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1-Cinnamoyl-3,11-dihydroxymeliacarpin is a natural bioactive compound with antiviral and nuclear factor-κB modulating properties

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Abstract

We have reported the isolation of the tetranortriterpenoid 1-cinnamoyl-3,11-dihydroxymeliacarpin (CDM) from partially purified leaf extracts of *Melia azedarach* L. (MA) that reduced both, vesicular stomatitis virus (VSV) and Herpes simplex virus type 1 (HSV-1) multiplication. CDM blocks VSV entry and the intracellular transport of VSV-G protein, confining it to the Golgi apparatus, by pre- or post-treatment, respectively. Here, we report that HSV-1 glycoproteins were also confined to the Golgi apparatus independently of the nature of the host cell. Considering that MA could be acting as an immunomodulator preventing the development of herpetic stromal keratitis in mice, we also examined an eventual effect of CDM on NF-κB signaling pathway. CDM is able to impede NF-κB activation in HSV-1-infected conjunctival cells and leads to the accumulation of p65 NF-κB subunit in the cytoplasm of uninfected treated Vero cells. In conclusion, CDM is a pleiotropic agent that not only inhibits the multiplication of DNA and RNA viruses by the same mechanism of action but also modulates the NF-κB signaling pathway.

Keywords: *Melia azedarach* L.; 1-Cinnamoyl-3,11-dihydroxymeliacarpin; Natural antiviral; Antiinflammatory; NF-κB; Triterpenoids; Herpes simplex virus type 1

Biologically active natural products are useful in the development of novel drugs with pharmacological actions. Particularly, triterpenoids isolated from plants are attracting considerable interest owing to their variety of structures and their broad range of biological activities. Recently, some compounds showing significant anti-tumor promoting activities in an in vivo assay have been reported [1]. Avicins, a family of triterpenoidal saponins, reduce both oxidative and nitrosative cellular stress and thereby suppress the development of malignancies and related diseases [2]. Triterpenoids exhibit an antiinflammatory effect when assayed by a paw edema model in vivo [3] and also have been screened for their antiviral activity. In the last years, limonin and nomilin proved to inhibit human immunodeficiency virus type 1 (HIV-1) replication on infected human mononuclear cells, with different mechanisms of action [4]. Besides, some triterpenoidal saponins have been reported to show anti-herpes simplex virus type 1 (HSV-1) activity [5].

Previously, we have reported the isolation of the tetranortriterpenoid 1-cinnamoyl-3,11-dihydroxymeliacarpin (CDM) from partially purified leaf extracts of *Melia azedarach* L. (MA) that reduced both, vesicular stomatitis virus (VSV) and HSV-1 infectivity, in vitro conditions [6]. Likewise, it was demonstrated that CDM exerts its antiviral action by blocking VSV entry and the intracellular transport of VSV-G protein, confining it to the Golgi apparatus, by pre- or post-treatment, respectively [7].

Topical administration of MA in the corneas of HSV-1-infected mice exerts its therapeutic effect by reducing the viral load in the eye as well as by abolishing the ocular inflammatory reaction [8].

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The nuclear transcription factor NF-κB has been shown to play a pivotal role in triggering immune and inflammatory responses to a range of stimuli, including viral infections. Multiple families of viruses, such as HIV-1, human T cell leukemia virus type 1, hepatitis B, and influenza viruses, have evolved to modulate the NF-κB pathway. This activation could have several functions: to promote viral replication, prevent virus-induced apoptosis, and mediate the immune response to the invading pathogen [9].

To increase our understanding about the mechanism of antiviral action of CDM, we decided to investigate its effect on HSV-1 glycoprotein transport in different cell cultures. Besides, considering that both viral and host immune factors determine the severity of the inflammatory response in the eye infected with HSV-1, together with the fact that MA would act as an immunomodulator preventing the development of murine HSV-1-induced ocular disease, we also examined whether CDM would display an antiinflammatory activity through the modulation of NF-κB.

Materials and methods

Cells and viruses. Vero and IOBA-NHC cells [10] were grown in Eagle's minimal essential medium (Gibco) supplemented with 5% inactivated fetal bovine serum (MEM 5%) and 50 μg/ml gentamycin, and maintained after monolayer formation in MEM supplemented with 1.5% inactivated fetal bovine serum (MEM 1.5%).

The Indiana strain of VSV and HSV-1 strain F were propagated at low multiplicity of infection (m.o.i.) and plaque-assayed on Vero cells.

Chemicals and reagents. TNF-α mouse recombinant expressed in *E. coli* was purchased from Sigma. Actinomycin D (Act D) was obtained from Sidus. The mouse monoclonal anti-gB, anti-gC, and anti-gD antibodies were kind gifts of Dr. Josefina Carlucci, FCEN, UBA, Buenos Aires, Argentina. The rabbit polyclonal anti-G antibody was kindly provided by Dr. Pablo Grigera, CEVAN, Buenos Aires, Argentina. The rabbit polyclonal anti-p65 antibody was obtained from Santa Cruz.

