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1-Cinnamoyl-3,11-dihydroxymeliacarpin delays glycoprotein transport restraining virus multiplication without cytotoxicity

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ABSTRACT

The 1-cinnamoyl-3,11-dihydroxymeliacarpin (CDM), isolated from extracts of *Melia azedarach* L., displays antiviral and immunomodulating properties. CDM is the first reported tetranortriterpenoid responsible for the alkalinization of intracellular compartments affecting both, viral endocytic and exocytic pathways. Considering that viral glycoprotein synthesis is completely dependent upon cellular membrane trafficking, we questioned whether CDM might also interfere with the normal transport of cellular glycoproteins. This study demonstrates that CDM promoted a transient block in the transport of two cellular glycoproteins, the transferrin receptor (TfR) and TNF-α. Nevertheless, CDM did not affect the transferrin binding ability of TfR and did not impede the TNF-α secretion. On the other hand, CDM disturbed the intracellular localization of capsid, glycoprotein and tegument proteins simultaneously in the same HSV-1 infected cells. Besides, we show that concanamycin A and monensin provoke a permanent blockage of viral and cellular glycoproteins, in contrast to the delay observed after CDM treatment. Thus, the delay on glycoprotein transport caused by CDM would account for the strong inhibition on virus multiplication without interfering with the bioactivity of cellular glycoproteins.

Introduction

Most of the current drugs that target viral proteins have a relatively narrow spectrum of action, often lead to the development of viral resistance, and are ineffective to control latency and recurrence in immunocompromised host. Therefore, there is an imperative need to develop novel antiviral drugs that could overcome some of the limitations of the ones available. One alternative to the traditional approach of affecting virally-encoded molecules is targeting cellular proteins since viruses possess limited genetic capacity and must exploit multiple host-cell functions for successful infection. The principle drawback of these strategies is the greater potential for cellular toxicity; however, a plethora of compounds commonly used as therapeutic human drugs target cellular proteins and have no toxic effects. Actually, the genome-wide RNA interference screen and microarray analysis are used to identify such cellular genes important for viral replication [1].

The 1-cinnamoyl-3,11-dihydroxymeliacarpin (CDM) isolated from leaf extracts of *M. azedarach* L. is a natural tetranortriterpenoid with antiviral activity [2]. CDM is able to induce the alkalinization of intracellular compartments triggering an antiviral state in a variety of continuous and primary cell cultures when added before infection. This alteration inhibits the entry of Vesicular stomatitis virus (VSV) nucleocapsids into the cytoplasm by the endocytic pathway. Likewise, we have found that a later step in VSV and Herpes simplex virus type 1 (HSV-1) multiplication cycles is hindered by CDM, since glycoproteins (g) B, gC and gD of HSV-1, as well as gG of VSV, are confined to the Golgi apparatus when CDM is added after infection [3,4].

CDM also exhibits immunomodulating properties because it impedes nuclear factor-κB (NF-κB) activation in HSV-1 infected macrophages, corneal and conjunctival cells, and leads to the decrease in IL-6 production. Besides, CDM enhances levels of TNF-α production, decreased IL-6 secretion and did not inhibit NF-κB translocation when macrophages were induced with lipopolysaccharide (LPS), suggesting that an alternative cell signaling pathway would be affected [5].

Considering that viral glycoprotein synthesis is completely dependent upon cellular membrane trafficking, we questioned whether CDM might also interfere with the normal transport of cellular glycoproteins. To address this, we studied the transport of both fluorescently tagged transferrin receptor in transfected cells, and endogenous TNF-α in activated macrophages. In addition, we evaluated if CDM has an effect on the bioactivity and/or functionality of cellular and viral glycoproteins. We also compared the action of CDM with the effect of monensin (MON) and concanamycin A (CON).

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Materials and methods

Cell culture and viruses. IOBA-NHC (human conjunctival cells) and Vero cells were grown in Eagle's minimal essential medium supplemented with 5% inactivated fetal bovine serum (FBS) (MEM 5%), and 50 μg/ml gentamicin. Murine macrophage cell line J774A.1 was grown in RPMI 1640 medium supplemented with 10% inactivated FBS (RPMI 10%).

