Antiviral effect of a synthetic brassinosteroid on the replication of vesicular stomatitis virus in Vero cells

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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 replication of vesicular stomatitis virus (VSV) in Vero cells was investigated. Time-related experiments showed that 6b mainly affects a late event of the virus growth cycle. Virus adsorption, internalisation and early RNA synthesis are not the target of the inhibitory action. Results obtained indicate that the antiviral compound adversely affects virus protein synthesis and viral mature particle formation.

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1. Introduction

Vesicular stomatitis virus (VSV), a member of the Rhabdoviridae family, is an enveloped single-strand RNA virus that causes an economically important disease in cattle, horses and swine. The VSV genome encodes five proteins: the nucleocapsid protein (N), the phosphoprotein (P), the large protein (L), the glycoprotein (G) and the matrix protein (M). The P protein in combination with the L protein forms the viral RNA-dependent RNA polymerase [1].

VSV is one of the most carefully studied viruses. Its replication strategy forms a valid model for the replication of all Mononegavirales viruses and provides important insights for the study of replication of other viruses with negative-sense RNA genomes [2].

Brassinosteroids (BRs) are a group of naturally occurring polyhydroxy steroidal plant hormones [3]. To study their biological activities, it is necessary to obtain them by chemical synthesis, as their concentration in plants is very low [4]. In previous reports, we demonstrated that synthetic BRs inhibit the in vitro replication of several DNA and RNA viruses [5–7]. Among the tested compounds, the derivative (22S,23S)-3β-bromo-5α,22,23-trihydroxystigmastan-6-one (6b) was the most active against the assayed viruses [7–9]. Preliminary studies performed with brassinolide, a naturally occurring plant hormone [3], have shown significant in vitro antiviral activity against VSV [10].

The aim of the present study was to characterise the antiviral activity of the BR 6b using VSV replication in cell cultures as a model to ascertain the mode of action of this kind of synthetic derivative.

2. Materials and methods

2.1. Cells and virus

Vero cells were grown in Eagle’s minimal essential medium (MEM; Gibco, Carlsbad, CA) containing 5% inactivated calf serum and 50 μg/mL gentamicin. Maintenance medium (MM) consisted of MEM supplemented with 2% inactivated calf serum and gentamicin.

VSV (Indiana serotype) was obtained from the American Type Culture Collection (ATCC, Rockville, MD). Virus stock was propagated and plaque assayed in Vero cells.
of 6b (10 mg/mL) was prepared in ethanol, stored at −20 °C and further diluted in MM shortly before use [5].

2.3. Cytotoxicity assay

To determine the cytotoxic concentration of 6b, monolayers of confluent Vero cells were exposed to various concentrations of the derivative in MM. After 24 h of incubation, cell viability was examined by the MTT colorimetric assay [12] following the procedure described elsewhere [5]. The 50% cytotoxic concentration (CC50) was defined as the concentration (µg/mL) required to reduce cell viability by 50%. These values were calculated by regression analysis.

2.4. Virus yield reduction assay

Confluent Vero cells were infected with VSV at a multiplicity of infection (MOI) of 1. After 1 h of adsorption at 37 °C the cells were covered with MM containing varying concentrations of 6b. At 24 h post infection (p.i.), cultures were subjected to two cycles of freeze–thawing followed by centrifugation at low speed (1000 × g) and the supernatants were titrated by a plaque assay. Antiviral activity was expressed as 50% effective concentration (EC50), i.e. the concentration (µg/mL) required to reduce virus yield by 50% compared with the untreated control cultures.

2.5. Effect of time of 6b addition on VSV production

Compound 6b (60 µg/mL) was added to confluent monolayers of Vero cells infected with VSV at a MOI of 1 at time 0 of infection (simultaneously with virus inoculum) or at 2, 4, 6 and 8 h p.i. Cultures were further incubated at 37 °C until 24 h p.i. and at that time extracellular virus yields were measured from supernatants by plaque assay. To determine total infectivity, cells were subjected to two cycles of freeze–thawing followed by centrifugation at low speed and the supernatants obtained were titred by plaque assay.

