



Characterization of Junín virus particles inactivated by a zinc finger-reactive compound

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ABSTRACT

Our previous studies reported the inhibitory action against arenaviruses of antiretroviral zinc finger-reactive compounds provided by the National Cancer Institute (USA). These compounds were able to inactivate virions as well as to reduce virus yields from infected cells. Here, the inactivation of the arenavirus Junín (JUNV), agent of Argentine hemorrhagic fever, by the aromatic disulfide NSC20625 was analyzed. The treatment of purified JUNV with this compound eliminated infectivity apparently through irreversible modifications in the matrix Z protein detected by: (a) alterations in the electrophoretic migration profile of Z under non-reducing conditions; (b) an electrodense labeling in the internal layer beneath the envelope and around the matrix Z protein, in negatively stained preparations; (c) changes in the sub-cellular localization of Z in cells transfected with a recombinant fusion protein JUNVZ-eGFP. The infection of Vero cells with JUNV inactivated particles was blocked at the uncoating of viral nucleocapsid from endosomes, providing new evidence for a functional role of Z in this stage of arenavirus cycle. Furthermore, the inactivated JUNV particles retained the immunoreactivity of the surface glycoprotein GP1 suggesting that this disulfide may be useful in the pursuit of an inactivating agent to obtain a vaccine antigen or diagnostic tool.

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1. Introduction

The family *Arenaviridae* comprises several human pathogens capable of causing severe diseases, which are included in the biodefense Category A priority viral agents by the National Institute of Allergy and Infectious Diseases (NIAID), such as Junín virus (JUNV), Machupo virus (MACV), Guanarito virus (GTOV) and Lassa virus (LASV) (Rotz et al., 2002). JUNV is the agent of Argentine hemorrhagic fever (AHF), an endemo-epidemic disease with annual outbreaks affecting the population of the richest agricultural zone of Argentina (Damonte, 2002). No reliable drug therapy is presently available for the treatment of AHF and the other arenaviral human diseases (Damonte and Coto, 2002). Ribavirin is the only compound showing partial efficacy against JUNV and LASV infections but with a high level of undesirable secondary reactions (Enrúa and Maiztegui, 1994; Fisher-Hoch et al., 1992; McCormick et al., 1986).

Arenaviruses contain a bisegmented single-stranded RNA genome with an ambisense coding strategy. The small (S) segment encodes the nucleocapsid protein (NP) and preGPC, a pre-envelope

glycoprotein precursor which is processed posttranslationally into a signal peptide, the external glycoprotein GP1 and the trans-membrane GP2, whereas the large (L) fragment encodes the RNA polymerase L and an 11 kDa protein called Z. The Z protein is conformed by a conserved RING finger domain of 60 amino acids flanked by a N-terminal portion with a myristoylation site (Perez et al., 2004) and a C-terminal portion, which contains proline-rich motifs found in the called late domains that were identified in matrix proteins of enveloped viruses (Eichler et al., 2004; Freed, 2002). Thus, although the precise functional role of this small protein in arenavirus infections is not totally elucidated, many studies have proposed for Z the structural functions of a matrix protein (Compans, 1993; Neuman et al., 2005; Perez et al., 2003; Salvato et al., 1992; Salvato, 1993; Strecker et al., 2003) together with a regulatory role through inhibition of viral RNA synthesis (Cornu and de la Torre, 2001, 2002; López et al., 2001). These characteristics have turned the Z protein an attractive target for arenavirus antiviral chemotherapy.

Given the abovementioned properties of Z, a series of disulfides and azoic derivatives known to be reactive against the zinc finger motifs of the human immunodeficiency virus type 1 (HIV-1) nucleocapsid protein NCp7, causing Zn ejection from the protein and inhibition of HIV-1 multiplication (Rice et al., 1996, 1997), were evaluated against the New World arenaviruses JUNV, Tacaribe (TCRV), and Pichinde (PICV) and the Old World arenavirus prototype

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lymphocytic choriomeningitis virus (LCMV). These compounds were very effective arenavirus inhibitors and, according to their inhibitory action, could be classified in two categories: (i) virucidal agents able to inactivate cell-free virions and (ii) antiviral agents that blocked the intracellular viral multiplication cycle (García et al., 2000). The most effective inactivating agent was NSC20625 (Fig. 1A), an intermolecular aromatic disulfide that quickly inactivates arenaviruses in a concentration and time dependent manner (García et al., 2002). Inactivated virions appeared to maintain the functional integrity of the viral glycoproteins, since they were able to bind and enter into the host cell (García et al., 2002, 2006). By contrast, the treatment of a recombinant LCMV Z protein with this virucidal compound showed the formation of Z multimers probably due to zinc ejection from the molecule, confirming that this protein is the main target (García et al., 2006).

Here, the mechanism of JUNV inactivation by this compound was further investigated demonstrating alterations in Z properties and that the infection of Vero cells with inactivated JUNV virions is abolished by blockade at the uncoating of viral nucleocapsid in the endocytic pathway, providing new evidence for a probable functional role of Z as a matrix protein during this step of arenavirus multiplication cycle.

2. Materials and methods

2.1. Compounds

The compound 1-(2-guanidine) phenyldisulfide (NSC20625) was provided by the National Cancer Institute, Frederick, USA. Azodicarbonamide (ADA) was purchased from Sigma–Aldrich (USA). A 100 mM stock solution of each compound was prepared in dimethylsulfoxide.

