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ORIGINAL INVESTIGATION

Natural and semisynthetic diterpenoids with antiviral and immunomodulatory activities block the ERK signaling pathway

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Abstract The pathogenesis of many viral infections lies on the damage caused by the immune response against the virus. Current antiviral drugs do not act on the inflammatory component of the disease. Thus, new compounds that inhibit both viral multiplication and the immunopathology elicited by the virus are an approach that should be considered. In the present study, we identified two jatropholones (2A and 5B) and one carnosic acid derivative (9C) that significantly inhibited multiplication of TK+ and TK− strains of HSV-1 in Vero cells. Compounds 2A, 5B and 9C also prevented HSV-1- and TLRs-induced inflammatory response in cultivated murine macrophages. In macrophages infected with HSV-1, the inhibitory effect of compounds 2A, 5B and 9C on TNF-α and IL-6 production could be associated with the block of ERK pathway, whereas NF-κB pathway was not hampered by any of the compounds. Besides, 2A, 5B and 9C also inhibited ERK pathway and reduced TNF-α production in macrophages stimulated with TLR2, TLR4 or TLR9 agonists and were able to hinder IL-6 secretion after activation with TLR2 or TLR4, but not with TLR9. The immunomodulatory effect of 2A, 5B and 9C in macrophages infected with HSV-1 may be a consequence of the inhibition of ERK pathway activated by TLRs. The availability of compounds with both antiviral and immunomodulatory properties which affect TLR signaling pathways might be a useful strategy to control the progress of virus-induced disease.

Keywords Antiviral · Immunomodulatory · Carnosic acid · Jatropholones · HSV-1 · ERK pathway

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Introduction

Viral and host components may contribute to the pathogenesis of different viral infections. Many of these viruses are of public health significance because they cause damage through triggering an immunopathology. Most of the current antiviral drugs target viral proteins but are ineffective to act on the inflammatory component of the disease. To ameliorate the symptoms of disease, the use of antiinflammatory compounds is recommended, in combination with antiviral drugs to treat many virus-induced diseases, such as herpetic infections [1–3]. The symptoms of the herpetic disease can be alleviated by immunosuppressive reagents such as systemic corticosteroids and cyclosporine A, and treatments also include acyclovir (ACV) to mitigate viral reactivation due to the immunosuppression caused by these drugs [4]. Nevertheless, the use of commercial antivirals against herpesviruses, particularly during viral recurrences and in immunosuppressed individuals, entails the occurrence of viral mutant populations resistant to such drugs. In consequence, and taking into account the adverse side effects described for corticosteroids, there is an urgent need for new drugs effective against these immunopathologies of viral origin [2].

Thus, the finding of novel compounds that inhibit both viral multiplication and the immunopathology triggered by the virus is an approach that should be considered.

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Medicinal plants have been used to treat viral infections for decades. The chemical diversity and broad spectrum of antiviral activity of natural products make them ideal candidates for new therapeutics [5]. In fact, we have reported that meliacine (MA), an antiviral principle present in partially purified leaf extracts of *Melia azedarach* L., reduces the viral load and abolishes the ocular inflammatory reaction and neovascularization along the development of herpetic stromal keratitis (HSK) in mice [6]. Bioassay-guided purification of MA has led to the isolation of the tetranortriterpenoid 1-cinnamoyl-3,11-dihydroxymeliacarpin (CDM), which hinders herpes simplex virus type 1 (HSV-1) multiplication and exhibits immunomodulatory properties in vitro and antiangiogenic activities both in vitro and in vivo [7–10].

A great number of plant-derived substances, such as di- and triterpenes with antiviral and/or immunomodulatory properties, have been described [5]. Two diterpenes isolated from *Rosmarinus officinalis* L. and *Salvia officinalis* L., carnosic acid and carnosol and their derivatives, show antiviral and antiinflammatory activities [11, 12]. Likewise, diterpenes isolated from *Tripterygium wilfordii* L., and the diterpene compound taxol extracted from *Taxus brevifolia* L., which is commercially used as an antitumoral drug, also display antiviral and immunomodulatory effects [13, 14].

Jatropha species (*Euphorbiaceae*) contain compounds with remarkable biological activity [15]. *Jatropha isabelli Muell Arg* possesses a gastroprotective effect ascribed to its diterpene constituents, which have been identified as jatrophone and jatropholones A and B [16, 17].

The aim of the present study was to examine the antiherpetic activity of natural and semisynthetic jatropholones and carnosic acid derivatives and their ability to modulate the production of different cytokines and signaling pathways in macrophages stimulated by HSV-1 and TLR ligands.

Materials and methods

Cells and viruses

The murine macrophage cell line J774A.1 was kindly provided by Dr. Osvaldo Zabal (INTA—Buenos Aires, Argentina) and grown in RPMI 1640 medium supplemented with 10 % inactivated fetal bovine serum (FBS) and maintained in RPMI supplemented with 2 % inactivated FBS.

Vero cells were grown in Eagle's minimal essential medium (MEM) supplemented with 10 % inactivated FBS and maintained in MEM supplemented with 1.5 % inactivated FBS.

HSV-1 KOS, thymidine kinase-deficient (TK−) B2006 and Field strains of HSV-1 were used and propagated at low multiplicity of infection (m.o.i.).