2.6. Indirect immunofluorescence assays

Vero cells grown on glass coverslips were infected with VSV (MOI = 0.1) and 6b (60 µg/mL) was added or not at 0 h or 3 h p.i. and incubated at 37 °C until 4 h p.i. At that time, supernatants were removed and cells were washed with phosphate-buffered saline (PBS), fixed with methanol (10 min at −20 °C) and stained for total immunofluorescence (IF) using polyclonal serum anti-G protein (kindly provided by Dr Pablo Grigera, CEV AN, Buenos Aires, Argentina) and the interaction of antibody was revealed using fluorescein isothiocyanate (FITC)–anti-rabbit conjugate (Sigma–Aldrich, St Louis, MO). For membrane IF assay, cells were washed with PBS and incubated with polyclonal serum anti-G protein at 4 °C. Afterwards, cells were fixed with methanol (10 min at −20 °C) and incubated with FITC–anti-rabbit conjugate.

2.7. Adsorption and penetration assay

Approximately 100 plaque-forming units (PFU) of VSV were adsorbed for 1 h at 4 °C on Vero cells in the presence or absence of 60 µg/mL of the BR 6b. Cultures were then washed twice with cold PBS and overlaid with MM containing 0.7% methylcellulose to quantify virus adsorption. For the internalisation assay, following virus adsorption at 4 °C for 1 h, cells were incubated at 37 °C to maximise virus penetration. At various time periods, the monolayers were washed and treated with citrate buffer (pH 3) to inactivate any remaining attached virus. Cultures were then overlaid with MM containing 0.7% methylcellulose to determine the plaque number.

2.8. Analysis of radiolabelled proteins

Compound 6b (40 µg/mL) was added or not, simultaneously with virus inoculum, to confluent monolayers of Vero cells infected with VSV at a MOI of 5. After viral adsorption, cell cultures were radiolabelled at different times p.i. (0, 2 and 4 h). For that purpose, monolayers were washed with PBS and incubated for 1 h with methionine-free medium. Then, 10 µCi/mL of 35S-methionine (sp. Act. 1031 Ci/mmol; New England Nuclear Corp., Beverly, MA) were added and cells were incubated for another 2 h at 37 °C. Afterwards, cells were harvested, treated with a lysing buffer containing 0.125 M Tris–HCl (pH 6.8), 4% sodium dodecyl sulphate (SDS), 20% glycerol, 10% β-mercaptoethanol and 0.02% Bromophenol blue for 2 min at 100 °C. Cell lysates were subjected to electrophoresis on a 10% SDS-polyacrylamide slab gel [13]. Molecular weight markers were used. After electrophoresis, gels were analysed by fluorography and visualised by autoradiography.
2.9. RNA extraction, cDNA synthesis and polymerase chain reaction (PCR) analysis

Confluent Vero cells, grown in six-well plates, were infected with VSV (1.10⁷ PFU) and after 1 h of adsorption at 37°C cultures were covered with MM or MM containing 6b (60 μg/mL) at 2 h p.i., total RNA was extracted with the TOTALLY RNA kit (Ambion, Austin, TX) and re-suspended in 20 μL of water. Detection of RNA by PCR was performed from cDNA synthesised with the sense primer ILS (5'-GAGACCTTCAACACCAGCCG-3'). PCR was conducted using specific primers: the sense primer ILS and the antisense primer ILA (5'-GGTGTTGCAGACTATGTTGGAC-3'), which generated a 358 bp amplification fragment corresponding to the L gene.

Synthesis of cDNA was carried out as follows. RNA was heated to 65°C for 5 min in the presence of 0.5 mM VSV-specific primer (ILS) and 0.5 mM of dNTPs. Then, buffer RT and 0.01 M of DTT were added and incubated for 2 min at 42°C. Finally, 200 units of the enzyme RT-Super Script (Invitrogen, Carlsbad, CA) were added and incubated for 50 min at 42°C. For each RNA sample, β-actin mRNA was retrotranscribed in cDNA using the primer 5'-GAGACCTTCAACACCAGCCG-3' (0.5 mM).