Antiviral compound. CDM was purified from leaves of *M. azedarach* L., as described by Alché et al. [6], solubilized in MEM 1.5% to a final concentration of 1 mg/ml (1.5 M), and stored at −20 °C.

Antiviral activity. The antiviral activity was performed by a plaque reduction assay. Confluent Vero cells grown in 24-well culture plates (Falcon, NJ, USA) were treated with CDM during 2 h and then infected with 200 plaque forming units (PFU) of VSV per well. The plates were incubated in 5% CO_2 at 37 °C for 1 h and then overlaid with a 1:1 mixture of MEM 1.5% and 0.7% methylcellulose. Plaques were counted at 24 h post-infection (p.i.) after cell monolayers were fixed with 10% formaldehyde and stained with 1% crystal violet.

Indirect immunofluorescence assay (IFI). Subconfluent cells grown on glass coverslips were fixed with methanol for 10 min at −20 °C. After three washes with PBS, the coverslips were inverted on a drop of diluted primary antibody for 30 min at 37 °C, and then returned to culture dishes and subjected to three additional washes with PBS. Afterwards, cells were incubated with goat anti-rabbit or anti-mouse IgG secondary antibodies (1:50) conjugated to FITC (Sigma), for 30 min at 37 °C. Finally, coverslips were rinsed, mounted, and photographed with a Zeiss microscope with epifluorescence optics or analyzed with an Olympus FB300 confocal microscope.

Image analysis. Images were collected with a 40× objective from the tissue locations and imported into the NIH ImageJ 1.34s program (written by Wayne Rasband, NIMH, Bethesda). The immunofluorescence images were converted to 8-bit gray scale from 0 (black) to 255 (white) and magnified. Individual cells were mapped for total or nuclear fluorescence and the mean density from each one was obtained. This process was repeated for several cells in the field, and the average was calculated to represent the total and/or nuclear mean density for an individual cell. To compare fluorescence distribution within the cell, numerical results were put in a table in Excel. Total or nuclear intensity was calculated as total or nuclear mean density per total or nuclear area, respectively. Then, the percentage of intensity in the nucleus with respect to total intensity was estimated. Student's *t* test was used for statistical analysis of the data.

Double fluorescence assay. Vero cells grown on coverslips in a 24-well plate were transfected with a cDNA coding for galactosyltransferase T2 fused with the enhanced green fluorescent protein (GalT2-GFP) [11], provided by Dr. Hugo Maccioni. Lipofectin Reagent (Gibco) was used for transfection of Vero cells with 2 μg of plasmid DNA per well. After 24 h transfection, cells were infected with HSV-1 at a m.o.i. of 1 PFU/cell. At 13 h p.i., cells were processed for gD-protein IFI staining using a TRITC-conjugated goat anti-mouse secondary antibody (Sigma). Coverslips were mounted and analyzed with an Olympus FB300 confocal microscope. Images were collected and processed using Fluoview version 3.2 and Adobe Photoshop software.

Acridine orange staining of living cells. Vero cells grown on coverslips were stained with acridine orange (1 μg/ml) for 15 min at 37 °C. Then, cells were washed twice with cold PBS, mounted on PBS and visualized on a Zeiss microscope with epifluorescence optics.

Results

Intracellular localization of HSV-1 glycoproteins in CDM-treated Vero cells

When unravelling the mechanism of action, we found that CDM exerts its antiviral effect on the endocytic and exocytic pathway of VSV. Hence, we decided to use HSV-1 glycoproteins in addition to VSV-G glycoprotein as a model to investigate the effect of CDM on protein trafficking.

We visualized the intracellular localization of gB, gC, and gD in Vero cells infected with HSV-1 (m.o.i. = 1) and treated with 75 μM CDM, by total IFI staining. No significant differences between the number of fluorescent cells from untreated and CDM-treated cultures were detected at 13 h p.i., although a differential pattern of fluorescence was observed. In untreated cells, gB, gC, and gD fluorescence appeared widely distributed throughout the cytoplasm and the plasma membrane, showing the characteristic foci of HSV-1 cytopathic effect. However, fluorescence revealed a compact juxtanuclear staining pattern in the majority of treated cells, no matter which was the glycoprotein involved (Fig. 1A). To determine whether this pattern was associated with the accumulation of viral glycoproteins in the Golgi apparatus, Vero cells were transfected with a cDNA coding for GalT2-GFP as a Golgi marker. After 24 h transfection, cells were infected with HSV-1 at a m.o.i. of 1 PFU/cell. At 13 h p.i., cells were processed for gD-protein IFI staining. The processing of images with a confocal microscope evidenced that gD co-localized with the Golgi marker (Fig. 1B).

