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Major Article

Prostaglandin A_1 triggers Mayaro virus inhibition and heat shock protein 70 expression in an epithelial cell model

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Abstract

Introduction: The Mayaro virus (MAYV), which is an arbovirus closely related to the Chikungunya virus, causes a dengue-like acute illness that is endemic to Central and South America. We investigated the anti-MAYV activity of prostaglandin A_1 (PGA_1), a hormone which exhibits antiviral activity against both ribonucleic acid (RNA) and deoxyribonucleic acid (DNA) viruses. Further, we examined the effects of inducting the stress protein HSP70 following PGA_1 treatment. **Methods:** Hep-2 cells infected with MAYV were treated with PGA_1 (0.1-6µg/ml) 12h before infection and for different periods post-infection. Inhibition of viral replication inhibition was analyzed via viral titer determination, whereas the effect of PGA_1 on viral morphogenesis was examined via transmission electron microscopy (TEM). Autoradiography (with ^{35}S methionine labeling) and western blotting were used to assess the effect of PGA_1 treatment on viral and cellular protein synthesis, and on HSP70 induction, respectively. **Results:** PGA_1 strongly reduced viral replication in Hep-2 cells, particularly when added during the early stages of viral replication. Although PGA_1 treatment inhibited viral replication by 95% at 24 hours post-infection (hpi), viral structural protein synthesis was inhibited only by 15%. TEM analysis suggested that PGA_1 inhibited replication before viral morphogenesis. Western blot and densitometry analyses showed that PGA_1 treatment increased HSP70 protein levels, although this was not detectable via autoradiography. **Conclusions:** PGA_1 inhibits MAYV replication in Hep-2 cells at early stages of viral replication, prior to production of viral structural proteins, possibly via HSP70 induction.

Keywords: Mayaro virus. Prostaglandin A. Heat shock proteins.

INTRODUCTION

Genus *Alphavirus* includes human and animal pathogens for which no effective antiviral treatment is yet available. This genus includes the Mayaro virus [(MAYV); genus *Alphavirus*, family *Togaviridae*], the causative agent of Mayaro fever (MF), a dengue-like disease characterized by acute fever, maculopapular rash, intense arthralgia and nausea[1]. MAYV is an Arbovirus, transmitted mainly by mosquitoes (*Haemagogus* spp.), which was first described in Trinidad, in 1954[2]. Birds are secondary hosts of MAYV and may spread the infection; this is observed in several countries in Central and South America[3]. Both acute and chronic phases of MF often present nonspecific symptoms that may confound diagnosis. Joint pain, characteristic of chronic MF, may persist for at least a year after infection. In MAYV endemic areas, patients with joint pain who are seronegative for arthritis may be infected with MAYV[4,5].

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In Brazil, attention first focused on MAYV in 1955, due to an outbreak in the State of Pará[6]. Since then, MAYV infections have been detected in several states[7-9]. MAYV is endemic to the Amazon region and Central Brazil, as confirmed by a high incidence of immunoglobulin M (IgM) against MAYV in febrile patients in the Goiás State[10]. Additionally, Terzian et al.[11] isolated a genetically distinct Brazilian MAYV lineage in the State of Acre.

MAYV detection in equids, sheep and caimans provided indirect evidence of MAYV circulation in the Brazilian Western region[4], reinforcing the outbreak potential of this virus, and revealing previously unknown hosts. MAYV infection rates may be underestimated, as MF is commonly mistaken for dengue, in the absence of specific diagnostics[12]. Some cases of a dengue-like viral disease, attributed to yet another alphavirus, the Chikungunya virus (CHIKV), during a recent outbreak in Brazil, may have been caused by MAYV infection[13].

Similar to CHIKV, and all other alphaviruses, MAYV genome consists of a single strand of positive-sense RNA, which is 12kb in length and encapsulated in an icosahedral capsid composed of a single protein (the C protein) and enveloped

by a lipid bilayer derived from host cell plasma membrane. The genome is arranged in two sections: the 5′ two-thirds encodes non-structural proteins required for transcription and replication of RNA, with the 5′ terminus capped with a 7-methylguanosine; the 3′ one-third encodes structural proteins translated from sub-genomic messenger RNA (mRNA), with a polyadenylated 3′ terminus. Viral replication occurs in host cell cytoplasm, and virions mature by budding through the plasma membrane, where virus-encoded surface glycoproteins E2 and E1 are embedded[14,15].