Reagents

LPS (TLR4 ligand) from *Escherichia coli* serotype 055:B5 was obtained from Sigma. Pam2CSK4 (TLR2 ligand) and ODN2395 (TLR9 ligand) were purchased from InvivoGen. The rabbit polyclonal anti-IκBα antibodies, anti-ERK1 and anti-p-ERK1/2, the mouse monoclonal antibody anti-gD of HSV-1 and the peroxidase-conjugated goat anti-rabbit or anti-mouse antibodies were obtained from Santa Cruz Biotechnology. The anti-actin antibody was obtained from Calbiochem. Secondary goat anti-rabbit FluoroLinkTM CyTM2 and anti-mouse FluoroLinkTM CyTM3 antibodies were purchased from GE Healthcare Bio-Sciences.

Compounds

Jatropholones A and B were isolated from the rhizomes of *Jatropha isabelii* as described by Pertino et al. [16, 17]. The derivatives were synthesized according to the procedures of Pertino et al. [16] and Theoduloz et al. [18]. Carnosic acid was isolated from the leaves of *R. officinalis* L. and used to synthesize the different derivatives following the procedures previously reported [19, 20]. The purity of all derivatives was over 98 % as assessed by ^{1}H NMR spectrometry.

Cytotoxicity assay

Cell viability was determined using the tetrazolium salt MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (Sigma) according to the manufacturer's instructions. J774A.1 and Vero cells were seeded at a concentration of 10^4 cells/well in 96-well plates and grown at 37 °C for 24 h. The culture medium was replaced by medium containing the compounds in triplicate, and cells were incubated for 24 h. The absorbance was measured on a Eurogenetics MPR-A 4i microplate reader using a test wavelength of 570 nm and a reference wavelength of 630 nm. Results were expressed as a percentage of absorbance of treated cell cultures with respect to untreated ones. The cytotoxic concentration 50 (CC_{50}) was calculated as the concentration of compounds required to reduce cell viability by 50 % relative to untreated cells.

Antiviral activity

Cytopathic effect assay

Cells grown in 96-well plates were infected or not with HSV-1 at a m.o.i. of 0.1 PFU/cell. After 1-h adsorption at 37 °C, the inoculum was removed and medium containing or not the compounds was added, in triplicate. The plates were incubated at 37 °C until 24 h post-infection (p.i.),

when 100 % of cell death was observed in virus control. Then, cells were fixed with 10 % formaldehyde for 15 min at room temperature, washed once with distilled water and stained with 0.05 % crystal violet in 10 % ethanol over 30 min. Afterward, cells were washed once and eluted with a solution of 50 % ethanol and 0.1 % acetic acid in water. The absorbance of each well was measured on an Eurogenetics MPR-A 4i microplate reader using a test wavelength of 590 nm. Results were analyzed as the percentage of absorbance of treated and infected cells compared with control (untreated/uninfected) cells. We considered the untreated/uninfected control cells as 100 % of cell survival.

Viral yield assay

Cells grown in 96-well plates were infected with HSV-1 at a m.o.i. of 0.1 PFU/cell. After 1-h adsorption at 37 °C, the inoculum was removed and medium containing the compounds was added, in triplicate. The plates were incubated at 37 °C until 24 h p.i. After cell disruption by three cycles of freezing and thawing, supernatants were harvested and pooled. Virus yields were titrated by plaque assay in Vero cells, and the effective concentration 50 (EC_{50}) was calculated as the concentration of compounds required to reduce viral yields by 50 % relative to the untreated virus control.

Indirect immunofluorescence assay (IFI)

Subconfluent cells grown on glass coverslips in 24-well plates were fixed with methanol for 10 min at −20 °C. After three washes with PBS, coverslips were inverted on a drop of diluted primary antibody for 30 min at 37 °C, then returned to culture dishes and subjected to three additional washes with PBS. Afterward, cells were incubated with diluted secondary antibody for 30 min at 37 °C. Finally, coverslips were rinsed, mounted and photographed with an Olympus BX51 microscope with epifluorescence optics.

Virucidal effect

HSV-1 KOS (10^7 PFU) was diluted in culture medium containing or not each compound and incubated for 30, 60 and 120 min at 37 °C. Aliquots were diluted to a non-inhibitory drug concentration and titrated by plaque assay on Vero cells.

Adsorption and penetration assay

Subconfluent Vero cells grown in 24-well plates (2×10^5 cells) were inoculated with 100 PFU of HSV-1 and adsorbed for 1 h at 4 °C with or without the compounds. To quantify adsorbed virus, cells were washed twice with cold PBS, overlaid with medium containing 0.7 % methylcellulose and incubated at 37 °C for 3 days.

To determine internalized virus, cells were incubated at 37 °C to maximize virus penetration after viral adsorption at 4 °C for 1 h, with or without the compounds. At 120 min, monolayers were washed twice with PBS and treated for 1 min with citrate buffer (pH 3). To quantify internalized virus, cells were washed twice with cold PBS, overlaid with medium containing 0.7 % methylcellulose and incubated at 37 °C for 3 days.

Time-of-compound-addition studies

Compounds were added to confluent monolayers of Vero cells infected with HSV-1 at a m.o.i. of 1, together with viral inocula, or at 2, 6 and 12 h after infection. Cells were further incubated at 37 °C till 24 h p.i. and subjected to three cycles of freeze–thawing followed by centrifugation at low speed. Supernatants were titrated by plaque assay in Vero cells.

Western blot analysis

Whole extracts of cells grown in 24-well plates for 24 h were loaded on 10 % sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene fluoride membranes for 60 min at 75 mA. Membranes were blocked in PBS containing 5 % unfitted milk overnight and then incubated with diluted primary antibodies overnight at 4 °C. After washing, membranes were incubated with diluted peroxidase-conjugated antibodies for 1.5 h at 37 °C. The immunoreactive bands were visualized using an enhanced chemiluminescence system (ECL, PerkinElmer).

