Arenavirus Z protein as an antiviral target: virus inactivation and protein oligomerization by zinc finger-reactive compounds

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Several disulfide-based and azoic compounds have shown antiviral and virucidal properties against arenaviruses in virus yield-inhibition and inactivation assays, respectively. The most effective virucidal agent, the aromatic disulfide NSC20625, was able to inactivate two strains of the prototype arenavirus species *Lymphocytic choriomeningitis virus* (LCMV). Inactivated viral particles retained the biological functions of the virion envelope glycoproteins in virus binding and uptake, but were unable to perform viral RNA replication. Furthermore, in inactivated virions, the electrophoretic profile of the Z protein was altered when analysed under non-reducing conditions, whereas the patterns of the proteins NP and GP1 remained unaffected. Treatment of a recombinant LCMV Z protein with the virucidal agents induced unfolding and oligomerization of Z to high-molecular-mass aggregates, probably due to metal-ion ejection and the formation of intermolecular disulfide bonds through the cysteine residues of the Z RING finger. NSC20625 also exhibited antiviral properties in LCMV-infected cells without affecting other cellular RING-motif proteins, such as the promyelocytic leukaemia protein PML. Altogether, the investigations described here illustrate the potential of the Z protein as a promising target for therapy and the prospects of the Z-reactive compounds to prevent arenavirus dissemination.

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INTRODUCTION

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The family Arenaviridae comprises several virus species included in a single genus, Arenavirus (Salvato et al., 2005). Based on geographical distribution, antigenic cross-reactivity and phylogenetic analyses, the genus is divided into two groups (Wulff et al., 1978; Howard, 1993; Clegg, 2002; Charrel et al., 2003): (i) the Old World group, including four African arenaviruses and *Lymphocytic choriomeningitis virus* (LCMV), the prototypic and most widely distributed species, and (ii) the New World group, which comprises 18 viruses distributed in South and North America. Several arenaviruses are human pathogens: LCMV has teratogenic effects and can cause aseptic meningitis, whereas five members of the family, Junín virus (JUNV), Machupo virus, Guanarito virus, Sabiá virus and Lassa virus (LASV), can cause severe haemorrhagic fever (HF) (McCormick & Fisher-Hoch, 2002; Peters, 2002). The danger of arenaviruses for human health, their increased emergence and the absence of either effective chemotherapy or approved vaccines support their consideration as potential agents of bioterrorism (Damonte & Coto, 2002; Rotz et al., 2002).

The virions contain two single-stranded RNA molecules known as L (large) and S (small), arranged as helical nucleocapsids and enclosed in a lipid envelope. Each genome segment presents an ambisense coding strategy, with two genes in opposite orientations and separated by an intergenic non-coding region (Auperin *et al.*, 1984). The L fragment encodes the RNA-dependent RNA polymerase L at its 3' end from an antigenome-sense mRNA and a zincbinding protein named Z at the 5' end from a genome-sense mRNA (Salvato & Shimomaye, 1989). Similarly, the S fragment encodes the nucleocapsid protein (NP) at its 3' end and the glycoprotein precursor preGPC at the 5' end. preGPC is cleaved co-translationally into a stable signal peptide and GPC, and GPC is then processed posttranslationally into GP1 and GP2 (Eichler *et al.*, 2003a, b).

The Z protein was the last gene product to be discovered and, although its precise role is poorly understood, its importance in the virus life cycle is indisputable. An early report suggested that Z was required for mRNA synthesis and genome replication of the arenavirus *Tacaribe virus* (TCRV) (Garcin *et al.*, 1993), but more recent studies have

shown that Z exerts an inhibitory effect on viral RNA synthesis (Cornu & de la Torre, 2001, 2002; López *et al.*, 2001). In addition to this regulatory role, Z was also implicated as a virion component with matrix functions, similar to other enveloped, negative-stranded viruses (Salvato *et al.*, 1992; Salvato, 1993; Perez *et al.*, 2003; Strecker *et al.*, 2003; Neuman *et al.*, 2005). Furthermore, Z has also been shown to interact with cellular proteins such as the ribosomal protein P0, the eukaryotic translation initiation factor eIF4E, the promyelocytic leukaemia protein PML (Borden *et al.*, 1998a, b; Campbell Dwyer *et al.*, 2000) and the proline-rich homeodomain protein (Djavani *et al.*, 2005), providing evidence for numerous virus–host interactions.

Z contains a conserved RING-finger domain composed of 60 aa that is flanked by a N-terminal hydrophobic domain with a myristoylation site and a phosphorylation site (Franze-Fernández *et al.*, 1993; Perez *et al.*, 2004). The C-terminal portion of the Z protein contains proline-rich motifs that were identified as late motifs in matrix proteins (Freed, 2002; Eichler *et al.*, 2004). It was found that the integrity of the late motifs and the RING-finger domain is necessary for biological functions (Cornu & de la Torre, 2002; Perez *et al.*, 2003; Strecker *et al.*, 2003), making this protein an attractive target for arenavirus chemotherapy.

In the search for agents reactive with the Z protein, a series of compounds with diverse chemical structures, including disulfides and azoic and hydrazide derivatives, was evaluated and they were found to be very effective inhibitors of JUNV and TCRV (García *et al.*, 2000). These compounds had previously been shown to target the retroviral zinc-finger motifs of the human immunodeficiency virus (HIV) nucleocapsid protein NCp7, causing zinc ejection from the protein and inhibition of HIV multiplication (Rice *et al.*, 1996; Tummino *et al.*, 1997). The studies reported here have extended the spectrum of virus susceptibility to the Old World arenavirus LCMV and have implicated Z as the target for these compounds.

