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Antiviral mode of action of a synthetic brassinosteroid against Junin virus replication

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Abstract

The antiviral mode of action of the synthetic brassinosteroid (22S,23S)-3β-bromo-5α,22,23-trihydroxystigmastan-6-one (**6b**) against Junin virus replication in Vero cells was investigated. Time-related experiments showed that **6b** mainly affects an early event of virus growth cycle. Neither adsorption nor internalization of viral particles was the target of the inhibitory action. The analysis of the effect of **6b** on viral RNA synthesis demonstrated that the presence of the compound adversely affects virus RNA replication by preventing the synthesis of full length antigenomic RNA. Although **6b** was most effective the earlier it was added to the cells after infection with JV, a high level of inhibition of JV yield and fusion activity of newly synthesized viral glycoproteins was still detected when the compound was present during the last hours of infection. Therefore, we cannot rule out an inhibitory action of **6b** on later events of JV replicative cycle.

Keywords: Arenavirus; Hemorrhagic fever virus; Brassinosteroid; Antiviral activity

1. Introduction

Junin virus (JV), a member of the *Arenaviridae* family, causes a severe disease in humans known as Argentine hemorrhagic fever (AHF). This is an endemo-epidemic disease geographically restricted to the most fertile areas of the country, the habitat of JV main reservoir in nature being the cricetid *Calomys musculinus*. Virions are enveloped particles containing a bipartite single stranded RNA genome, with ambisense coding strategy. The small (S) fragment encodes the nucleocapsid protein, N, and the precursor of virus glycoproteins, GPC. Post-translational cleavage of GPC gives origin to the two envelope glycoproteins G1 and G2. The large (L) fragment encodes the RNA-dependent RNA polymerase, L, and a Zn-binding protein named Z (Romanowski, 1993). Other arenaviruses, such as Lassa virus from West Africa, Machupo virus from Bolivia, Guanarito virus from Venezuela and Sabia virus from Brazil, also cause severe hemorrhagic diseases in man (Clegg, 2002; Peters, 2002). The importance of these viruses as human pathogens and the continuous emergence of new arenaviruses during the last years in North and South America (Peters, 2002) have demanded renewed efforts to develop effective antiviral agents against them.

Although several compounds inhibit in vitro arenavirus replication (Damonte and Coto, 2002), to date ribavirin is the only drug that has shown a partial efficacy against JV infection in animal models (McKee et al., 1988). Ribavirin has been used in Lassa fever patients (McCormick et al., 1986) and has been tried in AHF patients late in infection but without success (Enria and Maiztegui, 1994). Hence, the current therapy for AHF is based upon the early administration of immune plasma, however, a drawback of this therapy is the development of a late neurological syndrome in about 10% of the treated patients (Enria and Maiztegui, 1994).

Brassinosteroids (BRs) are a group of naturally occurring polyhydroxy steroidal plant hormones that play essential roles in modulating the growth and differentiation of plant cells at nanomolar to micromolar concentration (Clouse and Sasse, 1998). In previous reports, we demonstrated that synthetic BRs inhibit in vitro replication of the arenaviruses Junin, Tacaribe and Pichinde (Wachsman et al., 2000). Among the tested compounds, the derivative (22S,23S)-3β-bromo-5α,22,23-trihydroxystigmastan-6-one (**6b**) was the most active

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against JV infection in Vero cells, with a 50% cytotoxic concentration (CC_{50}) of 277 μM (using confluent non-growing cells) and a 50% effective concentration (EC_{50}) of 0.4 μM for IV_{4454} JV strain. A higher level of cytotoxicity was exerted in growing cell cultures (CC_{50} = 55 μM) (Wachsman et al., 2002), however, even under these conditions, **6b** showed a better selectivity index (SI = 137) than ribavirin (SI = 18) against JV.

Since it was demonstrated that the compound **6b** did not cause direct inactivation of JV particles (Wachsman et al., 2000) the aim of the present study was to elucidate the mode of antiviral action of the synthetic analogue **6b** on JV replication in cell cultures.

2. Materials and methods

2.1. Cells and virus

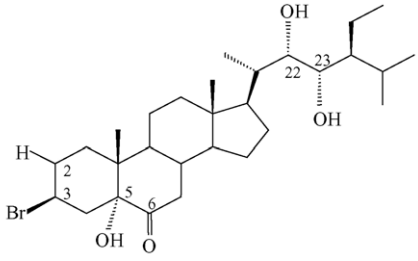
Vero and BHK-21 cells were grown in Eagle's minimal essential medium (MEM, GIBCO) containing 5% inactivated calf serum and 50 μg/ml gentamicin. Maintenance medium (MM), pH 7.5, consisted of MEM supplemented with 2% inactivated calf serum and gentamicin. IV_{4454}, an attenuated strain of JV isolated from a mild human case of AHF (Candurra et al., 1989), was propagated on BHK-21 cells. Virus stock (titer: 2×10^7 PFU/ml) was plaque-assayed on Vero cells.