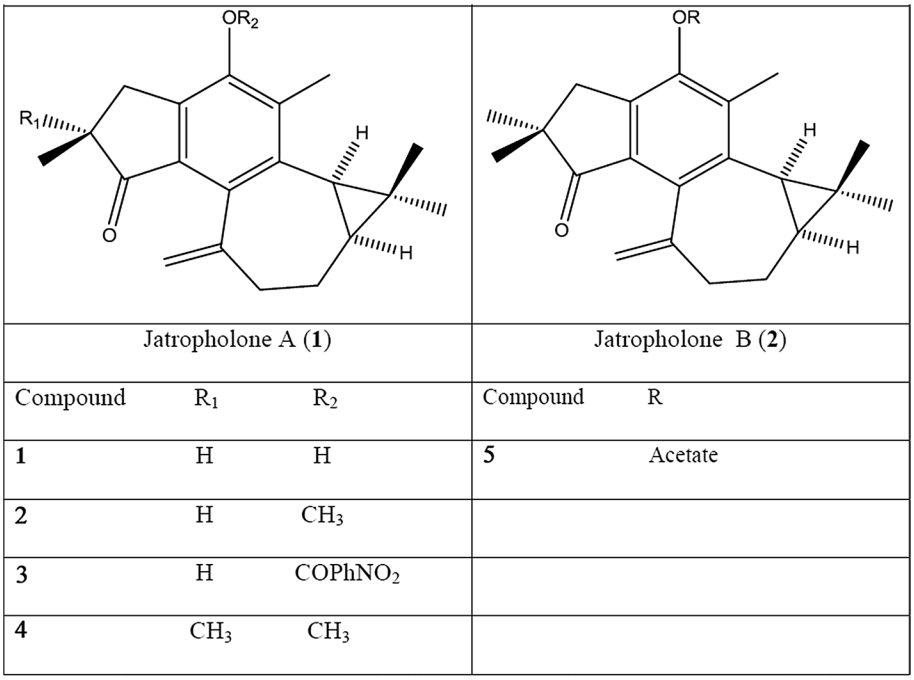
Cytokine determination

Cells were frozen and thawed, and then, supernatants were harvested, centrifuged at 1,000 rpm for 10 min, and cytokines were quantified by ELISA, in triplicate. Mouse TNF-α and IL-6 were quantified by commercial ELISA sets (BD OptEIATM, Becton–Dickinson) according to the manufacturer's instructions.

Transfections

Transfection assays with Lipofectamine 2000 reagent (Invitrogen) were performed according to the manufacturer's instructions. The NF-κB-LUC reporter vector and RSV-β-gal plasmid were kindly provided by Dr. Susana Silberstein (Universidad de Buenos Aires). RSV-β-gal, which codes for the bacterial β-galactosidase gene under the control of the viral RSV promoter, was used as second reporter control plasmid.

Fig. 1 Structure of the jatropholone derivatives



Jatropholone A (**1**)			Jatropholone B (**2**)	
Compound	R_1	R_2	Compound	R
1	H	H	**5**	Acetate
2	H	CH_3		
3	H	$COPhNO_2$		
4	CH_3	CH_3		

Statistical analysis

Statistical analysis was done by one-way ANOVA followed by a Tukey's multiple comparison test.

Results

Compound screening

One natural jatropholone (2A), four semisynthetic jatropholones (1A, 3A, 4A and 5B) and five carnosic acid derivatives (6C, 7C, 8C, 9C and 10C) were selected to analyze their antiviral activity (Figs. 1, 2). First, the cytotoxic effect of all compounds was evaluated. For that purpose, Vero cells were treated with the jatropholones and carnosic acid derivatives (1–300 μM) and, after incubation at 37 °C for 24 h, a MTT assay was performed. No cytotoxicity was found at the concentrations tested, being the CC_{50} value higher than 300 μM (Table 1). This result was in accordance with already reported findings which showed that most of these compounds were no cytotoxic for other cell lines [16–20].

Next, to screen the antiviral activity of the compounds, Vero cells grown in 96-well plates were infected with HSV-1 KOS and treated or not with 300, 150 and 75 μM of all compounds for 24 h. From ten compounds assayed, only compounds 2A, 5B and 9C prevented 100 % of the cytopathic effect induced by HSV-1 at all concentrations tested. The other compounds did not protect the cytopathic effect even at 300 μM (Table 1).

Antiviral effect of three hit compounds

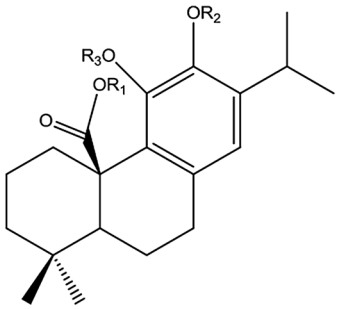
Identification of antiviral effect by indirect immunofluorescence assay (IFI)

First, we corroborated the antiviral effect observed by means of an IFI staining. For that purpose, Vero cells infected with HSV-1 KOS (m.o.i = 1) treated or not with 75 μM of 2A, 5B and 9C for 24 h were analyzed by an IFI staining using a mouse monoclonal anti-glycoprotein D (gD) antibody. We found that the majority of infected control cells expressed gD protein, most of them clustered in characteristic HSV-1 foci (92 %). A limited appearance of fewer and scattered foci expressing gD was observed when infected cells were treated with any of the three compounds. Thus, the number of fluorescent cells expressing gD was reduced to 29, 27 and 25 % in infected cells treated with 2A, 5B and 9C, respectively ($p < 0.05$) (Fig. 3a).

Virucidal assay

To establish whether these compounds produced a direct effect on the viral particle, we performed a virucidal assay.