As it occurs in the case of VSV-G glycoprotein, we have found that HSV-1 glycoproteins were also confined to the Golgi apparatus when CDM was added after infection.

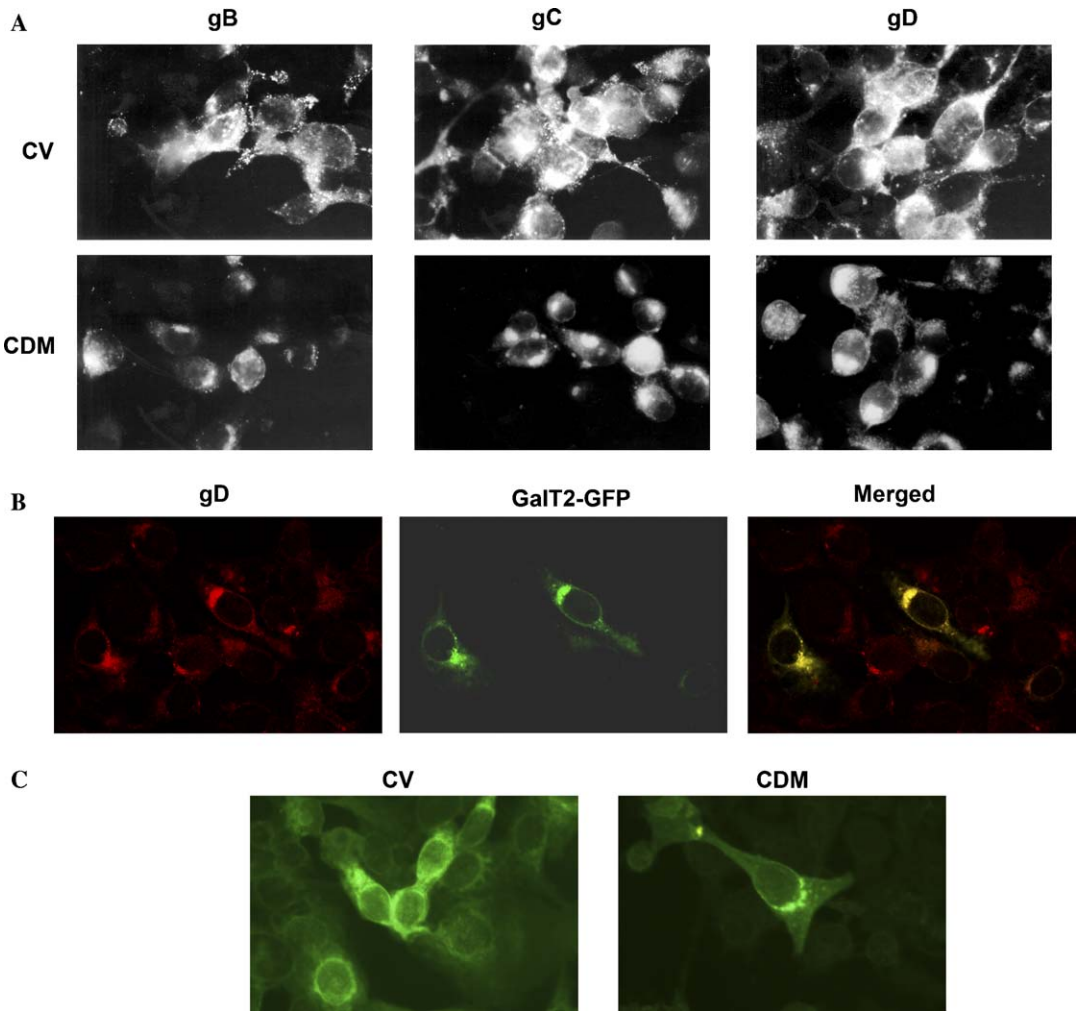

Fig. 1. Intracellular localization of HSV-1 glycoproteins in CDM-treated cells. (A) HSV-1-infected Vero cells were treated with CDM or not (CV) and after 13 h p.i. the intracellular localization of gB, gC, and gD was done by IFI staining. Magnification 400×. (B) Vero cells transfected with a plasmid containing the GalT2-GFP cDNA (green) were infected with HSV-1 and treated with 75 μM CDM. gD-protein was detected by using an anti-rabbit TRITC secondary antibody (red). Cells were analyzed by confocal microscopy and merged images appear yellow. Magnification 1000×. (C) HSV-1-infected NHC cells were treated with CDM or not (CV) and after 24 h p.i. the intracellular localization of gD was done by IFI staining. Magnification 400×. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

Antiviral effect of CDM on a human conjunctival cell line

Considering that MA proved to exert an antiviral action on the development of HSV-1-induced ocular disease [8], we investigated the antiviral activity of CDM in NHC cells, a human conjunctival cell line, where HSV-1 multiplies [12].