METHODS

Compounds. Six disulfide-based compounds and one azoic compound, shown in Fig. 1, were provided by the National Cancer Institute, Frederick, MD, USA. Stock solutions at a concentration of 100 mM were prepared in DMSO. Aldrithiol-2 (AT-2) and azodicarbonamide (ADA) were purchased from Sigma-Aldrich.

Cells and viruses. Vero E6, HepG2 and BHK-21 cells were grown as monolayers in Eagle's minimum essential medium (MEM; Gibco) containing penicillin G (100 U ml⁻¹), streptomycin (100 U ml⁻¹), 10% heat-inactivated fetal bovine serum and 2 mM L-glutamine. Maintenance medium (MM) consisted of MEM supplemented with 5% fetal bovine serum. The WE and ARM strains of LCMV were used. Virus stocks were prepared in BHK-21 cells.

Cytotoxicity assay. Cytotoxicity was measured by the 3-(4,5dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma-Aldrich) method in Vero E6 cells as described previously (García



Fig. 1. Chemical structures and National Cancer Institute code numbers of disulfide-based and azoic compounds.

et al., 2000) and the cytotoxic concentration 50 (CC₅₀), i.e. compound concentration required to reduce the MTT signal by 50 % compared with controls, was calculated.

Virus yield-inhibition assay. Vero E6 cells were infected at an m.o.i. of 0·1 p.f.u. per cell. After 1 h adsorption, cells were washed and re-fed with MM containing serial twofold dilutions of each compound. After 48 h incubation at 37 °C, extracellular virus yields were determined by a plaque assay. The effective concentration 50 (EC₅₀) was calculated as the concentration required to reduce virus yield by 50 % in the compound-treated cultures compared with untreated ones. Each value is the mean of duplicate determinations.

Virus-inactivation assay. Equal volumes of a virus suspension containing approximately 1×10^6 p.f.u. LCMV and serial twofold dilutions of compounds in MM were mixed and incubated for 1.5 h at 37 °C. A virus control was also performed by incubation of the virus suspension with MM under the same conditions. Then, samples were chilled and diluted further with MM before being placed on Vero E6 cell cultures for plaque assay, to assess the titre reduction due only to cell-free virion inactivation. The inactivating concentration 50 (IC₅₀), i.e. concentration required to inactivate virions by 50 %, was calculated.

Virion binding and internalization assays. The preparation of ³⁵S]methionine-labelled purified virus was described previously (Damonte et al., 1994). Radiolabelled virions $(2 \times 10^7 \text{ p.f.u. ml}^{-1};$ 5×10^8 d.p.m. ml⁻¹) were mixed with MM or MM containing 50 μM NSC20625, AT-2 or ADA and incubated at 37 $^\circ C$ for 1.5 h. Then, the viruses were collected by ultracentrifugation and unbound compound was removed. For the binding assay, Vero E6 cells were incubated with virus samples at 4 °C for 40 min. Then, cells were washed with PBS, lysed with a 0.1 M NaOH solution containing 1% SDS and cell-bound radioactivity was quantified. For the internalization assay, after virus adsorption at 4 °C for 40 min, cells were incubated at 37 °C for 60 min to allow virus penetration. Then, cultures were washed with PBS and treated with 1 mg proteinase K ml⁻¹ in PBS to remove external adsorbed virus. Protease treatment was stopped by adding 1 mM PMSF in PBS containing 3 % BSA. Cells were then pelleted and lysed in NaOH/SDS solution as above, and cell-associated radioactivity was determined.

Effect of inhibitors on viral RNA synthesis. Vero E6 cells were infected with NSC20625-inactivated and control LCMV at an m.o.i. of 1 p.f.u. per cell. 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, LCMV cDNA was generated by using AMV (avian myeloblastosis virus) reverse transcriptase (Promega) and a Z primer, gZ (F89) (5'-AAGCTTATGGGCCAAGGCAAGTC-3'). This cDNA was amplified by direct PCR using the following arenavirus-specific oligonucleotides: gZ (F119), 5'-GACACCAGCAATACAGGCAG-3', and agZ, 5'-CTCGAGTTACTCCTCATAGGGAGGTAGG-3', generating a 284 bp amplification fragment comprising the 5' end of the L RNA. The final products were resolved by 1.5% agarose-gel electrophoresis in TAE buffer (40 mM Tris/acetate, 1 mM EDTA, pH 8.0) with 0.2 μ g ethidium bromide ml⁻¹.

To analyse the antiviral properties of NSC20625 on viral RNA synthesis, HepG2 cells were infected with LCMV at an m.o.i. of 1 and, after adsorption, cells were re-fed with MEM containing 40 μ M NSC20625. At 48 h after infection, total RNA was extracted as described before and amplification of cDNA was performed with random primers to avoid false-negative results and analysed by direct or real-time PCR using the following oligonucleotides: gZ (F119) and agZ; gapdhF, 5'-GTTGCCATCAATGACCCCTTCATTG-3', and gapdhR, 5'-CAGCCTTCTCCATGGTGGTG-3'. The level of Z mRNA evaluated by real-time PCR was done by using SYBR Green technology

and amplification plots were expressed as $C_{\rm T}$ values to be analysed with 5700 SDS software (Perkin-Elmer). $C_{\rm T}$ is the reaction cycle at which PCR products reach a threshold level of detection. $C_{\rm T}$ values were normalized by using glyceraldehyde-3-phosphate dehydrogenase (*gapdh*) as standard. Dissociation analysis of the PCR products was used to confirm specificity.