Serologically, MAYV is most closely related to the Semliki Forest virus (SFV) and to CHIKV. All three viruses are considered members of the Semliki Forest Complex. Similar to CHIKV, MAYV is transmitted from the adult mosquito to its progeny. Intrinsically high copy error rates of viral RNA polymerases have resulted in an extension of the CHIKV geographical range[16]. This phenomenon may also occur with MAYV. Therefore, the severe public health risk of a MAYV geographical spread due to adaptation to other mosquito species should not be underestimated, as there is currently no vaccine or effective treatment against MF.

This study aimed to increase the understanding of MAYV morphogenesis, by examining MAYV/host cell interactions and the effect of prostaglandin (PG) on viral replication. PGs are naturally occurring cyclic fatty acids that are synthesized from polyunsaturated fatty acid precursors, induced by cell injury and inflammation[17]. PGs play regulatory roles in physiological processes such as eukaryotic cell proliferation, differentiation, inflammation and febrile conditions[17-19]. Antiviral properties of PGs are evident, mainly among A and J type PGs[20]. A_1 and J_2 type PGs are well known for their antiviral effect. Chemical analysis of these PGs indicated that the presence of a carbonyl group at the unsaturated (α, β) cyclopentane ring might be responsible for this property[21,22]. PGs inhibit production of MAYV[23] and other viruses, including Sendai[24], Vesicular stomatitis virus (VSV)[25,26], human immunodeficiency virus (HIV)[27], Rotavirus[28] and Classical swine fever virus[29].

Despite the broad antiviral effects of PGs, mechanisms underlying antiviral activity of PGs, which vary among different virus-cell systems, are not yet understood. Heat shock proteins (HSPs), known for their chaperone function, are induced under PG treatment, representing a potential mechanism underlying antiviral effects of PGs[30,31]. We evaluated the effect of PGA_1 on MAYV production, and the relevance of the heat-shock protein, HSP70, to this process.

METHODS

Chemicals

PGA_1 was purchased from Sigma Chemical Company, St Louis, USA. It was diluted in ethanol (1mg/mL) and cultured in Dulbecco's Modified Eagle Medium (DMEM, at 20µg/mL). Both stock solutions were stored at -20°C.

Cell culture and virus

Human cervix carcinoma (Hep-2 cells, ATCC®CCL-23™) were grown in DMEM (Gibco) supplemented with 10% fetal bovine serum, and incubated at 37°C and 5% CO_2 in 25cm^2 cell culture flasks.

MAYV (lineage TR 4675) was stored at -80°C. The virus was obtained from the American Type Culture Collection (Rockville, MD, USA), and propagated *in vitro* in Hep-2 cells.

Virus Infection and PGA_1 treatment

Hep-2 cell monolayers (~80% confluency) were inoculated with MAYV at a multiplicity of infection (MOI) of 1, for drug effect evaluation using viral titer assays, or 5, for electron microscopy and viral protein analysis. Following the virus adsorption period (1h, at 37°C and 5% CO_2), inoculum was removed and fresh medium added. Depending on the assay, cells were pre-treated with PGA_1 for 12h prior to infection, or treated with different PGA_1 concentrations for different post-infection periods under standard culture conditions (37°C and 5% CO_2). At specific treatment times, culture supernatants were collected for virus titer determination via plaque assay, as per Baer & Kehn-Hall[32].

Cell viability assay

Cell monolayers were incubated in a solution containing 5µg/mL neutral red diluted in phosphate buffered saline (PBS), for 3h at 37°C, in 96-well plates. After discarding the solution, cells were incubated with 4% formaldehyde (in PBS) for 1 min, and 100% methanol for 20 min. Samples were analyzed for absorbance at 490nm, using an enzyme-linked immunosorbent assay (ELISA) plate reader (SpectraMax M2).

