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An Antiviral Principle Present in a Purified Fraction from *Melia Azedarach* L. Leaf Aqueous Extract Restrains Herpes Simplex Virus Type 1 Propagation

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Meliacine (MA), an antiviral principle isolated from leaves of *Melia azedarach* L., exhibits potent antiviral activity against herpes simplex virus type 1 (HSV-1) by inhibiting specific infected-cell polypeptides (ICPs) produced late in infection. Some of these are involved in DNA synthesis and in the assembly of nucleocapsids. The present report provides additional evidence to elucidate the mode of action of MA against HSV-1. Time-of-addition experiments confirmed that MA affects a late event in the multiplication cycle of HSV-1. We showed that MA diminished the synthesis of viral DNA and inhibited the spread of infectious viral particles when HSV-1 that expresses β-galactosidase activity was used. In addition, the lack of a protein with an apparent MW of 55 KD was detected in MA-treated cell extracts. Ultrastructural analysis of infected cells showed that, in the case of MA treatment, a large number of unenveloped nucleocapsids accumulated in the cytoplasm and a minor proportion of mature virus was found in cytoplasmic vesicles.

These findings suggest that MA exerts an antiviral action on both the synthesis of viral DNA and the maturation and egress of HSV-1 during the infection of Vero cells. Copyright © 2002 John Wiley & Sons, Ltd.

Keywords: meliacine; herpes simplex virus type 1; antiviral; *Melia azedarach* L.

INTRODUCTION

Herpes simplex virus (HSV) causes a wide range of infections which are usually self-limiting in the immunocompetent host. HSV-1 is generally associated with primary and recurrent mucocutaneous facial, ophthalmic or genital lesions. The virus establishes a latent ganglionic infection in the neurons that innervate the area of primary infection and it is periodically reactivated causing repeated lesions at or near the site of initial infection.

Until now, a number of nucleoside analogues have been developed as antiherpetic agents, i.e. thymidine analogues (idoxuridine, trifluridine), guanosine analogues (acyclovir, gancyclovir, pencyclovir) and arabinonucleosides (vidarabine/Ara-A and Ara-T). The therapeutic limitation of these nucleoside analogues is that drug resistance strains develop readily through mutations in the thymidine kinase and/or polymerase viral genes (Alrabiah and Sacks, 1996; Hirsch *et al.*, 1996). For this reason, the search for new types of antiviral agents effective for viral strains resistant to current antiviral compounds that may act against different targets along the herpesvirus replication cycle has been intensified.

Several phytochemicals isolated from different plant species have been extensively studied for their potential therapeutic effect against viral infections (Ngan *et al.*, 1988; Cordell, 1995; Mahmood *et al.*, 1997). Such compounds are particularly useful because of their relative abundance in nature and, in many cases, their low cytotoxicity.

In our laboratory, we found that crude extracts of leaves of members of the *Meliaceae* family exhibited an antiviral activity against different viruses (Wachsman *et al.*, 1988; Córdoba *et al.*, 1991; Benencia *et al.*, 1997). Thus, meliacine (MA), a bioactive principle detected in a purified fraction from *Melia azedarach* L. leaf aqueous extracts, inhibited the replication of HSV in Vero cells when added before or after infection (Andrei *et al.*, 1994). It was demonstrated that MA exerts this antiviral effect with high selectivity, since no cytotoxicity was observed (Villamil *et al.*, 1995).

Likewise, MA proved to be equally active against TK^+ and TK^- strains of HSV-1, suggesting a different mode of action from that of acyclovir (Barquero *et al.*, 1997).