Effect of inhibitors on viral protein synthesis. Vero E6 cells were infected with NSC20625-inactivated and control LCMV at an m.o.i. of 1. At 48 h after infection, cells were lysed in RIPA buffer [10 mM Tris/HCl (pH 7·4), 0·15 M NaCl, 0·1% SDS, 1% Triton X-100, 1% sodium deoxycholate and 0·4 mM PMSF]. The cell lysates were solubilized by boiling in sample buffer [5% SDS, 2% 2-mercaptoethanol (2-ME), 10% glycerol, 0·005% bromophenol blue, 0·0625 M Tris/HCl (pH 6·8)], electrophoresed by SDS-PAGE (15% gel) and then transferred to PVDF membranes (Millipore). Membranes were incubated with guinea pig polyclonal anti-LCMV serum for 2 h. Blots were washed in Tris-buffered saline (TBS) containing 0·1% Tween 20, incubated with anti-guinea pig IgG conjugated to horseradish peroxidase (Sigma-Aldrich) for 1 h and revealed by enhanced chemiluminescence (Amersham Biosciences).

Effect of inhibitors on virion proteins. Suspensions of purified LCMV were obtained according to a procedure described previously for JUNV (Damonte *et al.*, 1994). Samples of purified virions were incubated at 37 °C for 1·5 h in the presence or absence of 10 mM AT-2. Control and inactivated particles were lysed in sample buffer either with or without 2-ME and fractionated by SDS-PAGE (11 or 18 % gel). Then, proteins were immunoblotted with guinea pig anti-LCMV and rabbit anti-Z hyperimmune sera and probed as above.

Effect of inhibitors on purified recombinant Z protein. To obtain a preparation of pure Z protein, 1 l Luria-Bertani medium supplemented with ampicillin (100 µg ml⁻¹) containing Escherichia coli harbouring the pGEX-2T/ARM-Z plasmid (Borden et al., 1998b) was grown to mid-exponential phase at 37 °C. The culture was allowed to grow for an additional 3 h at 37 °C in the presence of 0.4 mM IPTG to induce fusion-protein expression. Bacteria were harvested and lysed by freezing-thawing and sonicating. Then, 0.5 % Tween 20 was added to solubilize the fusion protein and the cleared lysate was adjusted to pH 7.4 and filtered through a 0.45 µm membrane. The filtrate was applied to a glutathione-bead column (Glutathione Sepharose 4B; Amersham Biosciences) and purified as recommended by the manufacturer. Finally, the glutathione S-transferase-ARM-Z fusion protein was treated with 500 µl thrombin (1 U ml⁻¹) in order to obtain purified ARM-Z protein. By this procedure, approximately 1 ml ARM-Z protein solution was obtained at a concentration of 220 µg ml⁻¹. The same procedure was carried out for LASV-Z protein purification. Samples of purified protein (1 µg) were incubated for 1.5 h at 37 °C with each compound. Then, mixtures were heated in sample buffer either with or without 2-ME and fractionated by SDS-PAGE (18% gel) and Z protein was detected by immunoblotting as described above.

Optically monitored metal-ion ejection. The full-length apo-ARM-Z protein was purified as described previously for RING motif-containing proteins (Roehm & Berg, 1997) and refolded at a concentration of 300 μ M in the presence of five equivalents (1·5 mM) of CoCl₂. The cobalt(II)-refolded ARM-Z protein was incubated with increasing concentrations of compound or with the appropriate amount of the carrier (DMSO) for 30 min at room temperature in the analysis buffer [10 mM NaH₂PO₄ (pH 7·5), 300 mM NaCl, 10 μ M Tris/carboxyethyl phosphine (TCEP), 10 μ M CoCl₂, 0·01 % DMSO]. The concentration of cobalt-refolded ARM-Z protein was kept constant at 50 μ M. During incubation, continuous UV/visible absorption spectra were recorded from 200 to 950 nm at room temperature by using a 1 cm path-length cuvette (Helma) on an Agilent 845 spectrophotometer (Agilent Technologies). Spectra in

the region of 600–900 nm were used to monitor cobalt(II) ejection, as this region is free of absorption from unbound cobalt(II). Furthermore, the absorption maxima in this spectral range correspond to the d-d transitions of tetrahedrally coordinated cobalt ions in the RING motif of ARM-Z. The experiments were repeated independently three times and the resulting absorption spectra were subtracted against appropriate controls, corrected for the dilution effect and averaged.

Circular dichroism (CD) spectroscopy. CD on Z protein treated with virucidal agents was performed as described previously (Kentsis et al., 2002). Purified ARM-Z protein (50 µM) was incubated for 30 min at room temperature in a folding buffer [10 mM NaH_2PO_4 (pH 7.5), 300 mM NaCl, 1 µM ZnCl₂, 10 µM TCEP (Pierce), 0.1 % (v/v) DMSO] that contained either no additions, 100 µM EDTA or between 10 nM and 100 µM of NSC10625 or ADA. The treatments were performed three times independently. Far-UV CD continuous spectra (197-257 nm) were collected by using a Jasco-810 spectropolarimeter with a 1 mm cuvette (Hellma) at room temperature. All of the measurements were repeated three times with a 1 nm bandwidth. CD spectra of the treated and untreated ARM-Z protein were normalized by subtraction against the spectra obtained for the corresponding concentration of the compound, EDTA or folding buffer, respectively, and averaged. Relative ellipticity was converted to mean residue molar ellipticity according to Fasman (1996).