2.2. Compounds

The compound (22S,23S)-3β-bromo-5α,22,23-trihydroxystigmastan-6-one (**6b**), synthesized from stigmasterol as previously described (Teme Centurión and Galagovsky, 1998), was kindly provided by Dr. L.R. Galagovsky (University of Buenos Aires, Argentina). Structural formula of compound **6b** is shown in Fig. 1. Stock solutions of **6b** (10 mg/ml) or cycloheximide (Cx) (Sigma Aldrich) (10 mg/ml) were prepared in ethanol, stored at −20 °C and further diluted in MM shortly before use.

2.3. Effect of time of *6b* addition on JV production

Compound **6b** (20 μM) was added to confluent monolayers of Vero cells infected with JV at a multiplicity of infection (moi) of 1 PFU/cell, at time 0 of infection (simultaneously with virus inoculum) or at 1, 2, 4, 6 or 8 h post-infection (p.i.). Cultures were further incubated at 37 °C till 12 h p.i. and at that time, extracellular virus yields were measured from supernatants by plaque assay on Vero cells. To determine cell-associated infectivity, cells were covered with fresh MM and they were subjected to two cycles of freeze-thawing followed by centrifugation at low speed and the supernatants obtained were titrated by plaque assay.



(22S,23S)-3 β-bromo-5α,22,23-trihydroxystigmastan-6-one

Fig. 1. Structural formula and IUPAC name of compound **6b**.

2.4. Radioimmunoprecipitation assay

Compound **6b** (20 μM) was added to confluent monolayers of Vero cells infected with JV (moi = 1 PFU/cell) at 0, 5 or 8 h p.i. and remained till 48 h p.i. At that time, cultures were pulse labelled with 35 μCi/ml of ^{35}S-methionine (sp. act. 1031 Ci mmol, New England Nuclear, Boston, MA) in methionine-free medium for 4 h. Then cells were washed three times with cold PBS, lysed in radioimmuneprecipitation assay buffer (RIPA: 10 mM Tris–HCl pH 7.4, 0.15 M NaCl, 0.1% SDS, 1% Triton X-100, 1% sodium deoxycholate and 0.4 mM phenylmethylsulfonyl fluoride (PMSF)) and incubated with hyperimmune rabbit anti-JV serum for 30 min at 37 °C and further incubated at 0 °C for 4 h. Antibody–antigen complexes were collected with protein A—sepharose and immunoprecipitated proteins were resuspended in sample buffer containing 5% SDS, 2% β-mercaptoethanol, 10% glycerol and 0.005% bromophenol blue in 0.0625 M Tris–HCl, pH 6.8. Samples were heated for 2 min in boiling water and SDS–PAGE was performed on 4% stacking/10% separating Laemmli gels (Laemmli, 1970). After electrophoresis gels were subjected to fluorography and visualized by autoradiography on AGFA films, as described previously (Bonner and Laskey, 1974).

2.5. Binding and internalization assays

JV labelled with ^{35}S-methionine was prepared by infecting BHK-21 cells at a moi of 0.02 PFU/cell. At 72 h p.i., MM was replaced by methionine-free medium with 10 μCi/ml of ^{35}S-methionine and cultures were further incubated at 37 °C for 24 h. After labelling, supernatants were collected, clarified by low-speed centrifugation and concentrated by ultracentrifugation at 100,000 × *g* for 2 h. For the binding assay, Vero cells grown in vials were infected with ^{35}S-methionine-JV (3×10^4 CPM/vial, moi = 1 PFU/cell) diluted in PBS containing 0.4% bovine serum albumin (BSA) and incubated 1 h at 4 °C in the presence or absence of **6b** (20 or 80 μM). Then, free virus was removed and cells were extensively washed with cold PBS containing 0.2% BSA. Monolayers were disrupted with 0.1N NaOH solution containing 1% SDS and cell-bound radioactivity was quantified using a liquid scintillation counter. For the internalization assay, after virus adsorption at 4 °C for 1 h, cells were incubated at 37 °C during 1 h to allow virus penetration in the presence or in the absence of **6b** (20 or 80 μM). Then, cultures were washed with PBS and treated with 1 mg/ml of proteinase K in PBS to remove external adsorbed virus. Protease treatment was then stopped by adding 1 mM PMSF in PBS containing 3% BSA. Cells were then pelleted, lysed in NaOH-SDS solution as above and cell-associated radioactivity was quantified.