The triply fluorescent recombinant HSV-1 YK608 was kindly provided by Dr. Yasushi Kawaguchi (The Institute of Medical Science, the University of Tokyo, Japan) [6]. The Indiana strain of VSV, HSV-1 strain KOS and YK608 were propagated in Vero cells at low multiplicity of infection (m.i.).

Reagents. LPS from *Escherichia* coli serotype 055: B5, goat polyclonal anti-TNF-α, MON, and CON were purchased from Sigma. The mouse monoclonal antibody anti-gD of HSV-1 was obtained from Santa Cruz Biotechnology, USA. The rabbit polyclonal anti-giantin was kindly provided by Dr. Graciela Bocaccio (Instituto Leloir, Buenos Aires, Argentina). The rabbit polyclonal anti-gG of VSV was kindly provided by Dr. Pablo Grigera (CEVAN Buenos Aires, Argentina). Secondary goat anti-rabbit FluoroLinkTM CyTM2 and anti-mouse FluoroLinkTM CyTM3 antibodies were purchased from GE Healthcare Bio-Sciences, Argentina. Secondary anti-goat FITC from Vector was kindly provided by Dr. Dante Paz (FCEyN, UBA). Plasmid coding for transferrin receptor fused to green fluorescent protein (TfR–GFP) was kindly provided by Dr. Alfredo Cáceres (Instituto Investigación Médica Mercedes y Martín Ferreyra, Córdoba, Argentina). Transferrin-rhodamine (Tfn-Rho) was kindly provided by Dr. Elsa Damonte (FCEyN, UBA). Cicloheximide (CHX) was kindly provided by Dr. Susana Mersich (FCEyN, UBA).

Antiviral compound. CDM was purified from leaves of *M. azedarach* L., as described by Alché et al. [2], solubilized in MEM 1.5% to a final concentration of 1 mg/ml (1.5 mM).

Indirect immunofluorescence assay (IFI). For total glycoprotein staining, subconfluent cells grown on glass coverslips in 24-well plates were fixed with methanol for 10 min at −20 °C. After washes with PBS, the coverslips were incubated with primary antibody for 30 min at 37 °C, and then returned to culture dishes and subjected to additional washes with PBS. Afterwards, cells were incubated with secondary antibody for 30 min at 37 °C.

For surface glycoprotein staining, subconfluent cells grown on glass coverslips in 24-well plates were incubated with primary antibody for 30 min at 37 °C. Next, cells were subjected to washes with PBS and fixed with methanol. Then, cells were incubated with secondary antibody for 30 min at 37 °C.

Finally, coverslips were rinsed, mounted and photographed with an Olympus FB300 confocal microscope or an Olympus BX51 with epifluorescence optics.

Transfection. Subconfluent cells grown on glass coverslips in 24-well plates were transiently transfected using Lipofectamine 2000 (Invitrogen) according to manufacturer's instructions. Cells were fixed with methanol for 10 min at −20 °C. After washes with PBS, coverslips were processed for an IFI staining or rinsed, mounted and photographed with an Olympus FB300 confocal microscope or an Olympus BX51 with epifluorescence optics.

ELISA. Cells were frozen and thawed, and then, supernatants were harvested, centrifuged at 1000 rpm for 10 min, and cytokines were quantified by ELISA, in triplicate. Mouse TNF-α were quantified by commercial ELISA sets (BDOptEIATM, Becton Dickinson, USA) according to manufacturer instructions.

Transferrin uptake. NHC cells grown on coverslips were transfected with TfR–GFP. After treatments, cells were rinsed with PBS, incubated with Tfn-Rho (15 μg/ml) during 30 min at 37 °C and fixed with methanol. Finally, coverslips were rinsed, mounted and photographed with an Olympus FB300 confocal microscope.

Results

Effect of CDM on TfR–GFP intracellular localization

Since CDM exerts its antiviral action by affecting the trafficking of viral glycoproteins [3,4], we decided to track the transit of cellular glycoproteins in cells transfected with the fluorescently tagged TfR in the presence of CDM.

