development of drug resistances in virus evolving under selective pressures [9, 19]. An alternative approach should target antiviral drugs against some cellular function required for virus replication, if a transient interference of the normal cell function is well tolerated and results in a retarding of virus replication and spreading [23]. Searching for an antiviral product with intracellular action, high inhibitory efficiency and low cellular toxicity, is a critical challenge raised to the scientific community at present. The possibility to obtain an antiviral with low specificity should help to control a wide range of possible virus infections in animals and humans.

Previous studies have shown that gallic acid (3,4,5-trihydroxybenzoic acid) and its alkyl ester derivatives exhibited a potential antitumoral activity on different cultured cells [1, 2, 20], inducing apoptotic processes selectively in rapidly growing cells [18] and inhibiting the activity of purified human protein tyrosine kinases [13, 17]. Also, the tumoricidal activity of lauryl gallate towards chemically induced skin tumours has been reported [15]. Some of these compounds, as propyl gallate (E-310), octyl gallate (E-311), and lauryl gallate (E-312) have been widely used as antioxidant food additives to prevent the rancidity of different foodstuffs. Regarding the antiviral activity of gallic acid esters, an early study described the inhibition of herpes simplex virus by methyl gallate, and its inefficiency against several RNA viruses [12], and two recent publications [21, 22] have examined the antiviral effect of several gallic acid esters, with particular attention to the octyl gallate, against one DNA (herpes simplex virus) and three RNA (vesicular stomatitis, influenza and polio) viruses.

We describe in this report the effect of another alkyl ester of gallic acid, the lauryl gallate, on the infectivity of three large DNA viruses (African swine fever, Herpes simplex and Vaccinia) and on another three RNA viruses (Influenza, Sindbis and Porcine transmissible gastroenteritis), its dependence on multiplicity of infection, the effect of time of addition and the step inhibited by the drug in the virus infection cycle, as determined in the model system of African swine fever virus infecting Vero cell cultures.

Materials and methods

Cells and viruses

Vero (African green monkey kidney), BHK (baby hamster kidney), MDCK (Madin Darbey canine kidney) and ST (swine testis) cells were obtained from the American Type Culture Collection and grown in Dulbecco's modified Eagle Medium (DMEM) supplemented with 2 mM L-glutamine, 100 U of gentamicin per ml and non essential amino acids. Swine alveolar macrophages were prepared by broncho-alveolar lavage as previously described [4]. Cells were cultured at 37ºC in medium supplemented with either 5% newborn calf serum (Vero), 5% heat-inactivated fetal calf serum (BHK, ST, MDCK) or 10% homologous porcine serum (swine macrophages).

The Vero-adapted African swine fever virus (ASFV) strain BA71V was propagated and titrated by plaque assay on Vero cells as described [8]. Field ASFV isolates (E70, Uganda-vir and Mozam'86) were propagated from frozen stocks on swine macrophages as indicated elsewhere [5, 10] and titrated by hemadsorption and plaque assay as previously described [4, 8]. Both the strain Kos of Herpes simplex virus (HSV), kindly supplied by Dr. Fernando Valdivieso (from the CBMSO, Spain) and the WR strain of Vaccinia virus (VV) were grown and titrated by plaque assay on Vero cells, as indicated for ASFV. Influenza virus (WSN strain), susceptible MDCK cells and the conditions for virus plaque assay, were kindly provided by Dr. Amelia Nieto from Centro Nacional de Biotecnología, Madrid, Spain. Porcine transmissible gastroenteritis virus (TGEV) was a generous gift from Dr. Luis Enjuanes, also from Centro Nacional de Biotecnología, Madrid, Spain.

Virus inhibition assay

To analyse the effect of lauryl gallate (Sigma) on virus infectivity, the sensitive cells were grown on multiwell plates to about 700,000 cells per cm^2. Stock solutions of lauryl gallate (LG) were made 40 or 10 mM in ethanol and stored kept away from the light at -20ºC, while the working solutions (1 and 0.1 mM) were freshly prepared in culture medium for each experiment. All the cells were preincubated for 1 h with culture medium containing the indicated concentration of LG (from 0 to 100 μM) before virus infection. Cultures were then infected with the corresponding virus at the indicated MOI in a reduced volume (about 30%) in the presence of the drug during 1-2 h. Virus inoculum was removed and washed twice with saline or culture medium, to eliminate non-adsorbed virus, and cells were further incubated in drug-containing fresh medium until a massive cytopathic effect was developed in control-infected cultures (in the absence of LG), normally at 24 h after infection in all the DNA viruses tested so far in this study, and 48 to 72 h after SV, TGEV or Influenza virus infection. Total virus (or extracellular for SV and Influenza virus) production was then titrated in duplicate samples, as above described. Control cultures were infected in parallel in the presence of the same amount of ethanol present in the LG-treated samples.

Cell viability and proliferation assay.

To determine the toxic effect of LG treatments in cell culture, we used the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Swine macrophages, Vero, ST, MDCK or BHK cells were seeded on 96-well plates and incubated for 24 h before the addition of LG to give final concentrations from 0 to 100 μM in a volume of 0.1 ml per well. Six wells were used for each concentration, and two wells were maintained in parallel with the corresponding medium in the absence of cells (to subtract the background value due to the drug in the culture medium). After 24 or 48

h of incubation, 90 μl of medium was removed from each well and replaced with 90 μl of fresh medium plus 10 μl of MTT solution (7.5 mg/ml in PBS). Cultures were further incubated at 37ºC for 2 h and lysed by adding 100 μl of SDS-containing lysis solution (20% SDS, 0.45 N acetic acid, 0.025 N HCl in N,N-dimethylformamide). After incubation for 15 min with gentle agitation, the absorbance at 550 nm was determined in a microplate reader (Model 680 Biorad). Background values were subtracted from the absorbance average value (6 wells) obtained for each LG treatment, and then referred to that scored in the absence of drug (100% viability).
Cell viability was also determined in LG-treated cultures by trypan blue-exclusion test, obtaining in all the cell lines similar results to those recorded by MTT assay.