2.6. RNA extraction, cDNA synthesis and PCR analysis

Confluent Vero cells grown in dishes (9 cm diameter) were infected with JV (moi = 0.1 PFU/cell) and after 1 h of adsorption at 37 °C, inocula were removed and cultures were covered with MM or MM containing **6b** (40 μM) or Cx (100 μg/ml) or **6b** in combination with Cx. At 6 or 24 h p.i., total RNA was extracted with the TOTALLY RNA kit (Ambion) using a modification of the method from Chomczynski and Sacchi (Chomczynski and Sacchi, 1987). Briefly, the cells were lysed with GTC solution (4 M guanidinium isothiocyanate, 0.5% sarkosyl, 25 mM sodium citrate) and lysates were extracted with acid phenol/chloroform/isoamylic alcohol, precipitated with one volume of isopropanol, reprecipitated with ethanol and resuspended in 20 μl of water.

The detection of genomic S RNA by PCR was performed from cDNA synthesized with the primer vcN (5′-CGCACAGTGGATCCTAGGC-3′). PCR reaction was conducted using arenavirus-specific primers vN (5′-GGCATCCTTCAGAACAT-3′) plus vcN that generated a 186 bp amplification fragment comprising the 3′ end of the S RNA containing N coding sequence (Ellenberg et al., 2002).

Strand-specific RT-PCR was used to monitor the presence of N mRNA and full length antigenomic S RNA. The viral sense primers vN or vG (5′-ATGGGGCAATTCATCAG-3′) were used in the cDNA synthesis. Primers vN plus vcN, which rendered a 186 bp amplification fragment, and primers vG plus vcG (5′-CCCCTTAATGTAAAGATGGC-3′), which amplified a 297 bp fragment, were used in the PCR reactions (Tortorici et al., 2001).

Synthesis of cDNA was carried out as previously described (Lozano et al., 1997; Ellenberg et al., 2002). RNA was heated to 95 °C for 5 min in the presence of 2 μM arenavirus-specific primers vcN, vN or vG. For each RNA sample β-actin mRNA was retrotranscribed in cDNA using the primer 5′-GAGACCTTCAACACCCCAGCC-3′ (2 μM). Synthesis of cDNA was performed using AMV reverse transcriptase (Promega) and after incubation of the reaction mixture for 2 h at 42 °C, cDNA was ethanol precipitated with 100 mM sodium acetate and 2.5 μg of linear polyacrylamide and resuspended in 15 μl of water.

PCR amplifications were carried out in a final volume of 10 μl, containing 1 μl of the cDNA reaction, 0.125 U of Taq DNA polymerase (Promega), 0.2 mM each dNTPs, 1.5 mM $MgCl_2$, 50 mM KCl and 10 mM Tris–HCl, pH 8.3. The PCR cycle progression for vcN/vN was as follows: 1 min at 94 °C and 35 cycles of 10 s at 94 °C (denaturation), 20 s at 48 °C (annealing) and 20 s at 72 °C (extension) followed by 5 min at 72 °C for final extension. Amplification conditions for vG/vcG were: 30 s at 94 °C and 35 cycles of 15 s at 94 °C, 20 s at 50 °C and 30 s at 72 °C followed by 5 min at 72 °C. β-actin primers used were 5′-GAGACCTTCAACACCCCAGCC-3′ and 5′-GGCCATCTCTTGCTCGAAGTC-3′ which generated a 309 bp fragment. Amplification conditions for β-actin were 35 cycles of 95 °C for 30 s, 60 °C for 45 s and 72 °C for 1 min. The whole PCR reaction volume was electrophoresed at 4 V/cm for 60 min onto 2% agarose gel in TAE buffer (40 mM Tris–acetate, 1 mM EDTA, pH 8.0) with 0.2 μg/ml ethidium bromide. Negative controls were performed with distilled water instead of cDNA.

2.7. Syncytium formation assays

Vero cells grown on coverslips were infected with JV (moi = 1 PFU/cell) and after 1 h adsorption, cells were incubated in MM at 37 °C. At different times after infection, supernatants were collected in order to determine extracellular virus titers and cells were washed with PBS and incubated in low pH medium, pH 5.0 (MEM containing 0.2% BSA, 10 mM HEPES) for 2 h at 37 °C. After that, cultures were washed and incubated for 12 h at 37 °C in MM, fixed with methanol, stained with 0.4% Giemsa and examined for the presence of multinucleated cells (Castilla and Mersich, 1996). Percentage of fused cells was calculated from 20 randomly selected fields as (number of polykaryocytes/total number of nuclei in the field) × 100. To analyze the effect of time of **6b** addition on syncytium formation, **6b** (20 μM) was added to JV-infected Vero cells at 2, 4, 6 or 8 h p.i and further incubated at 37 °C. Another set of infected cultures was incubated with medium containing **6b** at 1 h p.i. and the drug was removed by medium change at 2, 4, 6 or 8 h p.i. In all cases, at 10 h p.i., supernatants were removed and cells were incubated in buffered MEM at pH 5.0 and processed to visualize cell fusion as described above.