After incubation with 75 μM of 2A, 5B and 9C, for 30 min, HSV-1 titers were 6×10^6, 8×10^6 and 5×10^6 PFU/ml, respectively, whereas an infectivity of 7×10^6 PFU/ml was obtained in untreated viral suspensions. Regardless the time of incubation, no reduction in viral titers was observed after treatment with 2A, 5B and 9C with respect to untreated control, suggesting that none of them exerted a virucidal activity.



Compound	R_1	R_2	R_3
6C	CH_3	$COCH_2CH_2CH_3$	$COCH_2CH_2CH_3$
7C	CH_3		
8C	CH_3		
9C	CH_3		
10C	$CH_2CH_2CH_3$	CH_3	$CH_2CH_2CH_3$

Fig. 2 Structure of the carnosic acid derivatives

Viral adsorption/penetration and time-of-addition experiments

Next, we analyzed whether compounds 2A, 5B and 9C interfere with virus adsorption and penetration. No significant differences in the amount of adsorbed virus between untreated (7×10^7 PFU/ml) and infected cells treated with any of the compounds were observed ($5–8 \times 10^7$ PFU/ml). In the case of the internalization assay, no differences in viral titers corresponding to untreated (2×10^8 PFU/ml) and treated cells ($1–3 \times 10^8$ PFU/ml) were detected.

To further characterize the inhibitory action of 2A, 5B and 9C, a time-of-addition experiment has been done. For that purpose, 75 μM of 2A, 5B and 9C were added to HSV-1-infected Vero cells at different times after infection and, 24 h p.i., infectivity was determined. A significant two-log inhibition of viral yield was observed when the compounds were added until 2 h after infection ($p < 0.05$) (Fig. 3b). Only compound 9C reduced HSV-1 production when added up to 6–8 h after infection ($p < 0.05$). At later times, none of the compounds restrained virus infectivity (Fig. 3b).

Identification of antiviral effects by plaque reduction assay

To quantify the antiviral effect, the inhibition rates of the three compounds were determined. Thus, HSV-1-infected Vero cells were treated with different concentrations of compounds for 24 h. Virus replication was evaluated by plaque assay, and EC_{50} was calculated by a non linear regression. The inhibitory effects of all three compounds showed dose-dependent patterns. The corresponding EC_{50} values calculated were 20, 15 and 7 μM for 2A, 5B and 9C, respectively (Fig. 3c).

When compounds 2A, 5B and 9C were also evaluated against ACV-resistant HSV-1 strains, we found that all of them restrained Field and B2006 viral multiplication, with EC_{50} values of 28, 21 and 15 μM against Field strain and 40, 36 and 19 μM against B2006 strain, respectively (Fig. 3c).

Therefore, the three compounds exerted a potent antiviral activity against both HSV-1 TK+ and TK− strains in Vero cells by restraining an early event in HSV-1 multiplication.

Table 1 Screening of jatropholones and carnosic acid derivatives: cytopathic effect and cytotoxicity assays

Compound	Percentage of inhibition[a]			Cytotoxicity (CC_{50}, μM)
	300 μM	150 μM	75 μM	
1A	13	12	10	>300
2A	100	100	100	>300
3A	5	4	6	>300
4A	18	13	4	>300
5B	100	100	100	>300
6C	21	17	12	>300
7C	19	12	8	>300
8C	9	4	5	>300
9C	100	100	100	>300

[a] Percentage of absorbance of infected cells treated with 300, 150 and 75 μM of the compounds compared with untreated–uninfected cells, considered as 100 % of cell survival

Effect of 2A, 5B and 9C on cytokine production in HSV-1 infected J774A.1 cells

Since macrophages are involved in the inflammatory reaction elicited by HSK and play a crucial role as one of the dominant cell infiltrates in infected tissues, we have evaluated the effect of compounds 2A, 5B and 9C in J774A.1 cells infected with HSV-1 [21, 22].

First, we found that none of the three compounds were cytotoxic in J774A.1 cells at the concentrations tested, being the CC_{50} value higher than 300 μM (data not shown). When J774A.1 cells were infected with HSV-1 KOS (m.o.i. = 0.1) and treated with 2A, 5B and 9C (1–300 μM) to evaluate their antiviral activity, we found that all compounds inhibited HSV-1 multiplication with EC_{50} values of 27, 24 and 12 μM, respectively.

It is well known that HSV-1 infection triggers the production of proinflammatory cytokines in macrophages [8, 23]. Hence, we investigated whether 2A, 5B and 9C affected IL-6 and TNF-α secretion.

