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Mode of inactivation of arenaviruses by disulfide-based compounds

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Abstract

Several disulfide-based compounds, including intermolecular aromatic disulfides of the type Ph-S-S-Ph and dithianes with the sulfur atoms tethered in a ring structure, have shown effective inhibitory activity against the arenaviruses Junin (JUNV), agent of Argentine hemorrhagic fever, and Tacaribe (TCRV). These compounds showed a strong virucidal effect with inactivating concentration 50% (IC_{50}) values in the range 0.6–5.0 μM, and also were effective to reduce virus yields from infected cells. The mode of inactivating action of two active compounds, the aromatic bis disulfide NSC20625 and the dithiane NSC624152, was further studied. Both compounds were able to inactivate arenaviruses after a few minutes of direct contact with virions, in a concentration- and time-dependent manner. The ability of drug-treated virus to perform several steps of the replication cycle was analyzed. The killed virus particles were found to bind and enter to Vero cells with the same efficacy as infectious native virions, but the ability of inactivated virions to synthesize viral proteins in Vero cells was abolished. Thus, treatment of JUNV and TCRV with these compounds destroyed virion infectivity, generating particles which entered the host cell but were unable to complete the viral biosynthetic processes.

Keywords: Arenaviruses; Junin virus; Tacaribe virus; Antiviral; Disulfides; Virus inactivation

1. Introduction

The arenaviruses are enveloped viruses containing a bipartite, single-stranded RNA genome, with ambisense coding strategy. The large (L) fragment encodes the RNA-dependent RNA polymerase and a Zn-binding protein named Z, whereas the small (S) fragment encodes the nucleocapsid protein (NP) and the two envelope glycoproteins GP1 and GP2.

Based on serological cross-reactivity and geographic distribution of the viruses, the members of Arenaviridae were grouped in two complexes, and this classification was then confirmed by phylogenetic analyses of S RNA nucleotide sequence data (Buchmeier et al., 1995; Bowen et al., 1996). The Old World or lymphocytic choriomeningitis–Lassa complex includes the worldwide prototype lymphocytic choriomeningitis virus (LCMV) and four African viruses. The New World or Tacaribe

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complex includes the prototype Tacaribe virus (TCRV) and 14 other virus species of America.

The principal hosts of most arenaviruses are rodents, which are carriers of chronic viral infection. Five arenaviruses, Lassa (LASV), Junin (JUNV), Machupo (MACV), Guanarito (GTOV) and Sabiá (SABV), are known to cause severe hemorrhagic fevers in humans. In particular, JUNV is the agent of the annual outbreaks of Argentine hemorrhagic fever (AHF), an occupational disease affecting the population of the most rich agricultural zone of Argentina (Weissenbacher et al., 1987). The danger of arenaviruses for human health and the continuous emergence of new viruses during the last years in North and South America (Fulhorst et al., 1997, 2001; Enserink, 2000) demand efforts to develop preventable vaccines or effective antivirals. Attenuated and recombinant vaccines have been tried against JUNV and LASV (Auperin, 1993; Maiztegui et al., 1998; López et al., 2000). In fact, an attenuated live vaccine named Candid 1 has been successfully evaluated in the human population at high risk of the endemic area of AHF (Enria et al., 1999). However, vaccines probably will never be the complete answer to the control of arenavirus infections. At present, no reliable drug therapy is available. The administration of standardized doses of convalescent plasma is up today the best therapy against AHF whereas ribavirin is the preferred method of treatment for Lassa fever, but undesirable side effects were recorded (Maiztegui et al., 1979; McCormick et al., 1986; Enría and Maiztegui, 1994).

The search for new antiviral compounds against arenaviruses has been reported (Andrei and De Clercq, 1990; Smee et al., 1992; Candurra et al., 1996; Cordo et al., 1999; Bartolotta et al., 2001). A previous screening study identified a series of known antiretroviral Zn–finger compounds, including disulfides, azoic and hydrazide derivatives, as inhibitors of arenavirus infection in Vero cells (García et al., 2000). In this paper, we investigated the mode of action of disulfide compounds against JUNV and TCRV, an arenavirus closely related to JUNV but not pathogenic for human (Martinez Peralta et al., 1993).