In order to determine if the compound **6b** affects membrane fusion process through a non-specific mechanism, uninfected or JV-infected Vero cells (moi = 5 PFU/cell) were incubated, with MM containing **6b** (40 μM) or untreated, at 37 °C for 24 h. Then, monolayers were treated with trypsin (0,75 mg/ml), centrifuged at 1000 × *g* for 5 min, washed with PBS, resuspended in MM and co-cultivated by mixing infected untreated cells with uninfected untreated cells or with uninfected **6b**-treated cells or by mixing infected **6b**-treated cells with uninfected untreated cells or uninfected **6b**-treated cells. Mixtures were seeded on coverslips contained in a 24-well culture plate and after 1 h at 37 °C medium was replaced by MEM pH 5.0. After 2 h of incubation at 37 °C syncytium formation was examined as described above.

3. Results

3.1. Effect of time of addition of *6b* on JV production and synthesis of viral proteins

To characterize the antiviral mode of action of **6b** against JV we first examined the effect of time of **6b** addition on JV replication. Compound **6b** (20 μM) was added to JV-infected Vero cells at different times after infection and maintained till the end of the first virus growth cycle. At 12 h p.i., extracellular and cell-associated virus yields were determined by plaque assay. As it is shown in Fig. 2A, inhibition of JV multiplication is dependent on the time of addition of the BR. Maximum inhibitory effect (97% of inhibition of virus yield) occurred when **6b** was added with virus inoculum (time 0 h) or immediately after JV adsorption (1 h p.i.) indicating that the BR mainly affects an early step of the replication cycle. However, the addition of **6b** at 4, 6 or

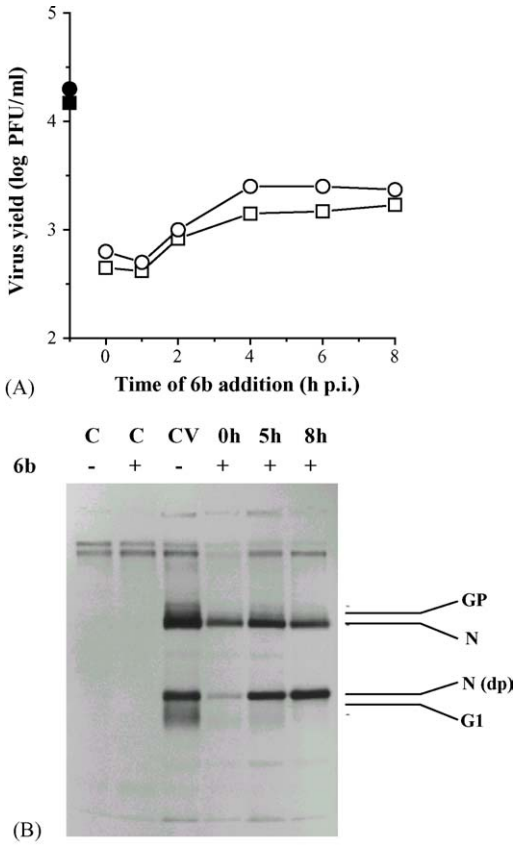


Fig. 2. Time-related effect of **6b** on virus replication and protein synthesis. (A) Vero cells infected with JV (moi = 1 PFU/cell) were incubated with culture medium (closed symbols) or culture medium containing **6b** (20 μM) (open symbols) at different times: time 0 (with virus inoculum), 1, 2, 4, 6 or 8 h p.i. At 12 h p.i., extracellular (squares) and cell-associated (circles) virus yields were determined by plaque assay. (B) Compound **6b** (20 μM) was added to JV-infected Vero cells at 0, 5 or 8 h p.i. At 48 h p.i., cultures were labelled with ^{35}S-methionine and viral proteins were immunoprecipitated with hyperimmune rabbit anti-JV serum and analyzed by SDS–PAGE. C: mock-infected cells, CV: untreated infected cells. The positions of bands corresponding to the main JV proteins are indicated: GPC (precursor of viral glycoproteins), N (nucleocapsid protein), N (dp) (N derived peptide), G1 (mature glycoprotein).

8 h p.i. still caused a significant reduction of virus production (90% inhibition of virus yield).