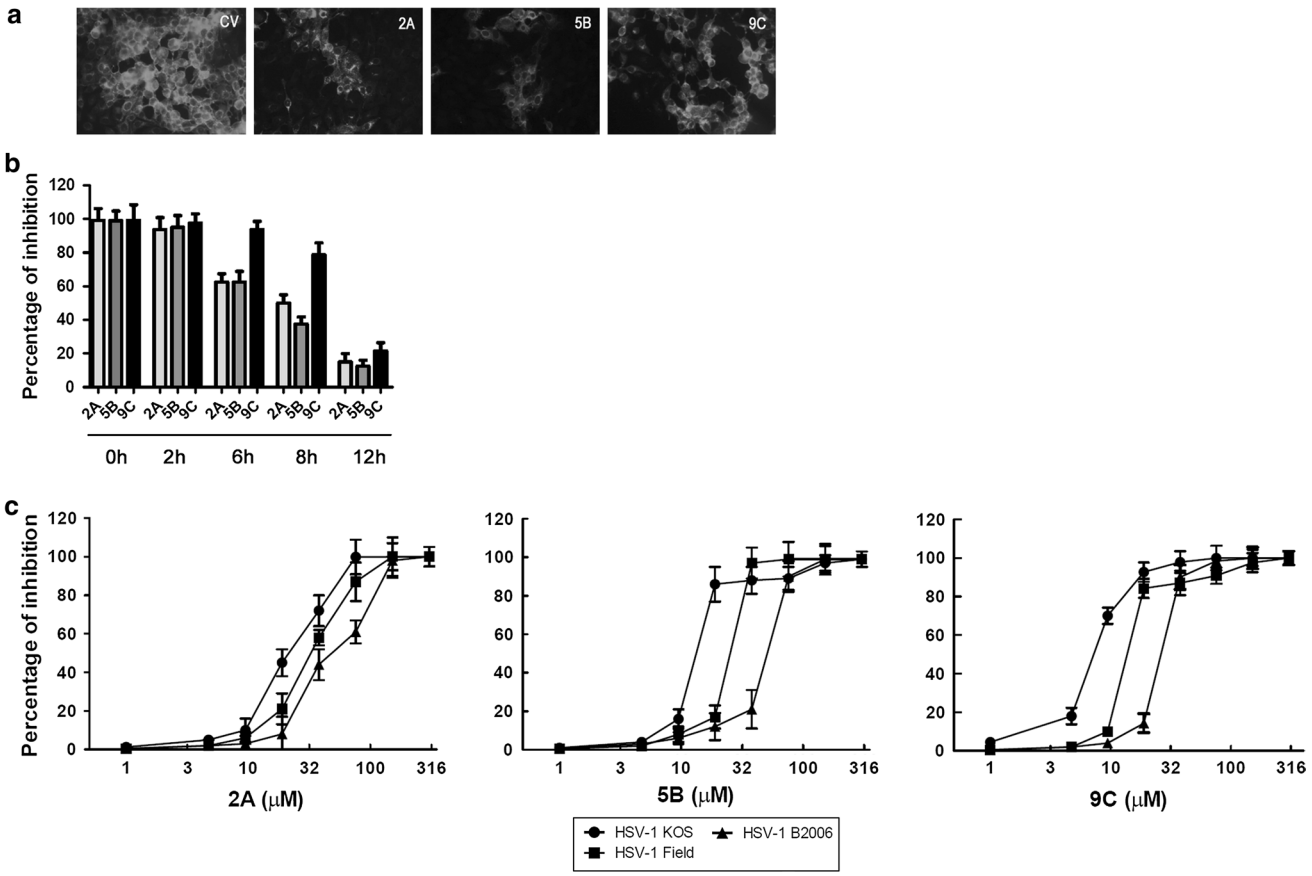


Fig. 3 Antiviral activity of 2A, 5B and 9C in Vero cells. **a** Effect of 2A, 5B and 9C on gD expression in Vero infected cells. Vero cells were infected with HSV-1 KOS (m.o.i. = 1) and treated with 2A, 5B and 9C or not (CV). At 24 h p.i., gD was localized by IFI staining. Magnification: 400×. **b** Vero cells infected with HSV-1 KOS (m.o.i = 1) were treated or not (control) with 75 μM of 2A, 5B and 9C at 0, 2, 4, 6, 8 or 12 h. After 24 h of incubation at 37 °C, virus yields were determined by plaque assay and plotted as the percentage of inhibition with respect to untreated–infected control. **c** HSV-1 KOS, Field and B2006 infected cells were treated with different concentrations of 2A, 5B and 9C. After 24 h, supernatants were harvested and titrated by plaque assay

J774A.1 cells infected or not with HSV-1 were treated or not with 75 μM of 2A, 5B and 9C for 24 h. Then, supernatants were harvested, and IL-6 and TNF-α were quantified by ELISA.

No significant differences between IL-6 and TNF-α release from non-treated and treated cells were detected in non-infected cells (Fig. 4). As previously reported, the production of IL-6 and TNF-α in infected cells was higher than that in non-infected cells ($p < 0.01$) [8]. Interestingly, we found that secretion of IL-6 and TNF-α was significantly reduced when any of the compounds were added to HSV-1-infected cells ($p < 0.05$) (Fig. 4a).

NF-κB and ERK activation induced by HSV-1 in J774A.1 cells

NF-κB is a major signaling pathway activated by HSV-1 and TLRs. A series of phosphorylation/recruitment/activation events, such as the degradation of IκB, lead to NF-κB nuclear translocation and transcription of inflammatory cytokine genes [23, 24]. Thus, to study the role of 2A, 5B and 9C in the NF-κB signaling cascade, we examined whether these compounds affected HSV-induced IκB degradation. For that purpose, macrophages infected with HSV-1 were treated with 75 μM of 2A, 5B and 9C for 24 h. Then, cells were processed through Western blot by using a rabbit polyclonal anti-IκB antibody.

None of the compounds degraded IκB in non-infected cells (Fig. 5a). HSV-1 infection induced IκB degradation while compounds 2A, 5B and 9C did not impede IκB disappearance after viral infection (Fig. 5a). However, we hypothesized that compounds 2A, 5B and 9C might inhibit any step downstream IκB degradation, such as NF-κB activation. Hence, we explored whether these compounds could affect NF-κB activation by using a NF-κB-LUC reporter plasmid.