NHC cells infected with HSV-1 (m.o.i. = 1) were treated with 75 μM CDM and, 24 h later, supernatants were collected and titrated on Vero cells; then, cells were analyzed by IFI staining. We found that CDM suppressed virus replication by 90% without cytotoxic effect (data not shown). Fig. 1C shows that gD localized mainly on the cell surface of untreated NHC cells, whereas it was confined to the perinuclear region of CDM-treated NHC cells.

Thus, CDM is able to block the transport of gD to the plasma membrane and to inhibit viral glycoprotein trafficking independently of the nature of the host cell.

Effect of CDM on NF-κB nuclear translocation induced by HSV-1

Given that human conjunctival cells are able to amplify inflammatory processes in the eye together with the fact that lesions corresponding to herpetic stromal keratitis (HSK) are due to a virus-induced immunopathological reaction whose development is impeded by MA [8], our next goal was to investigate whether CDM exhibits an immunomodulatory action. It was reported that HSV-1 induces a strong nuclear translocation of NF-κB in human cell lines [13]. Hence, we performed IFI experiments using anti-p65 antibodies to determine the effect of CDM on NF-κB intracellular localization, through confocal microscopic analysis.

CDM was added during 13 and 24 h to Vero and NHC cells infected with HSV-1 (m.o.i. = 1), respectively, and then, these cells were fixed for IFI staining. By visual

inspection of the images, we observed that p65 remained in the cytoplasm of either uninfected NHC or Vero cells, whereas p65 translocated to the nucleus of both, Vero and NHC-infected cells (Fig. 2A).

To corroborate these qualitative observations, a semi-quantitative analysis of nuclear translocation of p65 was obtained by counting 50 cells per coverslip, and calculating the percentage of translocation as the number of cells with fluorescence in the nucleus relative to the total cell number. In infected cells, a differential response depending upon the type of cultured cells was obtained. Whereas the majority of infected NHC cells exhibited p65 fluorescence in the nucleus (73%), the percentage of Vero cells showing NF-κB translocation to the nucleus was of 56% (Fig. 2A). To confirm these findings, we also quantified the percentage of intensity in the nucleus with respect to total intensity of individual cells by using image analysis of fluorescence patterns from the same photographic source. This process was repeated from several cells in the field ($n = 10$). Regardless of the type of cell and its condition (infected or not), those cells with essentially cytoplasmic NF-κB staining only showed $12.7 \pm 4.44\%$ of intensity into the nucleus, while those cells with nuclear NF-κB had $60 \pm 7.74\%$ of intensity into the nucleus. Furthermore, a low detection of NF-κB in both uninfected Vero and NHC cells was observed in comparison to infected Vero and NHC cells. This significant increase in staining was confirmed by calculating the mean density for either NHC or Vero individual cells (Fig. 2B).

As HSV-1 is able to induce a persistent NF-κB nuclear translocation in both NHC and Vero cells, we decided to examine the ability of CDM to prevent NF-κB nuclear translocation during HSV-1 infection. In treated infected cells, we found that 14% of NHC cells retained NF-κB in their nuclei, whereas 59% of Vero cells showed nuclear NF-κB fluorescence, which evidence that CDM is able to restrain NF-κB activation only in the conjunctival cell line.

Strikingly, infected and treated NHC and Vero cells showed a significantly enhanced mean density with respect to both control and untreated infected cell cultures (Fig. 2). We conclude that the amount of NF-κB could be increased by viral infection and, moreover, by the addition of CDM.