The effect of the time of **6b** addition on JV protein synthesis was also investigated. For this purpose, JV-infected cells, treated with **6b** at different times p.i. (0, 5 or 8 h p.i.) were labelled with ^{35}S-methionine at 48 h p.i. and viral proteins were immunoprecipitated and analyzed by SDS–PAGE. The presence of four viral polypeptides can be seen in lane CV (Fig. 2B) corresponding to infected untreated cell cultures. The major protein band (60–64 kDa) is the nucleocapsid N protein and the faint band above N represents GPC (68–72 kDa), the precursor of viral glycoproteins. The band called N (dp), of apparent molecular weight of 40–42 kDa, is a degradation product derived of N protein (Ellenberg et al., 2002) and the broad weak band of approximately 38 kDa corresponds to G1 mature glycoprotein. An extensive inhibition of viral protein synthesis was observed when **6b** was added at 0 h p.i. (simultaneously with virus inoculum) compared with untreated infected cultures (Fig. 2B). In accord with the results shown in Fig. 2A, lower levels of inhibition of viral protein synthesis were detected when the compound was added at 5 or 8 h p.i. As can be seen in Fig. 2B two cellular proteins were also precipitated with the anti-JV serum used in the radioimmunoprecipitation assay. Similar amounts of these cell proteins could be detected in uninfected cells, treated or not with **6b** during 48 h (Fig. 2B, lanes C (−**6b**) and C (+**6b**)), indicating that **6b** treatment did not affect host cell protein synthesis. However, in JV-infected cultures incubated with **6b** at time 0, these cell protein bands were fainter (Fig. 2B, lane 0 h). This decrease in the intensity of host cell protein bands was observed in two independent experiments and we have no explanation for these results since the same amount of sample was loaded on each lane of the gel.

The inhibitory effect of **6b** on viral protein synthesis was also investigated by immunofluorescence assays. In accord with the results obtained in the radioimmunoprecipitation assay, **6b** was much more effective in reducing the expression of viral proteins in infected cells, when present at early times of infection. Nonetheless, the addition of **6b** at 8 h p.i. still caused a significant reduction in the number of cells expressing viral antigens (data not shown).

Since the results of time of addition experiments suggested that **6b** exerts its inhibitory action mainly on an early event of JV replication cycle, we analyzed the effect of **6b** on JV adsorption and penetration steps by performing radiolabelled virion binding and internalization assays. The amount of bound radiolabelled virus (3.7×10^3 CPM) and internalized radiolabelled virus (3.2×10^3 CPM) was similar in the presence or absence of 20 μM of **6b**. Even at higher concentration (80 μM) of the compound, no significant differences on cell-bound or internalized radioactive virus were detected in **6b**-treated cultures compared with untreated ones, indicating the lack of effect of **6b** on the entry of JV into the cell.

3.2. *Effect of **6b** on JV RNA synthesis*

As was mentioned, JV genome is composed of two single stranded RNA species, S and L, with ambisense coding strategy. The genomic S RNA (v S RNA) is transcribed to only two antigenomic forms: the 1.8 kb N mRNA and the 3.4 kb full length antigenomic S RNA (vc S RNA) (Fig. 3). To analyze the effect of **6b** treatment on JV RNA synthesis we performed a semiquantitative RT-PCR analysis. First, JV-infected Vero cells were incubated in the presence or absence of **6b** (40 μM) at 37 °C. Total RNA was extracted at 24 h p.i. and cDNA synthesis was performed using the primer vcN, complementary to the 3′ end of viral S RNA. Further amplification by PCR using the primers vcN and vN renders a 186 bp fragment corresponding to the 3′ end of viral S RNA (Fig. 3). As can be seen in Fig. 4A, a strong inhibition of viral RNA synthesis was detected in **6b**-treated cultures at 24 h p.i.

In order to determine the effect of **6b** on viral RNA synthesis at early stages of infection, JV-infected cells were treated with **6b** (40 μM) and total RNA was extracted at 6 h p.i. The presence of N mRNA and antigenomic S RNA was detected by strand-specific RT-PCR. cDNA synthesis with primer vN followed by

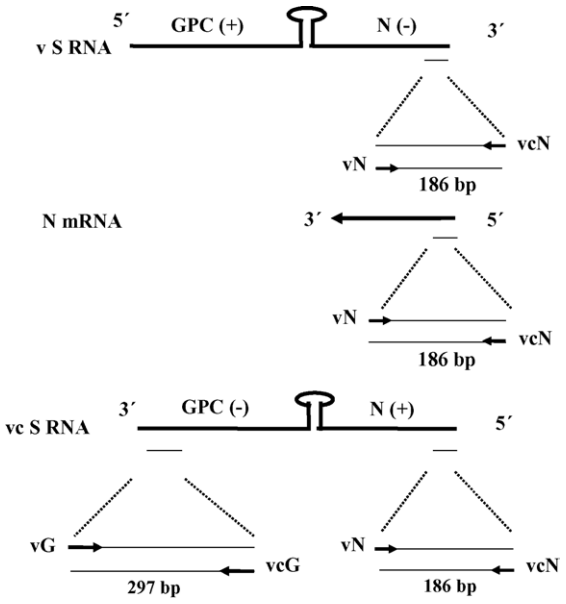


Fig. 3. Schematic representation of S RNA and PCR amplification fragments, The genomic S RNA (v S RNA) and the antigenomic forms, the N mRNA and the full length antigenomic S RNA (vc S RNA), are shown. The primers used for the RT-PCR detection of genomic S RNA or antigenomic forms are indicated as arrows. The sizes of the amplification products are indicated in base pairs (bp) underneath.