We visualized the intracellular localization of TfR–GFP in Vero and NHC cells treated with CDM. As shown in Fig. 1A, a punctuate Golgi-endosomal TfR pattern in control cells was observed, however, there was fluorescence concentrated mainly at a site adjacent to the nucleus of cells treated with CDM. The accumulation of fluorescence at this asymmetric juxtanuclear site indicated the possibility that this cytoplasmic structure was the Golgi complex. To confirm this, immunofluorescence assays were carried out by using antibodies to giantin, a Golgi marker protein. Indeed, the images obtained with a confocal microscope evidenced that TfR co-localized with giantin in CDM treated NHC cells (data not shown).

Thus, we have found that TfR was confined to the Golgi apparatus in CDM treated cells, as it occurs in the case of VSV-G and HSV-1 glycoproteins [3,4].

The next experiment was carried out to investigate the simultaneous localization of cellular and viral glycoproteins in the same cellular compartment. Initially, Vero cells transfected with TfR–GFP were infected with VSV, and an IFI staining using a rabbit polyclonal anti VSV-G antibody, was performed. Fig. 1B shows that VSV-G fluorescence was detected at the juxtanuclear domain of CDM treated cells, whereas the majority of infected treated cells showed TfR–GFP in a cytoplasmic punctuate pattern without

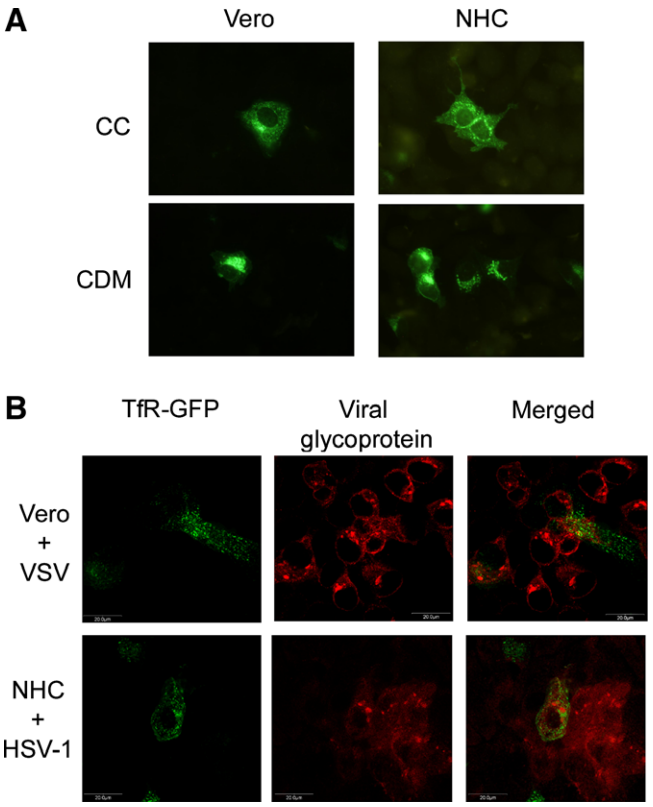

Fig. 1. Intracellular distribution of TfR in CDM treated cells. (A) Cells transfected with a cDNA encoding TfR–GFP were treated with CDM (40 μM) or not (CC) during 24 h. (B) Cells transfected with TfR–GFP were infected with VSV or HSV-1 (m.i. = 1) at 6 h p.t. After IFI staining, cells were analized by confocal microscopy.

colocalizing with VSV-G protein. Similarly, when TfR–GFP transfected NHC cells were infected with HSV-1, IFI staining revealed no overlap of TfR–GFP and HSV-1 gD glycoprotein either, since TfR displayed its typical punctuate pattern and gD protein localized at the perinuclear area (Fig. 1B).

Therefore, neither VSV-G at 6 h post-infection (p.i.) nor HSV-1 gD at 12 h p.i. coaccumulated with the TfR–GFP protein at the Golgi complex in CDM treated cells.