2.2. Cells, virus and plasmids

Vero cells were grown as monolayers in Eagle's minimum essential medium (MEM, GIBCO, USA) 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 on the same cells.

To obtain purified radiolabeled virions, Vero cells were infected with JUNV and at 48 h p.i. 25 µCi/ml of EXPRE³⁵S³⁵S (NEN Dupont, USA) was added in methionine–cysteine-free MM. Cell supernatants were harvested at 72 h p.i., and radiolabeling was repeated between 72 and 96 h p.i. After clarification by low-speed centrifugation, supernatants were concentrated by pelleting for 2 h at 100,000 × g. Then, the pellet was resuspended in TES buffer (Tris 0.01 M, NaCl 0.1 M, EDTA 0.001 M, pH 7.4) by brief sonication and layered onto a 15–60% sucrose gradient. After centrifugation at 100,000 × g for 2 h, the virus band was collected, pelleted again by centrifugation at 100,000 × g for 2 h, and finally resuspended in buffer TES by sonication.

To construct the reporter plasmid expressing the fusion protein JUNVZ-eGFP (enhanced green fluorescent protein), a truncated Z lacking a stop codon was obtained by PCR-amplification using plasmid pGEM containing a full-length cDNA copy of the Z gene as template DNA together with oligonucleotides Z-F (5' GGATCCATGGGCAACTGCAACGGGGCATC-3') and Z-trunc-R (5' AAAAGCGCCGCTGGTGGTGGTGGCT-3') containing a BamHI and NotI site, respectively (underlined). The insert DNA was subsequently recloned into the BamHI and NotI sites of the mammalian expression vector pcDNA3.1 to generate pcDNA3.1-JUNVZ. Oligonucleotides eGFP-F (5'-

AAAAGCGCCGCGCATGGTGAGCAAGGGCGAGGAG-3') and eGFP-R (5' AAAATCTAGATTACTTGTACAGCTCGTCCATGCC-3'), which contain a NotI and XbaI site, respectively (underlined), were used with plasmid pGEM-eGFP as template DNA to PCR-amplify the sequence encoding eGFP and generate pcDNA3.1-eGFP. The amplicon was cloned into the NotI–XbaI digested pcDNA3.1-Z vector to generate the reporter plasmid pcDNA3.1-JUNVZ-eGFP, in which the eGFP gene was fused in-frame with the 3' end of the Z truncated coding sequence.

2.3. Inactivation assay

Samples of purified virions (2×10^7 PFU/ml, 5×10^8 dpm/ml for radiolabeled preparations and 2×10^7 PFU/ml for nonlabeled virion samples) were incubated at 37 °C for 1.5 h in the presence of 40 µM NSC20625. A virus control was also performed by incubation of the virion suspension with MM under the same conditions. For infectivity titration, the mixtures were then chilled and diluted further with MM before being placed on Vero cell cultures for plaque assay, to assess that titer reduction was only due to cell-free virion inactivation.

2.4. Effect of inhibitor on virion proteins

Control and inactivated purified radiolabeled particles were lysed in sample buffer for electrophoresis (5% sodium dodecyl sulfate (SDS), 10% glycerol, 0.005% bromophenol blue, 0.0625 M Tris–HCl, pH 6.8) either with or without 2% 2-mercaptoethanol (2-ME). Then, proteins were fractionated by 18% SDS-polyacrylamide gel electrophoresis (PAGE) and visualized by fluorography.

For the immunoprecipitation assay, control and NSC20625-inactivated virus particles were incubated with polyclonal rabbit anti-JUNV serum in RIPA buffer (10 mM Tris–HCl pH 7.4, 0.15 M NaCl, 0.1% SDS, 1% Triton X-100, 1% sodium deoxycholate, 0.4 mM phenyl-methyl-sulphonyl-fluoride) for 30 min at 37 °C and 90 min at 4 °C. Antibody–antigen complexes were collected with protein A-sepharose, washed three times in RIPA buffer, and solubilized by boiling for 2 min in sample buffer containing or not 2% 2-ME. Viral polypeptides were then electrophoresed on 12% SDS-PAGE and visualized by fluorography.

2.5. Electron microscopy

Suspensions of control and inactivated JUNV particles were fixed overnight in 2.5% glutaraldehyde. A drop of the respective suspension (10 µl) was deposited on Formvar-carbon-coated nickel grids for 1 min. The excess fluid was blotted away with Whatman filter paper, and the samples were negatively stained for 1 min in 0.5% uranyl acetate. Dried specimens were examined with a Philips EM 301 transmission electron microscope.

2.6. Transfection and fluorescence

Vero cells grown on 12 mm diameter glass coverslips to 80% confluence were transfected with 1 µg of pcDNA3.1-JUNVZ-eGFP or pcDNA3.1-eGFP using Lipofectamine 2000 (Invitrogen, USA). After 6 h transfection, cells were incubated with MM containing or not 40 µM NSC20625. After 48 h, cells were fixed with cold methanol for 10 min at –20 °C and were mounted in a glycerol solution containing 1,4-diazabicyclo[2,2,2]octane (DABCO). Cells were observed for the expressions of eGFP in a confocal laser-scanning microscope Olympus Fluo View to follow the Z localization.