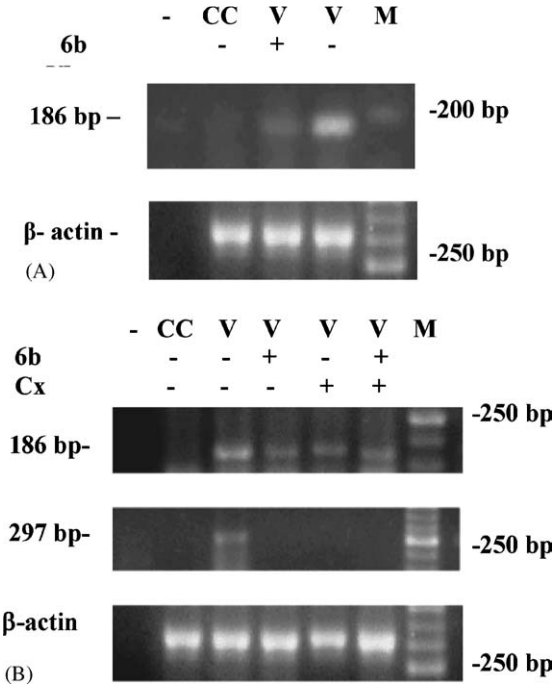


Fig. 4. Effect of **6b** on JV RNA synthesis. (A) RT-PCR analysis of S RNA synthesis in **6b**-treated cultures at 24 h p.i.: JV-infected Vero cells were incubated in culture medium containing (or not) **6b** (40 μM). At 24 h p.i., total RNA was extracted and detection of S RNA was performed after cDNA synthesis with the antisense primer vcN followed by PCR amplification of a 186 bp fragment using primers vcN plus vN. Expression of β-actin mRNA was detected in all samples. (−) Negative control; (cc) mock infected cells; (v) infected cells; (M) 50 bp DNA ladder. (B) RT-PCR analysis of N mRNA and antigenomic S RNA synthesis in **6b**-treated cultures at 6 h p.i.: JV-infected Vero cells were incubated in culture maintenance medium (MM), MM containing **6b** (40 μM), MM containing Cx (100 μg/ml) or MM containing both **6b** and Cx. Extraction of total RNA was performed at 6 h p.i. Detection of N mRNA and/or antigenomic S RNA was performed after cDNA synthesis with virus sense primer vN followed by PCR amplification of a 186 bp fragment using primers vN plus vcN. Detection of antigenomic full length S RNA was performed after cDNA synthesis with virus sense primer vG followed by PCR amplification of a 297 bp fragment using primers vG plus vcG. Expression of β-actin mRNA was detected in all samples. (−) Negative control; (cc) mock infected cells; (v) infected cells; (M) 50 bp DNA ladder.

PCR with primers vN and vcN allows the amplification of a 186 bp fragment indicative of the presence of N mRNA and/or antigenomic S RNA (Fig. 3). On the other hand, cDNA synthesis using primer vG followed by PCR reaction with primers vG and vcG results in the amplification of a 297 bp fragment indicative of the presence of antigenomic S RNA (Fig. 3). As can be seen in Fig. 4B, the 186 bp fragment could be detected in both untreated and **6b**-treated infected cultures, however, lower detectable levels of amplification were observed in **6b**-treated cells. In contrast, the amplification fragment of 297 bp was only detected in untreated infected cultures (Fig. 4B) indicating that **6b** mainly affects the synthesis of antigenomic full length S RNA.

It has been demonstrated that, in the presence of an inhibitor of protein synthesis, transcription of JV S RNA yields only the N mRNA, a phenomenon also observed for other arenaviruses (Tortorici et al., 2001; Meyer et al., 2002). The inhibitory effect of the protein inhibitor cycloheximide (Cx) on the synthesis of antigenomic S RNA was corroborated by performing the RT-PCR analysis from JV-infected cultures treated with 100 μg/ml of Cx (Fig. 4B). When JV-infected cultures were treated only with Cx or simultaneously with **6b** and Cx, a similar reduction on the amount of the186 bp fragment was observed. Since, in the presence of Cx only N mRNA is produced, these results also indicate that early transcription of N gene would not be affected by **6b**, confirming that the derivative prevents the synthesis of the antigenomic form of S RNA.