Determination of cellular and viral DNA synthesis
Cultures of Vero cells, pre-incubated or not for 1 h with 10 μM LG, were mock-infected or infected with ASFV (BA71V strain) at a MOI of 5 pfu per cell. After 2 h adsorption in the presence of the drug, cultures were washed away from the non-adsorbed virus and further incubated at 37ºC with LG-containing fresh medium. To study the kinetics of cellular and viral DNA synthesis, cultures were pulse-labelled for one hour with methyl-(^{3}H)-thymidine (Amersham) at 5 μCi/ml in culture medium. At different times after infection, cells were washed and fractionated with NP40 into nuclear and cytoplasmic fractions as previously described [16]. The acid-insoluble radioactivity was analysed in the nuclear or cytoplasmic fractions to determine the cellular or viral DNA synthesis, respectively.

Western Blot analysis
To determine the presence of specific proteins, cultures were collected at different times after virus infection, washed twice with PBS and lysed in TNT buffer (20 mM Tris-HCl, pH 7.5, 0.2 M NaCl, 1% Triton X100) supplemented immediately before use with protease inhibitor cocktail tablets (Roche). Protein concentration was determined by the bicinchoninic acid (BCA) method using the BCA Protein Assay Reagent from Pierce. Proteins (30 μg) were subjected to SDS-12% polyacrylamide gel electrophoresis, and then electroblotted onto an Immobilon (Amersham) membrane. After reacting with primary antibodies specific for early or late ASFV-induced proteins [7], for cellular protein p53 (sc-6243, Santa Cruz Biotechnologies) or for cellular active caspase-3 (#559565, BD PharMingen), membranes were exposed to horseradish peroxidase-conjugated secondary anti-species (rabbit or mouse) antibody (Amersham), followed by chemiluminescence (ECL, Amersham) detection by autoradiography.

Results

Evaluation of cellular toxicity and reversibility of LG treatments

The evaluation of cell viability by MTT assay in swine macrophages, Vero, ST, MDCK and BHK cells, after incubation with increasing concentrations of LG for 24 or 48 h, is shown in Figure 1A. As an estimation of the sensitivity of each cell line to the presence of the drug, we calculated the LG concentration maintaining ≥ 50% of cell viability (CC50, cytotoxic concentration 50%), which ranged from 20 (BHK, 48h), 25 (MDCK, 48h), 30 (ST or swine macrophages, 24h), to 60 μM (Vero, 24h). From these results, a working concentration of 10 μM LG was selected to analyse the effect of short-term (24-72 h) treatments of cell cultures. In the case of Vero cells, the effect of the presence of 10 μM LG for several days, in cell viability and proliferation, is shown in Figure 1B: LG inhibited the growth of Vero cells while maintained its viability for at least 3 days. To test the reversibility of LG treatment, cultures of Vero cells were incubated with 10 μM LG for 48 h and then released from the drug and further incubated in standard conditions: the recovered cells exhibited the same growth kinetics (Figure 1C) and ASFV-sensitivity (data not shown) than that of untreated control Vero cells.

Effect of LG on DNA and RNA virus productivity

We first analysed the effect of different concentrations of LG on virus productivity of three DNA viruses (ASFV, HSV and VV) infecting Vero cells (Figure 2A, left panel). An almost undistinguished dose-dependent inhibition curve was obtained in all of the viruses tested so far, suggesting that the same process was affected by the drug in all of the virus infections. The residual presence of solvent (ethanol) did not affect the virus production even at the concentration corresponding to 100 μM LG, while the antiviral activity was titrated in the range from 2 to 30 μM LG, concentrations which maintained the viability of Vero cells above 90%.

To determine if the antiviral drug might be used in *in vivo* ASFV infections, we studied the effect of LG on virus productivity of several ASFV field isolates grown in swine alveolar macrophages. Although the presence of the drug was slightly more toxic in these cultures (CC50: 30 μM) than in Vero cells (CC50: 60 μM), antiviral activity was still observed from 2 to 10 μM LG, a range already out from cell toxicity (Figure 2A, right panel). The effective concentration of LG to reduce 50% or 90% of virus productivity was estimated to be 2-3 μM (EC50) and 4 μM (EC90), respectively, for all the DNA viruses tested in Figure 2A.

The inhibition of the RNA-containing SV virus by the presence of the drug was then analyzed after incubation with increasing concentrations of LG of BHK cells infected at two different MOI (0.5 or 2 pfu per cell). As shown in Figure 2B (left panel), infective virus titres decreased about 2 log.u. after 48 h in the presence of 10 μM LG (CC50 for BHK cells was 20 μM LG in the assay conditions). A more dramatic effect was observed in another RNA virus, the strain WSN of Influenza virus, produced on MDCK cell cultures. Figure 2B (right panel) shows that the degree of virus inhibition was extended in this case to more than 5 log.u., in cells infected at MOI 0.001 in the range from 3 to 10 μM LG (non-toxic concentrations for MDCK cells, with a CC50 of 25 μM). However, the inhibitory effective concentration of LG estimated (Figure 2B) for both RNA viruses was quite similar (1-3 μM for EC50 and 4-5 μM for EC90) to that obtained in the infection by DNA viruses. The effect of LG on a third RNA virus is shown in the next section.

Effect of MOI and time of addition of LG on its inhibiting activity.