These patterns of TfR glycoprotein observed in CDM treated infected cells suggested that there was not a permanent blockade in the Golgi apparatus. To decipher this, CHX was added to the culture medium from 6 h until 24 h post-transfection (p.t.) to prevent further protein synthesis. The typical punctuate Golgi-endosomal TfR and the expected juxtanuclear accumulation patterns were already visualized at 6 h p.t. in untreated cells and CDM treated cells, respectively. At 24 h p.t., the TfR fluorescence pattern of control cells was not altered by the addition of CHX, while the perinuclear pattern observed in CDM treated cells was turned into a punctuate fluorescence spread all over the CDM–CHX treated cells (Fig. 2A). When CDM was removed or added at 6 h p.t., the TfR perinuclear fluorescence was also shifted to a punctuate pattern by the addition of CHX (Fig. 2B).

Thus, efficient mobilization of the TfR protein from the Golgi apparatus of CDM treated cells to recycling endosomes (RE) is only visualized after CHX addition, strongly suggesting that CDM promoted a transient block in the transport of cellular glycoproteins.

Considering that TfR protein is able to arrive at RE after its accumulation in the Golgi apparatus in CDM treated cells, we evaluated its biological activity by the transferrin uptake assay.

NHC cells transfected with TfR–GFP were treated with CHX at 6 h p.t. and Tfn-Rho uptake was done at 24 h p.t. In control cells, TfR–GFP was observed spatially localized with Tfn in RE. Similarly, both TfR and Tfn were colocalizing in RE of NHC cells treated with CDM (Fig. 2C).

In conclusion, CDM did not affect the Tfn binding ability of TfR when it reached its final destination.

Effect of CDM on TNF-α transport

Considering that most cytokines are released from cells through the secretory pathway, we examined the endogenous TNF-α transport in J774A.1 cells stimulated with LPS in the presence or absence of CDM for 6 h and 24 h, by immunostaining.

Upon stimulation of macrophages with LPS, a high level of TNF-α secretion is maintained for 4–6 h before declining [7]. Fig. 3A shows that newly synthesized TNF-α was observed in the Golgi complex at 6 h post-treatment, and then delivered to the plasma membrane, in LPS stimulated cells. By visual inspection of the images, we did not detect TNF-α fluorescence in cells treated with CDM for 6 h, while we observed a slight increase in TNF-α fluorescence after 24 h of treatment. When macrophages were treated with LPS in the presence of CDM, higher levels of TNF-α were detected within the Golgi complex compared to LPS alone, and later, fluorescence was widespread on the cell surface (Fig. 3A).

Hence, CDM did not impede the transport to the plasma membrane of active TNF-α allowing its secretion in LPS stimulated macrophages.

Effect of CDM on HSV-1 viral proteins

In view of the results obtained with cellular glycoproteins, we decided to investigate whether CDM also provokes a transient