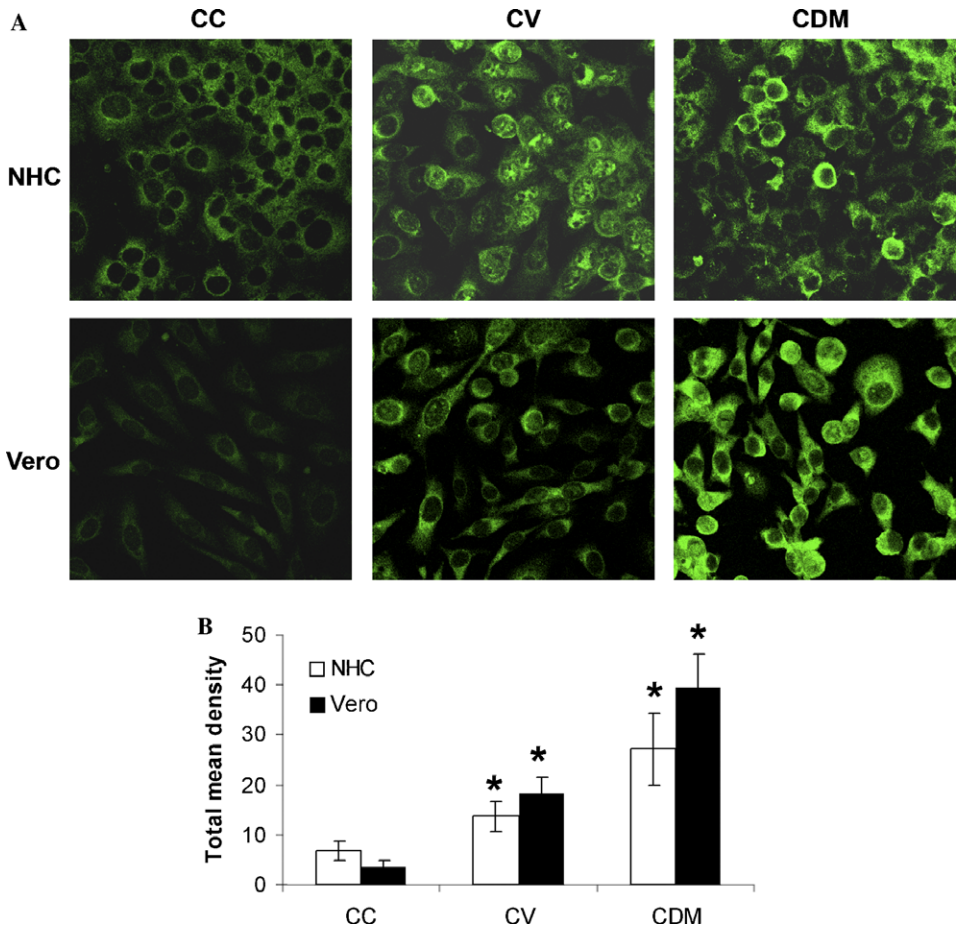

Fig. 2. Effect of CDM on NF-κB nuclear translocation induced by HSV-1. (A) NHC or Vero cells were infected with HSV-1 (CV) or not (CC) and treated with CDM after virus adsorption. IFI staining was performed by adding anti-p65 antibodies to methanol fixed cells and NF-κB translocation was analyzed by confocal microscopy. Magnification 400×. (B) Quantification of p65 fluorescence intensity observed in the cell types shown in (A). Values are the average of total mean density of individual cells ± SD, $n = 10$. $^{*}p < 0.001$, significantly different from uninfected untreated cells (CC).

p65 fluorescence intensity in CDM uninfected Vero cells

We next focused in determining whether the enhanced fluorescence corresponding to NF-κB in treated-HSV-1 infected Vero cells was also elicited by CDM in uninfected Vero cells. Cell monolayers treated with 75 μM CDM from 0 to 120 min were stained for p65 detection. A CDM time course dependent assay evidenced that p65 intensity gradually raised, reaching its maximum at 2 h post-treatment (Figs. 3A and C). This increase was only observed in the cytosol. Although CDM did not alter NF-κB intracellular localization in Vero cells, its addition significantly increased p65 fluorescence intensity in a time-dependent manner, even in the absence of HSV-1 infection.

To further investigate the mechanism of action involved in this event, we assayed CDM activity in the presence of the transcriptional inhibitor Act D. The p65 fluorescence enhancement induced by CDM was still observed even when Vero cells were preexposed to 5 μg/ml Act D (Figs. 3B and C).

