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)-3\(^{-}\)/H\(^{+}\)9252-bromo-5\(^{-}\)/H\(^{+}\)9251,22,23-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 (Romanowskis, 1993). Other arenaviruses, such as Lassa virus from West Africa, Machupio 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., 1998). 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)-3\(^{-}\)/H\(^{+}\)9252-bromo-5\(^{-}\)/H\(^{+}\)9251,22,23-trihydroxystigmastan-6-one (6b) was the most active...
against JV infection in Vero cells, with a 50% cytotoxic concentration (CC50) of 5.7 μM (using confluent non-growing cells) and a 50% effective concentration (EC50) of 0.4 μM for IVASSA JV strain. A higher level of cytotoxicity was exerted in growing cell cultures (CC50 = 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. IVASSA, 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: 10⁷ plaque-forming units/ml, moi = 1 PFU/cell) diluted in PBS was plaque-assayed on Vero cells.

2.2. Compounds

The compound (225,23S)-3β-bromo-5α,22,23-trihydroxy-stigmaster-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 for 4 h 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.

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 35S-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 radioimmunoprecipitation 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 35S-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 35S-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 35S-methionine-JV (3 × 10⁶ 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 protease 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 TOTAL 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, precipitated 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 vN (5′-GGCACAGTGGATCTAGGC-3′). PCR reaction was conducted using arenavirus-specific primers (5′-GGCATCTCTCAGAACAT-3′) plus vN 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 antigenic S RNA. The viral sense primers vN or vG (5′-GTCGACTGGGAACGTCG-3′) were used in the cDNA synthesis. Primers vN plus vN which rendered a 186 bp amplification fragment, and primers vG plus vG (5′-CTTGATGGAAGAAGTT CGG-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 vN, vN or vG. For each RNA sample β-actin mRNA was retrotranscribed in cDNA using arenavirus-specific primers vcN/vN or vG. The detection of genomic S RNA by PCR was performed from cDNA synthesized with the primer vN (5′-GGCACAGTGGATCTAGGC-3′). PCR reaction was conducted using arenavirus-specific primers (5′-GGCATCTCTCAGAACAT-3′) plus vN that generated a 186 bp amplification fragment comprising the 3′ end of the S RNA containing N coding sequence (Ellenberg et al., 2002).

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 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
An extensive inhibition of viral protein synthesis was observed 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 0h). 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 entry of JV into the cell. 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 semi-quantitative 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 vN and vN renders a 196 bp fragment corresponding 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
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 full length 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 g/ml of Cx (Fig. 4B). When JV-infected cultures were treated only with Cx or simultaneously with 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 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 vN. 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.

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
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 untreated 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.

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 Takatsuji, 1999). We have previously demonstrated that synthetic BRs inhibit the in vitro replication of several RNA and DNA viruses (Wachsmann 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).

Table 1

<table>
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<tr>
<th>Treatment period (h p.i.)</th>
<th>Percentage of cell fusion induced at 10 h p.i.</th>
<th>Percentage of inhibition of cell fusion</th>
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<tr>
<td>Control (untreated)</td>
<td>53.4</td>
<td>99.8</td>
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<tr>
<td>2–10</td>
<td>0.1</td>
<td>99.9</td>
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<tr>
<td>4–10</td>
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</tr>
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<tr>
<td>1–10</td>
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<td>100</td>
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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).
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 L mRNA and a more accurate quantification of N mRNA and JV L RNA synthesis, since lower levels of viral polymerase L protein have been identified as a BR receptor in plant cells, and are recognized by nuclear receptors, a transmembrane 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 L mRNA and a more accurate quantification of N mRNA and JV L RNA synthesis, since lower levels of viral polymerase L protein has been identified as a BR receptor in plant cells, and are recognized by nuclear receptors, a transmembrane

Acknowledgements

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