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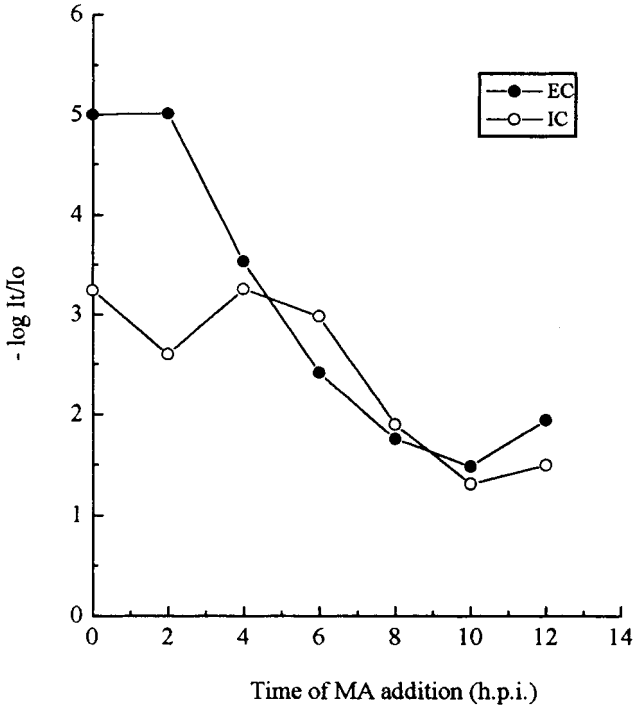

Figure 1. Effect of MA addition on HSV-1 yields. Vero cells grown in 24-well plates were infected with HSV-1 (m.o.i. = 1) and 50 μg/mL of MA was added at 0, 2, 4, 6, 8, 10 and 12 h after infection. At 24 h p.i., extracellular (EC) and cell-associated (IC) virus yields were determined

On studying which step of HSV-1 replication was the target for this antiviral effect, we found that viral adsorption and penetration were not affected. In the presence of MA, the analysis of infected-cell polypeptides (ICPs) revealed that all early ICPs were synthesized, whereas a strong inhibition of late ICPs was detected (Villamil *et al.*, 1995). Since late ICPs which were not synthesized in MA-treated cells are involved in DNA cleavage and packaging and in the assembly of HSV-1 nucleocapsids, the aim of the present study was to investigate whether MA affects these two late events of HSV-1 multiplication.

MATERIALS AND METHODS

Cells and viruses. Vero cells were grown in Eagle's minimal essential medium supplemented with 5% inactivated calf serum (MEM 5%) and 50 μg/mL gentamycin and maintained in monolayer formation in MEM supplemented with 1.5% inactivated calf serum (MEM 1.5%).

HSV-1 strain F was propagated at low multiplicity and plaque-assayed on Vero cell monolayers.

HSV-1 $Cgal^+$ (HSV-1 Cgal) containing the *Escherichia coli lacZ* reporter gene encoding β-galactosidase which was under the control of a strong heterologous promoter from the human cytomegalovirus immediate-early (IE) regulatory region (Johnson *et al.*, 1991) was kindly provided by Dr Alberto Epstein (Université Claude Bernard, Lyon, France).

Preparation of MA. MA was purified from crude leaf extracts of *Melia azedarach* L., as described by Alché *et al.* (2000). Briefly, an aqueous extract was prepared from lyophilized green leaves, and then extracted with ethyl acetate. The organic phase was chromatographed by molecular adsorption on silica gel (230–400 mesh) (E. Merck, Darmstadt, Germany) and fractions eluted with chloroform:methanol 95:5 were used. The bioactive product was solubilized in MEM 1.5% to a final concentration of 1 mg/mL and stored at −20 °C.

DNA hybridization assay. Vials containing 8×10^4 Vero cells were infected with HSV-1 strain F (m.o.i. = 10). At 8, 10 and 12 h p.i., cell monolayers were processed to evaluate viral DNA synthesis by using the Hybriwix Probe Systems Kit (Athens, OH, USA).

Measurement of β-gal activity. Vero cell monolayers grown in 24-well plates were infected with HSV-1 Cgal (m.o.i. = 1). After incubation for 1 h at 37 °C, inocula were eliminated and cells were covered with MEM 1.5%. At 24 h p.i., cells were stained *in situ* for β-gal. The medium from the cells was removed and 200 μL of a solution containing 1% formaldehyde and 0.2% glutaraldehyde in PBS was added for 30 min at 4 °C. Subsequently, supernatants were removed and the cells were washed twice with PBS. A β-gal activity staining solution (50 mM potassium ferrocyanide, 50 mM potassium ferricyanide, 20 mM $MgCl_2$ and 1 mg/mL X-gal) was added to the cells for 3 h at 37 °C. 'Blue' cells were observed at a 300x magnification and photographed.