As shown above, the average inhibition of DNA viruses productivity by LG treatment (10 μM) in our standard assay resulted in 3 log.u.. Since the standard test in DNA viruses was carried out in a relatively high MOI (about 2 pfu per cell), we thought of interest to know the effect of LG at lower MOI, considering that the first stages in virus diseases should correspond to infections with a very low ratio of pfu per susceptible cell. To achieve this, we have used the model system ASFV/Vero cell, in which we found that the efficiency of LG-mediated inhibition of virus productivity was higher when lower MOI were used. Thus, as shown in Figure 3A, decreases ≥ 3, 4 and 5 log. u. were obtained when the assay was performed at MOI 2, 0.2 or < 0.5 pfu per cell, respectively.

In the case of RNA viruses, the assay of inhibition was made at different MOIs depending on the particular virus system, as indicated in the previous section (and in the legend of Figure 2). Dose-dependent inhibition of virus production was detected (Figure 2B) in the presence of LG in cell cultures infected by SV (MOI 0.5 to 2) and Influenza virus (MOI 0.001). We also analysed another RNA virus (TGEV), in which preliminary experiments indicated that the virus production on ST cells was not largely affected by LG treatments in the standard virus inhibition assay (data not shown). However, when the experiment was done at different MOIs (Figure 3B) it was observed that the highest inhibition of virus production (90-95%) was obtained in ST cells infected at very low MOI (10^{-4} and 10^{-5}) in the presence of 5 or 15 μM LG, while MOIs ≥ 10^{-3} resulted in only weak reductions (to about one-half) of virus titres. The viability of ST cells (CC50 of 30 μM LG) remained close to 100 % at the LG concentrations used in the experiment (Figure 1A).

To determine the step of the virus cycle affected by the presence of LG, we performed an experiment in which the drug (10 μM) was added to cultures of ASFV-infected Vero cells at different times after infection (from -5 to 24 hpi), titrating de total virus produced at 24 hpi. As it is shown in Figure 3C, no virus production was detected when LG was added between -5 to 4 hpi (and maintained throughout the rest of the cycle), while the drug was totally ineffective when added from 12 to 24 hpi, indicating that the step affected by LG is critical between 5 to 8 hpi in ASFV infectious cycle.

Effect of LG on one-step ASFV cycle

We selected the model system ASFV/Vero cells to perform a more detailed study of the effect of LG on the virus infectious cycle. The analysis of the growth curve in a one-step viral infection (Figure 4A) showed that extracellular infective virus particles were not produced in the presence of the drug, suggesting that virus dissemination will be blocked by LG. However, the cells infected in the presence of the drug developed an extensive cytopathic effect, and eventually lyse at later times of infection.

To determine the precise step inhibited by LG in the infectious cycle, we analysed the presence of ASFV-specific early and late proteins induced in Vero cells infected by ASFV in the presence or absence of the drug. As shown in Figure 4B, both early (vp32) and late virus-specific proteins were detected, as expected, at different times of infection in control ASFV-infected cell cultures, while the presence of LG resulted in the inhibition of the late virus-induced proteins. Moreover, the induction of early proteins specific for ASFV should account for the cytopathic effect observed in LG-treated ASFV-infected Vero cell cultures, as it happens when the virus infection is

performed in the presence of, for example, cytosine arabinoside, which blocked the late stages of the ASFV infection.

We next examine if cellular and viral DNA synthesis was affected by the drug treatment: the results of pulse-labeling experiments in Vero cells infected by ASFV indicated that both nuclear and cytoplasmic DNA synthesis was drastically inhibited by LG (Figure 4C). It must be considered, that the viral DNA synthesis occurs mainly in the cytoplasm of the infected cell, and begins after 4-5 hpi: for this reason, it cannot be inhibited (because it is not induced) at very early times of infection, as it is observed in time 1 hpi in Figure 4C. The inhibition of cellular DNA synthesis by LG in mock-infected cultures should explain the cell growth arrest observed in Figure 1B, representing a very efficient, but reversible (Figure 1C), blockade of DNA replication.

Effect of LG on the induction of cellular proteins p53 and caspase-3.

We have previously described the induction of the tumour suppressor protein p53 (2) and the pro-apoptotic effector caspase-3 (1) in ASFV-infected Vero cells. To determine whether the LG treatment might affect the activation of these cellular factors, critical in the control of cell cycle and apoptosis, Vero cells were infected with ASFV in the presence or absence of the drug, and tested by Western blot for the expression of p53 and caspase-3 at 4 and 16 hpi. As shown in Figure 5A, the presence of LG did not affect the increase of p53 induced in Vero cells by ASFV infection. Interestingly, Vero cells incubated in the presence of LG resulted negative in caspase-3 activation, indicating that the apoptotic process was not induced by the drug in non infected cultures. Moreover, LG also inhibited the activation of caspase-3 induced by ASFV in Vero cells at late times of infection (16 hpi, Figure 5).

Discussion

We describe in this paper that short-term treatments with therapeutical dose (10 μM) of LG resulted in the inhibition of Vero cell proliferation, without affecting the cell viability (assayed by two independent methods, based on vital-staining or mitochondrial activity), a transient situation that could be recovered to normal parameters of cytoproliferation and virus-sensitivity by simply releasing the cell culture from the drug. The main observation in this report was the efficient inhibition of virus production observed in several cell lines incubated in the presence of LG. A wide spectrum of viruses, of veterinary and clinic importance, were inhibited by alkyl gallates, including members from many different families like Poxviridae, Asfarviridae, Herpesviridae, Orthomixoviridae, Togaviridae and Coronaviridae (all of them in this report), and Rhabdoviridae and Picornaviridae, besides Herpesviridae and Orthomixoviridae, in previous publications [12, 21, 22]. In the case of DNA viruses we have shown a very similar dose-dependent inhibition of the yield of infective ASFV, VV or HSV-1 virions in Vero cell cultures infected in the presence of LG, extending the inhibitory activity to several field isolates grown in swine macrophages in the case of ASFV. The similarity of the inhibition curves obtained for all the three DNA viruses tested on Vero cells, suggested that a cellular process (more than a specific virus component) was the target in LG treatments, although it cannot be completely excluded the possibility that a similar factor or process common to the three viruses might be blocked by the drug.