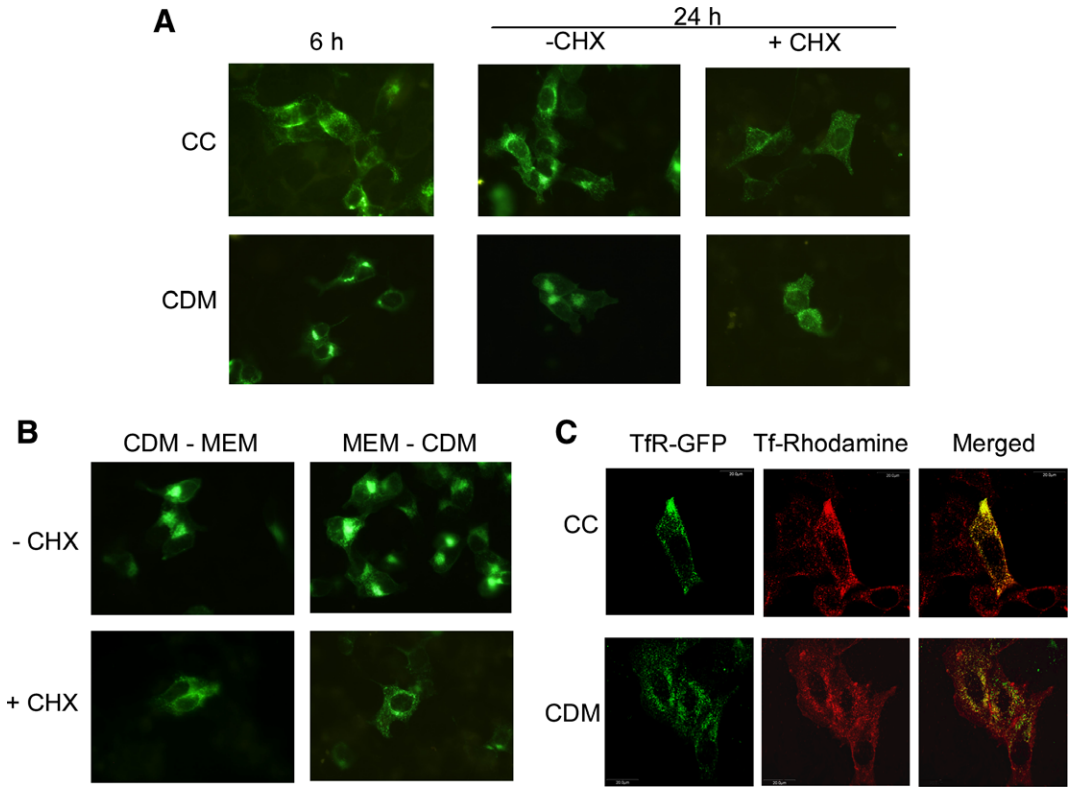

Fig. 2. TfR localization and bioactivity in CDM and CHX treated cells. (A) NHC cells transfected with TfR–GFP were treated with CDM (40 μM) or not (CC). At 6 h p.t. CHX (100 μg/ml) was added or not and cells were photographed at 6 and 24 h p.t. (B) CDM–MEM: cells were treated with CDM from 0 to 6 h p.t., then, CDM was removed and replaced with Opti-MEM with or without CHX. MEM–CDM: at 6 h p.t. Opti-MEM was replaced with CDM in the presence of CHX or not. At 24 h p.t. cells were visualized on an epifluorescence microscopy. (C) Transfected cells were treated with CDM (40 μM) or not (CC). At 6 h p.t. medium was replaced with Opti-MEM with CHX (100 μg/ml). Tf-rhodamine uptake assay was performed at 24 h p.t. and co-localization was analized by confocal microscopy.

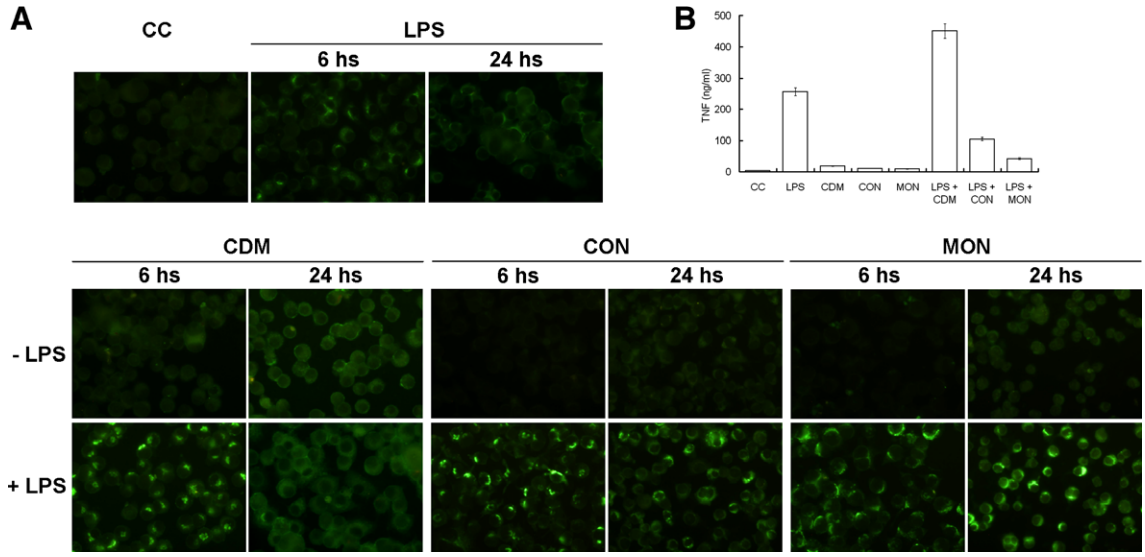

Fig. 3. Effect of CDM on TNF-α transport. Macrophage cells were treated with CDM (40 μM), CON (50 nM) or MON (20 μM) in the presence of LPS (100 ng/ml) or not. (A) After 6 and 24 h post-treatment, cells were fixed with methanol and TNF-α intracellular localization was done by IFI staining. (B) After 8 h post-treatment, TNF-α yield was quantified in the supernatants of treated cells, by ELISA. Data are expressed as the mean ± SD of three separate experiments.

block in the transport of HSV-1 gD glycoprotein by adding CHX to CDM treated infected cells at 12 h p.i., and performing a total IFI staining at 24 h p.i.