Western blot analysis. Vero cell monolayers grown in 24-well plates were infected with HSV-1 (F) (m.o.i. = 1). After incubation for 1 h at 37 °C, inocula were eliminated and cells were maintained with MEM 1.5% at 37 °C. At 8, 10 and 12 h p.i., cell lysates were electrophoretically separated on 10% SDS-polyacrylamide gels followed by electrical transfer to PVDF sheets in a Novablot System (LKB). The PVDF sheet was immunoblotted with a peroxidase-conjugated rabbit anti-herpes simplex virus type 1 (Dako) and the immobilized proteins were detected using a western-blot chemiluminescence reagent (NEN, Life Science Products, Boston, USA).

Electron microscopy. After 36 h p.i., Vero cell monolayers grown in 6-well plates infected with HSV-1 (m.o.i. = 5) or not were washed with PBS, then fixed in 4% paraformaldehyde–1% glutaraldehyde in 0.1 N cacodylate buffer, and post-fixed in 1% osmium tetroxide. Cells were scraped with a rubber policeman, embedded in Vestopal and observed in a Zeiss EM-109-T transmission electron microscope, at 80 KV.

RESULTS

Effect of MA addition at different times post-infection on HSV-1 yields

In a previous report, we have shown that MA strongly inhibited specific infected-cell polypeptides synthesized late in infection, when added immediately after viral adsorption (Villamil *et al.*, 1995). To establish the dependence of the inhibitory effect on the time of addition, a single-step HSV-1 growth experiment in which MA was added to infected cell cultures at

Table 1. Antiviral activity of MA on the synthesis of HSV-1 DNA

Hours post-infection	Cpm/vial	
	Control	MA treated
8	2876.3 ± 143.12	1574.7 ± 64.77
10	4234.05 ± 234.55	3644.5 ± 347.9
12	6348.7 ± 483.24	4959.85 ± 128.9

Vero cell monolayers were infected with HSV-1 (m.o.i. = 10) and treated or not with 50 μg/mL of MA. At 8, 10 and 12 h p.i., cell monolayers were lysed and DNA was denatured and captured on filter membrane supports. Membranes were then batch-hybridized with an ^{125}I radioiodinated DNA probe specific to HSV-1. The processed membranes were counted in a gamma counting device and the mean reactivity for control and MA treated samples was determined.

progressively later times throughout the virus replication cycle was performed.

Vero cells grown in 24-well plates were infected with HSV-1 (m.o.i. = 1) and 50 μg/mL of MA was added at 0, 2, 4, 6, 8, 10 and 12 h after infection. At 24 h p.i., extracellular and cell-associated virus yields were determined. As shown in Fig. 1, HSV-1 cell associated yields were reduced three log units for 6 h p.i., remaining 1 log unit lower than in the control cultures from 6 h p.i. onwards. A significant reduction in released virus yields was observed at 0 and 2 h p.i. that progressively diminished with the time of addition of MA. Therefore, MA inhibited cell associated and released virus yields when added at different times p.i.

Inhibition of viral DNA synthesis

Because MA proved to affect the synthesis of ICP 9 and ICP 18.5 (late ICPs) involved in DNA replication (Villamil *et al.*, 1995), we decided to investigate the effect of MA on HSV-1 DNA synthesis using a DNA hybridization assay. We quantified viral genome synthesis in MA-treated and untreated infected cells at 8, 10 and 12 h p.i. because the peak of HSV-1 DNA synthesis is generally observed between 8 and 12 h p.i. when a high multiplicity of infection is employed (Koyama and Uchida, 1988). As shown in Table 1, the mean cpm values decreased 45%, 14% and 22% in MA-treated infected Vero cells at 8, 10 and 12 h p.i. respectively, indicating that the amount of DNA was reduced with respect to HSV-1 untreated infected controls (ANOVA test, $p<0.05$). Thus, MA seems to interfere with DNA synthesis by provoking a slight reduction in the rate of ^{125}I incorporation, although this effect cannot explain the major antiviral action of MA observed in Fig. 1.