Since Herpes simplex virus has been studied in several reports of antiviral activity of alkyl gallates, we have calculated the antiherpetic effective concentration required to inhibit 50% of viral replication (EC50). The data obtained were as follows: 674-2354 μM (depending of the virus strain, [12]) or 3,5 μM [21] for octyl gallate, and 2,0 μM for LG in this report. Besides being the most effective against HSV, the alkyl ester LG has been also efficient against another important clinic pathogen like influenza virus, and even against viral agents involved in veterinary diseases (ASFV and TGEV in swine).

Furthermore, we have paid attention to the effect of putative antiviral drugs on host cell viability, to exclude side-effects due to cellular toxicity, and in the reversibility to normal status after releasing the treatment, to guarantee that cells are only transiently affected by the antiviral treatments. This is obviously an important issue when the antiviral product is directed against cellular components or functions, as it might be expected in a drug with such a broad spectrum antiviral activity as we have found here for LG. Therefore, we have included in this study the dose-response curve of cytotoxic effect of LG on each cell monolayer used, to determine the specific range of LG concentrations resulting in antiviral activity without cytotoxicity. It is important to note that this is an absolute requirement to assess the ability of any drug to be considered for antiviral therapy that, indeed, might be different in each particular cell and virus system. This information was not considered in previous reports on the antiviral effect of another alkyl (octyl) gallate against DNA and RNA viruses [21, 22], and it might be critical in the interpretation of i) results on assays of direct virucidal activity of octyl gallate, since they were performed at 100 to 400 μM in the presence of possibly toxic concentrations (dilution 1/10) of solvent DMSO, and ii) the inhibition to about 20% in poliovirus yield in Hep-2 cells, obtained in a concentration (40 μM) of octyl gallate that results, in the case of LG on BHK or MDCK cells, in a massive cell death (more than 50% cytotoxicity in 24 h incubation, as estimated by MTT assay). In fact, the authors pointed out that the inhibitory effect could be, at least in part, due to non-specific degeneration of the infected cells.

In order to consider future applications of LG in antiviral therapy, it was important to determine the degree of inhibition of infective virus yield, and the stage of the virus cycle blocked by the drug: these particular studies were performed in the model system ASFV in Vero cells. In this regard, the absence of extracellular infective virus particles in a one-step ASFV infection on Vero cells, suggests that no virus dissemination must be expected when the cell is infected in the presence of LG. Considering the low MOI associated to the *in vivo* infection in field conditions, this will represent a minimal damage in the whole organism, since no infectious virus will be produced after the first round of infection.

The inhibition of infective virus production was in agreement with the absence of induction of ASFV-specific late proteins in the presence of LG, likely as a direct consequence of the drastic inhibition of the synthesis of viral DNA. Interestingly, the DNA synthesis in the cell nucleus was also blocked by LG, indicating that DNA replication was completely prevented by the drug, and supporting the antitumoral activity associated to the drug [20]. From our results it can be concluded that the ASFV infection proceed normally in Vero cells in the presence of LG, from the attachment of virus particles, internalization, and uncoating of virus cores, to complete the transcription of early virus proteins, all along the first 5-8 h after infection. By this time, a LG-sensitive process is required to continue with the virus infection that, in the case of ASFV, will lead to the viral DNA replication at 10-12 hpi. In agreement with this interpretation, the addition of the drug was less effective at 8 hpi, and inefficient at 12 hpi, consistent to the reported data from octyl gallate-treated HEp-2 cells infected with HSV-1, that were fully productive if the drug was added at 8 hpi [21]. Moreover, Yamasaki et al. [22] described the inhibition by octyl gallate of a step in the middle stage of the multiplication of influenza virus. The inhibition of a similar step by two alkyl gallates in the infection of different cells by several DNA and RNA viruses, is almost incompatible with the hypothesis of a virus-specific component as target for the antiviral activity: most probably, a cellular factor/function that is affected by the drug treatment, is required in all of the viral infections susceptible to that kind of antiviral drugs. According to this interpretation, it could be expected that different viruses will display varied sensitivities to the drug, in correspondence to the specific requirement of each virus on the affected cell function. In fact, differences in the efficiency of alkyl gallates to inhibit two RNA viruses like VSV and poliovirus, or Influenza and TGEV, have been described by Uozaki et al. [21] and by us in this report.

Regarding the mechanism by which the alkyl gallates might inhibit the virus productivity, previous reports described the induction of apoptosis in tumoral cell lines by derivatives of gallic acid [18, 20], and the concomitant inhibition of the protein tyrosine phosphorylation detected in the presence of pervanadate. In the case of Vero cells, we have not been able to detect the activation of caspase-3 after incubation of cell cultures with non-toxic concentrations of LG, indicating that the apoptotic process was not triggered by the drug. This interpretation was also supported by the reversibility of the effects of LG on Vero cells (growth arrest and inhibition of DNA synthesis) when the cultures were released from the drug. As expected, incubation with LG, that, as shown in this work, completely inhibits ASFV infection, resulted in the blocking of the caspase-3 activation induced after 13 hpi in Vero cells [14]. Taking together all the results, and assuming that the target for LG is a cellular factor, we consider that the inhibition of protein kinases is a possible effect of LG, since the blockade of protein phosphorylation might produce many of the reported results: activation of proteins by kinases is one of the most important regulatory event in the eukaryotic cell, and the determination of the precise kinase(s) specifically affected by the antiviral drugs is the

main objective of our investigation in the next future, as well as the study of the effects of alkyl gallates on the specific cells and organs that are the targets of the *in vivo* virus infection.