2.7. Virion cellular uptake

Radiolabeled infectious or NSC20625-inactivated JUNV were adsorbed to Vero cells at 4 °C for 1 h, and then cells were incu-

bated at 37 °C during 2 h to allow virus penetration. 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 phenyl–methyl–sulphonyl–fluoride (PMSF) in PBS containing 3% bovine serum albumin. Cells were then pelleted, lysed in NaOH–SDS solution and cell-associated radioactivity was quantified.

2.8. Viral RNA transcription and replication

Vero cells were infected with NSC20625-inactivated and control infectious JUNV, at a MOI of 1 PFU/cell. At 48 h p.i. total RNA was extracted by using Trizol (Invitrogen, USA), according to the manufacturer instructions. The Qiagen RNase-free DNase supplement kit was used to ensure that the RNA had no DNA contamination. Quantitation of purified RNA was done by measuring absorbance at 260 nm. A strand specific RT-PCR was used to monitor the presence or absence of NP mRNA (transcription) and full-length antigenomic S RNA (replication) in RNA samples. The genome sense primers vN (186) (5'-GGCATCCTTCAGAACAT-3') and vG (GPC1) (5'-ATGGGGCAATTCATCAG-3') were used in the JUNV cDNAs synthesis. All the cDNAs were generated by using the AMV reverse transcriptase (Promega, USA) and analyzed by direct (NP) or nested-PCR (GPC) using the following arenavirus specific oligonucleotides: vcN (ARENA) 5'-CGCACAGTGGATCCTAGGC-3' and vN (186) primers generate a 186 bp amplification fragment comprising the 3' end of the S RNA containing N coding sequences; vcG (GPC2) 5'-CCCCTTAATGTAAGATGGC-3' and vG (GPC1) plus the internal primers vcG (GPC3) 5'-CAACCACTTTGTACAGGT-3' and vG (GPC1) generate a 129 bp amplification fragment comprising the 5' end of the S RNA. The PCR cycle progression for the vN (186)/vcN (ARENA) was as follows: 30 s at 94 °C and 35 cycles of 10 s at 94 °C, 20 s at 48 °C and 20 s at 72 °C followed the final extension. The PCR cycle progression for vG (GPC1)/vcG (GPC2) was 30 s at 94 °C and 40 cycles of 15 s at 94 °C, 20 s at 50 °C, 30 s at 72 °C followed by the final extension. The PCR cycle progression for vG (GPC1)/vcG3 was the same except for 20 s at 52 °C at the annealing step and 20 s at 72 °C at the extension step. The final products were resolved by electrophoresis on 1–1.5% agarose gel electrophoresis in TAE buffer (40 mM Tris–acetate, 1 mM EDTA, pH 8.0) with 0.2 µg/ml ethidium bromide.

2.9. Viral protein synthesis

Vero cells grown in microplates were infected with NSC20625-inactivated and infectious virus at a MOI of 1. At 48 h p.i. the infected cells were incubated in methionine–cysteine-free medium for 1.5 h and then labeled with 100 µCi/ml of EXPRE³⁵S-³⁵S (NEN, Dupont, USA) for 3.5 h. After labeling, cells were lysed in RIPA buffer. The clarified cell lysates were incubated with polyclonal rabbit anti-JUNV serum for 30 min at 37 °C, 90 min at 4 °C. Antibody–antigen complexes were collected with protein A-sepharose and solubilized by boiling in sample buffer. Polypeptides were then electrophoresed on 12% SDS-PAGE and visualized by fluorography.

2.10. Nucleocapsid penetration assay

Radiolabeled infectious and inactivated JUNV were bound to Vero cells at 4 °C for 1 h and then internalized at 37 °C for 2 h. Vero cells infected with infectious JUNV were treated with concanamycin A (Sigma–Aldrich, USA) during the 2 h internalization period as positive controls for the assay (Castilla et al., 2001). Cells were then harvested in homogenization buffer (10 mM Tris–HCl pH 7.4, 1 mM MgCl₂, 300 mM sucrose) and manually lysed on ice by 20 passes through a 25-gauge needle. This treatment disrupts the plasma membrane but leaves 95% of endosomes intact

(Marsh et al., 1983; Singh and Helenius, 1992). The lysates were treated with 1 mg/ml proteinase K, and the digestion was allowed to proceed on ice for 30 min. Then, 2 mM PMSF was added to inhibit total proteolysis. Samples were subjected to SDS–PAGE and fluorography to detect viral proteins. An aliquot of each sample was electrophoresed in the same conditions to assure the integrity of endosomes through the presence of an endosomal cellular marker. To this end, after SDS–PAGE, proteins were transferred to polyvinylidene difluoride membrane (PerkinElmer, USA). Membrane was incubated overnight with rabbit polyclonal anti-EEA1 (early endosome antigen 1) serum (Abcam, UK) at 4 °C. Blot was washed in Tris-buffered saline (TBS) containing 0.1% Tween 20, incubated with anti-rabbit IgG conjugated to horseradish peroxidase (Sigma–Aldrich, USA) for 1 h, and revealed by enhanced chemiluminescence (Amersham-Pharmacia).

