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Comparative inhibitory activity of the stilbenes resveratrol and oxyresveratrol on African swine fever virus replication

I. Galindo [a], B. Hernáez [a], J. Berná [b], J. Fenoll [c], J.L. Cenis [c], J.M. Escribano [a], C. Alonso [a,*]

[a] Dpt. Biotecnología, Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria, INIA, Autovia A6, Km 7.5, 28040 Madrid, Spain
[b] Departamento de Química Orgánica, Facultad de Química, Universidad de Murcia, Campus de Espinardo, E-30100 Murcia, Spain
[c] Instituto Murciano de Investigación y Desarrollo Agrario y Alimentario (IMIDA), c/ Mayor, 1, 30150 La Alberca (Murcia), Spain

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ABSTRACT

Stilbenols are polyphenolic phytoalexins produced by plants in response to biotic or abiotic stress. These compounds have received much attention because of their significant biological effects. One of these is their antiviral action, which has previously been documented for two members of this class, namely resveratrol and oxyresveratrol. Here we tested the antiviral effect of these two compounds on African swine fever virus, the only member of the newly created family *Asfarviridae* and a serious limitation to porcine production worldwide. Our results show a potent, dose-dependent antiviral effect of resveratrol and oxyresveratrol *in vitro*. Interestingly, this antiviral activity was found for these synthetic compounds and also for oxyresveratrol extracted from new natural sources (mulberry twigs). The antiviral effect of these two drugs was demonstrated at concentrations that do not induce cytotoxicity in cultured cells. Moreover, these antivirals achieved a 98–100% reduction in viral titers. Both compounds allowed early protein synthesis but inhibited viral DNA replication, late viral protein synthesis and viral factory formation.

1. Introduction

Natural trans polyphenolic stilbenes or stilbenols are products of secondary metabolism that are produced by plants in response to biotic and abiotic stress. Resveratrol, piceatannol and oxyresveratrol are the main representatives of this group of compounds. Stilbenes have numerous remarkable biological properties, pointing to their potential as therapeutic agents in human health. The most known stilbene is resveratrol (trans-3,5,4′-trimethoxystilbene), which is present in the skin of grape berries and in more than 70 other plants (Langcake and Pryce, 1976). The most frequent commercial source of this compound is found in the roots of *Polygonum cuspidatum*, a plant that has been used in traditional Chinese medicine for centuries. Resveratrol is claimed to protect against cancer, heart disease, neurodegenerative disease and inflammation. In addition, it exerts free radical scavenging, antiviral and antioxidant activity (Baur and Sinclair, 2006; Cucciolla et al., 2007; Marques et al., 2009).

Another relevant stilbene is oxyresveratrol (trans-2,3′,4,5′-tetramethoxystilbene). This hydroxylated analog of resveratrol with high structural and biological similarity is present mainly in *Morus alba* bark (Shin et al., 1998) as well as in a few other plants (Sritulakuk et al., 1998; Ban et al., 2006). However, as the natural sources of oxyresveratrol are much more limited than those of resveratrol, its effects have not been as extensively addressed.

Oxyresveratrol is a potent antioxidant and free radical scavenger (Lorenz et al., 2003), and an effective tyrosinase inhibitor (Sritulakuk et al., 1998; Shin et al., 1998; Li et al., 2007). The most documented biological effect of oxyresveratrol is in the field of neuroprotection (Andrabi et al., 2004; Ban et al., 2006; Chao et al., 2008).

One of the many biological effects of these stilbenols is their antiviral action. Resveratrol shows potent antiviral activity against various families of DNA and RNA viruses and it seems that this compound interferes with viral infection by altering cellular pathways rather than by acting directly against the virus itself (Campagna and Rivas, 2010). The first report on resveratrol was published in 1999. The authors showed that the addition of this drug in the first 6 h of herpes simplex virus (HSV), HSV-1 and HSV-2 infection blocked viral replication in a dose-dependent manner (Docherty et al., 1999). Other members of the *Herpes viridae* family are also susceptible to resveratrol treatment. Moreover, replication of VZV (varicella-zoster virus), HCMV (human cytomegalovirus) and EBV (Epstein–Barr virus) is inhibited by resveratrol in a dose-dependent manner (Docherty et al., 2006; Evers et al., 2004; Kapadia et al., 2002). This drug also inhibits polyomavirus replication by blocking the synthesis of viral DNA *in vitro* (Berardi et al., 2009). It has also been shown to exert strong antiviral activity against the influenza virus *in vitro* and *in vivo* by inducing a decrease in the translation of late viral proteins (Palamara et al., 2005).

