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Inhibition of Junin virus RNA synthesis by an antiviral acridone derivative

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ABSTRACT

There are no specific approved drugs for the treatment of agents of viral hemorrhagic fevers (HF) and antiviral therapies against these viruses are urgently needed. The present study characterizes the potent and selective antiviral activity against the HF causing arenavirus Junin virus (JUNV) of the compound 10-allyl-6-chloro-4-methoxy-9(10*H*)-acridone, designated **3f**. The effectiveness of **3f** to inhibit JUNV multiplication was not importantly affected by the initial multiplicity of infection, with similar effective concentration 50% (EC_{50}) values in virus yield inhibition assays performed in Vero cells in the range of 0.2–40 plaque forming units (PFU)/cell. Mechanistic studies demonstrated that **3f** did not affect the initial steps of adsorption and internalization. The subsequent process of viral RNA synthesis was strongly inhibited, as quantified by real time RT-PCR in compound-treated cells relative to non-treated cells. The addition of exogenous guanosine rescued the infectivity and RNA synthesis of JUNV in **3f**-treated cells in a dose-dependent manner, but the reversal was partial, suggesting that the reduction of the GTP pool contributed to the antiviral activity of **3f**, but it was not the main operative mechanism. The comparison of **3f** with two other viral RNA inhibitors, ribavirin and mycophenolic acid, showed that ribavirin did not act against JUNV through the cellular enzyme inosine monophosphate dehydrogenase (IMPDH) inhibition whereas the anti-JUNV activity of mycophenolic acid was mainly targeted at this enzyme.

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1. Introduction

Arenaviridae are a family of enveloped viruses containing a bisegmented single-stranded RNA genome with an ambisense coding strategy. The large RNA fragment (L, ca. 7.3 kb) encodes the viral RNA-dependent RNA polymerase L and a RING finger matrix protein Z whereas the small fragment (S, ca. 3.5 kb) encodes the nucleoprotein NP and the glycoprotein precursor GPC, post-translationally cleaved to yield the mature virion glycoproteins. Several human pathogens that represent a serious threat for public health are included in this family. Lassa virus (LASV) in Africa as well as Junin (JUNV), Machupo (MACV), Guanarito (GTOV) and Sabia viruses (SABV) in South America are etiological agents of viral hemorrhagic fevers (HF) with significant level of morbidity and mortality (McCormick and Fisher-Hoch, 2002; Peters, 2002). The prototype and worldwide distributed arenavirus lymphocytic choriomeningitis virus (LCMV) is also a human pathogen associated with acute central nervous system (CNS) disease, but concern about human infection with LCMV has recently increased, due to its identification as causal agent in congenital pediatric infections (Jamieson et al., 2006) and in immunocompromised patients through transplantation of LCMV-infected tissues (Fischer et al., 2006). In addition, an increased emergence of new tentative species, occasionally with pathogenic potential, has frequently occurred in recent years (Briese et al., 2009; Delgado et al., 2008), leading to the estimation that a new arenavirus may emerge and be recognized on average every 3 years.

Although different types of compounds have been studied for inhibitoring anti-arenavirus activity (Andrei and De Clercq, 1993; Damonte and Coto, 2002; García et al., 2011), no specific and safe chemotherapy for any arenavirus is currently available. The only approved drug for human treatment is ribavirin (RIB), a guanosine analog with a broad spectrum of antiviral activity against RNA viruses. However, this drug is not equally effective against all HF-causing arenaviruses and treatment, recommended only within the early phase of clinical disease, can produce undesirable secondary reaction (Enría and Maiztegui, 1994; Fisher-Hoch et al., 1992; McCormick et al., 1986).

In the search of novel antiviral compounds effective against these pathogenic agents, a previous screening study of antiviral

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activity of diverse *N*-substituted acridone derivatives identified a group of 10-allyl-9(10*H*)-acridones as effective and very selective inhibitors of arenaviruses (Sepúlveda et al., 2008). In particular, the 10-allyl-6-chloro-4-methoxy-9(10*H*)-acridone, designated **3f** (Fig. 1a), was the most active compound that blocked replication of the pathogenic arenaviruses JUNV and LCMV as well as that of the four serotypes of dengue virus, another HF-causing RNA virus belonging to the family *Flaviviridae*. Here, we investigated the mode of action of **3f** (Fig. 1a), against JUNV, agent of Argentine hemorrhagic fever.

2. Materials and methods

2.1. Compounds

The acridone derivative **3f** was synthesized as previously described (Fascio et al., 2007). RIB and mycophenolic acid (MPA) were purchased from Sigma–Aldrich. Stock solutions of all compounds were prepared in dimethylsulfoxide.