PCR amplifications were carried out in a final volume of 10 μL containing 1 μL of the cDNA reaction, 0.4 units of Go-Taq DNA polymerase (Promega, Madison, WI), 0.2 mM of each dNTP and 1 μM of each primer. The PCR cycle progression was as follows: 10 min at 93°C and 30 cycles of 1 min at 93°C (denaturation), 1 min at 37°C (annealing) and 2 min at 72°C (extension) followed by 10 min at 72°C for final extension. Amplification conditions for β-actin have been described previously [9]. The whole PCR reaction volume was electrophoresed at 4 V/cm for 60 min onto 2% agarose gel in TAE (40 mM Tris-acetate, 1 mM EDTA, pH 8.0) buffer with 0.2 μg/mL ethidium bromide. Negative controls were performed with distilled water instead of cDNA.

3. Results

3.1. Effect of 6b on VSV infectious particle production

Compound 6b inhibited the multiplication of VSV in confluent Vero cells in a dose-dependent manner. CC₅₀ and EC₅₀ values were 150 μg/mL and 12.3 μg/mL, respectively. To establish whether 6b produces a viricidal effect, 10⁷ PFU of VSV were diluted in culture medium containing or not 6b (600 μg/mL) and incubated for 0, 30, 60 or 90 min at 37°C. At the indicated times, aliquots were diluted in MM to a non-inhibitory drug concentration and titred by plaque assay. As shown in Fig. 2, no differences in virus titres were found between treated and untreated samples, indicating that 6b antiviral action is not due to direct inactivation of virus particles.

To ascertain whether 6b affects an early or late event in virus multiplication, a time-of-addition experiment was performed. For that purpose, 6b (60 μg/mL) was added to VSV-infected Vero cells at different times after infection and, at 24 h p.i., cell-free and total virus infectivity were determined. As shown in Fig. 3, yields of released VSV were reduced by 3 log units when the compound was added at 0 or 2 h p.i. and by ca. 2 log units when 6b was added at 4 h p.i. or later. Total virus yields were also reduced in a significant manner, but to a lesser extent than cell-free virus (Fig. 3), indicating that 6b may affect the release of infectious particles to the extracellular medium. Similar results were obtained...
under one-step VSV growth cycle conditions. Compound 6b was added at 0 h or 3 h p.i. to Vero cells infected with VSV at a MOI of 5 and at 6 h following infection supernatants were harvested and titrated by plaque assay. A decrease of 2 log units in virus yield was obtained in cultures treated at 0 h or 3 h p.i. compared with untreated infected cultures (data not shown). These results indicate that the inhibitory effect of the BR can be detected even when the compound was added late during infection, suggesting that the BR may affect a late step of the replication cycle.

The effect of time of addition of 6b on VSV antigen expression was further analysed. To this end, Vero cells were infected with VSV at a MOI of 0.1 and were treated or not with 6b (60 µg/mL) at 0 h or 3 h p.i. At 4 h p.i. supernatants were collected and cells were subjected to total or membrane IF assays. As shown in Table 1, maximum reduction in the number of fluorescent cells was observed when 6b was added simultaneously with the virus, suggesting that the presence of 6b from the beginning of infection affects viral protein expression. Furthermore, the percentages of inhibition found in the membrane IF assay were higher than those obtained in the total IF assay, indicating that 6b alters the transport of glycoprotein G to the cell membrane. In addition, under these experimental conditions, cell-free virus yield was highly inhibited (98% inhibition) by 6b treatment (Table 1).