* Corresponding author. Tel.: +34 91 347 6896, fax: +34 91 347 8771.
E-mail address: calonso@inia.es (C. Alonso).

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Several authors have analyzed the effect of resveratrol on HIV-1 (human immunodeficiency virus-1) infection and various mechanisms of action have been proposed (Heredia et al., 2000; Krishnan and Zeichner, 2004; Wang et al., 2004; Zhang et al., 2009). Recent publications have demonstrated that resveratrol inhibits the growth of vaccinia virus (Cheltsov et al., 2010). Nevertheless, in contrast to its antiviral activity against other viruses, resveratrol enhances HCV (hepatitis C virus) replication (Nakamura et al., 2010).

Oxyresveratrol also shows antiviral activity. The inhibitory activity of this drug in HSV-1 (Chuanasa et al., 2008) and in VZV infections (Sasivimolphan et al., 2009) has been well documented.

African swine fever virus (ASFV) is a large enveloped DNA virus with a genomic composition similar to that of poxviruses, although the virion structure and morphology resemble those of iridoviruses (Dixon et al., 2000). It causes an acute haemorrhagic fever in domestic pigs, causing mortality rates approaching 100%. Unfortunately, no effective vaccine against ASFV is available. Consequently, the development of new antiviral agents against this devastating virus is crucial.

Here we studied the effect of the stilbenols resveratrol and oxyresveratrol on the replication of ASFV. Given the limited availability of oxyresveratrol on the market, we used two protocols to obtain the compound, namely extraction from *M. alba* twigs, and chemical synthesis. The efficiency of these approaches was compared. We then analyzed the capacity of resveratrol and oxyresveratrol to protect Vero cells from ASFV infection.

2. Material and methods

2.1. Source of stilbenols

The oxyresveratrol used was obtained by two protocols. One was based on the isolation from *M. alba* twigs, and the other by chemical synthesis of the compound.

The isolation procedure was performed as described previously (Li et al., 2007). Briefly, 200 g powdered dried mulberry twigs was extracted with methanol. After combination and concentration, about 7 g of dried extract was dissolved in deionized water. It was subsequently successively partitioned with chloroform, ethyl acetate, and n-butanol. The ethyl acetate fraction (3.5 g) was further isolated on a Sephadex LH-20 column and silica gel column to obtain pure oxyresveratrol.

Oxyresveratrol was also prepared by standard synthetic procedures, as outlined in Fig. 1, starting from the commercially available 3,5-dimethoxybenzyl bromide (2), which was efficiently converted into the corresponding 3 phosphonate by a Michael–Arbuzov reaction with triethyl phosphite. Subsequently, a Horner–Emmons–Wadsworth reaction of the 3 phosphonate with 2,4-dimethoxybenzaldehyde led to an 80% yield of the mixture of stilbenes 4 but with modest trans selectivity (75:25). After treatment with iodine, the cis isomer was fully converted into the stilbene with the desired trans geometry E-4. Finally, (E)-3,5,2′,4′-tetramethoxystilbene (E-4) was demethoxylated by thermal treatment with methylmagnesium iodide, providing the tetraol 1 in a 21% yield. This compound showed identical spectroscopic data to those shown by a natural sample of oxyresveratrol isolated from mulberry twigs using the above described protocol and purities of 96–98% were obtained with both preparation procedures. Our data matched those reported for this compound (Choi et al., 2006).

2.2. Cell culture and viruses

Vero (African green monkey kidney) cell lines were obtained from the American Type Culture Collection (ATCC) and grown and maintained at 37 °C in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 5% calf fetal serum, 100 IU/ml penicillin, 100 μg/ml streptomycin and 2 mM L-glutamine.

ASFV isolate BA71V was used. In fluorescence experiments we used an infectious recombinant ASFV, B54GFP-2, which expresses and incorporates into the virus particle a chimera of the p54 envelope protein fused to the enhanced green fluorescent protein (EGFP) (Hernáez et al., 2006). Preparation of viral stocks, titrations, and infection experiments were carried out in cells as previously described (Enjuanes et al., 1976).