Immunofluorescence and confocal microscopy. Microscopy was performed as described previously (Topisirovic *et al.*, 2002). Briefly, HepG2 cells grown on coverslips were incubated in MM containing 40 μ M NSC20625 or not. At 72 h, cells were fixed with methanol and probed for 2 h with mouse monoclonal anti-PML (mAb 5E10; 1:20), followed by incubation with a Cy5-conjugated donkey anti-mouse (Jackson Laboratory) for 1 h. Thereafter, cells were dried and mounted in Vectashield mounting medium with DAPI (4',6'-diamidino-2-phenylindole; Vector Laboratories) to visualize nuclei. Fluorescence was observed at a magnification of \times 400 with a zoom of 2 under a Leica TCS-SP (UV) confocal microscope. All channels were detected separately, with no cross-talk between channels.

RESULTS

Antiviral and virucidal activities of disulfide and azoic compounds against LCMV

The antiviral and virucidal properties of the disulfide-based and azoic compounds were evaluated against two strains of LCMV by determining EC₅₀ and IC₅₀ values in virus yieldinhibition and virus-inactivation assays, respectively. All six tested disulfide compounds were able to inhibit the multiplication of LCMV in Vero E6 cells after 48 h infection. They were also able to destroy viral infectivity after direct treatment of the virion suspension with the compounds (Table 1). The two classes of disulfides, the intermolecular structures with the disulfide bridge linking two aromatic molecules and the dithianes with the sulfur atoms included in the ring structure, both exhibited inhibitory activities with a considerable level of selectivity with respect to their effect on cell viability, expressed as CC₅₀. By contrast, the azoic derivative ADA was found to be effective at inactivating LCMV, but had no ability to affect virus production in Vero E6 cells (Table 1). These results are in agreement with previous reports for JUNV and TCRV (García et al., 2002a, 2003b). A little variation was observed in the susceptibility of the two LCMV strains to these compounds, as reported previously among JUNV strains (García et al., 2000). Ribavirin was tested as a reference substance. As expected,

 Table 1. Cytotoxicity and antiviral and virucidal activities against LCMV

Each value is the mean \pm SD of duplicate determinations.

Compound	$CC_{50}~(\mu M)^{\star}$	EC ₅₀	(μM)†	IC ₅₀ ($IC_{50}~(\mu M)\ddagger$	
		WE§	ARM§	WE	ARM	
Disulfides						
NSC4493	$363 \cdot 2 \pm 19 \cdot 1$	$24 \cdot 6 \pm 2 \cdot 4$	9.85 ± 1.1	45 ± 0.9	41.6 ± 3.5	
NSC20625	$94 \cdot 4 \pm 7 \cdot 4$	11.6 ± 1.5	$8 \cdot 7 \pm 0 \cdot 5$	$4 \cdot 8 \pm 0 \cdot 3$	$3\cdot 5\pm 0\cdot 2$	
NSC203	$143 \cdot 1 \pm 9 \cdot 8$	$13 \cdot 1 \pm 1 \cdot 1$	$33 \cdot 9 \pm 2 \cdot 3$	$32 \cdot 3 \pm 1 \cdot 9$	12.5 ± 0.8	
AT-2	$> 400 \cdot 0$	$25 \cdot 1 \pm 3 \cdot 1$	14.0 ± 2.2	12.6 ± 1.3	$6 \cdot 2 \pm 0 \cdot 4$	
NSC624151	$> 400 \cdot 0$	$7 \cdot 9 \pm 0 \cdot 5$	6.8 ± 0.9	22.5 ± 1.7	$25 \cdot 0 \pm 2 \cdot 1$	
NSC624152	$352 \cdot 8 \pm 28 \cdot 4$	$14 \cdot 0 \pm 1 \cdot 2$	4.5 ± 0.2	$15 \cdot 9 \pm 1 \cdot 2$	$19 \cdot 3 \pm 2 \cdot 0$	
Azoic						
ADA	$> 200 \cdot 0$	$> 100 \cdot 0$	$> 100 \cdot 0$	$27 \cdot 9 \pm 1 \cdot 5$	12.5 ± 0.8	
Reference substance						
Ribavirin	>200.0	18 ± 1.5	21 ± 2.5	>100.0	>100.0	

*Compound concentration required to reduce Vero E6 cell viability by 50%, measured by MTT method. †Compound concentration required to reduce virus yield by 50%, measured by virus yield-inhibition assay at 48 h after infection in Vero E6 cells.

 \pm Compound concentration required to inactivate virus particles by 50 %, assayed by inactivation assay at 37 °C for 1.5 h.

§WE and ARM are LCMV strains.

ribavirin did not inactivate LCMV, whereas the antiviral EC_{50} values were about 20 μ M, in accordance with a previous report (Smee *et al.*, 1992). Thus, in addition to their virucidal activity, the most active antiviral disulfides (NSC20625, NSC624151 and NSC624152) showed higher efficacy *in vitro* than ribavirin.