Effect of MA on HSV-1 propagation

Considering that the synthesis of ICPs 30 and 35 (late ICPs) involved in the assembly of HSV-1 nucleocapsids is inhibited (Villamil *et al.*, 1995), we investigated whether viral propagation was impeded by MA. For this purpose, Vero cell monolayers grown in 24-well plates were infected with HSV-1 Cgal and incubated for 1 h at 37 °C. Then, inocula were eliminated and cells were covered with

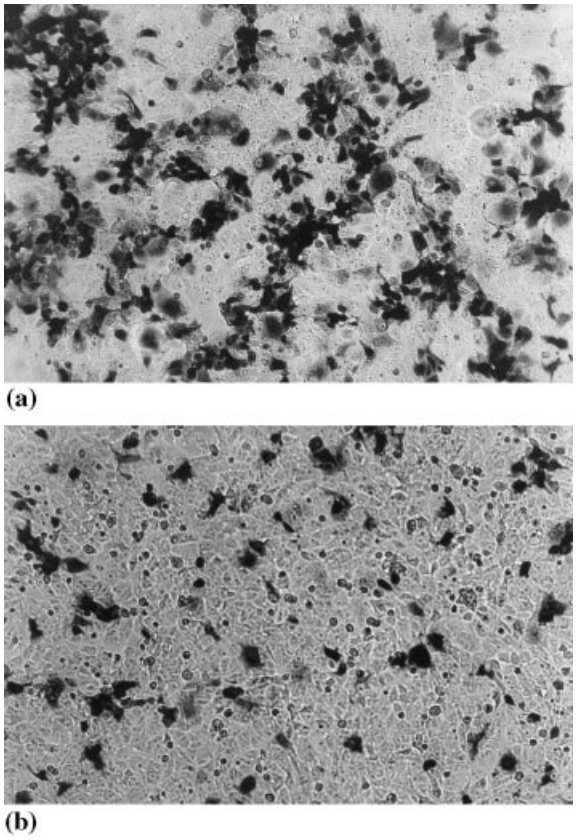

Figure 2. Effect of MA on HSV-1 propagation. Vero cell monolayers infected with HSV-1 C-gal at m.o.i. = 1 were treated or not with 50 μg/mL of MA. After 24 h p.i. at 37 °C, cells were washed and stained for β-gal, as described in the Materials and Methods (A: HSV-1 infected cells; B: HSV-1 infected MA-treated cells)

MEM 1.5% containing or not 50 μg/mL of MA per well. After incubation for 24 h at 37 °C, the cells were stained *in situ* for β-gal (see Materials and Methods).

In the absence of MA, 'blue' cells clustered in characteristic HSV-1 foci were observed (Fig. 2A), whereas MA treated-cultures exhibited only spared 'blue' cells expressing β-gal (Fig. 2B). Besides, the number of 'blue' foci determined in untreated-infected cells correlated with the number of single 'blue' cells from MA treated-infected cultures (data not shown), suggesting that MA prevents further viral spread.

Another series of HSV-1 infected cultures treated or not with MA were processed to corroborate the inhibitory effect on the virus yield exerted by MA. We found a reduction of 3 orders-of-magnitude in viral titre yields from MA-treated cells, in comparison with untreated cultures (data not shown).

Therefore, these results indicate that HSV-1 replicates in 'blue' cells from MA-treated infected cultures without being able to infect neighbouring cells.

Expression of HSV-1 proteins in MA-treated infected cells

In order to study the expression of accumulated viral proteins, HSV-1 infected Vero cells treated or not with MA were harvested at 8, 10 and 12 h p.i. and processed