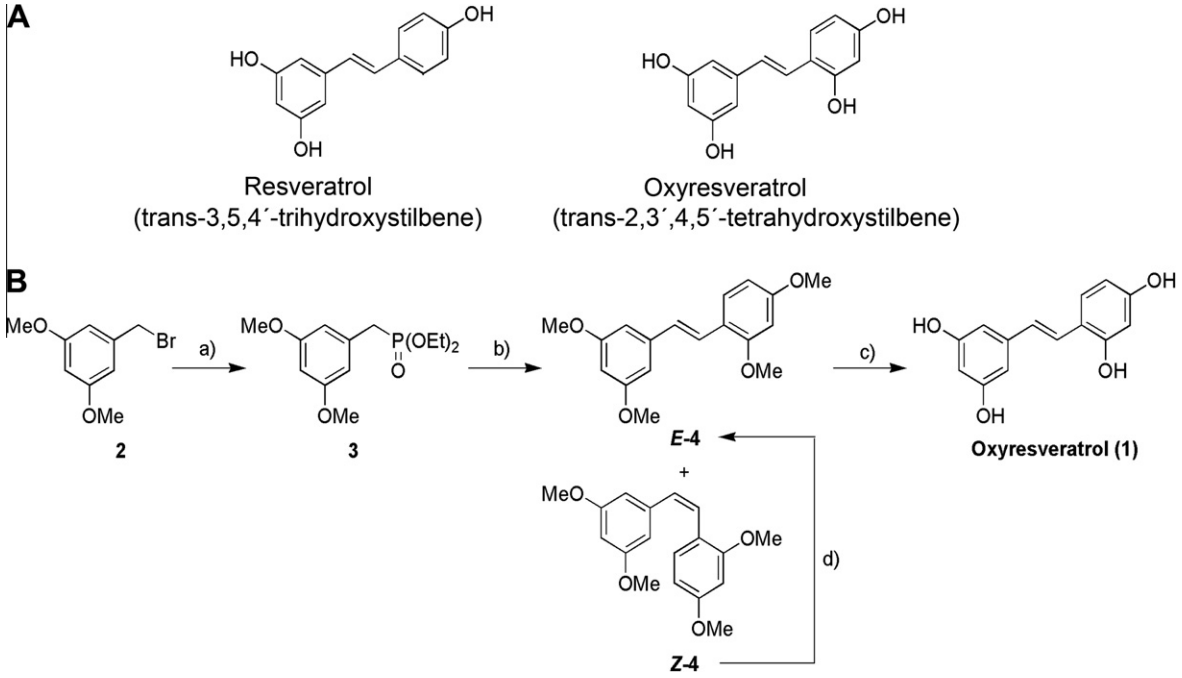

Fig. 1. (A) Structure of Stilbenols. (B) Synthesis of oxyresveratrol (1) Reagents and conditions: (a) $P(OEt)_3$, TBAI, 130 °C; (b) NaH, 2,4-$(MeO)_2C_6H_3CHO$, THF, from 0 °C to room temperature; (c) MeMgI, heat; (d) I_2, heptane, reflux.

2.3. Drug concentrations

Drugs were dissolved in DMSO, resulting in a final concentration of 40 mg/ml to form stock solution. Resveratrol was purchased from Sigma. The cytotoxic effect of resveratrol and oxyresveratrol was examined. Cells were seeded onto 96-well plates in triplicates for each assay. After 24 h cells were treated with resveratrol or oxyresveratrol at final concentrations ranging from 1 to 100 μg/ml. After incubation for 48 h, viable cells were counted using the CellTiter 96 Non-radioactive Cell Proliferation Assay (Promega) and following the Manufacturer's instructions. We also studied the cytotoxic activity of the organic solvent DMSO.

2.4. Effect of resveratrol and oxyresveratrol on viral production

Cells were infected with ASFV at a multiplicity of infection (moi) of 1 pfu/ml and resveratrol or oxyresveratrol (extracted and synthetic) were added at the indicated final concentrations after the absorption phase (30 min). Infection was allowed to proceed for 48 h and then total virus was collected and titrated by plaque assay in triplicate samples on cells. The infection assays were carried out at non-toxic concentrations of the drugs. The organic solvent DMSO has been reported not to modify ASFV infectivity at non-cytotoxic concentrations (Hernáez and Alonso, 2010).

2.5. Effect of the drugs on viral protein synthesis

To analyze the effect of resveratrol and oxyresveratrol (extracted and synthetic) on viral protein synthesis, cells were infected with 1 pfu per cell of ASFV. After a 2-h incubation for virus adsorption, cells were incubated for 2 days in the absence or presence of various concentrations of the two drugs. The infected cells were dissociated in Laemmli buffer, boiled for 5 min at 95 °C, resolved by SDS–PAGE, blotted onto nitrocellulose membranes, and incubated with mouse monoclonal antibodies anti p30, anti p72 or anti-tubulin (Sigma) diluted 1/1000, 1/500 or 1/2000, respectively. Bound antibodies were detected with a second peroxidase conjugated antiserum before detection by ECL system (Amersham), following the Manufacturer's instructions. Band densitometry was performed with Image Lab software (BioRad) and data were normalized to control values.