Mode of inactivation: entry, RNA replication and protein expression

The most effective virucidal agents against both LCMV strains were NSC20625 and AT-2; these two drugs, together with ADA, were chosen for further characterization of the mechanism of arenavirus inactivation. First, a radio-labelled virion-binding assay was performed. The binding of compound-inactivated LCMV to Vero E6 cells was comparable to the binding of infectious virions (Fig. 2a). The ability of treated virions to penetrate Vero E6 cells was evaluated by exposing cells to radiolabelled virions at 4 °C and cultures were then incubated at 37 °C for 1 h to allow uptake. As seen in Fig. 2(b), the amount of internalized



LCMV was similar for compound-treated and control virus preparations.

Next, the ability of inactivated virions to conduct RNA synthesis was analysed to identify the blockade to intracellular multiplication of LCMV. To this end, Vero E6 cells were infected either with an NSC20625-treated or control untreated LCMV preparation. After 48 h infection, RNA was extracted from cells and Z gene cDNA was synthesized and then amplified with Z-specific primers (Fig. 2c). In cells infected with untreated LCMV, a band of 284 bp corresponding to the amplified Z gene fragment was clearly detected (Fig. 2d, lane VC). No amplification product was observed in cells infected with treated virions (Fig. 2d, lane IN), indicating an absence of viral RNA replication after infection with NSC20625-inactivated LCMV.

The failure of compound-inactivated virus to replicate the viral RNA was corroborated indirectly by analysis of the expression of viral proteins. At 48 h after infection, no viral proteins were observed in cells infected with inactivated LCMV (Fig. 2e), whereas the main viral proteins (NP, GPC and GP1) were detected in control LCMV-infected cells.

Altogether, these studies showed that NSC20625 is able to produce inactivated arenavirus particles incapable of undergoing viral RNA replication, while apparently preserving the binding and uptake functions of the virion envelope glycoproteins.

Fig. 2. Mode of inactivation. (a) Binding of radiolabelled LCMV-ARM (shaded bars) and LCMV-WE (filled bars). Cells were incubated for 40 min at 4 °C with ³⁵S-labelled virus previously treated with compound or MM, then cell-bound radioactivity was determined. CC, Cell control; VC, virion control; NSC20625, AT-2 and ADA, virion samples inactivated with 50 μ M of each compound. Each value is the mean ± SD of duplicate, independent experiments. (b) Internalization assay: cells were incubated with labelled infectious or inactivated virus at 4 °C for 40 min, then for 60 min at 37 °C, and internalized virus was determined. Symbols as in (a). (c) Gene arrangement in L RNA and primers used for the amplifications. The RNA fragment is shown divided into two regions with opposite polarity. 'g' and 'ag' are genome and antigenome strands, respectively. Filled arrow indicates position and direction of the arenavirus-specific primer used for cDNA synthesis. Open arrows indicate position and direction of the arenavirus-specific primers used for PCRs. (d) Effect on viral RNA synthesis. Cells were infected with NSC20625-inactivated or control LCMV. At 48 h post-infection (p.i.), total RNA was extracted and cDNA was synthesized with a Z-specific primer and analysed by PCR. Lanes: M, molecular markers; C-, negative control; CC, cell control; VC, virus control; IN, NSC20625-inactivated virus. (e) Effect on viral protein expression. Cells were infected with NSC20625-inactivated or control LCMV. At 48 h p.i., viral proteins were analysed by electrophoresis in a 12% SDS-PAGE gel and Western blotting with anti-LCMV serum. Molecular mass markers are indicated on the right; arrows indicate the positions of the main viral polypeptides. Lanes as in (d).



Fig. 3. Effect on virion proteins. Purified LCMV suspensions were incubated with or without 10 mM AT-2 for 1.5 h at 37 °C. Then, virions were lysed and viral polypeptides were analysed under reducing (+ 2-ME) or non-reducing (- 2-ME) conditions by electrophoresis in an 18% SDS-PAGE gel and immunoblotting with anti-Z serum (a) or by electrophoresis in an 11% SDS-PAGE gel and immunoblotting with anti-LCMV serum (b). Molecular mass markers are indicated on the right; arrows indicate the virion proteins.

Target of inactivation: effect on virion proteins

The first approach to show that Z is the main target for these compounds was the analysis of their effects on virion proteins. Purified LCMV suspensions were inactivated with AT-2 and the virion proteins were studied by SDS-PAGE and Western blotting. When analysed under non-reducing conditions, the inactivated virion Z protein was not detected as the 11 kDa band observed in untreated purified virions. On the other hand, under reducing conditions, the treated and untreated Z profiles were similar (Fig. 3a). By contrast, treatment with this compound did not affect the electrophoretic patterns of either the NP or GP1 proteins under reducing or non-reducing conditions (Fig. 3b).