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FIGURE LEGENDS

Fig.1. Effect of LG on cell viability. A) Swine macrophages, Vero, ST, MDCK or BHK cells grown on 96-well plates were incubated for 24 or 48 h in the presence of increasing concentrations of LG, and then analyzed for cell viability by the MTT assay. B) Vero cells were grown in the presence (closed symbols) or absence (open symbols) of 10 μM LG, and, at the indicated times, analyzed for cell number and viability (MTT assay). C) Vero cells were incubated for 48 h in the presence (closed symbols) of 10 μM LG, then released from the drug and further incubated for 5 additional days in the absence of LG. Control cultures incubated in the absence (open symbols) of LG were maintained in parallel.

Figure 2. Effect of LG on the productivity of several animal viruses. A) DNA viruses, left panel. Vero cell monolayers were incubated for 1 h with the indicated concentration of LG (from 0 to 100 μM) before infection with ASFV-Ba71V strain (●), HSV (■) or VV (▲) at a MOI of about 2 pfu per cell. Control cultures infected in the presence of the same amount of ethanol present in the LG-treated samples were processed in parallel and represented (○) as mean values for the three virus infections ("Mean Virus (-LG)"). Total virus produced at 24 hpi was titrated by plaque assay on Vero cell monolayers in duplicate samples. The viability of non-infected Vero cells incubated in the same conditions, assayed by MTT, is also represented (x). A) DNA viruses, ASFV field isolates, right panel. Cultures of swine macrophages were incubated for 1 h with the indicated concentration of LG and then infected with different ASFV field isolates (Mozam'68 (●), E70 (▲) or Uganda-vir (■)) at a MOI of 2-4 HADU per cell. Total virus produced at 27 hpi was titrated by hemadsorption assays on swine macrophages. The viability (MTT) of non-infected macrophages incubated in the same conditions is also represented (x). B) RNA viruses, left panel. Cultures of BHK cells, preincubated with LG as above indicated, were infected with SV at two MOIs (2 (▲) or 0.5 (●) pfu per cell). Extracellular virus produced at 48 hpi was titrated by plaque assay on BHK monolayers. The viability (MTT) of non-infected BHK cells incubated in the same conditions is also represented (x). B) RNA viruses, right panel. Cultures of MDCK cells, preincubated with LG as above indicated, were infected with Influenza virus, WSN strain, at a MOI of 0.001 (●) pfu per cell. Extracellular virus produced at 48 hpi was titrated on MDCK monolayers. The viability (MTT) of non-infected MDCK cells incubated in the same conditions is also represented (x).

Fig.3. Effect of MOI and time of addition of LG on its inhibiting activity. A) Vero cells were preincubated or not with 10 μM LG and infected as above indicated with the ASFV-Ba71V strain, at different MOIs (from 0.005 to 2 pfu per cell), either in the presence (●) or absence (○) of LG. Cultures were harvested when the cytopathic effect in control-infected (in the absence of LG) samples was massive, from 24 to 120 hpi, depending of the MOI used. Total virus production was titrated on Vero cell monolayers. B) Cultures of ST cells were preincubated with different concentrations of LG (0, 5 or 15 μM) for 1 h and then infected with TGEV at MOI from 10^{-5} to 10^{-1} pfu per cell. Total virus produced at 24 hpi was titrated by plaque assay on ST monolayers. C) Vero cells were infected with the ASFV-Ba71V strain at a MOI of 2 pfu per cell, and LG was added at a concentration of 10 μM at different times before or after virus infection, maintaining the presence of the drug up to the completion of the virus cycle

(24 hpi), when total virus was titrated on Vero monolayers. Time 0 hpi represents the end of the virus adsorption (from -2 to 0 hpi).

Figure 4. Effect of LG on one-step ASFV cycle. A) Growth curve. Vero cells were preincubated or not for 1 h with 10 μM LG and infected as above indicated with the ASFV-Ba71V strain at a MOI of 0.5 pfu per cell. Non-adsorbed virus was washed away and the cultures maintained in the presence or absence of LG to complete the virus cycle. At the indicated times after infection the extracellular virus production was titrated by plaque assay on Vero monolayers. B) Protein expression. Cell extracts obtained at different hpi from samples infected as indicated in A, were subjected to Western blot analysis, to detect early (vp32) or late ASFV-induced proteins in the presence or absence of LG, as described under Materials and Methods. The presence of early (⇦) or late (🡐)virus-induced proteins is indicated by arrows. C) DNA synthesis. To determine the specific cellular and viral DNA synthesis, cultures of Vero cells were mock-infected or infected with the ASFV-Ba71Vstrain at a MOI of 5 pfu per cell, in the presence or absence of LG, as indicated in A. Cultures were pulse-labeled for 1 h at different times after infection with methyl-^{3}H-thymidine, and separated into nuclear and cytoplasmic fractions. It is represented the ratio between the acid-insoluble radiactivity obtained in the LG-treated mock (m+LG) or infected (V+LG) samples versus the corresponding non-treated cultures (m or V), referring as 100% the value obtained at 0 hpi in each series. Cellular DNA synthesis corresponds to the data in nucleus from non-infected cultures (m+LG/m, ○), while viral DNA synthesis is best represented in the cytoplasm of infected samples (V+LG/V, ●).

Figure 5. Effect of LG on the induction of p53 and caspase-3. Vero cells were mock-infected or infected with the ASFV-Ba71V strain at a MOI of 5 pfu per cell, either in the presence or absence of 10 μM LG. Cell extracts were obtained at 4 and 16 hpi and subjected to Western blot analysis, to detect the cellular proteins p53 and caspase-3, as described under Materials and Methods. A sample as positive control for the induction of p53 and caspase-3 was run in parallel (C+).