2.2. Virus and cells

Vero cells were grown as monolayers in Eagle's minimum essential medium (MEM, GIBCO) containing 5% inactivated fetal bovine serum and 50 μg/ml gentamycin. Maintenance medium (MM) consisted of MEM supplemented with 1.5% fetal serum.

The attenuated strain IV4454 of JUNV was used. Virus stocks were prepared in Vero cells and titrated by plaque assay in the same cells.

2.3. Virus yield inhibition assay

Cells grown in 24-well microplates were infected at a different multiplicity of infection (m.o.i.) with JUNV. After 1 h adsorption at 37 °C, cells were washed and refed with MM containing serial two-fold concentrations of the compound. After 48 h of incubation at 37 °C, supernatant cultures were harvested and diluted at least 100-fold before titration of virus yield by plaque assay in Vero cells. This dilution assessed that compound concentration was sufficiently low as not to interfere during the yield determination. The effective concentration 50% (EC_{50}) was calculated as the concentration required to reduce virus yield by 50%, in the compound-treated cultures compared with untreated ones. All determinations were performed thrice, and each time in duplicate.

2.4. Cytotoxicity assay

Cytotoxicity was measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT, Sigma–Aldrich) method in Vero cells using conditions equivalent to those used in antiviral assays, as previously described (Sepúlveda et al., 2008). The cytotoxic concentration 50% (CC_{50}) was calculated as the compound concentration required to reduce the MTT signal by 50% compared to untreated controls. All determinations were performed twice, each time in duplicate.

2.5. Time of addition assay

Vero cells grown in 24-well microplates were infected with JUNV (m.o.i. 0.1 PFU/cell) and adsorption was allowed for 1 h at 4 °C. After removal of the inocula, the cells were washed with phosphate-buffered saline (PBS), refed with MM and incubated at 37 °C. Duplicate wells were treated with 25 μM **3f** at various times after infection. A control infected culture without drug treatment was performed simultaneously. In all cases, extracellular virus yields were determined by plaque formation at 24 h post-infection.

2.6. Virus adsorption

Vero cells were infected with JUNV (m.o.i. 1 PFU/cell) under four different treatment conditions. Compound during adsorption: cells were infected in MM containing 25 μM **3f**; after 1 h adsorption at 4 °C, inoculum was discarded, cells were washed thrice with PBS and compound-free MM was added. Compound after adsorption: cells were infected in the absence of compound, and after 1 h adsorption at 4 °C the unadsorbed virus was removed by washing with PBS, and MM containing 25 μM **3f** was added. Compound always: the compound was present during JUNV adsorption at 4 °C and in MM added after adsorption. Virus control: cells were infected and maintained always in MM without compound. For all treatments, extracellular virus yields were determined after 48 h of infection by plaque formation.

2.7. Virus internalization

Vero cells were infected with JUNV (m.o.i. 1 PFU/cell). After 1 h adsorption at 4 °C, unadsorbed virus was removed and cells were washed with PBS and incubated at 37 °C during 1 h in the presence or absence of 25 μM **3f**. Then, cells were washed with PBS and treated with 0.1 ml of citrate buffer (citric acid 40 mM, KCl 10 mM, NaCl 135 mM, pH 3) for 1 min to inactivate adsorbed virus but not internalized virus. Cells were then washed with PBS and treated with trypsin for 5 min at 37 °C. After trypsin inactivation with

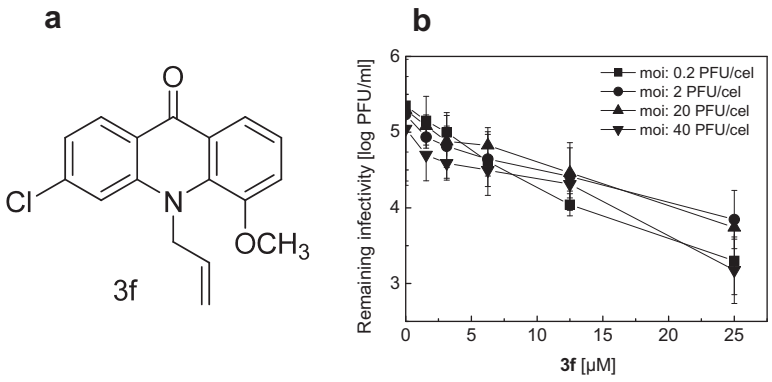

Fig. 1. (a) Chemical structure of compound **3f**. (b) Inhibition of JUNV infection by **3f** at different m.o.i. Vero cells were infected with JUNV at a range of m.o.i. from 0.2 to 40 PFU/cell in the presence of increasing concentrations of **3f**. Virus yields were determined at 48 h p.i. by plaque assay. Results are expressed as remaining infectivity (PFU/ml) in compound-treated cultures compared to untreated ones. Each value is the mean of triplicate assays ± standard deviation (SD).