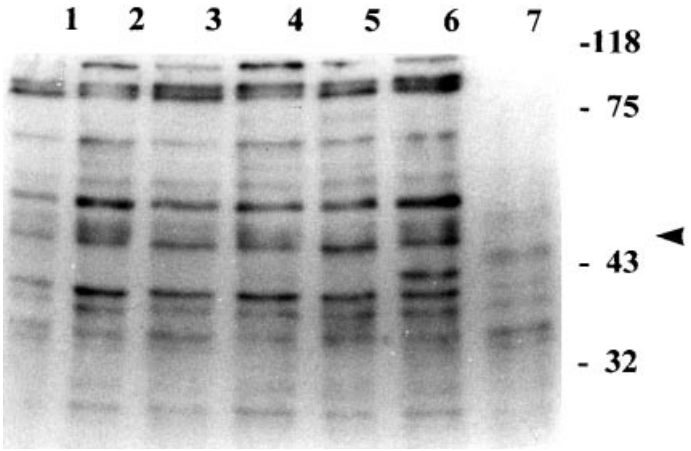

Figure 3. Effect of MA on the expression of HSV-1 proteins. Vero cell monolayers grown in 24-well plates were infected with HSV-1 (F) (m.o.i. = 1) (lanes 1–6) and treated with 50 μg/mL of MA (lanes 1, 3 and 5). At 8 (lanes 1 and 2), 10 (lanes 3 and 4) and 12 h p.i. (lanes 5 and 6), cell lysates were electrophoretically separated on 10 % SDS-polyacrylamide gels followed by electrical transfer to PVDF sheets in a Novablot System (LKB). The PVDF sheet was immunoblotted with a peroxidase-conjugated rabbit anti-herpes simplex virus type 1 (DAKO) and the immobilized proteins were detected using a western-blot chemiluminescence reagent (NEN, Life Science Products, Boston, USA). Lane 7: Uninfected control cells. The positions of the molecular weight markers are also indicated

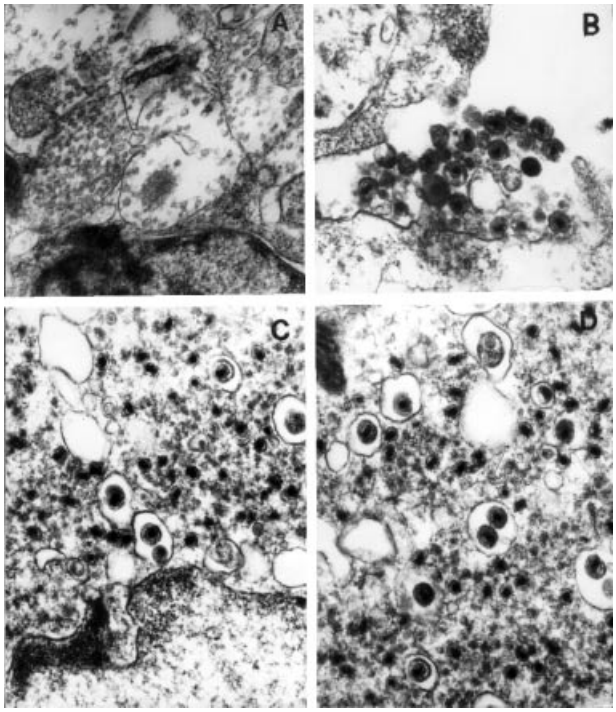

Figure 4. Electron microscopy of MA-treated and untreated cells. Monolayers of Vero cells grown in 6-well plates infected with HSV-1 (m.o.i. = 5) were treated or not with 50 μg/mL of MA. At 36 h p.i, cell monolayers were rinsed, fixed in glutaraldehyde, embedded and sectioned. A: MA-treated cells. Tiny, round-shaped structures can be observed into cytoplasmic vesicles; B: HSV-1 infected cells. Mature, enveloped virions are seen in the intercellular space after budding; C and D: MA-treated-HSV-1 infected cells. A few capsids are enveloped and located into cytoplasmic vesicles, probably belonging to the Golgi apparatus or the endoplasmic reticulum, and a large mass of unenveloped capsids are also observed in the cytoplasm. A, B, C and D: 40 000×

for immunoblotting, as described in Materials and Methods.

Figure 3 shows no significant differences between HSV-1 protein patterns corresponding to untreated and MA-treated cells, at 8 and 10 h p.i.. Nevertheless, in the lane containing the untreated cell extract, the appearance of a protein band with an apparent MW of 55 KD was detected at 12 h p.i., whereas it was absent from the MA-treated cell extract. Thus, the HSV-1 protein profile was affected by MA at 12 h p.i..

Ultrastructural studies of MA-treated HSV-1 infected cells

Considering that HSV-1 propagation is hindered by MA, we decided to investigate whether MA would act by allowing the accumulation of intracellular viral particles.

For that purpose, HSV-1 infected cells treated or not with MA were processed for electron microscopy, at 36 h p.i.. In the case of untreated cells, a classic pattern of HSV maturation was observed (Fig. 4B). Enveloped nucleocapsids were found in the intercellular space, whereas unenveloped nucleocapsids were detected in the nuclei (data not shown). In MA-treated infected cells, mature virus was seen in cytoplasmic vesicles, probably belonging to the Golgi apparatus or the endoplasmic reticulum, but strikingly, a great amount of unenveloped nucleocapsids were found in the cytoplasm (Fig. 4C and D).