2.6. Detection of ASFV DNA by quantitative PCR

DNA was purified from cells infected with ASFV (moi of 1 pfu/ml) and treated with the indicated concentration of resveratrol or natural oxyresveratrol for 48 h, using the kit "Dneasy blood and tissue" (Quiagen, UK), following the Manufacturer's instructions. Uninfected cells and cells infected in the absence of drug were used as controls. The PCR assay used fluorescent hybridization probes to amplify a region of the p72 viral gene, as described previously (King et al., 2003). For real-time PCR, 300 ng of the DNA template was added to a final reaction mixture of 20 μl comprising 1× PCR quantimix (Biotools), 50 pmol sense, 50 pmol anti-sense primers and 5 pmol probe, and then amplified with a RT-PCR instrument (Corvette). DNA from ASFV viral stock was extracted, diluted from 10^{-1} to 10^{-6} and then used as a quantification control. Amplification conditions included an initial denaturation step of 94 °C for 10 min, followed by 45 cycles of 94 °C for 15 s and 58 °C for 60 s.

2.7. Detection of viral factories by fluorescence

At 8, 16, 24 and 48 h postinfection (hpi), cells growing on coverslips were infected with a recombinant ASFV Ba54GFP-2 in the absence or presence of resveratrol (1, 5 or 10 μg/ml) or synthetic oxyresveratrol (5, 10 or 30 μg/ml). Cells were fixed with 4% paraformaldehyde for 12 min, permeabilized with PBS–0.1% Triton X-100 for 15 min and then incubated with Topro-3 (Invitrogen) 1/1000 in PBS1X for DNA staining. ASFV Ba54GFP-2-infected cells show a characteristic viral factory at 16 hpi (Hernáez et al., 2006). The number of viral factories were counted in duplicate experiments of treated and control cells in a total of 5×10^4 cells per well. Confocal microscopy was carried out in a Leica TCS-SPE confocal microscope.

3. Results

3.1. Evaluation of the cytotoxicity of resveratrol and oxyresveratrol

The cytotoxicity of resveratrol and other potent antioxidant substances has previously been documented (Delmas et al., 2006; Juan et al., 2008; Saiko et al., 2008). We tested a range of concentrations at which resveratrol and oxyresveratrol may exhibit potential cytotoxic activity on cells. After treatment for 48 h with resveratrol or oxyresveratrol, the number of viable cells was determined using the MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H tetrazolium) reduction assay (Promega). The organic solvent DMSO had no effect on cell survival (Fig. 2). Resveratrol inhibited cell growth at concentrations above 10 μg/ml and the cytotoxic concentration 50 (50% of cell survival or CC_{50}) was 30 μg/ml. The cytotoxic effect of both extracted and synthetic oxyresveratrol on cells was lower (Fig. 2). In fact, oxyresveratrol inhibited cell growth at concentrations above 30 μg/ml, with a cell death rate of about 50% at concentrations of 75 and 50 μg/ml, respectively. Resveratrol exhibited higher cytotoxicity than the two sources of oxyresveratrol. Next, we selected optimal non-toxic working concentrations to assay for an antiviral effect.

3.2. Effect of resveratrol and oxyresveratrol on viral production

Semiconfluent cells were infected with ASFV and after the absorption phase resveratrol or oxyresveratrol from the two sources were added at the final concentrations indicated. At 48 h, cells and media were harvested and viral production was determined by a plaque assay.

ASFV production in cells in the presence of resveratrol at a non-cytotoxic concentration of 5 μg/ml decreased viral production by 100% (Fig. 3A). Oxyresveratrol (synthetic and natural extracted) also showed a strong inhibition of viral titers in a dose-dependent

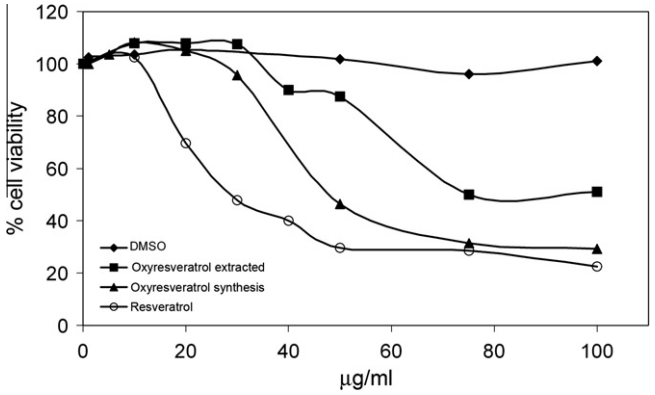

Fig. 2. The cytotoxicity of resveratrol and oxyresveratrol was analyzed and compared in Vero cells after 48 h of exposure to the drugs. The mean and standard deviation shown correspond to three independent experiments.