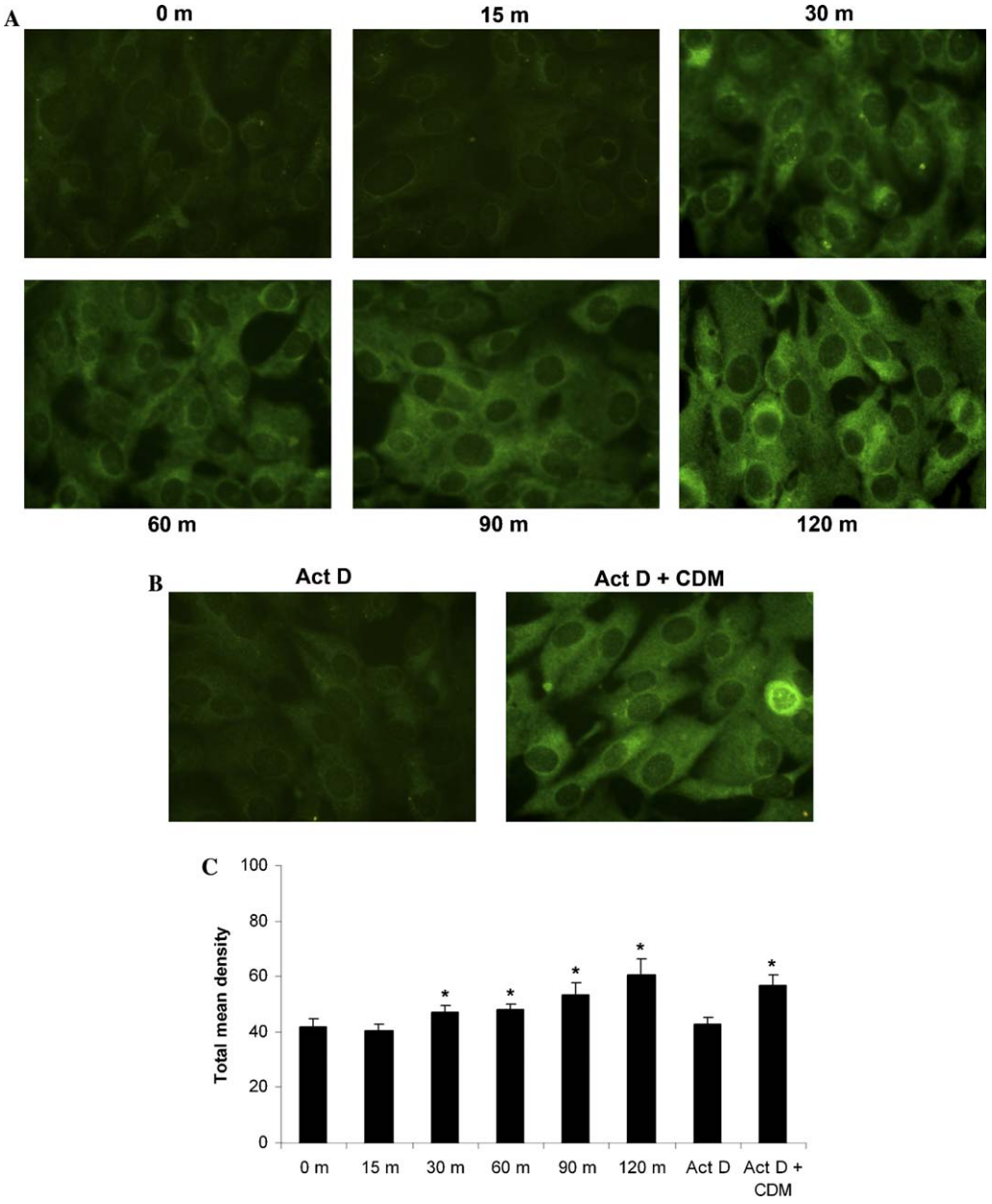

Fig. 3. NF-κB modulation in CDM uninfected Vero cells. (A) Cell monolayers were treated with CDM for 0, 15, 30, 60, 90, and 120 min at 37 °C, and then fixed with methanol. The localization of p65 was detected by IFI staining. Magnification 400×. (B) Vero cells were preexposed to 5 μg/ml Act D during 30 min at 37 °C. Then, CDM was added to the culture medium (Act D + CDM) or not (Act D) and monolayers were incubated for 2 h at 37 °C. IFI staining was performed by adding anti-p65 antibodies to methanol fixed cells. (C) Quantification of p65 fluorescence intensity observed in the cell types shown in (A,B). Values are average of total mean density of individual cells ± SD, $n = 10$. $^{*}p < 0.001$, significantly different from uninfected untreated cells.

We thus wondered whether the increment in fluorescence intensity is associated with an effect of CDM on NF-κB signaling pathway. To avoid the interference of the antiviral activity of CDM with NF-κB activation caused by HSV-1 infection, we used TNF-α, a well-known inducer of NF-κB translocation to the nucleus. IFI experiments were performed using Vero cells treated with 75 μM CDM for 2 h and further exposed or not to 10 ng/ml TNF-α during 2 h. Fig. 4A shows that the enhancement of p65 fluorescence intensity is still detected after 2 h of drug removal, either with or without TNF-α. However, the expected NF-κB translocation to the nucleus was not observed when TNF-α was added after CDM treatment. Interestingly, the simultaneous addition of CDM and TNF-α hampered the effect of CDM on p65 fluorescence intensity. These observations were confirmed through statistical analysis of the immunofluorescence quantification data (data not shown).

Taken together, these results demonstrate that the cellular transcriptional activity is not required by CDM to increase p65 fluorescence intensity, and lead us to postulate that CDM could be sequestering NF-κB in the cytoplasm of treated Vero cells.

These findings prompted us to evaluate if TNF-α also interferes with the antiviral activity of CDM. Vero cells grown in 24-well plates were treated with 75 μM CDM during 2 h, and, after its removal, infected with 200 PFU VSV. At 24 h p.i., the reduction in the number of plaques was