In an attempt to demonstrate the interaction between Z and the inactivating agents more thoroughly and to overcome the difficulties of visualizing Z in purified viral preparations, we examined the effects of the compounds on a recombinant Z protein obtained from the ARM strain of LCMV (ARM-Z), as described in Methods. Purified samples of ARM-Z were incubated with 10 mM solutions of NSC20625, AT-2 or ADA for 1.5 h at 37 °C, as in the virion-inactivation assay, and the samples were then analysed by SDS-PAGE and Western blotting. The compounds did not alter the profile of the Z protein when it was analysed in the presence of the reducing agent 2-ME, with a prominent band of the 11 kDa monomer protein and various amounts of larger forms at the top of the gel (Fig. 4a). By contrast, under non-reducing conditions, the three compounds eliminated the band corresponding to the Z monomer protein and promoted accumulation of a prominent, heterogeneous band of high-molecular-mass species reactive with the Z-specific antiserum (Fig. 4a). These results strongly suggest that Z multimers are produced, due to intermolecular disulfide bonds formed by cross-linkage through the cysteine residues of the RING fingers. The cross-linking reaction was concentration-dependent, as revealed by the increasing intensity of the multimer band after treatment of Z protein with different concentrations of ADA compound (Fig. 4b). The interaction between these compounds and Z, leading to protein cross-linking and multimer formation, was also demonstrated for another purified recombinant arenavirus Z protein, LASV-Z, which multimerized after treatment with AT-2 or ADA under the same conditions (Fig. 4c).

The consistent observation of the alterations in Z profile in non-reducing conditions, from a monomer to a heterogeneous, high-molecular-mass, smeared band, in both arenaviruses (LCMV and LASV) allows us to conclude that Z forms multimers after treatment with the compounds.

Compounds induce metal-ion ejection from Z RING motif and consequent unfolding of the Z protein

Unlike the optically inactive metal Zn(II), Co(II) has an incomplete outer shell, allowing d-d transitions that absorb in the UV/visible region of the spectrum. These optical spectral properties of cobalt have been deployed extensively in investigating dynamics of metal-ion binding of proteins containing RING-finger motifs (Roehm & Berg, 1997). The same strategy is often the method of choice for investigating the ability of certain Zn-ejecting agents to cause loss of metal coordination by the HIV zinc-finger protein NCp7 (Chen et al., 2000; Miller Jenkins et al., 2005). We used the same approach to investigate the ability of ADA and NSC20625 to induce metal-ion ejection from the RING-finger motif of the ARM-Z protein. After verifying the native folding of Co(II)refolded protein by utilizing far-UV CD spectroscopy (Fig. 5a), we have monitored the 600-900 nm absorption spectra of Co(II)-refolded ARM-Z. These spectra showed absorption maxima at wavelengths between 640 and 700 nm (Fig. 5b), which correspond to the d-d transitions of Co(II) in the four-coordinate tetrahedral geometry of a RING motif (Roehm & Berg, 1997). As expected, optically inactive, Zn(II)-refolded ARM-Z did not absorb light in this wavelength range significantly (Fig. 5b). Thereafter, Co(II)refolded ARM-Z (50 µM) was incubated with increasing concentrations of ADA and NSC20625. The resultant spectra showed marked reduction in absorbance in the 640-700 nm range, indicating that the compounds induced the ejection of Co(II) ions from the RING motif of ARM-Z (Fig. 5c, d). The reduction of the absorption was apparent when compound and protein concentrations were equimolar. Complete loss of the Co(II) coordination by ARM-Z was observed when the compounds were used in twofold excess.

Finally, far-UV CD spectroscopy was used to investigate whether the ADA- and NSC20625-induced loss of metal coordination affects the folding of the ARM-Z protein. A prominent loss in the far-UV CD spectra of ARM-Z was evident when the compounds were applied in a 1:1 ratio



Fig. 4. Effect of inhibitors on the Z protein. (a) ARM-Z was incubated with or without 10 mM NSC20625, AT-2 or ADA, and then analysed under reducing (+2-ME) or nonreducing (-2-ME) conditions by electrophoresis in an 18% SDS-PAGE gel followed by immunoblotting with anti-LCMV-Z serum. {*BLOB:S*}(b) ARM-Z was incubated with different concentrations of each compound and then analysed under non-reducing conditions as in (a). {*BLOB:S*}(c) LASV-Z was incubated with or without 10 mM AT-2 or ADA and then analysed under reducing (+2-ME) or non-reducing (-2-ME) conditions by electrophoresis in an 18% SDS-PAGE gel followed by immunoblotting with LASV-Z antiserum. Molecular mass markers are indicated on the right; U, untreated Z protein.

and diminished when the compounds were used in twofold excess (Fig. 5e, f). In the latter case, CD spectra are indistinguishable from those obtained for ARM-Z that was treated with twofold excess of EDTA. It was previously reported that, when in excess, EDTA completely disrupts the native structure of ARM-Z by chelating Zn(II) and disrupting the folding of its RING motif (Kentsis *et al.*, 2002). Taken together, the data obtained in CD and UV/visible spectroscopy experiments strongly suggest that ADA and NSC20625 induce Zn(II) ejection from the RING motif of the Z protein, which is followed by the loss of its native structure and the consequent aggregation of the unfolded protein.