3. Results

3.1. Effect of NSC20625 on purified virions and proteins

Since the mechanism of arenavirus inactivation by zinc finger-reactive compounds is proposed to occur via modification of the RING finger of Z protein, by ejection of Zn ion and destabilization of the protein (García et al., 2006), the effect of the compound NSC20625 on the proteins of purified ³⁵S-radiolabeled JUNV virions was analyzed. The effectiveness of inactivation of radiolabeled purified JUNV particles by NSC20625 was first assessed. Virion treatment with 40 µM NSC20625 completely eliminated the infectivity of purified JUNV, as determined by the inability of treated virus to form plaques in Vero cells (Fig. 1B), providing more than 3 log units of virus inactivation. Then, proteins of purified control and inactivated virions were studied by SDS-PAGE under reducing and non-reducing conditions in the sample buffer. The two main virion proteins, the nucleocapsid protein NP and the external surface envelope glycoprotein GP1, were detected as two prominent bands both in infectious and inactivated particles under reducing as well as non-reducing conditions (Fig. 1C). On the other hand, the electrophoretic pattern of Z protein was similar in compound treated and untreated virions in the presence of 2-ME, but under non-reducing conditions the 11 kD band corresponding to the Z protein was not detected in NSC20625 treated virions (Fig. 1C).

To further analyze virion proteins integrity, the immunoprecipitation of inactivated and untreated particles with hyperimmune anti-JUNV serum was performed and the precipitated proteins were then resolved by SDS-PAGE. As shown in Fig. 1D, the immunoprecipitation of NP and GP1 proteins was not affected by NSC20625 treatment and the electrophoretic pattern of both virion proteins was similar in the presence or absence of 2-ME. The immune serum was not able to react with Z protein neither in native intact virions nor in JUNV infected cells (data not shown). Altogether, results shown in Fig. 1C and D were indicative of some structural alteration in Z protein after compound inactivation with apparent preservation of the integrity of nucleocapsid and envelope proteins.

To identify any other structural change in virus particles after treatment with NSC20625, the morphology of native and inactivated virions was investigated by electron microscopy. Negatively stained preparations of native infectious JUNV showed the typical roughly spherical particles with an external unit membranous envelope previously described for other arenaviruses (Compans, 1993; Murphy and Whitfield, 1975; Tesh et al., 1994). By contrast, NSC20625-inactivated JUNV particles exhibited an electron-dense labeling in a layer beneath the envelope distinguishable from infectious control virions (Fig. 2), in an ultrastructural location assigned to Z protein according to its function of arenavirus matrix responsible to connect the lipid envelope with the internal

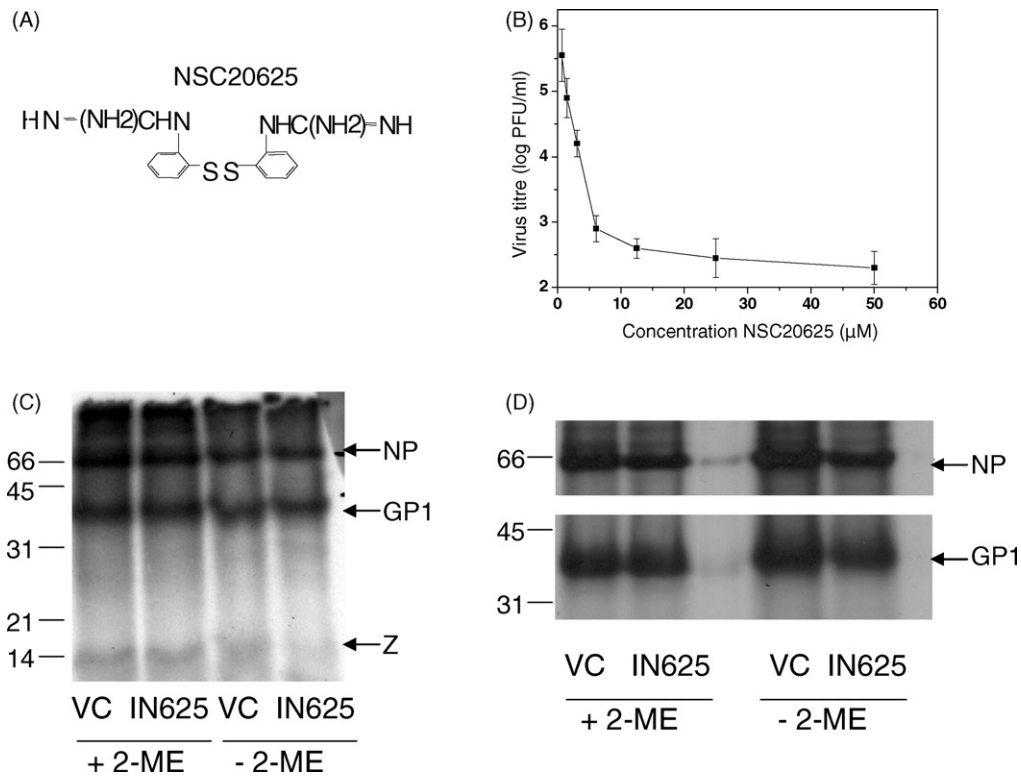


Fig. 1. Effect of NSC20625 on infectivity and proteins of purified JUNV virions. (A) Chemical structure of NSC20625. (B) Purified ^{35}S -labeled JUNV suspensions were incubated with MM containing or not NSC20625 for 1.5 h at 37 °C, then samples were diluted and infectivity was determined by PFU in Vero cells. (C, D) Purified ^{35}S -labeled JUNV suspensions were incubated with (IN625) or without (VC) 40 μM NSC20625 as in B. Then, half of each virion sample was lysed in sample buffer and viral polypeptides were analyzed under reducing (+2-ME) or non-reducing (-2-ME) conditions, in a 18% SDS-PAGE, followed by fluorography (C). The remaining fractions of virion samples were immunoprecipitated with polyclonal rabbit anti-JUNV serum and virion polypeptides were resolved on 12% SDS-PAGE and visualized by fluorography (D). The molecular mass markers are indicated in the right; arrows indicate the positions of the virion proteins.

nucleocapsid (Compans, 1993; Neuman et al., 2005). This morphological alteration observed in compound inactivated JUNV virions is also suggestive of Z protein modification after treatment with NSC20625, confirming conclusions obtained from SDS-PAGE of purified virions.