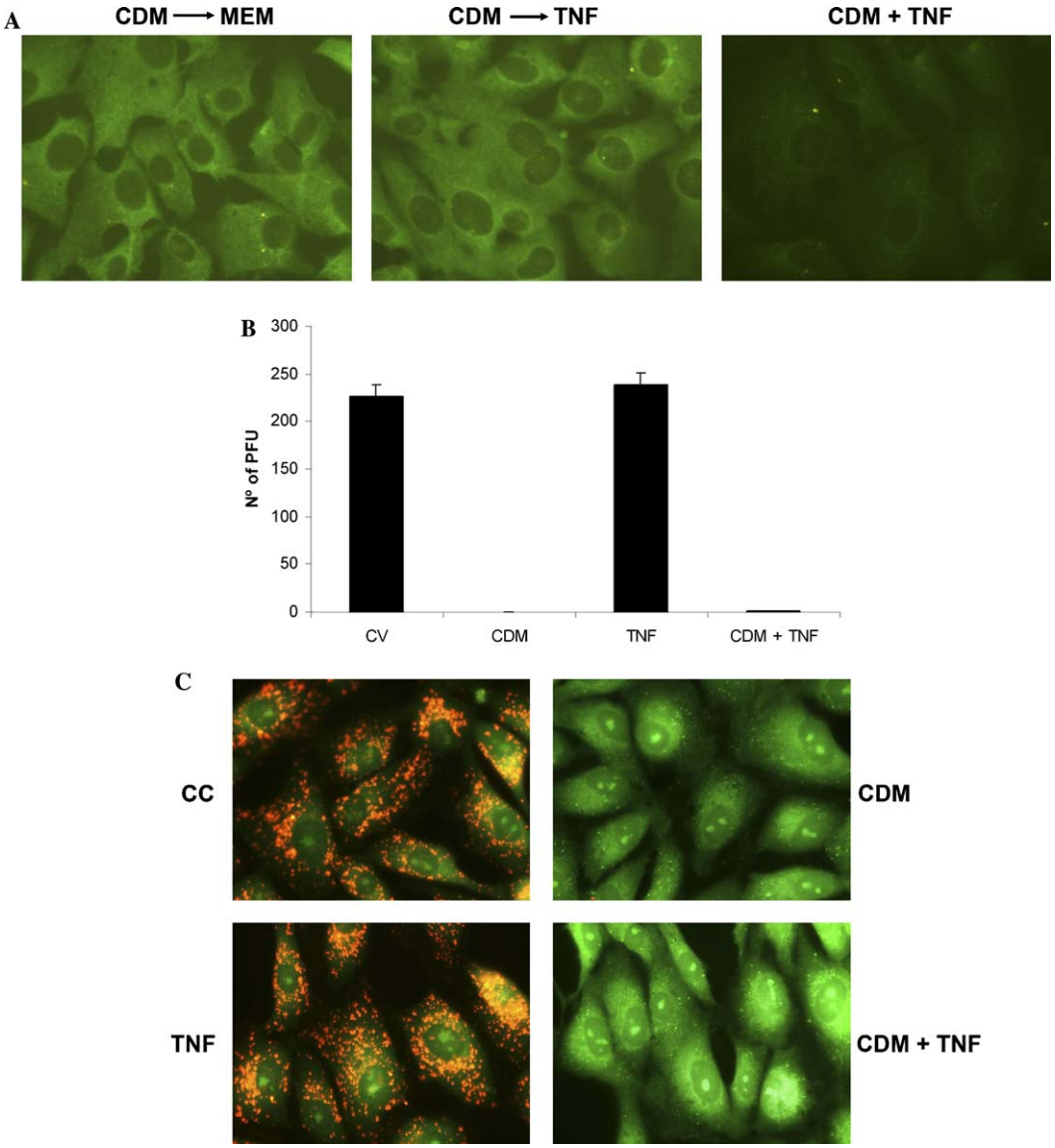

Fig. 4. Effect of TNF-α in NF-κB modulation and antiviral action of CDM. (A) Vero cells were treated with CDM for 2 h at 37 °C and, after drug removal, were incubated with TNF-α or MEM 1.5% for 2 h at 37 °C. CDM + TNF-α, cells simultaneously treated with both compounds during 2 h. After cell fixation, the localization of p65 was detected by IFI staining. Magnification 400×. (B) Vero cells treated with CDM, TNF-α or CDM plus TNF-α during 2 h at 37 °C were infected with 200 PFU VSV. At 24 h p.i., the number of PFU was quantified and plotted. CV, untreated infected cells. (C) Vero cells treated with CDM, TNF-α or CDM plus TNF-α during 2 h at 37 °C were stained with acridine orange. CC, untreated cells. Magnification 400×. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

quantified. Fig. 4B shows that while CDM completely abrogated VSV infectivity, neither viral multiplication nor the antiviral activity of CDM was affected by TNF-α. To further substantiate the latter finding, and considering the ability of CDM to induce a refractory state to VSV infection in Vero cells by causing cytoplasmic alkalinization [7], a vital fluorescence microscopy study was done in Vero cells under the same experimental conditions. Fig. 4C shows that TNF-α-treated cells exhibited a bright orange punctuate fluorescence pattern similar to untreated Vero cells. As expected, the effect of CDM on the pH of acidic intracellular vesicles was not affected by the addition of TNF-α, confirming that TNF-α did not restrict the anti-VSV activity of CDM.