3.3. *Effect of **6b** on JV-fusion activity*

Our findings indicate that **6b** would impair early RNA replication, however, the delayed addition of the compound at 8 h p.i. still caused a significant reduction in JV yield measured at 12 h p.i. (Fig. 2A). Since the time required for a complete JV replication cycle ranges from 10 to 12 h, these results might indicate an inhibitory action of **6b** on later events of the growth cycle. Unfortunately, the intrinsically poor efficiency of JV replication impeded the analysis of the effect of **6b** on viral protein synthesis within a single cycle of replication. To overcome this difficulty, we carried out an alternative approach to investigate the effect of **6b** on the expression of JV glycoproteins at cell membrane. We have previously demonstrated that newly synthesized JV glycoproteins, expressed at the cell surface of infected cells, are able to mediate low-pH-induced cell fusion with surrounding uninfected cells leading to the production of syncytia (Castilla and Mersich, 1996); so, we first examined which was the minimum time after infection required to detect fusion activity on infected cultures, indicative of the presence of JV glycoproteins exposed at cell membranes. For this purpose, Vero cells were infected with JV (moi = 1 PFU/cell) and at different times after

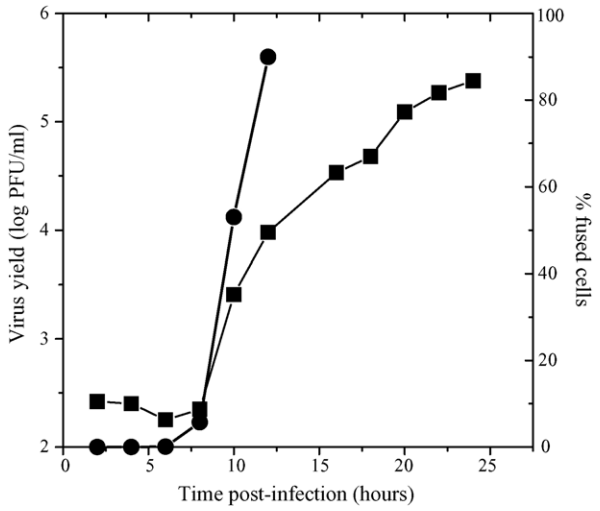


Fig. 5. JV-mediated fusion activity and extracellular virus production at different times post-infection, Vero cells grown on coverslips were infected with JV at a moi of 1 PFU/cell. At different times after infection extracellular virus yields (squares) were quantified from culture supernatants and cells were exposed to low-pH medium, fixed with methanol and stained with Giemsa to visualize syncytium formation by light microscopy. Percentages of cell fusion (circles) were calculated as described in Section 2.

infection supernatants were collected to measure virus yield and cells were exposed to a pulse of low pH in order to induce syncytium formation (Fig. 5). The release of infectious particles was first observed at 10 h p.i.; thereafter, virus yield increased in a continuous manner until 24 h p.i. On the other hand, a low fusion activity was first detected at 8 h p.i. indicating an incipient expression of JV glycoproteins at the surface of infected cells. At 10 h p.i., 50% of the cells in the culture were fused, while at 12 h p.i. more than 90% of fused cells were observed. In order to analyze membrane expression of JV glycoproteins along a single multiplication cycle, we decided to examine the effect of time of addition or removal of **6b** on syncytium formation induced at 10 h p.i. As can be seen in Table 1, in accordance with the results obtained in adsorption-penetration studies, the presence of **6b** within the period 1–2 h p.i. did not affect fusion activity. Maximum inhibitory effect was detected when **6b** was present between 2 and 8 h p.i. However, a significant reduction in syncytium production was also achieved when the compound was present in the period 8–10 h p.i. (Table 1). Taking into account the results shown in Fig. 5, the insertion of newly synthesized JV glycoproteins into the cell membrane appears to occur after 8 h p.i., therefore, the inhibitory effect of **6b** on fusion activity when the compound was present in the period 8–10 h p.i. (Table 1) might indicate a possible adverse effect of **6b** either on intracellular transport or fusion activity of JV glycoproteins.

In order to rule out any effect of **6b** on cellular membranes that might cause a non-specific inhibition of cell fusion, we examined syncytium production induced after co-cultivation of cells obtained from infected cultures, previously treated or untreated with **6b** (40 μM), with cells obtained from uninfected cultures, previously treated or untreated with **6b** (40 μM). Similar levels of fusion activity were obtained when untreated infected cells were co-cultivated with uninfected untreated cells (95% of fused cells) or uninfected **6b**-treated cells (93% of fused cells), thus, treatment of uninfected cells with **6b** did not affect syncytium formation. In contrast, only 13.5% of cell fusion was detected after co-cultivation of **6b**-treated infected cells with either uninfected untreated cells or uninfected **6b**-treated cells, indicating that inhibition of fusion activity is not due to a non-specific interference of the derivative on the membrane fusion process.