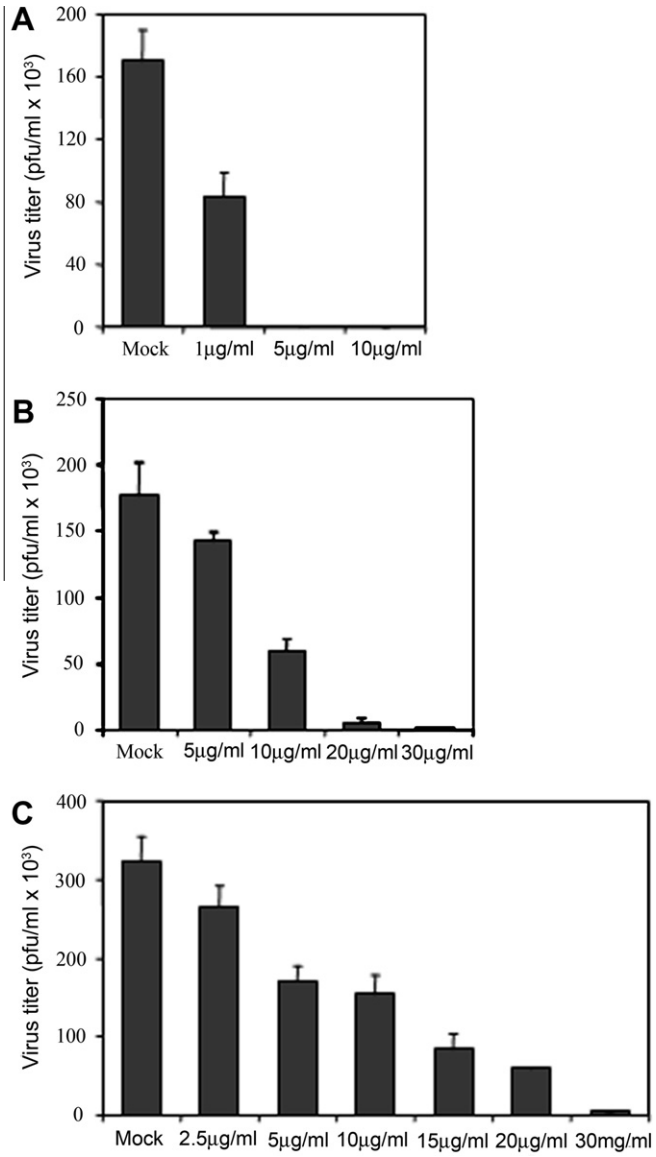

Fig. 3. ASF virus productivity in Vero cells in the presence of resveratrol (A), synthetic oxyresveratrol (B), or purified oxyresveratrol (C). Vero cells were infected with ASFV in the presence of resveratrol or oxyresveratrol for 48 h and infective virus production was titrated by plaque assay. Infected cells incubated with compound-free medium (mock) were used as control. Data represent the average of three independent experiments and standard deviation is shown.

manner, with a reduction of 98.5% for synthetic and 99.15% for natural oxyresveratrol at concentrations of 30 μg/ml (Fig. 3B and C).

The inhibitory concentration to reduce 50% of virus production (IC_{50}) was estimated to be 1 μg/ml for resveratrol and 10 μg/ml for both synthetic and natural extracted oxyresveratrol.

3.3. Effect of resveratrol and oxyresveratrol on viral protein synthesis

Western blot was used to analyze the synthesis of the early and late viral proteins p30 and p72, respectively, in the presence of resveratrol and the two sources of oxyresveratrol. The amount of p30 was similar in treated and untreated infected cells, while the amount of p72 was decreased in the infected cells in a dose-dependent manner as compared with untreated infected cells. Both drugs showed similar inhibition rates in late protein synthesis but at different concentrations. The presence of drugs during the infection had a dramatic effect on the synthesis of p72 but not on p30, thereby indicating that the drugs severely affect a later stage of the virus replication cycle (Fig. 4).