2. Materials and methods

2.1. Compounds

Seven disulfide-based compounds with the chemical structures and code numbers shown in Fig. 1 were provided by the National Cancer Institute, Frederick, MD. Stock solutions at a concentration of 100 mM were prepared in dimethylsulfoxide.

2.2. Cells and viruses

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

The naturally attenuated IV4454 strain of JUNV obtained from a mild human case and the TRLV 11573 strain of TCRV were used. Virus stocks were prepared in Vero cell cultures and titrated by plaque formation.

2.3. Cytotoxicity assay

Cytotoxicity was measured with MTT (3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide; Sigma-Aldrich) method (Mosmann, 1983) in Vero cells. Confluent cultures in 96-well plates were exposed to 2-fold dilutions of the compounds, with three wells for each dilution, during 48 h of incubation at 37 °C. Then, 10 μl of MM containing MTT (final concentration 5 mg/ml) was added to each well. After 2 h of incubation, the supernatant was decanted and 200 μl ethanol was added to each well to solubilize the formazan crystals. After vigorous shaking, absorbance was measured in a microplate reader and cytotoxicity was calculated as the 50% cytotoxic concentration (CC_{50}), compound concentration required to reduce the MTT signal by 50% compared to controls.

2.4. Virus yield inhibition assay

Vero cells grown in 24-well microplates were infected at a MOI of 0.1. After 1-h adsorption at

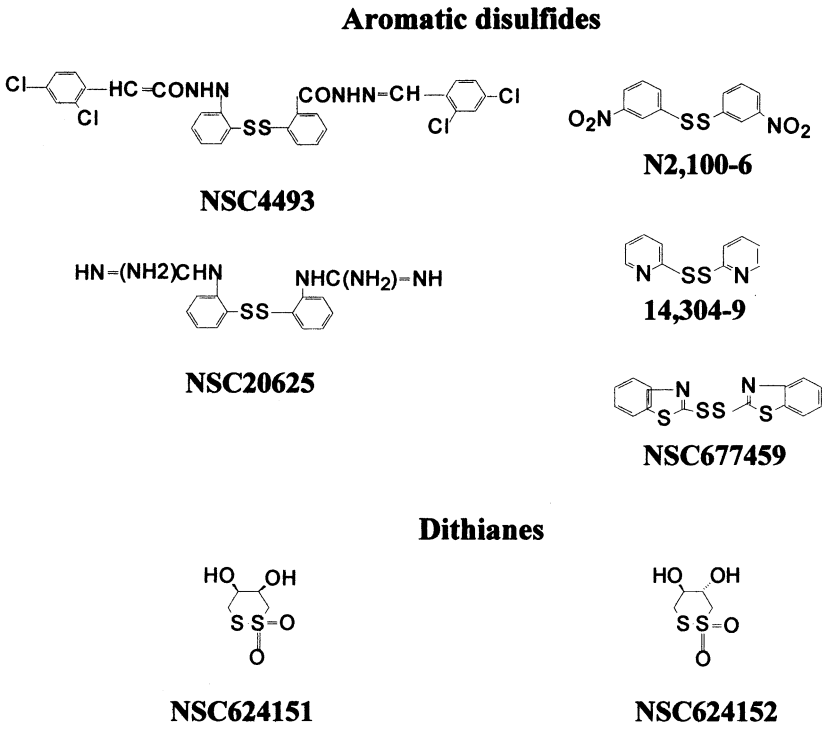

Fig. 1. Chemical structure and code number of disulfide-based compounds.