3.3. Effect of 6b on VSV protein synthesis

To reveal the effect of 6b on the synthesis of polypeptides within uninfected and VSV-infected cells, the following experiments were performed. The effect of the BR on uninfected cellular protein synthesis was analysed first. For this purpose, Vero cells were treated or not with different concentrations of 6b ranging from 10 µg/mL to 60 µg/mL. After 4 h of incubation with the compound, cultures were labelled with [35S]-methionine and cell polypeptide synthesis was analysed by SDS-polyacrylamide gel electrophoresis (PAGE) and autoradiography. As shown in Fig. 4A, none of the assayed concentrations of 6b affected the pattern of cell polypeptide synthesis. To determine the effect of 6b on VSV protein synthesis, cells infected with VSV at a MOI 5, treated or not with 6b (40 µg/mL), were labelled with [35S]-methionine at different times p.i. (0, 2 or 4 h) and viral polypeptides were analysed by SDS-PAGE and autoradiography. No virus polypeptides were observed in the lane corresponding to the labelling period 0–2 h p.i. However, later the presence of four near viral polypeptide bands can be seen in untreated VSV-infected cells labelled between 2–4 h or 4–6 h p.i. (Fig. 4B). The major band (60–64 kDa) is glycoprotein G. Two bands of apparent molecular weight 30–45 kDa called N/P are the nucleocapsid protein N and phosphoprotein P, whereas a broad weak band of ca. 26 kDa corresponds to matrix protein M. By contrast, extensive inhibition of viral protein synthesis within the periods 2–4 h and 4–6 h p.i. was shown in 6b-treated cultures.

3.4. Effect of 6b on VSV RNA synthesis

Inhibition of viral protein synthesis by VSV could be due to a blockage of RNA transcription. To investigate this possibility, experiments were performed to detect whether viral positive-stranded RNA is newly synthesised in the presence of 6b.

The earliest time after infection that allowed the detection, by a semiquantitative reverse transcription (RT)-PCR assay, of positive-stranded viral RNA was determined first. For that purpose, Vero cells were infected with VSV (MOI = 0.1) and at different times after infection total RNA was extracted. Afterwards, cDNA was synthesised using a sense primer (ILS) complementary to the L gene sequence and further PCR was performed, using the primers ILS and the antisense primer ILA, which render a 358 bp amplification fragment indicative of the presence of positive-sense RNA. Synthesis of positive-sense RNA could be detected from 2 h p.i. onwards (data not shown). To determine the effect of 6b on viral RNA synthesis, RNA from infected Vero cells treated or not with 6b (60 µg/mL) was extracted at 2 h p.i. and a
Fig. 4. (A) Effect of (22S,23S)-3\(^{-}\)bromo-5\(^{-}\),22,23-trihydroxystigmastan-6-one (6b) on cellular polypeptide synthesis. Vero cells treated with different concentrations of 6b for 4 h were labelled with \(^{35}\)S-methionine and cellular polypeptides were analysed by SDS-PAGE and autoradiography. (B) Effect of 6b on vesicular stomatitis virus (VSV) protein synthesis. Compound 6b (40 \(\mu\)g/mL) was added or not to VSV-infected Vero cells. At different times (0, 2 or 4 h post infection), cultures were labelled with \(^{35}\)S-methionine and viral proteins were analysed by SDS-PAGE and autoradiography. VC, untreated infected cells. The positions of bands corresponding to the main VSV proteins are indicated: G, viral glycoprotein; N, nucleocapsid protein; P, phosphoprotein; and M, matrix protein.

RT-PCR assay was performed as described above. As can be seen in Fig. 5, no inhibition of viral RNA synthesis could be observed in 6b-treated cultures compared with control infected cultures.

4. Discussion

In the present study, we analysed the effect of the synthetic BR 6b on different steps of VSV replication cycle. Previously, it was shown that the antiviral effect of the compound could not be attributed to direct inactivation of virus particles (Fig. 2). Time-of-addition experiments revealed that 6b inhibits late steps of the VSV replication cycle, mainly affecting the release of progeny virus to the extracellular medium (Fig. 3). Although extracellular virus production was affected by 6b even when the BR was added after 3 h p.i., viral antigen expression was maximally reduced when 6b was present from the beginning of infection (Table 1). These findings indicate that besides its adverse effect on viral particle release, the compound could also affect an earlier event in the viral multiplication cycle. The observed reduction in antigen expression was not caused by an impairment of virus adsorption or penetration into the cell. In contrast, we determined that 6b inhibits VSV protein synthesis (Fig. 4B) at concentrations that do not affect cellular protein synthesis (Fig. 4A). These findings are similar to those obtained in 6b-treated Vero cells infected with herpes simplex virus-1 or Junin virus (JUNV) [8,9]. As shown in Fig. 4B, between 2 and 4 h and 4 and 6 h p.i. the amount of individual VSV polypeptides in 6b-treated cells was decreased in comparison with infected control cells. These results cannot be attributed to inhibition of VSV transcription, since positive-strand RNA was detected by RT-PCR in 6b-treated infected cells (Fig. 5). VSV primary transcripts are synthesised by a viral transcriptase, which is a component of the virus particle [14], whereas secondary transcripts are synthesised on progeny genomes by nascent transcriptase. This latter process depends upon protein synthesis and takes place at 4 h p.i. [15]. Since the RT-PCR assay was done at 2 h p.i., the positive-strand RNA detected would mainly correspond to L mRNA.