Radioactive labelling, SDS-PAGE and densitometry

Cells were infected as described (section 2.2), and treated with PGA_1 for different periods of time, following which the culture medium was replaced by DMEM devoid of L-cysteine and L-methionine and containing 30µCi/mL ^{35}S-methionine. After 60 min of incubation, the medium was removed and 80µL of sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer (25mM Tris-HCl containing 10% glycerol, 0.02% bromophenol blue, 100mM dithiothreitol and 2% SDS) was added to each flask. Samples were heated for 5 min at 100°C, and subjected to SDS-PAGE on a 12% polyacrylamide gel. Radioactivity was detected by exposing gels directly to X-ray films (BioMax TranScreen LE intensifying screen, Eastman Kodak Company, USA). Densitometric analysis of autoradiography films was performed using the Bromma 2202 laser densitometer (LKB-Produkter).

Transmission electron microscopy

Hep-2 monolayers grown in cell culture flasks of 25cm^2 (infected and treated as described in section 2.3) were fixed for 2 h at room temperature, in 2.5% glutaraldehyde in 0.1M cacodylate buffer (pH 7.2), and post-fixed at room temperature in 1% OsO_4/0.8% potassium ferrocyanide in the same buffer for 1h. Samples were dehydrated in ethanol and flat-embedded in Polybed resin (Polysciences®). Ultrathin sections (60nm thick) were stained with uranyl acetate and lead citrate, and observed using a Zeiss 900 transmission electron microscope.

Western blot

Samples subjected to SDS-PAGE as described above (section 2.4) were transferred to nitrocellulose membranes in

a SemiPhor transfer system (Hoefer Scientific Instruments). Membranes were immersed in Towbin Solution (25mM Tris-HCl, pH 8.3, with 192mM glycine, 20% methanol, and 1.3mM SDS) for 20 min, and transferred using the same buffer for 1h, at 0.8mA/cm^2. Membranes were blocked overnight (at 4°C) in 10% non-fat milk in TBST (50mM Tris-HCl, pH 7.5, with 150mM NaCl and 0.05% Tween 20), and incubated with a monoclonal antibody against HSP70 (clone BRM-22, Sigma Chemical Co.) at room temperature at a dilution of 1:5,000 for 1h. After washing thrice (10 min each) in TBST, membranes were incubated with anti-mouse peroxidase-linked secondary antibodies (ECL, Amersham, UK) diluted at 1:1,000, for 1h at room temperature. Labelling was visualized via ECL (Amersham) using Kodak X-Omat K film.

RESULTS

PGA$_1$ inhibits MAYV replication in Hep-2 cells

To evaluate the effect of PGA$_1$ on MAYV replication in Hep-2 cells, virus proliferation in these cells was analyzed under different concentrations of PGA$_1$. Following the adsorption period with an MOI of 1, cells were treated with PGA$_1$ at specific times, and culture supernatants were collected for titration.

At 48 hours post-infection (hpi), PGA$_1$ inhibited MAYV replication by 50% and 83% at 0.1 and 1µg/mL PGA$_1$, respectively (**Figure 1A**). Effect of higher concentrations of PGA$_1$ on MAYV replication at was examined (**Figure 1B**). While 1µg/mL PGA$_1$ inhibited MAYV production by 50% at, higher concentrations inhibited viral production by 82 (2µg/mL), 90 (4µg/mL) and 95% (6µg /mL) at (**Figure 1B**). Viability tests using neutral red showed that PGA$_1$ is not cytotoxic under tested conditions (data not shown).

Effect of PGA$_1$ on MAYV replication at different times post-infection was evaluated (**Figure 1C**). The inhibition of MAYV replication was high (~90%) when PGA$_1$ (5µg/mL) was added soon (within 2h) after adsorption (**Figure 1C**). When PGA$_1$ was added post-infection, its inhibitory potential decreased (**Figure 1C**).

Effect of PGA$_1$ treatment before infection initiation was examined (**Table 1**). Cells were infected and treated with PGA$_1$ (5µg/mL) 12h before infection initiation, and at 0, 1, and 3 hpi (**Table 1**). For pre-treatment with PGA$_1$, cells were washed with PBS prior to MAYV infection, which was performed for 24 hpi, and supernatants were processed for virus titration. PGA$_1$ treatment at 1 and 3 hpi yielded similar inhibition values, confirming our earlier results (**Figure 1C**), but PGA$_1$ treatment 12h before infection did not inhibit MAYV production (**Table 1**).