MEM 5% inactivated fetal bovine serum, cells were washed with PBS by low-speed centrifugation, and the pellet was resuspended in MM. Different serial dilutions of the cell suspension were plated onto Vero cell monolayers to quantify productive internalized virus by infectious center formation.

2.8. Viral RNA synthesis

To analyze the inhibitory effect of **3f** on total viral RNA synthesis, Vero cells were infected with JUNV at a m.o.i. of 0.1 PFU/cell and, after adsorption, cells were re-fed with MM containing or not **3f**. At 48 h after infection, total RNA was extracted by using TRIzol (Invitrogen Life Technologies) according to the Manufacturer's instructions. To monitor RNA replication, cDNA was generated by using murine reverse transcriptase M-MLV (200 U/μl, Invitrogen) and random primers. This cDNA was amplified by real time PCR using SYBRGreen (Roche) detection. The mix reaction volume was 25 μl including 1 μl of cDNA, DNA polymerase GoTaq (5 U/μl, Promega) and specific primers to amplify the genes *n* (nucleoprotein NP), *gpc* (glycoprotein precursor GPC) and *z* (Z protein) (Table 1).

Real time PCR was carried out with an initial incubation at 94 °C during 1 min followed by 40 cycles of 30 s at 95 °C, 45 s at 60 °C, 45 s at 72 °C, 2 s at 79 °C and a final incubation of 15 s at 60 °C. Amplification plots were expressed as C_T values to be analyzed with Opticon Monitor 3.1. software, where C_T values represent the reaction cycle at which PCR products reach a threshold level of detection. C_T values were normalized by using *actin* as standard.

For viral RNA kinetics studies, Vero cells were infected with JUNV (m.o.i. 1 PFU/cell) and at different time points RNA was extracted with TRIzol. The cDNA was synthesized using *gpc* forward primer, specific for antigenomic RNA, and amplified by real time PCR as above mentioned. The effect of **3f** on early RNA synthesis at 5 and 7 h p.i. was also determined in the same experimental conditions.

2.9. Indirect immunofluorescence

Vero cells grown in coverslips were infected with JUNV at a m.o.i. of 0.1 PFU/cell and MM containing or not 25 μM **3f** was added after adsorption. At 48 h p.i., cells were fixed in methanol for 15 min at −20 °C and cytoplasmic immunofluorescence staining was carried out by using the monoclonal antibody (mAb) SA02-BG12 for NP (Sanchez et al., 1989), followed by fluorescein isothiocyanate (FITC)-goat anti-mouse IgG (Sigma–Aldrich). After a final washing with PBS, cells were stained with Evans Blue and mounted in a glycerol solution containing 1,4-diazabicyclo[2,2,2]octane (DABCO).

2.10. Reversal by guanosine

To address the ability of guanosine to reverse the effect of inhibitors on JUNV infection, JUNV was adsorbed to Vero cells for 1 h and then cultures were incubated with MM containing a fixed concentration of **3f** (25 μM), RIB (80 μM) or MPA (10 μM) and variable amounts of guanosine. After 48 h, extracellular virus yields were determined by PFU.

The reversal by guanosine of viral RNA synthesis inhibition was analyzed in JUNV infected cells incubated during 48 h after infection with MM containing **3f** (25 μM), RIB (80 μM) or MPA (10 μM) and 200 μM guanosine. Then, RNA was extracted with TRIzol, cDNA was synthesized using random primers and amplified by real time PCR using *z* primers (Table 1) as described above.

Table 1
List of primers for JUNV genes.

Gene	Primer sequences corresponding to genomic/antigenomic RNA
n	Forward 5′-GGCATCCTTCAGAACATC-3′ (genomic RNA)
	Reverse 5′-CGCACAGTGGATCCTAGGC-3′ (genomic/antigenomic RNA)
gpc	Forward 5′-ATGGGGCAATTCATCAG-3′ (genomic RNA)
	Reverse 5′-CCCCTTAATGTAAAGATGGC-3′ (antigenomic RNA)
z	Forward 5′-ATGGGCAACTGCAACGGGGCATC-3′ (genomic RNA)
	Reverse 5′-AGCCAACAGCACCACCACCATAG-3′ (antigenomic RNA)