Table 1
Inhibitory effect of **6b** on JV-mediated cell fusion

Treatment period (h p.i.)	Percentage of cell fusion induced at 10 h p.i.	Percentage of inhibition of cell fusion
Control (untreated)	53.4	–
2–10	0.1	99.8
4–10	0.4	99.2
6–10	0.9	98.3
8–10	7.2	86.5
1–2	51.0	4.5
1–4	12.2	77.2
1–6	9.1	83.0
1–8	1.2	97.6
1–10	0	100

Vero cells grown on coverslips were infected with JV at a moi of 1 PFU/cell. Compound **6b** (20 μM) was added to one set of infected cultures at 2, 4, 6 or 8 h p.i. and further incubated at 37 °C. Other set of infected cultures were incubated with **6b** (20 μM) at 1 h p.i. and the compound was removed by medium change at 2, 4, 6, 8 or 10 h p.i. In all cases, culture supernatants were removed at 10 h p.i. and cells were exposed to acid medium to induce syncytium production. Percentages of inhibition were calculated with respect to untreated infected cultures (control).

4. Discussion

Besides the well established importance of brassinosteroids (BRs) upon plant growth and development, new functions for these molecules are continuously discovered (Kamuro and Takatsuto, 1999). We have previously demonstrated that synthetic BRs inhibit the in vitro replication of several RNA and DNA viruses (Wachsman et al., 2000, 2002; Talarico et al., 2002). Though antiviral activity of other biologically active steroids, obtained from different natural sources, has been also reported in the last years (Kohen et al., 1991; Comin et al., 1999), their mode of antiviral action remains unknown.

Since arenaviruses are more susceptible than other viruses to the antiviral activity of BRs, in the present study, we analyzed the effect of the synthetic BR **6b** on different steps of JV replication cycle. Time of addition experiments revealed that **6b** was most effective the earlier it was added to the cells after infection with JV (Fig. 2). Similar levels of inhibition of both cell-associated and extracellular virus yields were detected (Fig. 2A) indicating that **6b** prevents the formation of mature viral particles rather than the release of progeny virus to the extracellular medium. The observed reduction in virus yield was not caused by an impairment of virus adsorption or penetration into the cell. The results showed that **6b** caused the inhibition of early viral RNA synthesis. At 6 h p.i. both N mRNA and antigenomic S RNA could be detected by RT-PCR in untreated infected cells, but only N mRNA was detected in **6b**-treated infected cultures (Fig. 4B).

The analysis of fusion activity, expressed at the surface of infected cells, confirmed that maximum inhibitory effect was exerted between 2 and 8 h p.i. (Table 1). In accord with data previously reported (Castilla et al., 1994; Romanowski, 1993), the period sensitive to **6b** activity comprises viral RNA and protein synthesis. However, high inhibition of JV yields (Fig. 2A) and JV-mediated cell fusion (Table 1) was also observed when the compound was present during the last hours of the infection. Given these results, we cannot rule out an adverse effect of **6b** on post-translational processing or proper insertion of JV glycoproteins into the cell membrane that would affect membrane fusion activity and the assembly of mature viral particles. Another possible explanation of these results is that, under the experimental conditions used, the infection was not fully synchronized and, in this case, the effect of the compound on RNA replication might account for the inhibitory effect detected when **6b** was present at the end of the virus replication cycle. It must be noted that even if a multiplicity of infection higher than 1 PFU/cell is employed, the low efficiency of JV adsorption process does not insure the synchronization of the infection (Castilla et al., 1994).

The evidence presented above supports the hypothesis that **6b** mainly impairs JV RNA replication. It has been proposed that the switch from transcription to replication during arenavirus infection might depend on the intracellular level of N protein. For JV S RNA it has been reported that at low levels of N protein only N mRNA transcripts are produced. When the N mRNA is translated, the newly synthesized N protein might act as a transcriptional antiterminator factor, allowing the synthesis of full length antigenomic S RNA (Tortorici et al., 2001). One possible explanation for the inhibitory action of **6b** might be related with a blockade of N protein synthesis or N protein function, however, at present, it is not known whether the arenavirus transcription–replication switch is defined simply by the concentration of N protein or implies a more complex scheme involving other viral or cellular factors. In addition, a better understanding of the inhibitory action of **6b** requires the analysis of the effect of **6b** on transcription and replication of JV L RNA and a more accurate quantification of N mRNA and L mRNA synthesis, since lower levels of viral polymerase L could also account for the decreased synthesis of antigenomic RNA.

On the other hand, meanwhile animal steroidal hormones are recognized by nuclear receptors, a transmembrane protein has been identified as a BR receptor in plant cells, and it seems that plants have no intracellular steroid receptors. Thus, the elucidation of the molecular mechanism responsible of **6b** antiviral activity requires further studies to understand the interaction of BRs with both viral components and animal cells.

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