To analyze effects of PGA$_1$ on MAYV replication, Hep-2 cells were infected (MOI = 5), treated with 5µg/mL PGA$_1$ for 24h, and examined via transmission electron microscopy. The treatment did not alter the overall ultrastructure of uninfected Hep-2 cells (**Figure 2A** and **Figure 2B**). Untreated, infected cells exhibited many mature viral particles adhering to the cell membrane at the extracellular domain, and others budding from the plasma membrane (**Figure 2C, Figure 2D** and **Figure 2E**). A marked reduction in the number of virus particles was observed in cells treated with 5µg/mL PGA$_1$. No viral precursors or modified vacuoles typical of viral morphogenesis inhibition were seen in the cytoplasm of treated cells, which had much fewer virus-like particles budding from the plasma membrane than untreated cells (**Figure 2F** and **Figure 2G**).

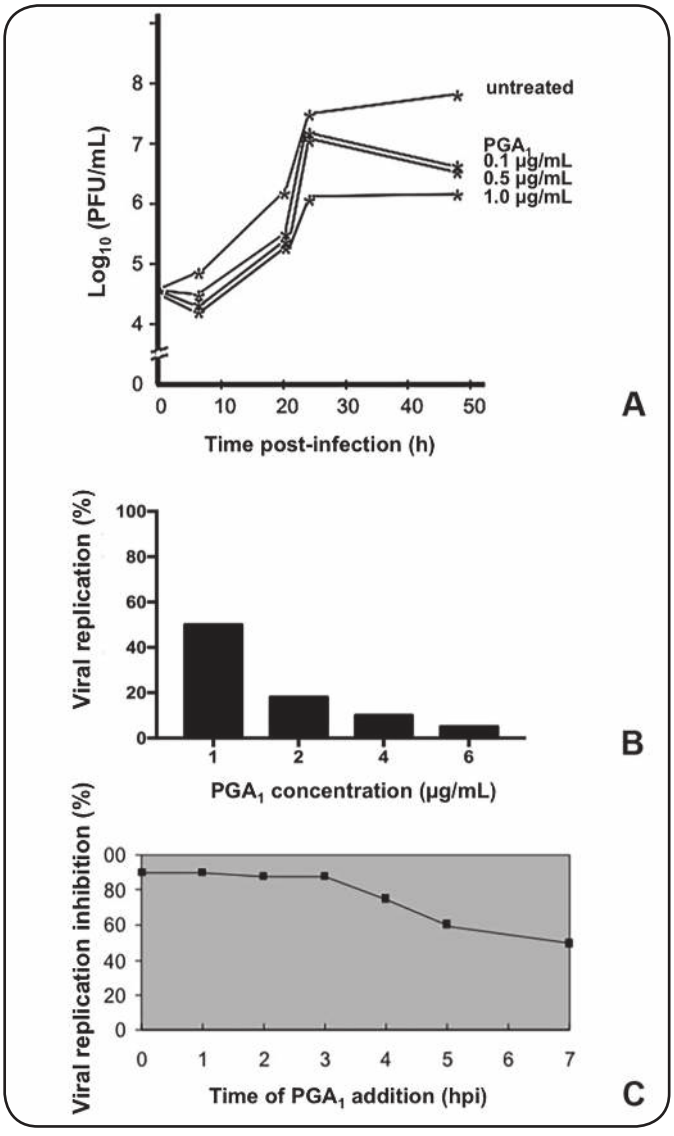

FIGURE 1: Effect of PGA$_1$ on MAYV replication. Hep-2 cells were infected with MAYV (MOI=1) and treated with PGA$_1$ at different concentrations and times post-infection. **A.** Lower PGA$_1$ concentrations (0.1, 0.5 and 1µg/mL) were added after the adsorption period, and the viral titer in the supernatant of infected cells was evaluated at 6, 20, 24 and 48 hpi. **B.** Effect of higher concentrations of PGA$_1$ (1 to 6µg/mL, for 24h) on viral replication in infected cells, relative to the controls - infected cells kept untreated. **C.** Inhibition of viral replication after treatment with 5µg/mL PGA$_1$ added at different times post-infection. **PFU/mL:** Plaque-forming unit; **PGA$_1$:** Prostaglandin A$_1$; **MAYV:** Mayaro virus; **MOI**: multiplicity of infection; **hpi:** hours post-infection.