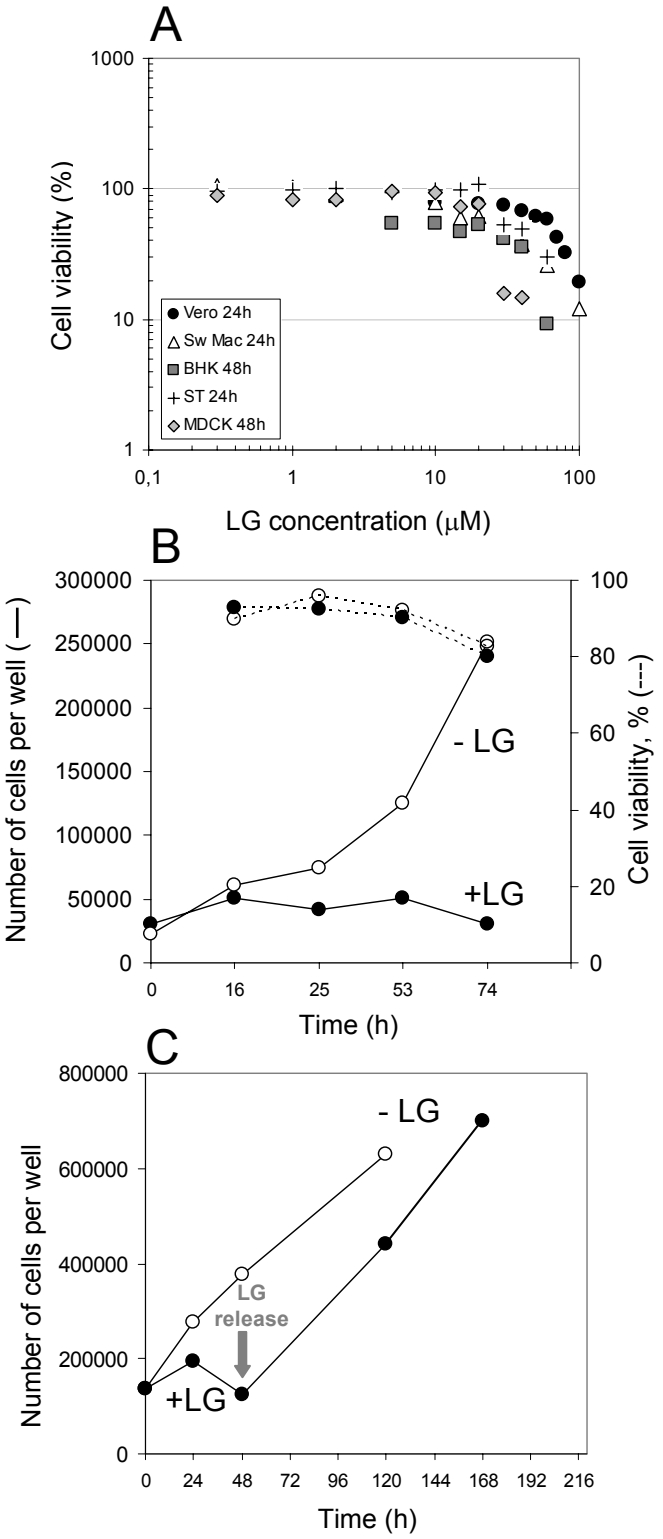

A
1000
100
10
1
Cell viability (%)
Vero 24h
Sw Mac 24h
BHK 48h
ST 24h
MDCK 48h
0,1
1
10
100
LG concentration (μM)
B
300000
250000
200000
150000
100000
50000
0
Number of cells per well (—)
100
80
60
40
20
0
Cell viability, % (---)
- LG
+LG
0
16
25
53
74
Time (h)
C
800000
600000
400000
200000
0
Number of cells per well
- LG
LG
release
+LG
0
24
48
72
96
120
144
168
192
216
Time (h)