Antiviral properties of NSC20625

The experiments reported above all deal with the effect of the compounds on free, infectious virions. In comparison with inactivating properties, we also studied the antiviral action of NSC20625 in LCMV-infected cells. As occurred with NSC20625-inactivated virions, radiolabelled particles of LCMV bound and entered compound-treated and untreated infected cells to the same extent (data not shown). Next, the effect of the compound on viral RNA synthesis was analysed by infecting cells with LCMV in medium containing NSC20625 or not. At 48 h postinfection, total RNA was extracted and Z cDNA was synthesized and amplified. As shown in Fig. 6(a), the 284 bp band corresponding to the amplified Z gene fragment was very reduced when RNA was extracted from infected cells treated with NSC20625, in comparison with untreated infected cells (V625 vs VC). Simultaneous amplification of the cellular gene gapdh showed no differences in the intensity of the corresponding bands between treated and untreated cells (Fig. 6b), indicating that NSC20625 did not affect transcription of the cellular gene. To assess the failure



Fig. 5. Loss of metal coordination and subsequent unfolding of ARM-Z by NSC20625 and ADA. (a) Far-UV CD spectra of ARM-Z refolded with CoCl₂ [Z Co(II)] or ZnCl₂ [Z Zn(II)]. (b) Absorption spectra of ARM-Z refolded with CoCl₂ [Z Co(II)] or ZnCl₂ [Z Zn(II)]. Absorption maxima in the 640-700 nm range correspond to the d-d transitions of tetrahedrally coordinated Co(II) ions of ARM-Z. (c, d) Resultant spectra for the optically monitored titration of ARM-Z Co(II) complexes with either NSC20625 (c) or ADA (d). The concentration of Co(II)-refolded ARM-Z was kept constant at 50 µM, whilst the concentration of the compounds was increased in a stepwise manner from 12.5 to 400 μ M. (e, f) Far-UV CD spectra of untreated ARM-Z (black line; e and f), ARM-Z treated with 100 µM EDTA (grey line; e and f) or ARM-Z treated with 10-100 µM NSC20625 (e) or ADA (f). $[\Theta]$, Mean residue molar ellipticity; λ , wavelength.

in viral RNA synthesis in treated infected cells, a real-time PCR was performed with RNA samples extracted from treated and untreated infected cells, using *gapdh* as cellular control. Quantification of RNA synthesis under these conditions showed more than 2-log inhibition of viral RNA synthesis in LCMV-infected cells after treatment with NSC20625 (Fig. 6c).

Based on data obtained with the arenaviruses TCRV (López *et al.*, 2001), LASV (Hass *et al.*, 2004) and LCMV (Cornu & de la Torre, 2001) showing that Z is not required for RNA synthesis, these results appear to contradict the proposal that Z is the NSC20625 target. However, as this compound has strong virucidal properties, the reduction in RNA synthesis shown in Fig. 6(a) and confirmed in Fig. 6(c) may be due to the failure of inactivated virions to reinfect cells after the first cycle of replication. Moreover, the inhibition of viral RNA synthesis was confirmed by analysis of the expression of the corresponding viral protein. By immunofluorescence

staining, a full inhibition of Z protein expression was observed in NSC20625-treated cells (data not shown).

When NSC20625 is used as antiviral agent in infected cells, it may affect not only the RING motif of Z protein, but also other cellular RING-motif proteins. To check the specificity of NSC20625 for the RING of Z protein, the PML protein was assayed. PML forms nuclear bodies that are dependent on the integrity of its RING motif: mutations of zincbinding residues in the RING finger disrupt nuclear-body formation and cause a diffuse pattern (Borden et al., 1995). Thus, HepG2 cells were treated with NSC20625 for 72 h and, thereafter, the distribution pattern of PML was evidenced by immunofluorescence staining. Fig. 7(a) shows the typical PML bodies present in the nucleus. In the presence of NSC20625, the pattern of PML expression was like that observed in untreated cells (Fig. 7b), indicating that the Zn-binding residues in the RING motif of this cellular protein are intact and unaffected by the compound.



Fig. 6. Antiviral properties of NSC20625. HepG2 cells were infected with LCMV and, after adsorption, cells were treated or not with 40 μ M NSC20625. At 48 h p.i., total RNA was extracted and cDNA was synthesized with random primers. Amplification of *z* (a) and *gapdh* (b) genes was done by using specific primers and analysed by electrophoresis. Lanes: M, molecular markers; CC, cell control; C625, uninfected cells treated with NSC20625. (c) The same samples were subjected to real-time RT-PCR analysis and the levels of *z* mRNA relative to those of *gapdh* were determined and are represented as fold mRNA change.

DISCUSSION

In this study, the mode of inactivation of arenaviruses by disulfide- and azo-based compounds was examined. The treatment of intact arenavirus particles with the zinc-reactive compounds has been shown to induce three concomitant effects: (i) viral infectivity was destroyed; (ii) virions retained the ability to enter the host cell, but were unable to synthesize Z viral RNA; (iii) the electrophoretic profile of the Z protein was altered when analysed under non-reducing conditions, whereas the pattern of the other major virion proteins NP and GP1 remained unaffected. In

addition, the compounds were able to induce metal-ion ejection from purified Z protein, with the consequent loss of its native structure and stability. The Z protein is mainly constituted by its RING structure and it is known that the integrity of this domain is required to achieve Z biological functions (Cornu & de la Torre, 2002; Perez *et al.*, 2003; Strecker *et al.*, 2003). Because the RING domain requires zinc coordination for folding and stability (Borden *et al.*, 1995; Roehm & Berg, 1997; Brzovic *et al.*, 1998; Capili *et al.*, 2001), the correlation of the effects observed on virions and on the purified protein supports the hypothesis that the Z protein, and in particular its RING-finger domain, is the most vulnerable target for these agents and is responsible for the resulting virion inactivation.