Effect of PGA$_1$ on cellular and viral protein synthesis

In addition to the effect of PGA$_1$ on virus production, we analyzed the effect of PGA$_1$ on viral and cellular protein production, via ^{35}S-methionine cell labeling (for 60 min, 24 and 48 hpi), followed by SDS-PAGE and autoradiography

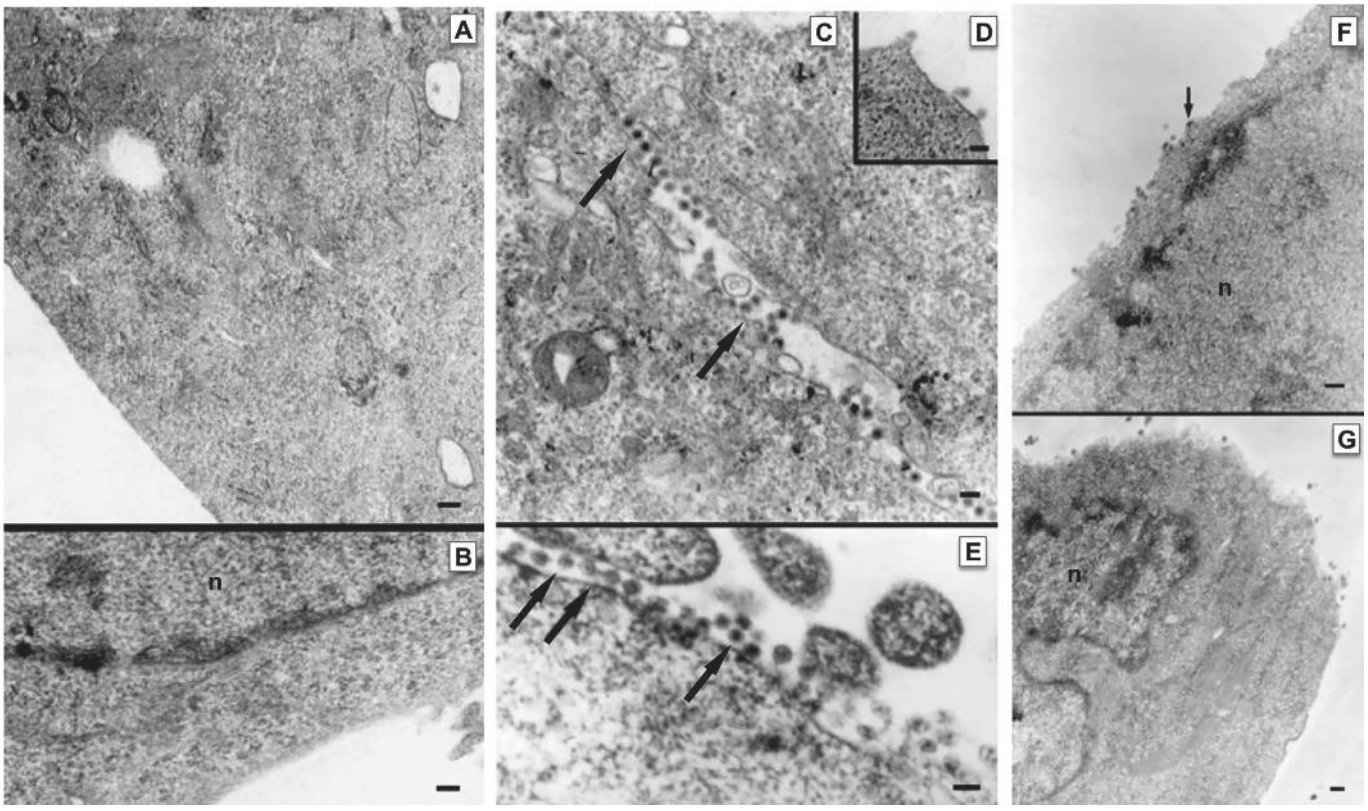

FIGURE 2: Transmission electron microscopy of Hep-2 cells infected with MAYV and treated with PGA_1. Cells were treated with PGA_1 (5µg/mL) for 24h concomitantly with infection with MAYV. **A** and **B** General views of the periphery from uninfected cells kept untreated **(A)** or treated with 5µg/mL PGA_1 (**B)** showing no clear structural changes after treatment. **(C, D** and **E)** Untreated cultures infected with MAYV (MOI=5) for 24h had a large number of viral particles being released to the intercellular region **(C)** after budding from the plasma membrane **D.** (arrows). **F** and **G.** Infected cells treated with PGA_1 for 24h, showing a drastic reduction in the release of viral particles (**F**, arrows), as well as the absence of nucleocapsids from the host cell cytoplasm **(G)**. n: nucleus. Scale bars: 100nm **(A-E)**; 200nm **(F-G)**. **Hep-2:** Human cervix carcinoma cells; **MAYV:**Mayaro virus; **PGA_1:** Prostaglandin A_1; **MOI**: multiplicity of infection.

analysis. As PG induces the expression of HSPs[30,31,33], we analysed whether HSP70 expression was induced after PGA_1 treatment. The controls consisted of uninfected and untreated cells subjected to heat-shock treatment (incubation at 42°C, for 1h) before ^{35}S-methionine labelling.

PGA_1 inhibited viral protein synthesis drastically at 48 hpi (p110, p62, E1/E2 and C), but could not reverse the inhibition of cellular protein synthesis induced by MAYV infection (**Figure 3A**). Densitometry analysis suggested that PGA_1 inhibited the production of MAYV proteins by ~15-30% and ~50%, at 24 and 48 hpi, respectively (**Figure 3B**).