3. Results

3.1. Antiviral effectiveness of *3f*: effect of multiplicity of infection

The derivative 10-allyl-6-chloro-4-methoxy-9(10*H*)-acridone, designated **3f**, (Fig. 1a) was found the most effective compound to inhibit JUNV infection in Vero cells among the diverse series of *N*-substituted acridones originally tested (Sepúlveda et al., 2008). In addition, as seen in Fig. 1b, the effectiveness of **3f** to inhibit JUNV infection was not importantly affected by the initial virus inoculum. When a virus yield inhibition assay was performed in Vero cells infected at different m.o.i., the profile of the dose–response curves was similar in the range 0.2–40 PFU/cell, with slight variations independently of the m.o.i. In fact, when the EC_{50}s were calculated from data presented in Fig. 1b, the values obtained were 2.6 ± 0.1, 1.6 ± 0.1, 2.3 ± 0.3 and 2.2 ± 0.3 μM for m.o.i. of 0.2, 2.0, 20.0 and 40.0 PFU/cell, respectively. Thus, within a 200-fold variation in virus inoculum, the EC_{50} showed a less than 2-fold change. Furthermore, cell viability was not affected after 48 h of Vero cell incubation with the compound, in the same conditions of the antiviral assay, up to a **3f** concentration of 1 mM (data not shown). Then, the selectivity indices (ratio CC_{50}/EC_{50}) were greater than 350 for all the JUNV multiplicities evaluated.

3.2. Mechanistic studies: early steps of replication

Time of addition experiments were conducted to estimate the timing of inhibition in JUNV replication exerted by **3f**. The compound was added to infected cultures at various times after adsorption at 4 °C and the reduction in virus yield relative to untreated cultures was determined at 24 h p.i. A strong inhibition in virus production greater than 3 log was observed when drug was added up to 5 h p.i., and a minor effect with 1–1.5 log reduction in virus titers was seen at later times (Fig. 2a). These data suggest that the main action of **3f** is mainly exerted within early and/or intermediate stages of virus replication.

The significant reduction observed in time of addition experiments shown in Fig. 2a was always obtained in the absence of the compound during virus adsorption. The lack of effect of **3f** on JUNV adsorption was confirmed by determining the inhibition produced when the compound was present only during the adsorption period of Vero cells with JUNV during 1 h at 4 °C. Under these treatment conditions, no reduction in virus production in the infected cell culture incubated without compound was observed, whereas the addition of the acridone after virus adsorption was as effective as its presence during both adsorption and the post-adsorption period (Fig. 2b).

Next, the action of **3f** on JUNV internalization into the cell was studied. To this end, Vero cells were infected with JUNV and incubated 1 h at 4 °C to allow virus adsorption; then, the compound was added to infected cultures and temperature of incubation was immediately raised to 37 °C to start virus penetration. Under these treatment conditions, the amount of internalized virus after 1 h at 37 °C determined by an infectious center assay was similar in compound-treated and -untreated cells (Fig. 2c).

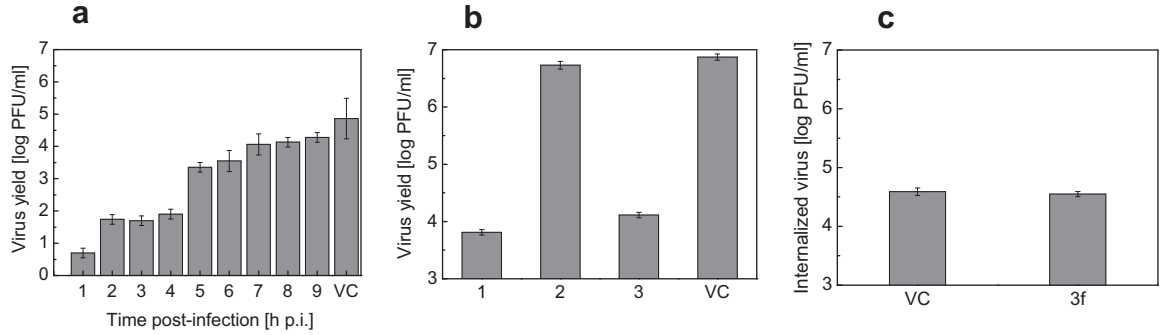

Fig. 2. Effect of **3f** on virus infection. (a) Time of addition assay. Vero cells were infected with JUNV and MM containing 25 μM **3f** was added at different times post-adsorption. Extracellular virus yields were determined by plaque formation after 24 h of incubation at 37 °C. (b) Adsorption. Vero cells were infected with JUNV under different treatment conditions with 25 μM **3f**: the compound was present during 1 h adsorption at 4 °C and during 48 h incubation at 37 °C after adsorption (1), only during 1 h adsorption at 4 °C (2) or only after adsorption, during 48 h incubation at 37 °C (3). Virus control (VC): cells were infected and maintained in MM without compound. For all treatments, extracellular viral titers were determined at 48 h p.i. by plaque formation. (c) Internalization. JUNV was adsorbed to Vero cells at 4 °C for 1 h. After incubation at 37 °C for 1 h in MM containing or not 25 μM **3f**, cells were treated with citrate buffer, trypsinized, and internalized virus was determined by an infectious center (IC) assay. Each value is the mean of triplicate assays ± SD.