J774A.1 cells were transfected with the NF-κB-LUC reporter vector and β-galactosidase control plasmid and,

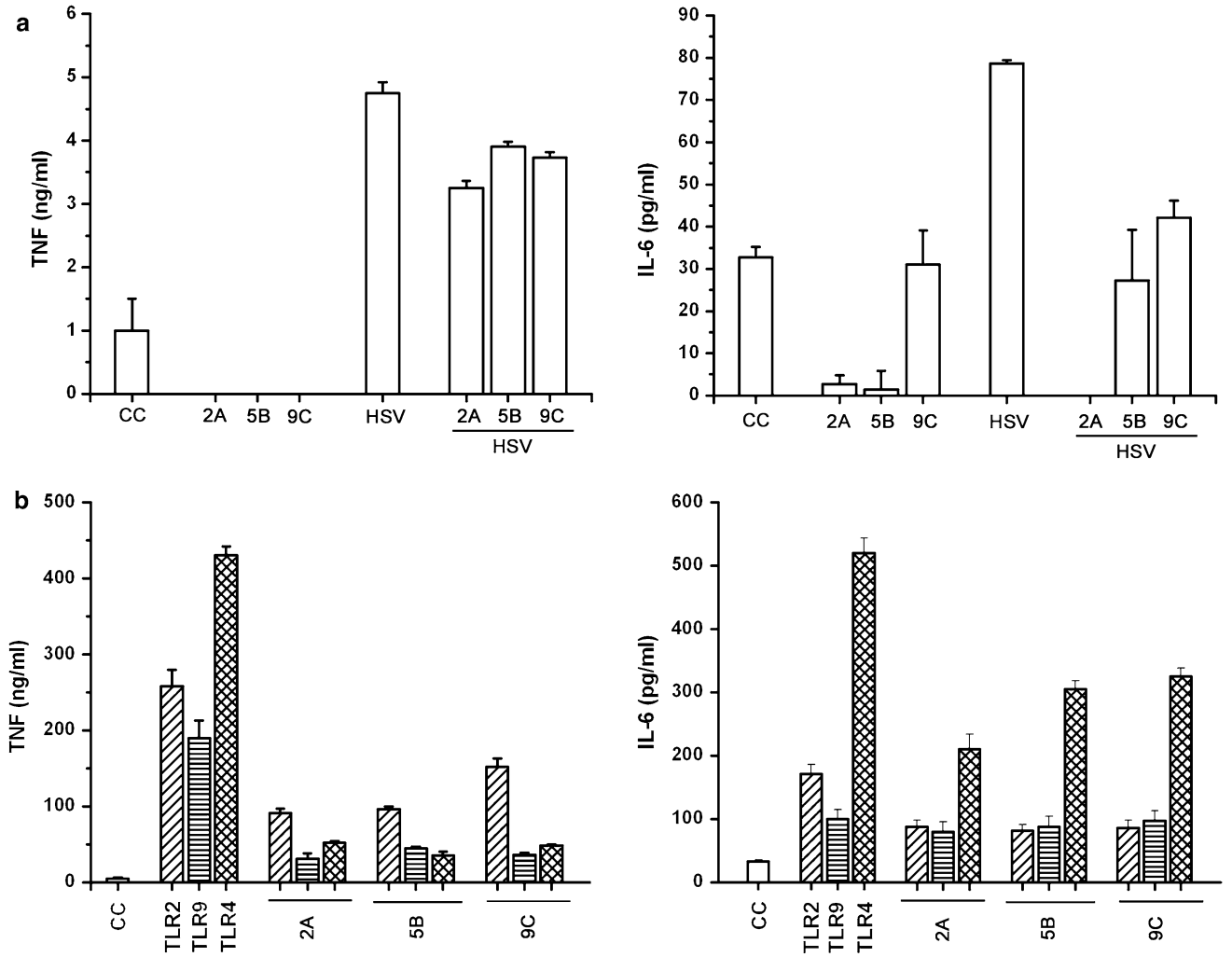


Fig. 4 Effect of 2A, 5B and 9C on cytokine production. J774A.1 cells were infected with HSV-1 (m.o.i. = 1) for 24 h (**a**), or stimulated with TLR2, TLR4 (100 ng/ml) and TLR9 (5 μg/ml) ligands for 8 h (**b**), and treated or not with 75 μM of 2A, 5B or 9C. TNF-α and IL-6 were determined by ELISA. Data are expressed as the mean ± SD of three separate experiments

24 h later, infected with HSV-1 and treated with 75 μM of 2A, 5B and 9C. After 16 h, luciferase activity was measured in cell extracts and each value was normalized to β-galactosidase activity. Although HSV-1 induced a strong NF-κB activation ($p < 0.01$), none of the compounds hampered its activation (Fig. 5a).

Considering that ERK pathway is also involved in the regulation of cytokine production, we examined whether 2A, 5B and 9C could modulate ERK phosphorylation. Thus, J774A.1 cells infected with HSV-1 were treated or not with 2A, 5B and 9C for 24 h. Subsequently, cells were lysed and subjected to Western blot with an anti-pERK antibody.

We found that none of the three compounds induced ERK phosphorylation in non-infected cells. We verified that the levels of ERK phosphorylation were increased within 24 h after HSV-1 infection and that, interestingly, they were strongly inhibited by 2A, 5B and 9C (Fig. 6a).

We concluded that the three compounds blocked ERK phosphorylation but none of them inhibited NF-κB activation induced by HSV-1.