Pronounced induction of HSP70 expression was observed when cells were subjected to heat shock (**Figure 3A**). To evaluate the effect of PGA_1 on HSP70 expression more precisely, western blot labelled with an anti-HSP monoclonal antibody was used. Heat-shock treatment increased HSP70 expression ~241 fold, relative to the untreated control, while 1 and 5µg/mL of PGA_1 increased HSP70 levels by 52% and 130%, respectively (**Table 2**).

TABLE 1: Effect of PGA_1 treatment on MAYV production by Hep-2 cells**.**

PGA_1 treatment (start time)	**PFU/mL**	**Replication inhibition (%)**
Before infection	5.3×10^6	1.85
Adsorption period	5.4×10^6	-
1 hpi	0.3×10^6	94.5
3 hpi	0.25×10^6	94.5

PGA_1 treatment (5µg/mL) started 12h before infection, during the virus adsorption period, or after virus adsorption (1 or 3hpi). Samples were collected at 24hpi for viral titration (PFU/mL), which was used to estimate viral replication inhibition (relative to untreated samples). **PGA_1:** Prostaglandin A_1; **MAYV:** Mayaro virus; **Hep-2:** Human cervix carcinoma cells**; PFU**: plaque-forming unit; **hpi:** hours post-infection.

DISCUSSION

Bird migration, and human migration and travel, combined with high mutation rates of RNA polymerases are some factors that may increase the risk of an MAYV outbreak in the Americas[3,13,34]. Neither vaccines nor effective treatments are available against human MAYV infection yet. Considering the epidemiological potential of MAYV, and Chikungunya-like symptoms that hinder differential diagnoses between Chikungunya and Mayaro[35], it is important to identify treatments capable of inhibiting MAYV replication.