Discussion

Since a long time, medicinal plants have been used for the treatment of many infectious diseases without any scientific basis supporting their employment. At present, there is an increasing emphasis on determining the scientific evidence and rationale for the use of preparations from medicinal plants. Thus, plants turn out to be a major source of new lead compounds for the reason that they are renewable in nature, locally available especially in developing and underdeveloped countries, and mainly, because they are often less prone to the emergence of drug resistance [14].

Despite the fact that the amount of information on anti-HSV plant extracts is very relevant, not all the bioactive anti-HSV molecules responsible for the activity of plant extracts have been identified, isolated, and tested. We have reported the chemical nature of CDM [6], and now, we have gone deep into the elucidation of its mechanism of action as an antiviral and/or cytoprotective molecule.

The direct antiviral activity of CDM achieved when the compound is supplied after infection has already been demonstrated for VSV: CDM affects G glycoprotein exocytic pathway from Vero cells, confining it to the Golgi complex [7]. In the present report, we have shown that CDM also affects the trafficking of, at least, gB, gC, and gD glycoproteins of HSV-1 by exerting an analogous antiviral effect in Vero cells (Figs. 1A and B). Thus, by arresting viral glycoproteins into the Golgi complex, CDM leads to the inhibition of the multiplication of viruses with different replicative strategies which accounts for the broad spectrum of antiviral activity reported for the partially purified leaf extract [15–17].

It is well known that many host cellular factors are necessary for a successful viral infection. HSV-1 entry as well as egress are complex multistep transport events which concite controversy nowadays, although it is widely accepted that viral glycoproteins whose synthesis is completely dependent upon cellular membrane trafficking, are required [18,19]. In consequence, these specific cellular functions essential for virus replication have attracted growing interest as targets for antiviral therapy because an altered cellular function is more difficult for the virus to adapt to, and besides, it should affect replication independently of the host cell type [20]. We have found that a recently established human conjunctival cell line is susceptible to both HSV-1 [12] and VSV multiplication (data not shown) and, hence, can be considered another useful experimental tool in the field of antiviral compounds. Results obtained with CDM in this particular cell system allow us to conclude that this molecule exhibits a common mechanism of antiviral action regardless of the cell substrate assayed (Fig. 1C).

It has been well documented that viral pathogens are able to alter host metabolism and modulate cellular signaling pathways and transcription factors to support replication [9]. In this sense, triggering of NF-κB activation is particularly relevant during HSV-1 infection because it harbors several consensus binding sites for NF-κB in their promoters, and this factor is utilized by HSV-1 to enhance its replication [21].

One might then expect that molecules interfering with NF-κB pathway would have anti-HSV activity. A good example of the effect of the block of NF-κB activation is given by the inhibition of IK kinase complex (IKK) function by cyclopentenone prostanoids during HSV-1 infection [22].

Experiments performed to investigate the effect of CDM on NF-κB pathway revealed that CDM blocks the HSV-1-induced activation of NF-κB by inhibiting its translocation to the nucleus of infected conjunctival cells (Fig. 2). Although these results are not enough to explain if the retention of NF-κB in the cytoplasmic compartment of treated-infected NHC cells is a consequence of the anti-HSV-1 effect or is due to an intrinsic antiinflammatory activity of CDM, we postulate that CDM would be able to abolish murine HSK by controlling viral spread and the associated immunopathology as well.

Whatever the cell substrate was, CDM is also associated with the accumulation of p65 in the cytoplasmic compartment, as pointed out by the enhanced intensity of fluorescence observed (Fig. 2). This fact seemed not to be related to viral infection, since CDM induces a gradual increase of p65 fluorescence intensity in uninfected Vero cells in a time-dependent manner (Figs. 3A and C). p65 cytoplasmic retention was also observed when cells were preexposed to Act D, indicating that CDM would withhold p65 in the cytoplasm through a nongenomic signaling pathway (Fig. 3B). This phenomenon is abrogated by the simultaneous addition of CDM and TNF-α (Fig. 4A). However, TNF-α neither restricted the anti-VSV activity nor the endosomal pH raising provoked by CDM (Figs. 4B and C) and, for this reason, p65 gathering in the cytosol is not implicated in alkalinization of acidic intracellular vesicles.

We hypothesized that CDM is modulating the NF-κB although the molecular reasons of this observation are unknown at present. The sequestration of p65 by CDM could be due to an inhibition of either the enzymatic activity of the IKK complex or the ubiquitination process, both

currently studied as potential targets for varied plant-derived products [23].

In conclusion, CDM displays a potent antiviral action affecting both DNA and RNA viruses by the same mechanism of action, and also comprises an additional biological property consisting in the alteration of the NF-κB pathway, which suggests an eventual role as an antiinflammatory agent.

This naturally based antiviral and NF-κB modulator deserves further studies to shed more light on its biological significance.

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