Effect of 2A, 5B and 9C on cytokine production induced by TLR ligands in J774A.1 cells

Toll-like receptors (TLRs) are innate immune sensors implicated in the control of infections through the recognition of pathogen-associated molecular patterns. Several TLRs, especially TLRs 2, 4 and 9, are involved in early recognition of HSV components [24–29]. Since the reduction of IL-6 and TNF-α production in 2A-, 5B- and 9C-treated and HSV-1-infected J774A.1 cells could be due

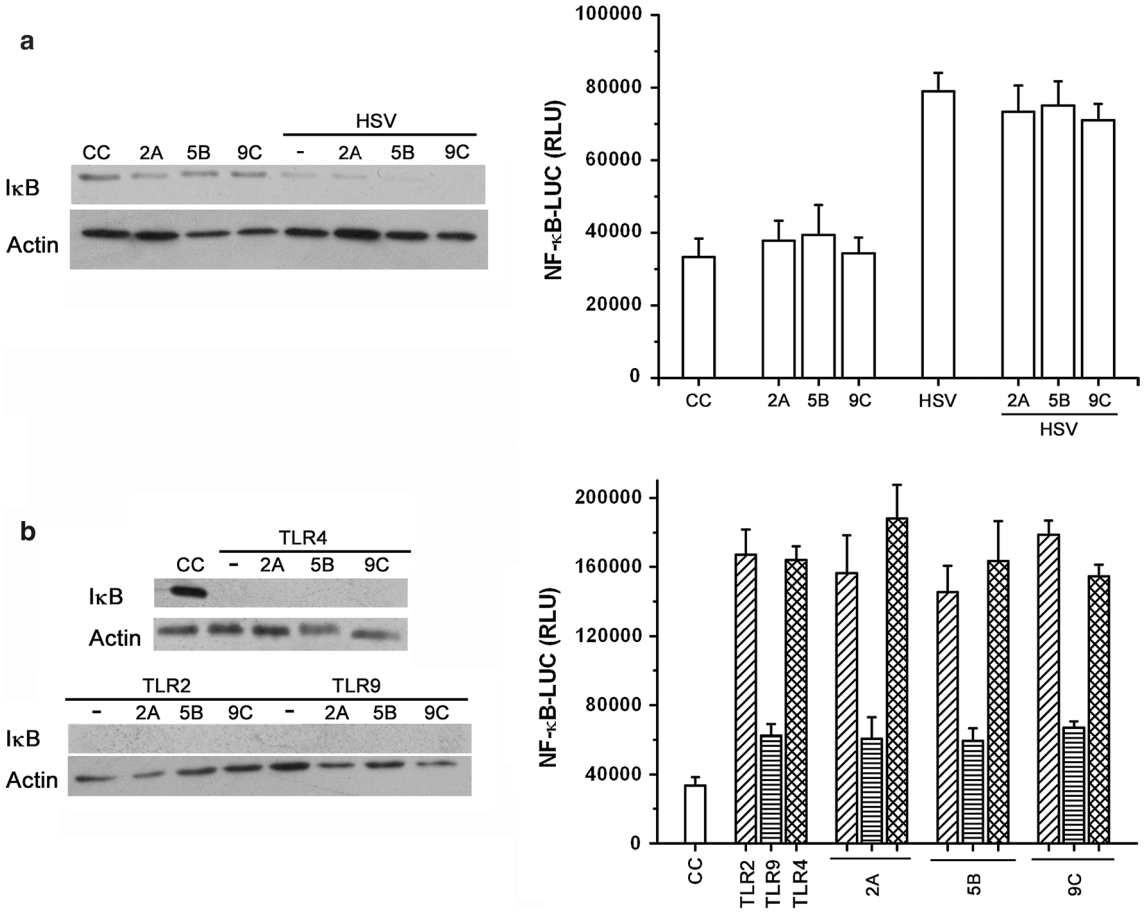


Fig. 5 Effect of 2A, 5B and 9C on IκBα degradation and NF-κB activation. J774A.1 cells were infected with HSV-1 (m.o.i. = 1) for 24 h (**a**), or stimulated with TLR2, TLR4 (100 ng/ml) or TLR9 (5 μg/ml) for 60 min (**b**), and treated or not with 75 μM of 2A, 5B or 9C. Macrophages were lysed and subjected to SDS-PAGE, followed by immunoblotting with antibodies against IκBα and actin. J774A.1 cells were transfected with 0.5 μg of NF-κB-LUC reporter vector and 0.5 μg of β-galactosidase control plasmid. After 24 h, J774A.1 cells were treated with 75 μM of 2A, 5B or 9C and infected with HSV-1 (m.o.i. = 1) for 16 h (**a**) or stimulated with TLR2, TLR4 (100 ng/ml) and TLR9 (5 μg/ml) ligands for 8 h (**b**). Luciferase activity was measured in cell extracts, and each value was normalized to β-galactosidase activity in relative luciferase units (RLUs). Data are expressed as the mean ± SD of three separate experiments

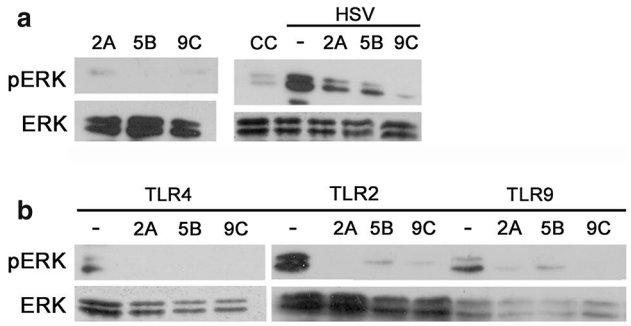


Fig. 6 Effect of 2A, 5B and 9C on ERK activation. J774A.1 cells were infected with HSV-1 (m.o.i. = 1) for 24 h (**a**), or stimulated with TLR2, TLR 4 or TLR 9 for 60 min (**b**), and treated or not with 75 μM of 2A, 5B or 9C. Macrophages were lysed and subjected to SDS-PAGE, followed by immunoblotting with antibodies against pERK and ERK

to the antiviral activity of these compounds, we used TLR ligands to evaluate solely their immunomodulatory effect.