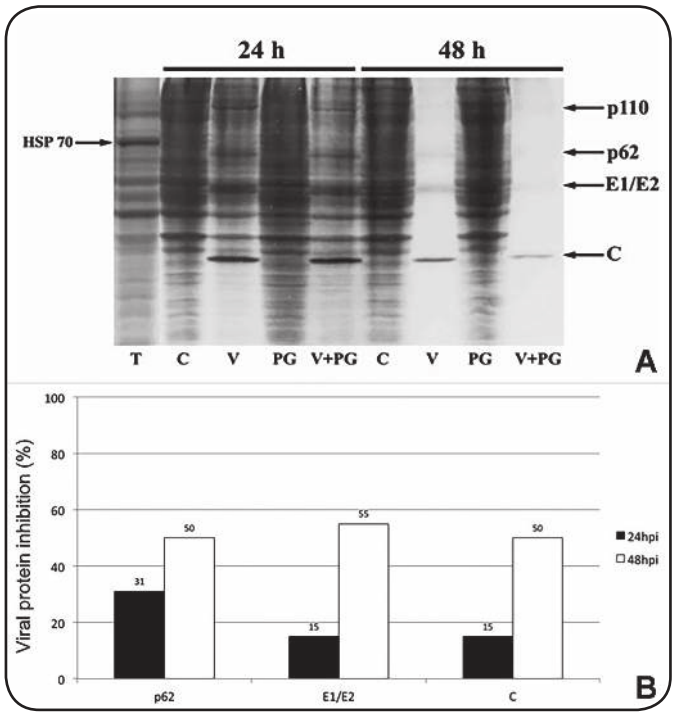

FIGURE 3: Cellular and viral protein synthesis during PGA_1 treatment of cells infected with MAYV. A. Cells were infected (MOI=1) and treated with PGA_1 (5µg/mL) for 24 and 48 hpi, incubated with ^{35}S-methionine (30µCi/mL) for 60 min and prepared for SDS-PAGE analysis. Samples are labeled as follows: C, control (mock infected, untreated) cells; V, MAYV infected cells; PG, uninfected cells treated with PGA_1; V+PG, infected cells treated with PGA_1; and T, uninfected and untreated cells subjected to heat shock (42°C, 1h), as a control for HSP70 induction. The expected positions of viral proteins (p110, p62, E1/E2, C) are indicated on the right. **B.** Densitometric analysis of MAYV protein levels detected a slight inhibition (15%) in the synthesis of viral structural proteins at 24hpi, and this effect increased to 45-55% at 48 hpi. **HSP:** heat shock protein; **PGA_1:** Prostaglandin A_1; **MAYV:** Mayaro virus; **MOI**: multiplicity of infection; **Ci/mL**: Curie; **SDS-PAGE**: sodium dodecyl sulfate polyacrylamide gel electrophoresis; **hpi:** hours post-infection.

TABLE 2: Densitometric analysis of HSP70 protein levels by western blot, after treatment of MAYV-infected cells with 1 and 5µg/mL of PGA_1 for 24h.

	Percentage of increase in HSP70 protein levels (relative to untreated control)
Untreated	-
Heat shock	241
PGA_1 (1µg/mL)	52
PGA_1 (5µg/mL)	130

Heat shock treatment (for 1h at 42°C) was used as a positive control for HSP70 induction. **HSP70:** heat shock protein; **MAYV:** Mayaro Virus; **PGA_1:** Prostaglandin A_1.

We demonstrated that PGA_1 may inhibit MAYV production in Hep-2 cells in a dose-dependent manner, with 2µg/ml PGA_1 inhibiting ~80% of virus production. PGA_1 inhibited MAYV replication at lower concentrations than those required for Sendai virus inhibition[24]. It is noteworthy that PGA_1 treatment had no detectable effect on cellular viability even at higher doses (10µg/ml), during the treatment times analyzed, (not shown).

Santoro et al.[24], reported that PGs do not significantly affect early stages of viral infection, namely adsorption, penetration and uncoating[24]. However, PGA_1 treatment at 4 hpi, reduced antiviral potential compared to PGA_1 treatment at earlier stages of infection (1-3 hpi). Similar results have been reported regarding PGA_1 inhibition of HIV replication[27], corroborating the hypothesis that initial stages of infection may be important targets for PG inhibition of MAYV replication.

Some antiviral molecules, including interferon[36], may inhibit viral replication even when cells are treated before infection. However, pre-treatment of cells with PGA_1 had no protective effect against MAYV infection.

We showed that some antiviral substances (e.g., interferon, monensin, and weak bases) induce remarkable defects in viral particle morphogenesis[37-39]. Superti et al.[28] observed that in monkey kidney cells (MA-104) infected with Rotavirus, and treated with PGA_1, normal cytoplasmic inclusions and particles budding from the endoplasmic reticulum were present, but viral maturation was impaired. Our analysis suggests, however, that PGA_1 does not disrupt MAYV morphogenesis in Hep-2 cells, as evidenced by the lack of nucleocapsid accumulations in the cytoplasm following PGA_1 treatment, and the drastic reduction in viral particles in intercellular spaces. These results indicate that the severe decrease in the viral titer after treatment, may, at least in part, be due to structural protein synthesis inhibition by PGA_1.