3.2. Effect of NSC20625 on expression and localization of JUNV Z protein

To further assess the interaction between JUNV Z protein and the inactivating agent, we next examined the reactivity of NSC20625 with Z inside the cell using a recombinant fusion protein

JUNVZ-eGFP. Vero cells were transfected either with the control plasmid pcDNA3.1-eGFP or the recombinant plasmid pcDNA3.1-JUNVZ-eGFP, expressing eGFP and JUNVZ-eGFP, respectively, in the presence or absence of 40 μM NSC20625. After 48 h of transfection, the expression and localization of eGFP and JUNVZ-eGFP was followed by confocal microscopy. The expression of control eGFP was not affected by the compound, whereas the pattern of JUNVZ-eGFP localization in treated transfected cells was completely altered, changing from a disperse punctuate distribution along the cytoplasm to aggregate formation close to the endoplasmic reticulum (Fig. 3). The reactivity between JUNV Z protein and the compound seemed to destabilize the viral protein leading to evident changes

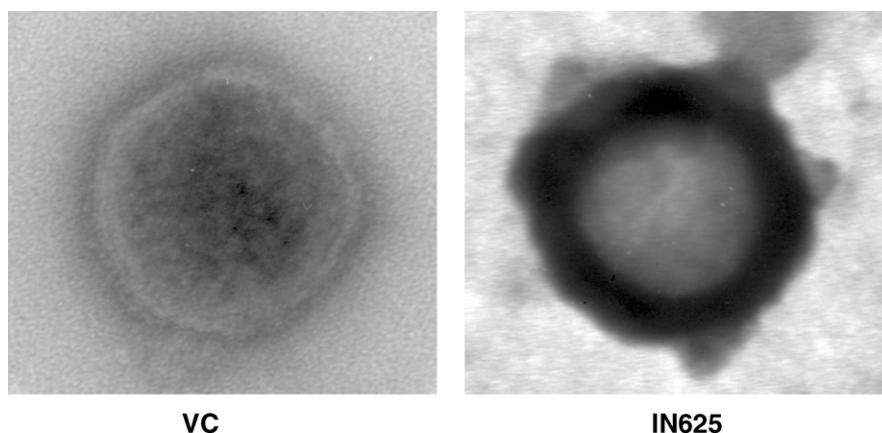


Fig. 2. Structure of native infectious (VC) and NSC20625 inactivated (IN625) JUNV particles. Suspensions of control and inactivated JUNV were fixed overnight in 2.5% glutaraldehyde, deposited on Formvar-carbon-coated nickel grids, and negatively stained for 1 min in 0.5% uranyl acetate. Dried specimens were examined with a Philips EM 301 transmission electron microscope. Magnification = 60,000 \times .

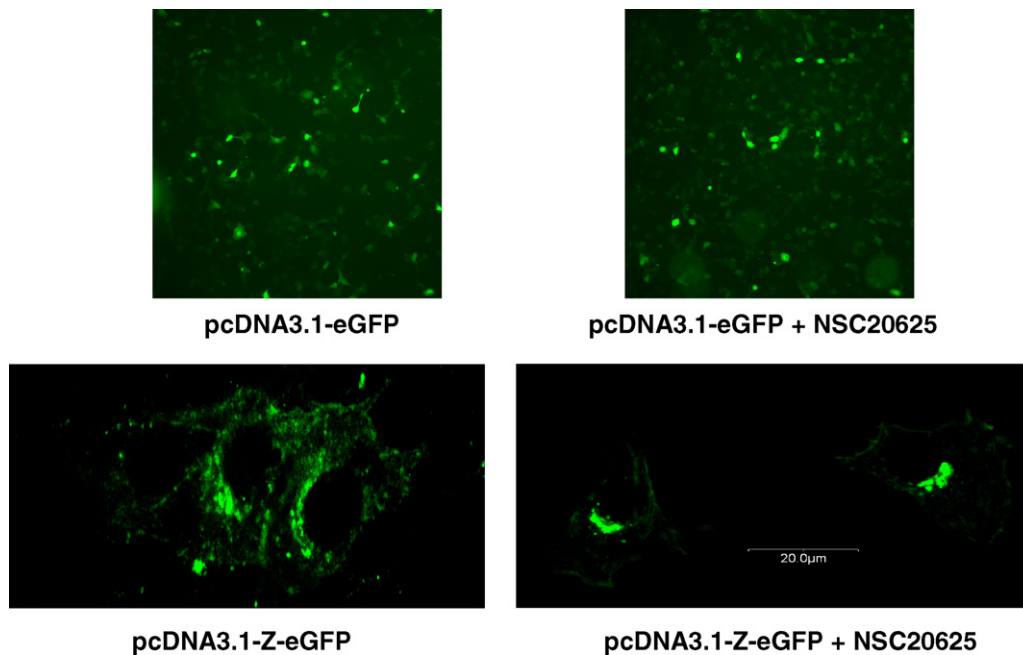


Fig. 3. Expression and localization of JUNV Z protein in Vero cells. Vero cells were transfected with 1 μ g of pcDNA3.1-JUNVZ-eGFP or pcDNA3.1-eGFP using Lipofectamine 2000. After 6 h transfection, cells were incubated with MM containing or not 40 μ M NSC20625. After 48 h, cells were fixed and observed in a confocal microscope. Magnification: upper panel = 100 \times , lower panel = 600 \times .

in the interaction of Z with the cellular components and its location inside the cell.