Fig. 1

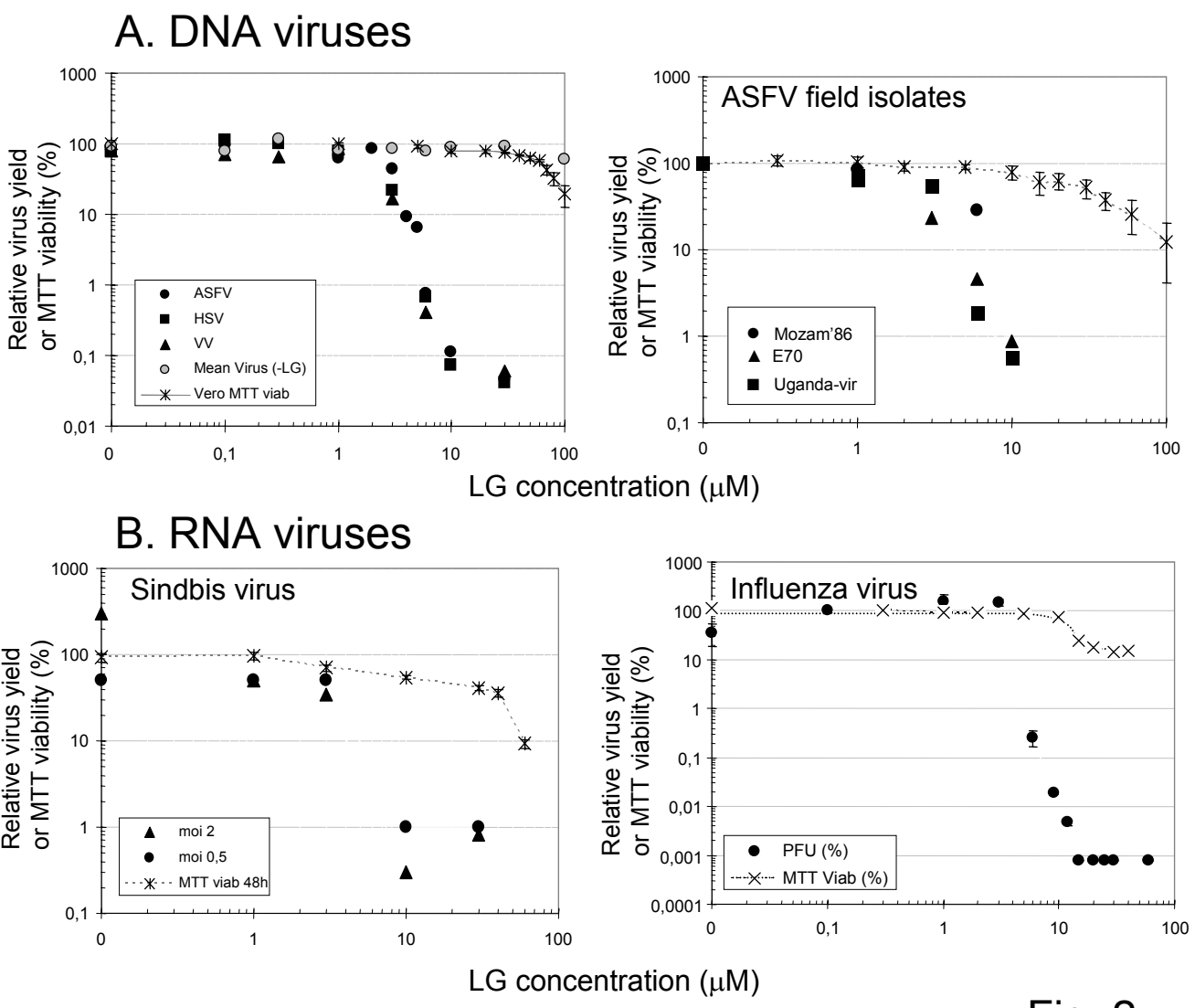

A. DNA viruses
Relative virus yield or MTT viability (%)
1000
100
10
1
0,1
0,01
0
0,1
1
10
100
ASFV
HSV
VV
Mean Virus (-LG)
Vero MTT viab
ASFV field isolates
Mozam'86
E70
Uganda-vir
LG concentration (μM)
B. RNA viruses
Sindbis virus
moi 2
moi 0,5
MTT viab 48h
Influenza virus
PFU (%)
MTT Viab (%)
0,0001
0,001
LG concentration (μM)

Fig. 2

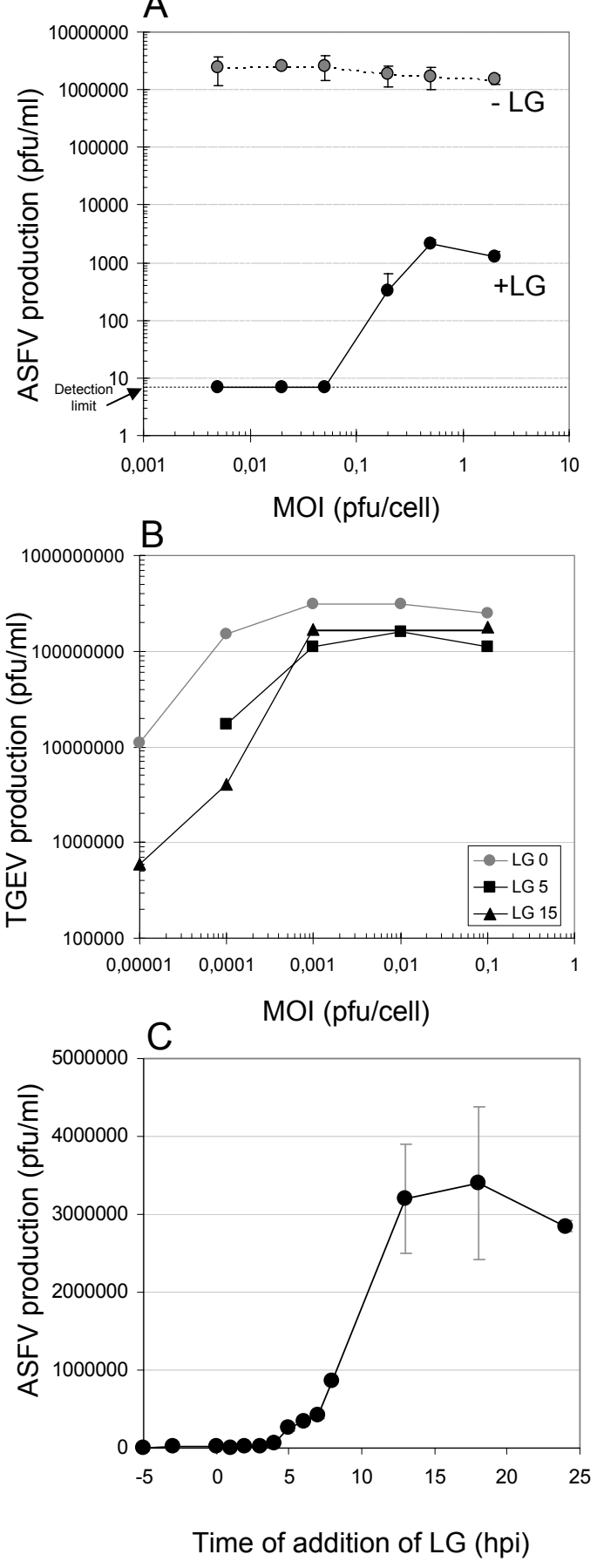
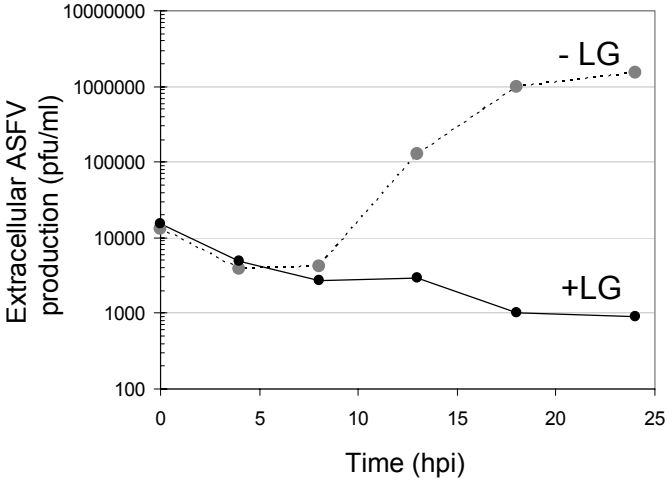
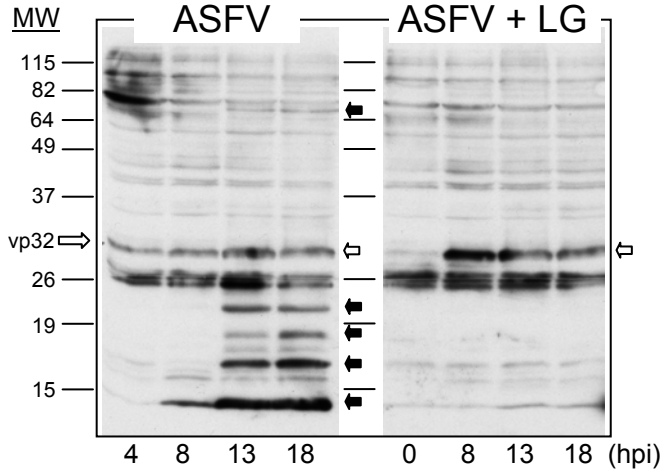