3.4. Detection of ASFV DNA by quantitative PCR

Inhibition of viral DNA replication was analyzed by real-time PCR at 48 h post-infection (hpi) after incubation with increasing concentrations of the drugs. DNA from viral stock, quantified by a nanodrop spectrophotometer, was used as a quantification control. Resveratrol and oxyresveratrol extracted from natural sources produced a dose-dependent inhibition curve (Fig. 5). The presence of these drugs during the infection decreased the viral DNA synthesis about 10- and 7.1-fold in the presence of 10 μg/ml of resveratrol and 30 μg/ml of oxyresveratrol, respectively.

3.5. Detection of viral factories in the presence of resveratrol or oxyresveratrol

Cells grown on coverslips were infected with a genetically manipulated ASFV expressing the green fluorescent protein (Ba54GFP-2) (Hernáez et al., 2006) at 8, 16, 24 and 48 hpi in the absence or presence of resveratrol or natural oxyresveratrol and then analyzed by confocal microscopy. Infection with this GFP-expressing virus allowed visualization of infection progression at several time points. Starting at 8 hpi, virus replication sites, the so-called "virus factories", were visualized as the characteristic intense perinuclear fluorescent clusters at the microtubule organizing center (MTOC; (Hernáez et al., 2006)). At 16 hpi, over 98% of untreated infected cells showed these densely packed accumulations (Fig. 6). Nevertheless, drug concentrations over 1 μg/ml for resveratrol or 5 μg/ml for oxyresveratrol resulted in a drastic decrease in the number of viral factories. In those cultures, the few remaining infected cells lacked the characteristic morphology and perinuclear location of the viral factories. These cells showed fragmented clusters of viral replication, which were dispersed in the cytoplasm (Fig. 6B and C).

4. Discussion

Resveratrol is one of the natural stilbenes found in grape skin and red wine (Soleas et al., 1997) and it has been widely studied because of its antioxidant activity. Oxyresveratrol differs from resveratrol because it has an extra hydroxyl group, which enhances its antioxidant activity. Both compounds show a broad range of antiviral activity against several infections. The exact mechanism of action exerted by these molecules is still unknown but it has been suggested that they inhibit viral infection by disrupting cellular functions rather than acting against the viruses themselves. Here we have shown that resveratrol and oxyresveratrol inhibit the growth of ASFV in Vero cells to achieve full inhibition of virus titers at low concentrations of resveratrol. Also, we report that mulberry twigs provide a new natural source of antiviral compound oxyresveratrol.

In our model system, the evaluation of possible cytotoxic effects of resveratrol and oxyresveratrol was a pre-requisite. The vital cell count observed allowed us to determine a specific range of drug concentrations below cytotoxic levels. It is interesting to note that oxyresveratrol showed lower cytotoxicity than resveratrol. This feature could be critical for compound selection. This lower toxicity has been previously reported in a different system based on a lactate dehydrogenase assay. In that assay, resveratrol was found to be several times more cytotoxic than oxyresveratrol, at concentrations over 10 μM (Chao et al., 2008).

At non-toxic concentrations (5 μg/ml resveratrol or 30 μg/ml oxyresveratrol), these compounds inhibited ASFV titers by