3.3. Blockade of NSC20625-inactivated virion uncoating

We next analyzed the infection of Vero cells with NSC20625-inactivated JUNV to determine which step is arrested in the inactivated virus life cycle. The incubation of cells with 35 S-labeled virions during 60 min at 4 $^{\circ}$ C followed by 120 min at 37 $^{\circ}$ C showed that inactivated JUNV particles were taken up by Vero cells with the same efficacy as control infectious virions (Fig. 4A). Since the cellular uptake of virions is dependent on functional envelope glycoproteins, it was a further indication of apparently preserved glycoprotein functionality on treated virus.

Next step in arenavirus multiplication cycle is virion uncoating by low pH-induced fusion of the viral envelope with the endosomal membrane leading to nucleocapsid release into the cytoplasm. To evaluate whether cell-associated NSC20625-inactivated JUNV was capable of undergoing virus uncoating in the endosomes, a nucleocapsid penetration assay was next performed. Vero cells were infected with 35 S-labeled virions and virion uptake was allowed as in the previously described experiment. Then, cell homogenization was performed in conditions to preserve endosome integrity, and then treated with proteinase K. The validation of the procedure was assessed by the presence in homogenates of EEA1, a protein marker of cell endosomes, detected by Western blot (Fig. 4B). The amount of viral NP resistant to proteinase K digestion was indicative of the presence of viral nucleocapsids retained in the endosomes and protected by the endosomal membrane. As shown in Fig. 4B, after infection with NSC20625-inactivated JUNV, NP was retained in the endosomes indicating that nucleocapsids were not able to enter into the cytoplasm. By contrast, the amount of labeled NP detected in virus control infected cells was very weak, as expected for a normal process of virion uncoating. As a positive control, concanamycin A, a drug known to inhibit vacuolar H $^{+}$ ATPase and raise the endosomal pH leading to the prevention of the fusion process in the endosome and the virion uncoating (Castilla et al., 2001; Guinea and Carrasco, 1994) was assayed in parallel. The endoso-

mal accumulation of NP in cells infected with JUNV in the presence of concanamycin A resembles that observed in cells infected with NSC20625-inactivated JUNV (Fig. 4B). Moreover, JUNV inactivated with the azoic derivative azodicarbonamide (ADA), another zinc finger-reactive drug with anti-arenavirus virucidal activity (García et al., 2006; Rice et al., 1997) was also tested in this nucleocapsid penetration assay, providing further evidence of uncoating blockade (Fig. 4B).

We next analyzed the subsequent steps of JUNV intracellular macromolecular biosynthesis to verify that uncoating of inactivated virions was prevented. Both processes of RNA transcription and replication of the S fragment were analyzed using appropriate primers. At 48 h p.i., total RNA was extracted from Vero cells infected with either NSC20625 treated or untreated JUNV and used to synthesize a cDNA corresponding to a fragment of the 5' end (NP gene) of the antigenomic S RNA and equivalent to the mRNA for NP. After amplification of this cDNA corresponding to the NP mRNA, a wide band of 186 bp was visualized in cells infected with infectious JUNV (Fig. 4C, lane VC). By contrast, when the material obtained from cells infected with inactivated JUNV was analyzed, the 186 bp fragment of the NP mRNA was not detected (Fig. 4C, lane IN625). This result was indicative of a failure in the viral transcription process in NSC20625-inactivated JUNV. As the specific primer used is complementary to both sequences, the NP mRNA sequence and the 5' end antigenomic sequence, it may be inferred that not only viral RNA transcription but also viral RNA replication is blocked. To verify the lack of viral RNA replication in JUNV inactivated virions, the RNA extracted from JUNV infected cells was used as template in a nested RT-PCR, to synthesize and amplify the cDNA of a fragment from the 3' end (GPC gene) of the antigenomic S RNA. The detection of the amplification products from this reaction is indicative of the performance of the RNA replicative process. Fig. 4C shows that the 129 bp fragment corresponding to the 3' end of the antigenomic S RNA (GPC gene) is not detected in Vero cells infected with NSC20625-inactivated JUNV. By real time PCR it was also demonstrated that JUNV inactivated virions were unable to perform the processes of RNA transcription and replication (data not shown).