3.3. Effect of **3f** on viral RNA synthesis and protein expression

After penetration into the host cell, the following step in arenavirus life cycle is viral RNA synthesis. To analyze the effect of **3f** on JUNV RNA synthesis, cells infected with JUNV were incubated in MM containing or not compound, and at 48 h p.i. total RNA was extracted. A real-time PCR was performed with RNA samples from treated and untreated cells which were amplified with primers corresponding to three viral genes, *n, gpc* and *z*, (Table 1) and using *actin* as cellular control. Quantification of total viral RNA (genomic and antigenomic RNA) synthesized under these conditions showed a strong level of inhibition, with almost 3 log reduction of viral RNA synthesis in JUNV infected cells after treatment with 25 μM **3f** (Fig. 3a). The degree of inhibition was similar for the three viral genes, and also a dose-dependent response was observed (data not shown).

To assess that RNA synthesis was the target of **3f** in JUNV replication cycle, the effect of this compound at earlier times was analyzed. It is known that the multiplication cycle of JUNV is completed in about 12 h (Mersich et al., 1981) and viral RNA is first detected in infected cells around 3–4 h p.i. (Ellenberg et al., 2007). As seen in Fig. 3b, the RNA viral kinetics assay performed to confirm the time course of JUNV RNA synthesis was in accordance with the mentioned studies. The kinetics patterns obtained by real