Thus, to stimulate macrophages, we used TLR2, TLR9 and TLR4 ligands which are involved in HSV-1-induced cytokine production and are associated with neuroinflammation, herpetic encephalitis and HSK [24–29].

For that purpose, J774A.1 cells were stimulated or not with TLR2, TLR4 and TLR9 ligands and treated or not with 2A, 5B and 9C for 8 h. Then, supernatants were harvested, and IL-6 and TNF-α were quantified by ELISA.

The three ligands were able to induce IL-6 and TNF-α in J774A.1 cells ($p < 0.01$) (Fig. 4b). Interestingly, TNF-α production was significantly inhibited by 2A, 5B and 9C ($p < 0.01$) (Fig. 4b). In addition, the three compounds significantly reduced IL-6 release after activation with TLR2

and TLR4 ligands ($p < 0.01$), even though they did not modify IL-6 when induced with TLR9 ligand (Fig. 4b).

NF-κB and ERK activation induced by TLR ligands in J774A.1 cells

Since 2A, 5B and 9C modulated TNF-α and IL-6 production induced by TLRs ligands, we next determined whether the NF-κB and ERK pathways were affected by them in cells stimulated with TLR ligands. Stimulation with TLRs ligands completely degraded IκB and activated NF-κB ($p < 0.01$), whereas 2A, 5B and 9C prevented neither TLR-induced IκB degradation nor NF-κB activation (Fig. 5b).

In contrast, the three compounds completely blocked ERK phosphorylation triggered in macrophages stimulated with TLR2, TLR 4 and TLR9 ligands (Fig. 6b).

Hence, 2A, 5B and 9C modulated TNF-α and IL-6 secretion which, in turn, could be associated with the inhibition of the ERK signaling pathway.

Discussion

The current standard of care for HSK includes topical antivirals to inactivate and prevent further viral replication and corticosteroids to combat the immunopathological component of stromal disease [2]. The combination of antivirals and corticosteroids for other HSV-1-induced diseases such as herpetic encephalitis has been also examined [30, 31]. However, corticosteroids have adverse side effects, and there is concern that they prolong viral shedding responsible for further virus-induced damage. Furthermore, prolonged virus replication would be likely to increase the risk of antiviral resistance development [1–3, 30, 31].

In the present study, we identified three diterpenes that hindered both HSV-1 multiplication and HSV-1- and TLR-ligand-induced inflammatory response in vitro.

Likewise, compounds 2A, 5B and 9C were effective to restrain multiplication of TK− strains of HSV-1 resistant to ACV, which indicated that they would have a different mechanism of action from that of ACV.

It has been previously demonstrated that the modulation of the Raf/MEK/ERK or NF-κB pathways impairs the multiplication of HSV-1 [32, 33]. Since 2A, 5B and 9C prevented ERK activation, they might exert their antiviral action by interfering host cell functions required for virus replication. Hence, we cannot discard that these compounds presented an antiviral action as a consequence of the inhibition of the ERK pathway. Moreover, the inhibitory effect of 2A, 5B and 9C on cytokine production could be associated with the ERK signaling, while the NF-κB pathway was not hampered by any of these compounds (Figs. 4a, 5a, 6a).

Considering that several viruses interact with TLRs involved in eliciting the innate immune response, it has been proposed that the inhibition of TLR signaling could have great therapeutic potential in virus-induced diseases, as it occurs with herpetic, arenavirus and influenza infections [25, 34–36].

When J774.A1 macrophages were stimulated by TLRs ligands, 2A, 5B and 9C inhibited ERK activation as well as reduced TNF-α production (Figs. 4b, 6b). Compounds were able to hinder IL-6 secretion after activation with TLR2 or TLR4 ligands, but not with TLR9 ligand (Fig. 4b). Therefore, the inhibition of the ERK pathway may not be sufficient to prevent IL-6 secretion in TLR9-stimulated cells and, consequently, other signaling pathways such as NF-κB should be involved (Figs. 4b, 5b, 6b). In fact, it has been reported that NF-κB is a key regulator of IL-6 expression following TLR9-mediated activation [28].

In the case of HSV-1-infected macrophages, the inhibition of both TNF-α and IL-6 production could be ascribed to the block of the TLR2 and TLR4 signaling pathways which would probably counteract the lack of IL-6 inhibition due to TLR9 activation (Figs. 4, 6).

Thus, we hypothesize that the immunomodulatory effect of 2A, 5B and 9C in macrophages infected with HSV-1 may be a consequence of the inhibition of the ERK pathway activated by those TLRs involved in viral infection.

The discovery of compounds with antiviral and immunomodulatory activities constitutes a relevant contribution to the treatment immunopathologies of viral origin. However, to our knowledge, scientific literature reveals that there are very few examples of this kind of bioactive diterpenes. Particularly, Krawczyk et al. [13] reported that three taxol derivatives possess biological activity since they inhibit HSV replication cycle and affect immune response by inhibiting PHA-induced T lymphocyte proliferation. Nevertheless, they were not assayed against ACV-resistant herpes virus.

Diterpenes of natural origin such as compounds 2A, 5B and 9C exhibited this dual property and, besides, are effective against ACV-resistant HSV populations. These compounds affected TLR signaling pathways and, hence, might be a useful strategy to control the progress of HSV-induced disease. Further in vivo studies will be needed to determine the potential therapeutic effects of 2A, 5B and 9C to treat HSV-infection-associated diseases.

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Conflict of interest The authors declare that they have no conflict of interest.

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