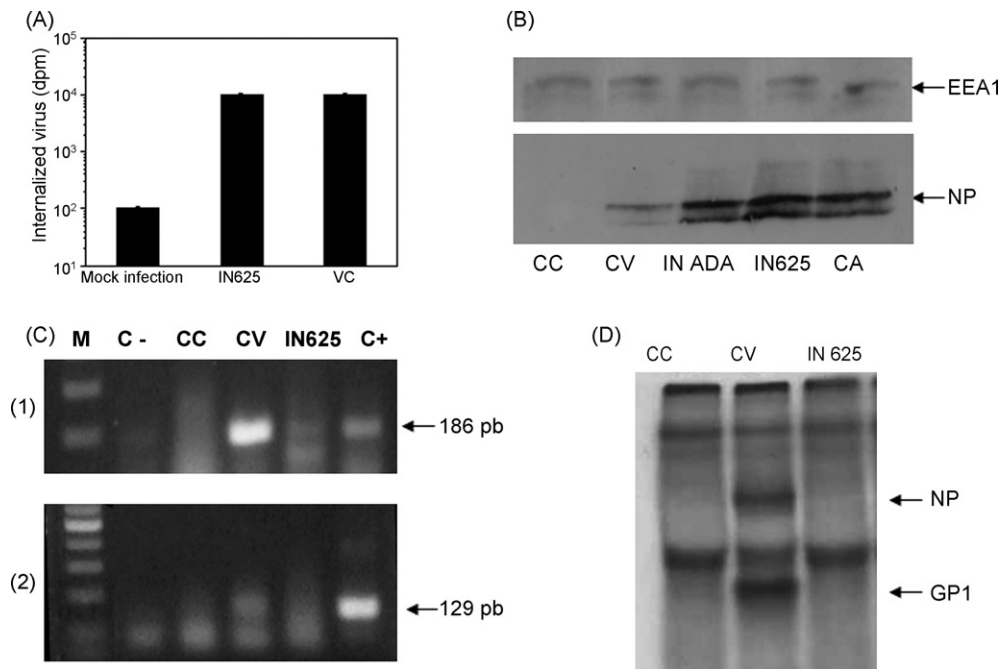


Fig. 4. Blockade in the virus multiplication cycle in Vero cells infected with NSC20625-inactivated JUNV. (A) Virion cellular uptake. Vero cells were incubated with infectious (VC) and NSC20625-inactivated (IN625) radiolabeled JUNV during 1 h at 4 °C, and then 2 h at 37 °C. Cellular internalized radioactivity was quantified. (B) Nucleocapsid penetration. Infectious (VC), NSC20625 inactivated (IN625), and ADA-inactivated (INADA) radiolabeled particles were bound to Vero cells at 4 °C and internalized at 37 °C for 2 h. Then, cells were lysed in homogenization buffer; the lysates were treated with proteinase K, and subjected to SDS-PAGE and fluorography to detect viral proteins. An aliquot of each sample was electrophoresed and immunoblotted with rabbit polyclonal anti-EEA1 (an endosomal cellular marker) serum. Concanamycin A treated infected cells (CA) processed under the same conditions were used as positive controls. CC: mock infected cells. (C) Viral RNA transcription and replication. Vero cells were infected with infectious or NSC20625-inactivated JUNV, at 48 h p.i total RNA was extracted and cDNA synthesis was performed with NP mRNA (1) or GPC antigenomic (2) specific primers and analyzed by PCR. Lanes: M, Molecular markers; C-, negative control; CC, cell control; VC, virus control; IN625, NSC20625-inactivated virus. (D) Viral protein synthesis. Vero cells were infected with infectious or NSC20625-inactivated JUNV, at 48 h p.i. cells were labeled with EXPRE³⁵S-³⁵S for 3.5 h, and then viral proteins were immunoprecipitated with rabbit anti-JUNV serum, and analyzed by 12% SDS-PAGE and fluorography. Lanes: CC, mock infected control cells; VC, virus control; IN625, NSC20625-inactivated virus. The molecular mass markers are indicated in the right; arrows indicate the positions of the main viral polypeptides.

The failure to transcribe and replicate the viral RNA in inactivated virions was indirectly corroborated by analysis of the synthesis of viral proteins in Vero cells. At 48 h after infection with NSC20625-inactivated and control infectious virions, no viral proteins were observed in cells infected with inactivated JUNV suspensions (Fig. 4D), whereas the main viral proteins (NP, the precursor GPC, and the surface glycoprotein GP1) were detected in samples from cells infected with the corresponding infectious viruses. Thus, the lack of viral RNA and protein synthesis in cells inoculated with inactivated JUNV was demonstrated supporting the blockade in nucleocapsid uncoating produced by the virion treatment with NSC20625.

4. Discussion

In the present report we analyzed the inactivation of JUNV by the zinc finger-reactive disulfide NSC20625. The treatment of purified JUNV particles with this compound completely eliminated their ability to form plaques in Vero cells. The loss of virion infectivity was joined to alterations in Z protein, the arenavirus counterpart to matrix proteins found in other negative-stranded RNA viruses. The effects of NSC20625 treatment on Z protein were detected by different experimental strategies. First, the SDS-PAGE profile of purified virion proteins showed that Z protein was modified in NSC20625-treated JUNV so that it did not migrate to the expected position on gel electrophoresis; however, the chemical reduction of virion suspensions with 2-ME in sample buffer resulted in the recovery of the Z band at the corresponding molecular weight of 11 kDa, suggesting that the compound may effectively remove the zinc ions from the RING finger leading the cysteines to adopt chemical linkages which

are sensitive to a reducing environment and apparently seem to destabilize the viral protein.