Fig. 4A shows that the gD fluorescence appeared at the perinuclear region in the majority of infected cells treated with CDM during 24 h p.i. and it was distributed throughout the cytoplasm and the plasma membrane in CDM and CHX treated cells, as it occurred with infected control cells. Consistent with this result and in order to visualize the localization of gD at the plasma membrane, we performed a surface IFI staining in HSV-1 infected and treated cells. The number of fluorescent cells expressing gD with respect to untreated cells was reduced to 62% at 12 h p.i. nevertheless this inhibition was not observed when the treatment with CDM was extended to 24 h p.i.

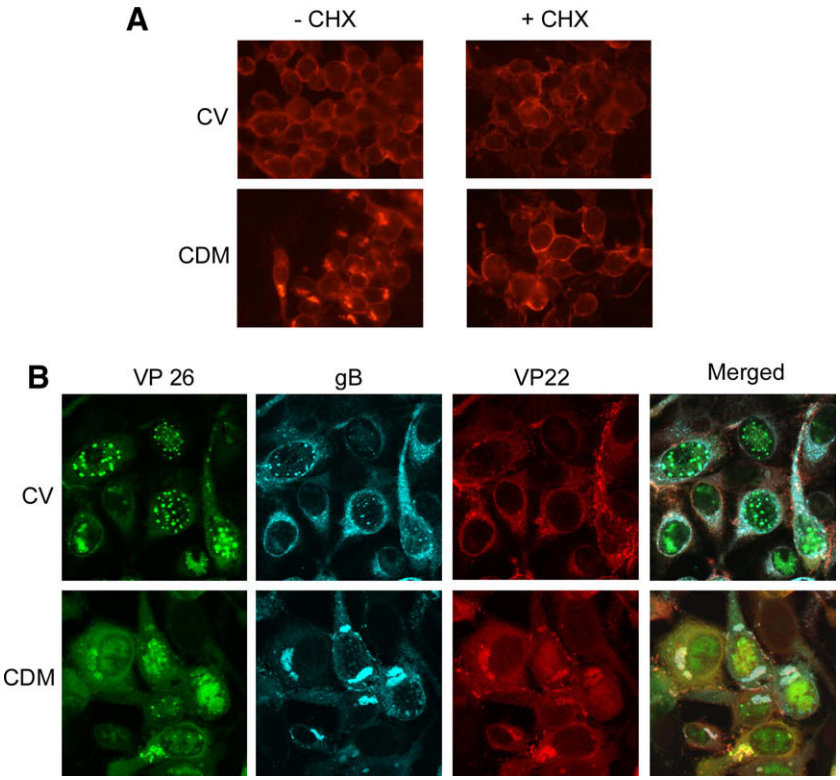

Fig. 4. Effect of CDM on HSV-1 proteins. (A) NHC cells infected with HSV-1 (m.i. = 1) were treated with CDM (40 μM) or not (CV) and at 12 h p.i., CHX was added or not. IFI staining was performed by adding anti-gD antibodies to cells fixed with methanol at 24 h p.i. (B) Vero cells infected with YK608 (m.i. = 1) were treated with CDM (40 μM) or not (CV). Single-color images were collected by confocal microscopy at 20 h p.i.

These findings suggested that CDM provoked a delay in both viral and cellular glycoprotein transport instead of a permanent blockage.

At present, we have investigated the effect of CDM on the subcellular localization of HSV-1 glycoproteins, solely. To extend our understanding, we decided to monitor its action on the intracellular transport of capsid, glycoprotein and tegument proteins simultaneously in the same HSV-1 infected cells.