Bader and Ankel[26] showed that inhibition of viral RNA polymerase activity may be a potential mechanism underlying VSV replication inhibition by PGA_1. This may explain the decrease in viral messenger RNA synthesis observed in cells treated with PGA_1[40]. Inhibition of primary transcript production may potentially explain the antiviral effect of PGA_1. However, RNA polymerase inhibition may not fully clarify other patterns of DNA and RNA virus replication inhibition by PGs, which may be mechanistically more complex.

Western blot analysis using anti-HSP70 monoclonal antibodies showed that PGA_1 treatment increases HSP70 expression. These results confirm those of other studies showing that viral replication inhibition by cyclopentane PGs (PGA_1 and PGJ_2) is associated with the ability of these molecules to trigger HSP70 synthesis in a large variety of animal cells[41,42]. This phenomenon is mediated by the heat shock factor (HSF), a transcriptional activator for HSPs[43,44]. Several studies demonstrated that HSP70 is required for viral replication inhibition by PGA_1. HSP70 inhibition decreased antiviral activity of PGA_1 against MAYV in Vero cells[45]. The inhibitory effect of PGA_1 against the Sindbis virus was abolished by HSP70 induction[46]. A variety of HSP70 inducers may block the replication of different virus types[47,48].

Due to different viral replication inhibition strategies of PGs, it is difficult to establish a general antiviral mechanism for HSP activation associated with PG treatment. HSPs may control viral replication by interacting with viral proteins, as observed in VSV and enterovirus, among others[49,50]. Alternatively, HSPs

may downregulate viral protein synthesis, as HSP70 synthesis triggered by PGA_1 treatment during initial stages of infection, has been associated with viral protein synthesis inhibition and a reversal of cellular protein synthesis inhibition induced by the virus[41]. We observed that PGA_1 inhibited MAYV protein synthesis by 50% at 48 hpi. However, the profile of cellular proteins was not altered by PGA_1, which did not reverse virus-induced inhibition of cellular protein synthesis, after 48h. While PGA_1 inhibited viral protein synthesis by 15% at 24 hpi, viral replication was inhibited by 95%, indicating that viral mRNA translation is not the main target of the compound, which corroborates previous data on the effects of PGA and PGJ on poliovirus infection[51].

Although ^{35}S-methionine labelling in non-infected cells after 24h of PGA_1 treatment did not clearly indicate HSP70 induction, western blot analysis revealed a 130 fold increase in HSP70 levels following 5µg/mL PGA_1 treatment. A low level of constitutive HSP70 expression was also detected in the control cells, and heat shock treatment induced higher HSP70 expression levels than those observed following PGA_1 treatment. Possibly, relatively low levels of HSP70 synthesis induced by PGA_1 in Hep-2 cells may be sufficient to inhibit MAYV replication. This result is different compared to that reported for numerous other cell lines, where PGA_1 treatment did not induce HSP70 synthesis[42,52].

PGA_1 and PGJ_2 inhibit viral mRNA transcription, leading to the identification of nuclear factor kappa B (NF-κB) as a target for PGs[30]. This factor remains inactive in eukaryotic cells unless activated by phosphorylation in response to different stimuli, including stimulation by cytokines as well as bacterial and fungal infections. NF-κB is then translocated to the nucleus where it induces the transcription of a variety of genes. In addition to being important immunological mediators, PGA_1 and PGJ_2 play a role in the inflammatory response by inhibiting NF-κB in human cells through blocking phosphorylation and degradation of the NF-κB inhibitor Iκ-B[19]. Inhibition of NF-κB by PGA_1 may explain some results of our present study, but further studies are needed to establish whether NF-κB modulation affects the observations made in our study.

Our results indicate that interaction between the Hep-2 cell line and MAYV may be different compared to a large number of cell lines examined in previous studies[23,45,53]. While we detected an increase in HSP70 expression in infected Hep-2 cells treated with PGA_1 via western blot, HSP70-independent PGA_1 modes of action may also be operating in this virus-cell system.

Conflict of interest

The authors declare that there is no conflict of interest.

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