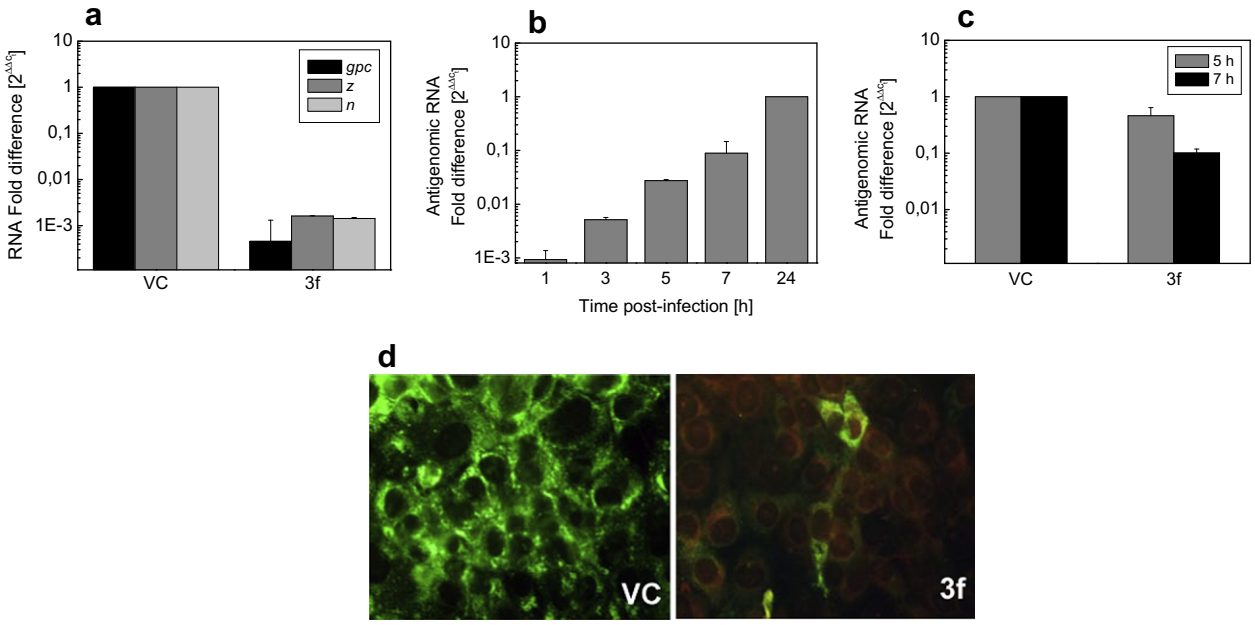

Fig. 3. Effect of **3f** on virus RNA synthesis and protein expression. (a) Vero cells were infected with JUNV and incubated during 48 h in the absence (VC) or presence of 25 μM **3f**. Then, total RNA was extracted and cDNA synthesized with random primers. These cDNAs were amplified by real time PCR using specific primers to amplify the viral genes *n*, *gpc* and *z*, and cellular *actin* amplification was used for normalization. Results are expressed as fold difference viral RNA level respect to viral control for each gene. b. Vero cells were infected with JUNV and total RNA was extracted at different time points: 1, 3, 5, 7 and 24 h p.i. Then, cDNA was generated using a forward *gpc* primer specific for viral antigenomic RNA, and amplified by real time PCR with *gpc* primers. Results are expressed as fold difference viral RNA level at each time point compared to the RNA level found at 24 h p.i. (c) Vero cells were infected with JUNV and incubated during 5 or 7 h in the absence (VC) or presence of 25 μM **3f**. Immediately thereafter, total RNA was extracted, cDNA was synthesized and amplified as in (b). Results are expressed as fold difference in viral RNA level with respect to viral control at 5 and 7 h, respectively. (d) Vero cells were infected with JUNV in the absence (VC) or presence (**3f**) of 25 μM **3f**. At 48 h p.i. immunofluorescence staining was performed using anti-JUNV mAb. Magnification: 400×.

time PCR for viral genes *n* and *z* were similar (data not shown). When the effect of **3f** was assayed at the initial times of RNA synthesis, a significant reduction was observed on viral RNA at 5 and 7 h p.i. (Fig. 3c). The level of inhibition was increasing with time since a higher inhibition was evident at 7 h p.i when a greater amount of viral RNA was detected in control infected cells. These results allow to conclude that there is a direct effect of the acridone on viral RNA synthesis and the inhibition observed at 48 h p.i. (Fig. 3a) is effectively due to the cumulative inhibitory action of **3f** on this step of the viral cycle.

The failure on RNA synthesis was indirectly corroborated by analyzing the effect of **3f** on the expression of JUNV proteins, studied by indirect immunofluorescence. In control infected cells, the presence of the nucleocapsid protein NP, the more abundant viral protein, was revealed with mAb SA02-BG12 exhibiting a finely dotted regular distribution (Fig. 3d). The amount of cells showing cytoplasmic fluorescence for NP was drastically reduced in the presence of **3f**. Thus, the lack of viral RNA mRNAs and, consequently, the absence of viral protein expression in cells inoculated with JUNV and treated with **3f** were further assessed.

3.4. Reversal by guanosine on JUNV infectivity and RNA synthesis

Acridone-based compounds have been reported to be potent and uncompetitive inosine monophosphate dehydrogenase (IMPDH) inhibitors (Watterson et al., 2007). IMPDH is a key enzyme responsible for catalyzing the conversion of inosine 5′-monophosphate to xanthosine 5′-monophosphate, a rate-limiting step for intracellular *de novo* synthesis of guanosine nucleotides. To explore whether the antiviral activity of **3f** might be due to GTP pool reduction, JUNV infected Vero cells were incubated with MM containing **3f** in the presence or absence of guanosine. As control RIB and MPA, two compounds with reported inhibitory activity against IMPDH (Sintchak and Nimmesgern, 2000), were simultaneously tested against JUNV infection in the same conditions. RIB has inhibitory activity against JUNV in vitro and in a rhesus macaque model (McKee et al., 1988), but the clinical evaluation in Argentine HF patients did not show efficacy in reducing mortality (Enría et al., 1987; Enría and Maiztegui, 1994), whereas MPA has been recently found active against LASV (Ölschläger et al., 2011). The EC_{50} values of RIB and MPA against JUNV, determined by a virus yield inhibition assay at a m.o.i. of 0.2 PFU/cell in Vero cells, were 18.5 ± 1.7 and 0.37 ± 0.04 μM, respectively, and the corresponding CC_{50}s were >400 and >500 μM, respectively. Then, the reversal assay was performed with a fixed concentration of each inhibitor and increasing concentrations of guanosine. The fixed concentration chosen for each inhibitor reduced significantly the virus titer by at least two orders of magnitude (Fig. 4a–c). The addition of exogenous guanosine rescued the infectivity of JUNV in **3f**-treated cells in a dose-dependent manner, but the reversal was partial as reflected in PFU levels: the virus yields were reduced from 3.6×10^5 PFU/ml in the control infected cells to 6.0×10^3 PFU/ml in **3f**-treated infected cells, and the simultaneous treatment with **3f** and 200 μM guanosine increased the titer to 6.1×10^4 PFU/ml. Thus, 1 log of infectivity was recovered but there still was a loss of approximately 80% of the production corresponding to untreated infected cells (Fig. 4a). The guanosine reversal for RIB was even more incomplete, with only about 0.5 log difference among titers of guanosine-rescued and non-rescued infected cells (Fig. 4c), and the highest recovery of infectivity by guanosine addition was observed in MPA-treated cells (Fig. 4b). The effect of exogenous guanosine addition on the inhibitory activity of the three compounds was also corroborated by determination of viral RNA synthesis: a similar profile of partial restoration for **3f** and RIB and high recovery for MPA was observed (Fig. 4d).