Furthermore, membrane IF assay showed that the intracellular transport of VSV G protein was partially affected by the derivative (Table 1). However, extracellular virus yields were more sensitive than antigen expression, indicating that the main target of 6b inhibitory action may be the formation of viral infectious particles. Since sterols found in all eukaryotic cells are membrane components that regulate the fluidity of phospholipid bilayers, it could not be excluded that 6b could affect viral assembly by changes at the cell membrane level. Inhibition of viral maturation and assembly is in accordance with results previously obtained with JUNV [9].

Fig. 5. Effect of (22S,23S)-3\(^{-}\)bromo-5\(\alpha\),22,23-trihydroxystigmastan-6-one (6b) on vesicular stomatitis virus (VSV) RNA synthesis. Reverse transcription polymerase chain reaction (RT-PCR) analysis of L mRNA synthesis in 6b-treated cultures at 2 h post infection (p.i.) was done. VSV-infected Vero cells were incubated in maintenance medium containing or not 6b (60 \(\mu\)g/mL). At 2 h p.i., total RNA was extracted and detection of L mRNA was performed after cDNA synthesis with the sense primer ILS followed by PCR amplification of a 358 bp fragment. Expression of \(\beta\)-actin mRNA was detected in all samples. CC, mock-infected cells; VC, infected cells; M, 50 bp DNA ladder.
Although 6b shows a broad antiviral spectrum, some viruses are more susceptible to the inhibitory effect of 6b than others, therefore we think that 6b could exert its antiviral action by affecting a specific viral factor and to a lesser degree a cellular function required for viral replication.

In animals, steroid hormones are recognised by nuclear receptors that transcriptionally regulate specific target genes. This is called genomic signalling because it involves the genome in generating a physiological response. Steroid hormones can also elicit cellular responses in the presence of inhibitors of transcription or protein synthesis and in enucleated cells. This mechanism is often referred to as non-genomic steroid signalling. Non-genomic steroid signalling pathways can also stimulate alterations in second messenger levels, ion fluxes and protein kinase activities and this mechanism would involve steroid membrane receptors. On the other hand, BRs are perceived at the plasma membrane of plant cells by direct binding to the extracellular domain of a transmembrane protein that has a serine–threonine protein kinase activity. BR perception initiates a signalling cascade and further kinases and phosphatases determine the phosphorylation state and stability of nuclear transcription factors. These factors mediate major BR effects in various plant physiological processes. In addition, alternative BR receptors, similar to steroid receptors in animal cells, have also been reported. Recently, we found that 6b exhibits antiviral activity against RNA viruses even in the presence of actinomycin D, suggesting that the inhibitory action of the BR derivative does not require cellular transcription (unpublished data). These findings suggest that 6b would exert its antiviral action via a non-genomic mechanism.

Although it is too premature to predict the clinical utility of this kind of compound, there is some evidence for the low level of toxicity of natural BRs. Being constituents of practically all plants, BRs are usually consumed by mammals and the safety of some natural BRs has been confirmed by toxicological studies in mice and rats (orally and dermally). In vivo studies, performed in a murine experimental model, have demonstrated a non-toxic effect after topical eye administration of 6b (20 μg/mL) three times a day during 3 consecutive days. Further studies are needed to increase our knowledge regarding the ability of BRs to inhibit in vivo replication of VSV and other BR-susceptible viruses.

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