Vero cells infected with a triple fluorescent-tagged HSV-1 expressing capsid protein VP26, tegument protein VP22 and enveloped protein gB as fusion proteins with monomeric yellow, red, and cyan fluorescent proteins, respectively, were treated with CDM and examined by confocal microscopy at 20 h p.i. Fig. 4B shows that VP26 was accumulated in the cytoplasm and as granular structures in the nucleus, gB was accumulated in the cytoplasm and the nuclear rim and VP22 was distributed mainly in the cytoplasm of YK608 infected cells. Notably, in CDM treated cells, intracellular localization of VP26, gB and VP22 proteins was markedly affected. The fluorescence pattern observed for gB was mainly adjacent to the nucleus, consistent with the anti-gD immunofluorescence studies of cells infected with wild type HSV-1 and treated with CDM, previously reported [3–5]. The VP22 protein was predominantly found at the perinuclear region colocalizing with gB protein. Besides, it appeared diffusely distributed in the nucleus of treated cells. In the case of VP26 protein, it was detected in the cytoplasm also colocalizing with both gB and VP22 proteins. Furthermore, the punctuate fluorescence pattern observed in the nucleus of control cells became compact and large in the majority of treated cells. Similar patterns of localization of capsid, tegument and envelope fusion proteins were also observed in NHC cell infected with YK608.

Therefore, the antiviral action of CDM not only altered the intracellular localization of gB but also disturbed that of other major virion components, such as VP26 and VP22.

Effect of acidotrophic agents on glycoprotein transport

Acidification of vacuolar compartments plays an important role in a variety of cellular processes. Perturbation of the acidic pH of various intracellular organelles by acidotrophic agents, such as weak bases and ammonium chloride, ionophores such as MON and specific inhibitors of vacuolar (V-) ATPase such as CON, affects both endocytic and exocytic pathways [8].

Previously, we have demonstrated that CDM induces an increase in the pH of intracellular acidic compartments in different cell systems [3–5]. Therefore, we decided to compare the effect of CDM on cellular and viral glycoprotein transport to those of MON and CON.

As expected, MON and CON affected the TfR–GFP transport since accumulation of fluorescence at juxtanuclear sites was visualized. When CHX was added, no difference on TfR fluorescence pattern was observed in both cases (data not shown).

In the case of macrophages treated with LPS in the presence of CON and MON, higher levels of TNF-α were detected within the Golgi complex compared with LPS alone, and this fluorescence pattern was still detected at 24 h post-treatment (Fig. 3A).

To assess the biological relevance of the inhibition in the TNF-α transport provoked by MON and CON, we measured its effect on TNF-α secretion. Supernatants harvested from J774A.1 cells stimulated with LPS and treated or not with MON and CON were used to quantify TNF-α by ELISA, in triplicate (Fig. 3B). We found that the production of TNF-α in LPS treated cells was significantly higher than that of control cells ($p < 0.01$, Student's t-test). This increase was significantly reduced by MON and CON ($p < 0.01$) in contrast with the strong enhancement induced by CDM ($p < 0.05$).

Next, we evaluated the effect of CON and MON on gD protein transport in HSV-1 infected cells. At 12 h p.i., both compounds inhibited the transport of gD to the plasma membrane showing an inhibition of 57% and 70% in the number of fluorescent cells with respect to untreated control cells, respectively, and these percentages of inhibition were even observed at 24 h p.i.

Thus, CON and MON provoked a permanent blockage of viral and cellular glycoproteins, in contrast to the delay observed after CDM treatment.

Discussion

Since viral infections remain the leading cause of death globally, the improvement of strategies for the development of new antiviral agents continues to be a challenge.

Lately, extensive attention has been paid to the identification of compounds that interfere with specific host-cell functions that are required for viral replication. Indeed, the Food and Drug Administration recently approved Maraviroc, the first antiretroviral agent targeting a host element that the virus uses for its entry [9]. Likewise, Debio 025, an oral cyclophilin A inhibitor with a potent anti-hepatitis C virus (HCV) activity *in vitro*, is under clinical evaluation. This synthetic cyclosporine analogue with no immunosuppressive capacity is also endowed with a strong anti-HIV activity [10].