4. Discussion

We recently reported a series of *N*-allyl acridone derivatives that had selective antiviral activity in cell cultures against arenaviruses (Sepúlveda et al., 2008). The studies shown here demonstrate that **3f**, the most potent inhibitor in the compound series, was able to interfere with JUNV intracellular multiplication through a strong inhibition of infectious virus production and viral RNA synthesis, with about 2.5–3 log reduction in the amount of infective PFU and viral RNA at 48 h p.i. in compound-treated cells relative to non-treated cells. Additionally, the antiviral activity of **3f** against JUNV was similar independently of the m.o.i. used, in the range 0.2–40.0 PFU/cell. The m.o.i. dependence reported for various agents-virus combinations is very variable. The lack of dependence of the antiviral potency of acridones with the infecting virus inoculum here shown is not unusual for several types of antiviral agents (Artuso et al., 2009; Rice et al., 1997; Stránská et al., 2002; Talarico and Damonte, 2007; Whitby et al., 2005), but represents a clear advantage for those compounds, such as **3f** against JUNV, as it is able to block infection even in the presence of high initial virus doses.

The initial steps of virus adsorption and internalization were not affected by treatment with the acridone and the subsequent process of virus RNA synthesis appeared to be the main target of **3f** antiviral action. An important inhibition on RNA synthesis was observed at early and late times after infection, affecting all tested virus genes. The mechanism of action of inhibitors targeted to viral RNA may involve inhibition of viral and/or host factors participating in RNA synthesis. Given the reported activity of certain acridone derivatives as IMPDH inhibitors (Watterson et al., 2007), interference of **3f** with this cellular enzyme was entertained. A partial reversion of the inhibitory effect was detected in the simultaneous presence of guanosine and **3f** (Fig. 4) suggesting possible involvement of IMPDH inhibition in the antiviral activity of this acridone. But, the recovery of infective virus particles as well as RNA synthesis was incomplete in the presence of guanosine indicating another mode of inhibition may also be operative in the antiviral action of **3f**. It was interesting to compare the effects of **3f** against JUNV with other IMPDH inhibitors with antiviral activity against several viruses, like RIB and MPA. RIB is a nucleoside analog which once inside the cell is first converted to RIB-5′-monophosphate and in this form becomes a competitive inhibitor of IMPDH (Streeter et al., 1973). Then, RIB-5′-triphosphate is formed by further phosphorylation and other antiviral mechanisms associated with the production of the triphosphate have been proposed: direct inhibition of viral RNA polymerase (Eriksson et al., 1977; Rankin et al., 1989; Toltzis et al., 1988), inhibition of the 5′ end capping of viral mRNAs (Goswami et al., 1979) and induction of error catastrophe as a result of accumulation of mutations (Graci and Cameron, 2002). Evidence exists to support each of the proposed RIB mechanisms of action, and distinct virus/host combinations may preferentially encourage one or more of these mechanisms during viral therapy. In the particular case of arenaviruses, there is evidence for LCMV and LASV that the mechanism of action of RIB is not mainly focused on IMPDH inhibition, as described for other viruses like flaviviruses and paramyxoviruses (Leyssen et al., 2005), and may involve the viral polymerase (Moreno et al., 2011; Ölschläger et al., 2011; Ruiz-Jarabo et al., 2003). Our experiments with RIB and guanosine also indicate that depletion of intracellular GTP is not dominant for RIB arenavirus inhibition (Fig. 4). MPA is a non-nucleoside non-competitive