A
ASFV production (pfu/ml)
10000000
1000000
100000
10000
1000
100
10
1
- LG
+LG
Detection limit
0,001
0,01
0,1
1
10
MOI (pfu/cell)
B
TGEV production (pfu/ml)
1000000000
100000000
10000000
1000000
100000
LG 0
LG 5
LG 15
0,00001
0,0001
0,001
0,01
0,1
1
MOI (pfu/cell)
C
ASFV production (pfu/ml)
5000000
4000000
3000000
2000000
1000000
0
-5
0
5
10
15
20
25
Time of addition of LG (hpi)

Fig. 3

A. Growth curve



B. Protein expression



⇦ Early virus protein vp32

⬅ Late virus proteins

C. DNA synthesis

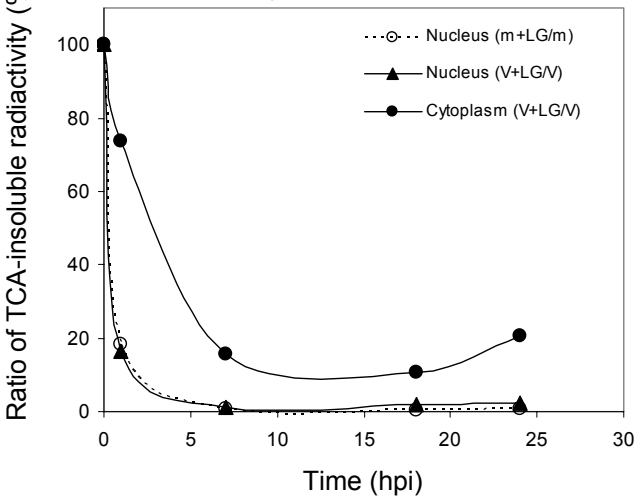


Fig. 4

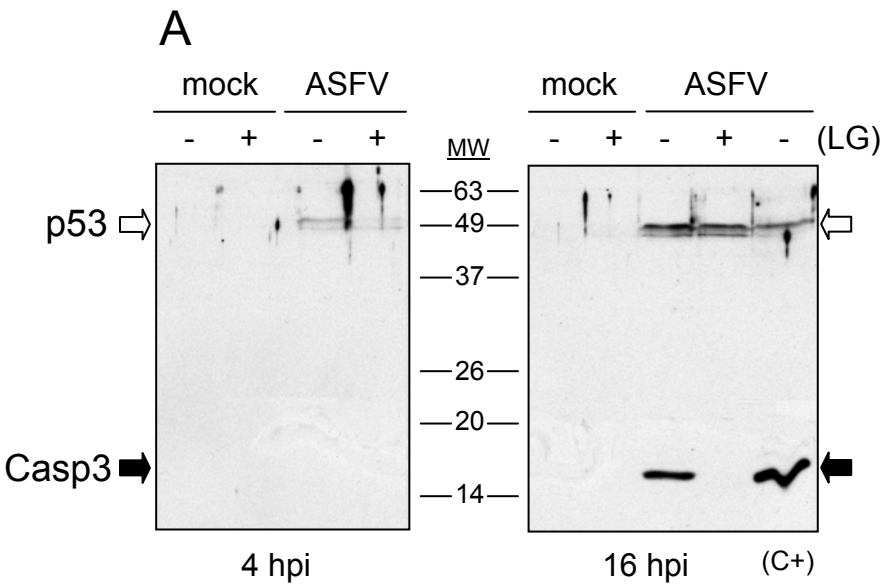

A
mock
ASFV
mock
ASFV
- + - + MW - + - + - (LG)
p53
Casp3
63
49
37
26
20
14
4 hpi
16 hpi
(C+)

Fig. 5