Recent studies have shown that LCMV and LASV Z proteins are strongly membrane-associated and are sufficient, in the absence of other viral proteins, to release virus-like particles (Perez et al., 2003; Strecker et al., 2003), indicating that Z functions as a matrix protein during arenavirus budding, localized beneath the lipid bilayer of the envelope. Thus, the interaction between the zinc-reactive compounds and the viral particles appeared to involve the entry of compounds into the virion by diffusion across the lipid envelope and the chemical modification of the Z protein, with loss of infectivity as the final consequence. The biological consequences of compound addition reflect the roles of the antiviral target during the replication cycle. At present, it is not totally clear why the oxidation of the Z protein's cysteine thiolates into cross-linked disulfide bridges should inactivate an infectious arenavirus particle. Inactivated particles are not blocked at the early steps of virus binding and uptake, confirming the preservation of these biological functions for the envelope glycoproteins. The fact that these particles are not able to synthesize viral RNA is consistent with the role of Z as a matrix protein, because Z oligomerization would presumably impair virus uncoating upon entering the cell. This possibility is supported by the results obtained when the antiviral effect of the compounds on LCMV-infected cells was analysed: a specific inhibition of viral RNA synthesis was detected without reduction in transcription of cellular mRNA.

The presence of classical zinc-finger domains has also been described in NP (Salvato & Shimomaye, 1989; Tortorici



Fig. 7. Effect of NSC20625 on PML expression. HepG2 cells were incubated in MM containing (b) or not (a) 40 μ M NSC20625. After 72 h, cells were fixed and probed with mouse monoclonal anti-PML and then with Cy5-conjugated donkey anti-mouse. Then, cells were mounted in Vectashield mounting medium with DAPI to visualize nuclei. By confocal laser microscopy, blue (DAPI) or red (PML) fluorescence was recorded independently (magnification, \times 400). CC. Control cells; C625, cells treated with NSC20625.

et al., 2001). This protein is responsible for maintaining the structure of the infecting nucleocapsid, which is the template for RNA transcription and replication. Although we cannot discard an interaction between the inhibitors and NP, at present, no alterations in NP pattern were detected after virion inactivation. Also, in comparing NSC20625treated and untreated cells, no alterations were observed in the pattern of PML, a cellular protein containing a RING motif (Fig. 7). Similarly, it has been reported that ADA and NSC624151, both HIV inhibitors reactive to NCp7 zinc fingers, did not impact the functions of cellular zinc-finger proteins, including poly(ADP-ribose) polymerase and transcription factors (Huang et al., 1998). It must be noted that the exact susceptibility of different zinc-finger motifs to compounds is still not fully understood, but selective inhibitors are clearly less damaging to the host cell than to extracellular virus (Table 1). Slight variations in atomicsurface geometrics, hydrophilic/hydrophobic characteristics and other accessibility factors may be affected by the intracellular environment and contribute to the observed differences (Rein et al., 1996; Turpin et al., 1999), supporting the strong virucidal activity of inhibitors without cytotoxicity.

Five members of the family Arenaviridae are able to cause severe HF and only limited strategies are available for patient treatment. The administration of standardized doses of convalescent plasma is the recommended treatment for Argentine HF, caused by JUNV; however, it is not efficient when initiated after 8 days of illness and a late neurological syndrome is observed in 10% of treated patients (Enría & Maiztegui, 1994). With respect to Lassa fever, chemotherapy with ribavirin is the preferred treatment, but with a high level of undesirable secondary reactions (McCormick et al., 1986; McKee et al., 1988). Other nucleoside analogues were assayed in vitro and in vivo, but without successful results (Huggins et al., 1984; Burns et al., 1988; Andrei & de Clercq, 1990; Smee et al., 1993; Guillerm et al., 2003; Uckun et al., 2004). Thus, the development and evaluation of effective agents directed to new targets in the viral cycle constitute a necessary approach for arenavirus chemotherapy.

In recent years, several natural and synthetic products have been reported as active *in vitro* inhibitors against arenavirus infection. The compounds under study include steroids (Wachsman *et al.*, 2000), polysulfates (Andrei & De Clercq, 1990; Witvrouw *et al.*, 1994), thiosemicarbazones (García *et al.*, 2003a), trichothecenes (García *et al.*, 2002b), phenothiazines (Candurra *et al.*, 1996), fatty acids and analogues (Cordo *et al.*, 1999; Bartolotta *et al.*, 2001) and peptides (Castilla *et al.*, 1998; Albiol Matanic & Castilla, 2004). Recent research on antiviral strategies against LCMV also describes the potential application of certain nucleoside analogues for increased mutagenesis (Grande-Pérez *et al.*, 2005), as well as the downregulation of gene expression produced by small interfering RNAs (Sánchez *et al.*, 2005).

The findings reported in this study provide evidence that the Z protein may be a promising target for arenavirus therapy. The spectroscopic studies showed that disulfide and azoic

compounds similarly disrupt the metal-bound structure of Z, thereby providing a mechanism for the freed cysteine thiolates to oxidize and form Z aggregates. We speculate that this mode of action is the basis for the compound effectiveness, as the spectral changes are most pronounced at the concentrations with optimal virucidal and antiviral effects. Although all of the compounds reported here could be potent virucidal agents for virion inactivation, the azoic derivative ADA was much less effective than the disulfides in reducing virus yields from infected cells. Thus, the disulfides, particularly NSC20625 and AT-2, are more promising antivirals that could be used to affect Z function during therapeutic treatments. For several biothreat agents, including arenaviruses, person-to-person transmission is not efficient and virus spread occurs primarily by contact with the reservoir or in hospital settings. Thus, disulfide compounds deserve further study to assure their utility as barriers to minimize human exposure.

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