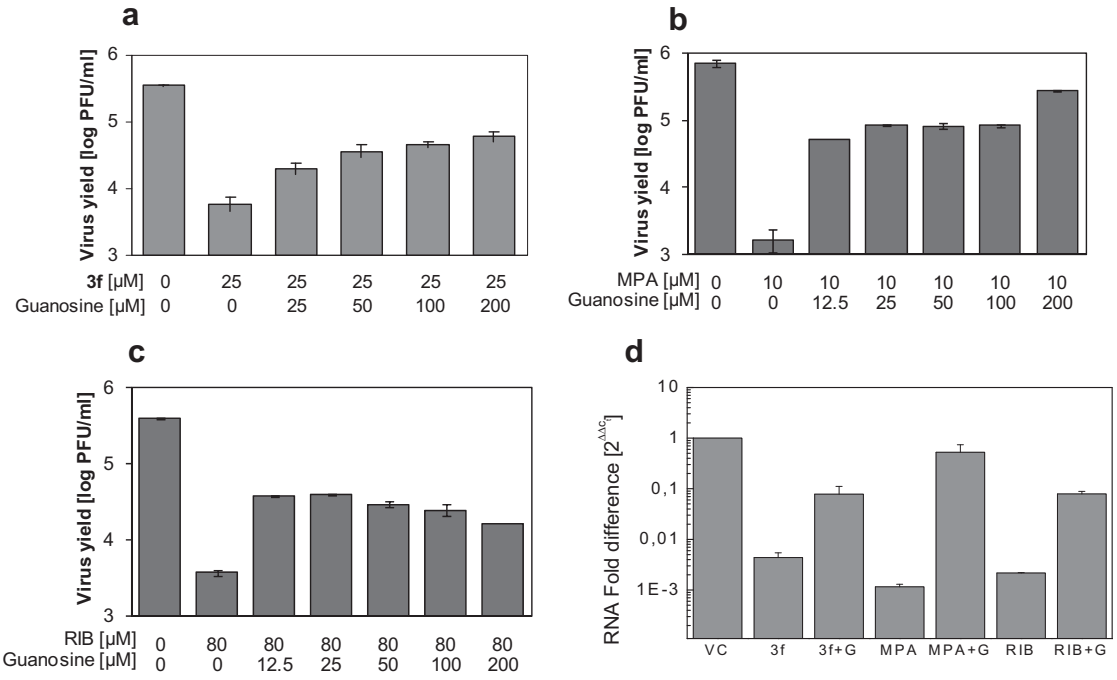

Fig. 4. Reversal of inhibition by exogenous addition of guanosine. Vero cells were infected with JUNV and treated with 25 μM **3f** (a), 10 μM MPA (b) or 80 μM RIB (c) in the presence or absence of guanosine. After 48 h, extracellular virus yields were determined by plaque formation. Each value is the mean of triplicate assays ± SD. (d) In addition, total RNA was extracted and cDNA synthesized with random primers. cDNAs were amplified by real time PCR using *z* specific primers and cellular *actin* amplification was used for normalization. Results are expressed as fold difference of viral RNA level compared to viral control.

inhibitor of IMPDH with well known immunosuppressive effects and effective against multiplication of several viruses (Kitchin et al., 1997). The very potent antiviral action of this drug against JUNV multiplication was here shown for the first time and this inhibition was highly reversed when guanosine was supplemented to the medium even at low concentrations (Fig. 4). Thus, the anti-JUNV activity of MPA was certainly associated with the depletion of GTP pool through IMPDH blockade, additionally demonstrating the high susceptibility of JUNV replication to this cellular pathway of inhibition. A similar mechanism of inhibition for MPA was recently demonstrated against the Old World HF arenavirus LASV (Ölschläger et al., 2011).

In recent years, diverse natural and synthetic acridone-based substances have been known as multi-targeted agents with anticancer and antimicrobial properties against parasites, viruses and fungi (Belmont et al., 2007; Hedge et al., 2004; Kelly et al., 2009; Singh et al., 2009; Vispé et al., 2007) renewing the interest on the therapeutic potential of these bioactive compounds. Concerning to antiviral activity, acridone-derivatives were shown to inhibit different viruses, including the DNA containing adenovirus (Zarubaev et al., 2003) and herpesviruses (Bastow, 2004), as well as RNA viruses like human immunodeficiency virus (Fujiwara et al., 1999), bovine viral diarrhea virus (Tabarrini et al., 2006) and hepatitis C virus (Manfroni et al., 2009; Stankiewicz-Drogon et al., 2010). For the last flavivirus, the antiviral action was also due to inhibition of viral RNA synthesis and the activity of viral enzymes like the RNA helicase and, probably, the RNA polymerase was affected. Additionally, acridone derivatives have the ability of nucleic acid intercalation and, consequently, disrupt enzyme recognition and/or association to the modified nucleic acid (Adams, 2002). Stankiewicz-Drogon et al. (2010) found a strong preference for double stranded RNA in the intercalating properties of some acridone-based HCV inhibitors, but the authors could still not elucidate if these compounds affect RNA synthesis only through this intercalation, if there is also a direct interaction of the compound with the viral enzymes or the combination of both effects.

In conclusion, a potent and selective antiviral activity against the HF causing arenavirus JUNV was detected in the acridone **3f**. The reversibility of anti-JUNV inhibitory action of **3f** by exogenous guanosine was intermediate between that observed for RIB and MPA and these results are consistent with a more than single mode of action for this compound, as reported for other acridones. A possible cellular target is represented by the enzyme IMPDH whereas another still unidentified target, presumably a viral target given the high selectivity of the compound, may also contribute to the antiviral activity of **3f** against arenaviruses.

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