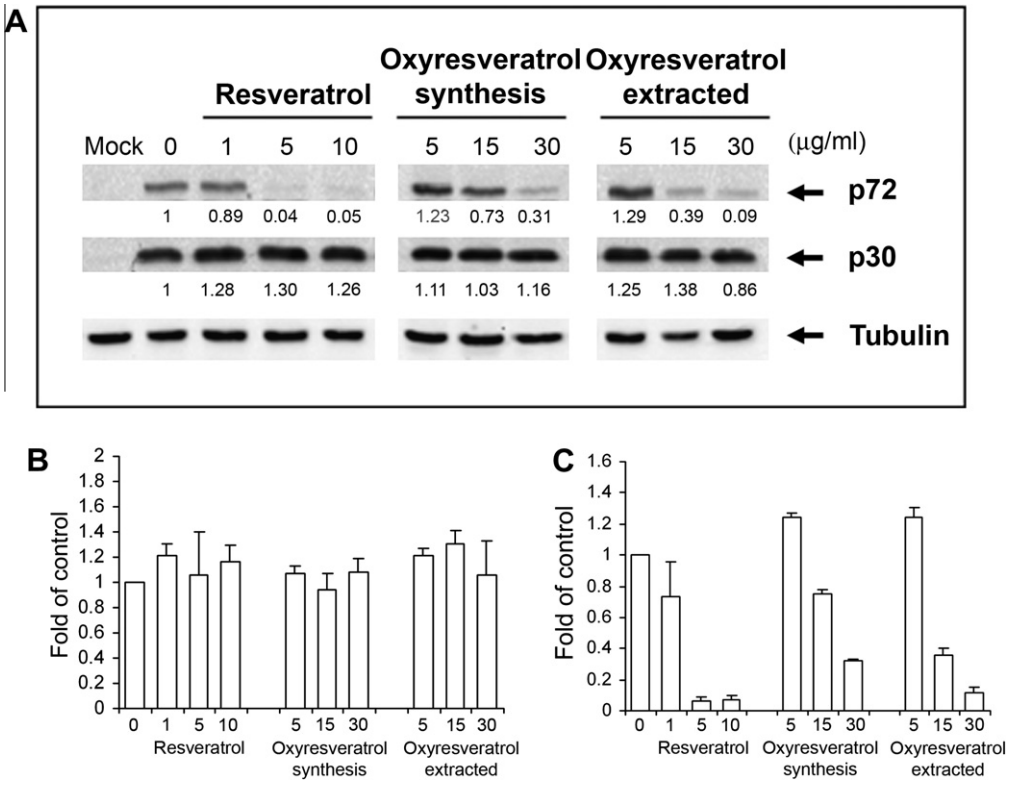

Fig. 4. Viral protein synthesis at increasing concentrations of resveratrol and oxyresveratrol. (A) Inhibition of ASFV proteins synthesis was analyzed by Western blotting of infected cell extracts with specific antibodies against viral proteins p30 (early) and p72 (late). Tubulin was used as protein load control. Quantification of the bands corresponding to p30 (B) and p72 (C) by densitometry was corrected to tubulin data and normalized to control values.

98–100%. The reduction of infective virus yields was dose-dependent and neither drug showed significant cytotoxicity. The inhibitory effect of the oxyresveratrol obtained by synthetic methods and that extracted from natural sources (mulberry twigs) was very similar.

Other chemical compounds, tested for anti-ASFV activity, were also found to reduce viral titers, such as lauryl gallate (Hurtado et al., 2008); however, a residual virus production of 10^3 pfu/ml was found when infecting at the same moi (1 pfu/ml). Others described antiviral activities in microalgae extracts (Fabregas et al., 1999) but those extracts were not able to reach full virus inhibition in a plaque reduction assay using 10^2 infective viruses.

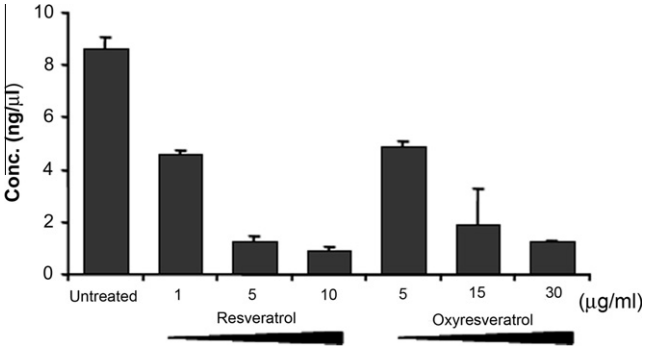

Fig. 5. Viral replication analysis: ASFV genome copy number was analyzed by real-time PCR. DNA from Vero cells infected in the presence of increasing concentrations of resveratrol (1, 5 and 10 μg/ml) or oxyresveratrol extracted from natural sources (5, 15 and 30 μg/ml) was used as template for qPCR. DNA from ASFV viral stock was used as quantification control.

Other efforts are underway to find new targets for antivirals against this virus (Hernáez et al., 2010).