Furthermore, many natural products have been explored as a source to discover new antiviral drugs, owing to its amazing structural diversity and broad range of bioactivities [11,12]. In particular, polyphenols, flavonoids, terpenes, etc., have been reported to inhibit several steps of viral replication cycle and certain cellular factors involved in the multiplication of many viruses [13,14]. We have reported the isolation from *M. azedarach* L. of the limonoid CDM which might be affecting a cellular factor involved in the transport of viral glycoproteins [2].

In the present paper, we demonstrate that CDM is able to interfere with both, cellular and viral glycoproteins, since the TfR–GFP was confined to the Golgi apparatus in uninfected and CDM treated cells (Fig. 1A), as it occurs in the case of VSV-G and HSV-1 glycoproteins (Fig. 1B). Nevertheless, there is no temporal overlap between TfR and viral glycoproteins at the Golgi complex in CDM treated cells (Fig. 1B). Besides, the TfR–GFP and gD perinuclear accumulation observed in CDM treated cells was shifted to a typical punctuate pattern by the addition of CHX (Figs. 2A, B and 4A).

These results highlight that the CDM block was transient instead of permanent, since the proteins accumulated in the Golgi complex may reach their final destination. This effect became evident when the viral shut-off transcription as well as the CHX treatment prevents new TfR–GFP synthesis.

Similarly to the results observed with TfR–GFP and viral glycoproteins, after 24 h of CDM plus LPS treatment in J774A.1 cells, endogenous TNF-α arrived at the plasma membrane for its secretion (Fig. 3A). In this case, it is not necessary to add CHX to display its final location because the expression of TNF-α is rapidly activated after LPS stimulation and declines after 4–6 h.

Taken together these findings suggest that both, viral and cellular glycoproteins are correctly sorted in the presence of CDM. Moreover, we confirmed that CDM did not affect the Tfn binding ability of TfR when it reached its final destination, by performing a Tfn uptake assay (Fig. 2C). Previously, it has also been demonstrated that CDM does not affect the TNF-α cytotoxic action by using a functional biologic assay [5]. In conclusion, the delay caused by CDM is not affecting the biological activity of the cellular glycoproteins.

It has been reported that CDM inhibits HSV-1 replication [3–5]. Now, we demonstrate that CDM dramatically changed the localiza-

tion pattern of viral proteins in cells infected with YK608 (Fig. 4B). During virus assembly, concentration of envelope, capsid and tegument proteins in the *trans*-Golgi network was found [6,15,16]. From these observations, it is reasonable to speculate that the CDM effect on the rate of glycoprotein transport through the Golgi, could be affecting the localization of the capsid and tegument proteins. However, we cannot rule out an intrinsic action of CDM on the transport of VP26 and VP22 proteins.

Acidification of vacuolar compartments plays an important role in a variety of cellular processes including the exocytic pathway. Two acidotrophic agents that elevate Golgi lumen pH by inhibiting the V-ATPase and promoting Na^+/H^+ exchange, CON and MON, respectively, block glycoprotein transport [8]. Likewise, one might think that CDM is affecting the glycoproteins transport as a consequence of raising the intracellular compartments pH [3]. Nevertheless, while CDM caused a delay in glycoprotein traffic, CON and MON permanently retained TfR–GFP, TNF-α and viral glycoproteins transport (Fig. 3).

Therefore, although CON, MON, and CDM induced the alkalinization of intracellular compartments, they affect the glycoprotein traffic by different mechanism. This might indicate that changes in Golgi lumen pH are not the sole cause of the transient nature of the CDM effect on glycoprotein distribution, and other cellular targets on the secretion pathway may be involved, for example the small GTPases of the Rab family [17].

In conclusion, CDM is able to slow down glycoproteins transport without impeding the ultimate localization and the bioactivity of cellular glycoproteins. This finding can explain why CDM is well tolerated in the normal cells unlike others compounds that permanently block the cellular transport machinery. Nevertheless, CDM is able to impede the virus multiplication of many DNA and/or RNA viruses despite the fact that viral glycoproteins may reach the plasma membrane in CDM treated cells. Therefore, unraveling its mechanism of action may lead to discover cellular targets for new antiviral agents that overcome some limitations of the ones currently approved for clinical therapy.

Acknowledgments

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