A second experimental approach to determine the effect of NSC20625 treatment on JUNV morphology consisted of electron microscopy studies of purified particles. Ultrastructural analyses of negatively stained preparations showed that treated virions maintained the morphology of the external envelope surface similar to untreated JUNV, but a more electrodense labeling was visualized in the internal layer beneath the envelope, around the location of the matrix Z protein. This conclusion is in accordance with recent data regarding the ultrastructure of arenavirus virions as determined by cryoelectron microscopy where Z was assigned to a region just below that of the lipid envelope of the virus particles (Neuman et al., 2005), consistently with the location of other RNA virus matrix proteins. Furthermore, the morphological alteration of treated JUNV particles here shown confirms the diffusion of NSC20625 through the virus lipid envelope proposed in our previous studies (García et al., 2006) and it is in agreement with the modification of the internal core localization caused by this kind of compounds in HIV particles (Berthoux et al., 1999).

As a complementary approach to assess the interaction of the compound with the Z protein, we examined the reactivity between a recombinant fusion protein JUNVZ-eGFP and the inactivating agent in transfected NSC20625-treated Vero cells. In accordance with reports from others (Eichler et al., 2004; Strecker et al., 2006), untreated Z was observed in a disperse punctuate pattern throughout the cytoplasm, extending from the plasma membrane to the perinuclear region whereas in treated cells the localization of Z changed to an aggregated pattern close to the endoplasmic reticulum.

Both electron microscopic and electrophoretic determinations evidenced modifications in the properties of Z protein after virus inactivation. By contrast, the main JUNV proteins NP and GP1 were detected as two prominent invariable bands both in infectious and inactivated particles under reducing as well as non-reducing conditions. These data were suggestive that inactivated virus may preserve the antigenic properties of the surface protein GP1, responsible to induce neutralizing and protective antibodies (López et al., 2000). To further examine this question, the binding of infectious and inactivated JUNV to hyperimmune anti-JUNV serum was examined by immunoprecipitation. Precipitated proteins resolved by SDS-PAGE showed that NSC20625-treated virus was recognized by antibodies against the unmodified JUNV with the same avidity. However, further research is needed to assure that treated virus antigenicity and immunogenicity are not altered, including studies about its reactivity against a panel of monoclonal antibodies and the ability to elicit antibody response after immunization of a JUNV animal model.

We finally followed the infection of Vero cells with inactivated JUNV to determine at which step in the virus life cycle the multiplication of inactivated particles was impaired. Only the initial uptake of NSC20625-treated virions by the cell appeared to occur, since then the virions appeared to be retained in endosome vesicles, as determined by the nucleocapsid penetration assay, and were not able to enter into the cytoplasm, with the consequent blockade in JUNV RNA transcription, RNA replication and protein synthesis.

The evidence gathered in our studies suggest that the inactivation of JUNV by NSC20625 treatment seems to involve irreversible modifications in the Z matrix protein, probably by Zn ejection from the RING finger motif, and this alteration would trigger the inability to complete virion uncoating and release the nucleocapsid into the cytoplasm. These results indicate for the first time that the Z protein appears to be critical for this early step of the arenavirus replication cycle. Similarly, the influenza virus matrix protein M1 is essential for virus uncoating, and the zinc bound to a highly conserved zinc finger motif in M1 was described as a determinant factor for a conformational transition of the protein in an acidic environment leading to uncoating (Okada et al., 2003). Probably, the function of Z as a matrix protein, connecting the lipid envelope with the internal nucleocapsid is affected by the compound, after penetration of the lipid envelope by simple diffusion. Recently it was shown that Z protein is the driving force for arenavirus budding (Perez et al., 2003; Strecker et al., 2003), a process requiring the interaction of Z with components of the cell vacuolar protein-sorting pathway (Perez et al., 2003; Urata et al., 2006). Similarly, the Z protein may be involved in completing virion uncoating in the endosome through an interaction with still not elucidated cellular proteins or other viral components. Moreover, a novel zinc-binding activity in the cytoplasmic domain of GP2, the arenavirus transmembrane glycoprotein, was recently identified (York and Nunberg, 2007). As GP2 is a viral protein not usually detected in purified virion preparations or by immunoprecipitation, at present we can not discard the possibility of an interaction of the compound NSC20625 with this unusual motif on GP2, resembling a zinc finger-like structure.

Finally, our results here also suggest that NSC20625-induced inactivation of JUNV, and potentially the inactivation of other human pathogenic arenaviruses, represents a promising attenuation approach for vaccine applications. The development of new strategies to achieve viral inactivation is still a challenge in basic and applied virus research. Despite the advances in the field of immunology, viral inactivation remains an important procedure since it is an easy and relatively non-expensive approach for producing new effective and safe vaccines. The most common approach for virus inactivation is the crosslinking of surface glycoproteins by formaldehyde; this method results in efficient inactivation, but it can severely distort the structure of immunogenic epitopes

(Bachmann et al., 1994). Thus, there is a need for inactivation approaches that retain the structural integrity of the virus. Here, it was shown that JUNV can be safely chemically inactivated by the zinc finger-reactive compound NSC20625 and the non-infectious particles retained the external ultra structure morphology and the immunoreactivity of surface glycoprotein. Furthermore, the immunogenic properties of NSC20625-inactivated JUNV in mice have been recently evaluated by our group with good perspectives (data not shown), suggesting that this aromatic disulfide may be useful as a prototype compound to be optimized in the pursuit of an inactivating agent to obtain a potential hemorrhagic fever vaccine antigen or diagnostic tool.

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