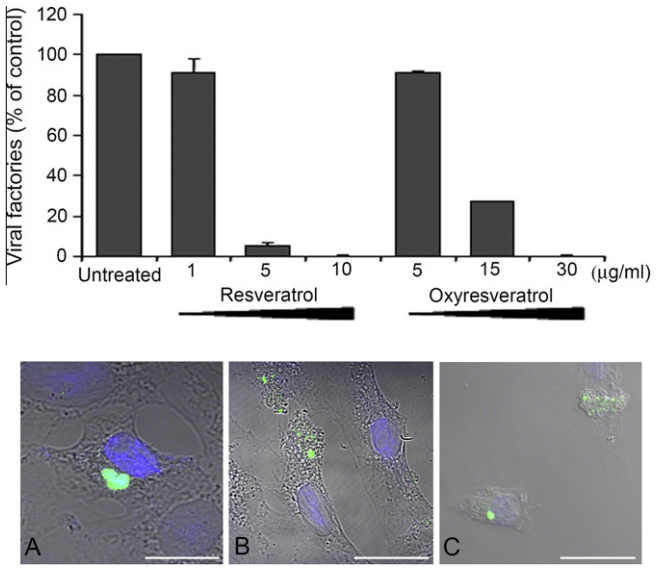

Fig. 6. The formation of viral replication sites was impaired in the presence of resveratrol or oxyresveratrol extracted from natural sources. Vero cells were infected with B54GFP-2 in the presence of a range of concentrations of resveratrol (1, 5 and 10 μg/ml) or oxyresveratrol (5, 15 and 30 μg/ml). At 16 hpi, cells were fixed and the formation of viral factories was analyzed by confocal microscopy. The percentage of viral factories found compared to the untreated control is shown in the graphics. Viral factories in infected cells untreated (A) and treated with resveratrol (B) or oxyresveratrol (C). Bar. 25 μm.

In order to examine the precise stage of the virus cycle blocked by stilbenes, we analyzed the presence of specific early and late proteins induced by ASFV in the presence or absence of the drugs. The synthesis of late protein p72 was markedly decreased while that of the early protein p30 was not affected. These findings strongly suggest that the early phases of ASFV replication are not targets for the antiviral effect of resveratrol or oxyresveratrol. As early infection steps are not affected, a later infection step, necessary for viral replication and subsequent infection rounds, should be impaired by these antivirals. Moreover, both resveratrol and oxyresveratrol had similar pattern of inhibition.

Quantitative PCR analysis showed an approximately 50% decrease in viral genome copy numbers in Vero cells infected in presence of 1 μg/ml of resveratrol or 5 μg/ml of oxyresveratrol. Thus, resveratrol and oxyresveratrol inhibited viral DNA replication, subsequently inhibiting late viral protein synthesis.

Protein p54 is a late virus protein essential for virus replication and it is incorporated into the external envelope of virions (Rodriguez et al., 1994, 1996). B54GFP-2 is an infectious recombinant ASFV that expresses and incorporates a chimera of the p54 protein fused to the enhanced green fluorescent protein (EGFP) into the virus particle. Like p54, B54GFP-2 shows an intracellular distribution that is fully coincident with the ASFV replication site, also called the viral factory, at late post-infection times (Hernáez et al., 2006). The ASFV replication site is characteristically found in a perinuclear area, coincident with the MTOC. We studied the formation of viral factories in Vero cells infected with Ba54GFP-2 in the absence and presence of resveratrol and oxyresveratrol. Both drugs significantly reduced the number of viral factories and modified morphology and localization of viral replication sites, which appeared dispersed. This finding indicates that the organization and formation of viral replication sites results was impaired by these antivirals.

There is an urgent need for countermeasures against ASFV. While vaccination is the first option in the prevention of animal viral diseases, other alternatives, such as antiviral agents, are starting to gain relevance in the control and eradication of these diseases. Despite the intense effort to find a vaccine for ASFV, none is yet available and the control of the disease relies on the early diagnosis and "stamping-out" of the entire pig population in farms in affected areas. In this scenario, there is an urgent need for alternatives and research should be oriented to antiviral research.

When an outbreak of ASF occurs, one option could be to apply antiviral prophylaxis to treat pigs in farms located close to the infected farm in order to create a "safety ring" around the outbreak focus and thus control the spread of the infection. Stabilizing the epidemic area by means of an antiviral agent may give the authorities time to organize the appropriate countermeasures and to reduce the number of animals to be slaughtered.

We consider that greater effort should focus on the development antiviral drugs that could be added to feed; this approach would facilitate the treatment of large numbers of animals in a very short time. Furthermore, the characterization of natural sources of antivirals that could be obtained at a reasonable cost could prevent shortage and would facilitate administration routes.

5. Conclusions

Our results demonstrate a potent, dose-dependent anti-ASFV effect of resveratrol and oxyresveratrol. We propose that mulberry twigs, which contain large amounts of oxyresveratrol, provide a new natural source of starting material for the development of antivirals against ASFV. Therefore, both resveratrol and oxyresveratrol might be potential tools for the treatment or prevention of ASFV infection.

Acknowledgments

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