Electron microscope photographs also show multivesicular bodies of round shape and about 50 nm size in cells treated with MA (Fig. 4A). The presence of these structures could not be taken as an indication of severe cellular cytotoxicity, since neither nuclear damage nor alterations in mitochondria and endoplasmic reticulum were observed. Furthermore, the metabolic activity and viability of cells after 36 h p.i of MA treatment were not affected, as demonstrated by MTT and trypan blue exclusion assays, respectively (data not shown).

DISCUSSION

Until now, chemically synthesized or modified compounds have been the major source of selective antiviral agents, particularly in the case of antiherpetic compounds (Prusoff *et al.*, 1990). Due to the need for compounds to combat resistance to the established drugs, Darby (1995) has suggested that the future for new antiherpes drugs will lie largely in the area of the non-nucleosides. Thus, significant progress has been made in the discovery of new leads for the development of herpesvirus protease-based therapeutic agents (Waxman and Darke, 2000). There is another important potential source of antiviral compounds, namely natural products (Souza Brito, 1996). Recently, plant-derived and semi-synthetic calanolide compounds isolated from the dried fruits and twigs of *Calophyllum lanigerum* were reported as non-nucleoside analogues capable of inhibiting human cytomegalovirus replication (Ze-Qi Xu *et al.*, 2000).

The specific effect of MA on HSV-1 multiplication proved to be the suppression of the synthesis of some late ICPs, in the absence of cytotoxicity (Villamil *et al.*,

1995). Our results of time-of-addition experiments confirm that MA affects a late event in the replication cycle of HSV-1 (Fig. 1). ICPs 9 and 18.5 are associated with HSV-1 DNA replication: the former carries out helicase and ATPase activities, and the latter is required for DNA cleavage and packaging. Prevention of the synthesis of both γ-proteins previously demonstrated (Villamil *et al.*, 1995) correlates with a decrease of HSV-1 DNA synthesis in MA-treated Vero cells observed through a DNA hybridization assay (Table 1). However, the extent of this effect is not enough to explain the strong inhibition displayed by MA on HSV-1 multiplication.

To investigate the antiviral effect of MA on HSV-1 propagation, we used HSV-1 containing the *Escherichia coli lacZ* reporter gene encoding β-galactosidase, whose expression is independent of DNA viral replication. The correlation between the number of 'blue' HSV-1 foci with the number of single 'blue' cells observed in control and treated cultures, respectively, corroborates that viral adsorption and penetration are not inhibited by MA. Since viral infectivity was recovered from treated cells, the appearance of isolated 'blue' cells instead of HSV-1 'blue' foci suggests that MA impeded viral spreading (Fig. 2A and B).

Ultrastructural observations seem to confirm this hypothesis. In Fig. 4, HSV-1 particles are observed outside the untreated cells. In contrast, mature virions are included in cytoplasmic vesicles from MA-treated cells, probably being responsible for the remaining infectivity. Noteworthy, nonenveloped nucleocapsids were clearly visible in the cytoplasm of MA-treated cells.

Campadelli-Fiume *et al.* (1991) have shown that cells infected with a herpesvirus carrying a mutation in glycoprotein D contained many more unenveloped capsids in the cytoplasm in comparison with the HSV-1 wild type, which were not released efficiently. This pattern of immature HSV-1 particles was also observed in infected cells treated with brefeldin A (BFA), though the mode of action of BFA and MA seems to be different (Cheung *et al.*, 1991).

Taking into account the inhibition of the synthesis of gD by MA (Villamil *et al.*, 1995) and the lack of a viral protein with a similar MW in MA-treated cell extracts at 12 h p.i. (Fig. 3), we inferred that, in the presence of MA, naked nucleocapsids were released to the cytoplasm and could not be enveloped by a Golgi-derived membrane. We speculate that MA would also act over two different steps during the last stages of the multiplication cycle, since accumulation of naked viral particles in the cytoplasm as well as the decrease in virus yield might be an independent phenomenon from that of reducing DNA synthesis.

It is hoped that the present contributions in antiviral drug discovery will lead to molecules targeting new functions, and that some of these molecules will possess an efficacy comparable to, or even better than that of the nucleoside analogues. Our efforts are being done to place MA in this context.

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