37 °C, cells were washed and refed with MM containing different concentrations of the compounds (2 wells per concentration). After 48 h of incubation at 37 °C, supernatant cultures were harvested and extracellular virus yields were determined by a plaque assay. The dilution of supernatant cultures required for titration of virus yields at 48 h after infection assessed that residual drug in the medium was below the active concentrations. The 50% effective concentration (EC_{50}) and 90% effective concentration (EC_{90}) were calculated as the concentrations required to reduce virus yield by 50 and 90%, respectively, in the compound-treated cultures compared with untreated ones.

2.5. Inactivation assay

Equal volumes of a virus suspension containing approximately 1×10^6 PFU of either JUNV or TCRV and various concentrations 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. According to the experiment, the mixtures were then processed in two different forms:

(i) to determine virucidal activity by infectivity titration, samples were 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. The 50% inactivating concentration (IC_{50}), and the 90% inactivating concentration (IC_{90}), the concentrations required to inactivate virions by 50 and 90%, respectively, were calculated.

(ii) for binding, internalization and protein synthesis assays, the mixtures were centrifuged at $100\,000 \times g$ during 2 h to remove the compound from the virus, the viral pellet was resuspended and inoculated to cell cultures.

2.6. Virion binding and internalization assays

The preparation of ^{35}S-methionine-labeled purified TCRV and JUNV was described previously (Damonte et al., 1994). Radiolabeled virions (2×10^7 PFU/ml, 5×10^8 dpm/ml) were mixed with MM or 20 μM of each compound and incubated at 37 °C for 1.5 h. After incubation, the viruses were collected by ultracentrifugation (Beckman SW55 Ti rotor, $100\,000 \times g$, 1.5 h, 4 °C) and unbound compound was removed. For the binding assay, the viruses were then added to Vero cells grown in a 24-well microplate and incubated at 4 °C for 40 min. Then, cells were washed extensively with PBS, lysed with a 0.1 M NaOH solution containing 1% sodium dodecyl sulphate (SDS), and cell-bound radioactivity was quantified using a liquid scintillation counter. For the internalization assay, after virus adsorption at 4 °C for 40 min, cells were incubated at 37 °C during 60 min 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 as above and cell-associated radioactivity was quantified.

2.7. Assay for viral protein synthesis

Vero cells grown in 24-well microplates were infected with compound-inactivated and control infectious TCRV at a MOI of 1. At 48 h post-infection, infected cells were incubated in methionine–cysteine-free medium for 1.5 h and then labeled with 100 μCi/ml of EXPRE^{35}S-^{35}S (NEN, duPont) for 3.5 h. After labeling, cells were lysed in a lysis buffer consisting of 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 clarified cell lysates were mixed with polyclonal rabbit anti-TCRV serum for 30 min at 37 °C and further incubated at 4 °C for 90 min. Antibody–antigen complexes were collected with protein A–sepharose and solubilized by boiling in sample buffer containing 5% SDS, 2% 2-mercaptoetanol, 10% glycerol and 0.005% bromophenol blue in 0.0625 M Tris–HCl, pH 6.8. Polypeptides were then electrophoresed on 12% polyacrylamide–SDS slab gels and visualized by fluorography.

3. Results

The first approach to elucidate the mode of action of the disulfide-based compounds against arenaviruses was the evaluation of their virucidal activity by direct incubation of virions with the compounds during 1.5 h at 37 °C, and subsequent dilution of the mixture to assay residual infectivity on Vero cells. Table 1 shows the values of inactivating concentrations against JUNV and TCRV. Two set of aromatic disulfide-based compounds were assayed: intermolecular disulfides of the type Ph-S-S-Ph, with the disulfide bridge linking two aromatic molecules, and dithianes, compounds in which the sulfur atoms are tethered in a ring structure (Fig. 1). Among the aromatic bis disulfides, NSC20625 was the most effective virucidal agent with the lowest values of IC_{50} (0.6–1.3 μM) and IC_{90} (1.6–3.1 μM) against JUNV and TCRV. The pyridine-derivative 14,304-9 (known as Adrithiol-2) and the nitro-derivative N2,100-6 were also very effective virucidal agents with IC_{50} values lower than 5.0 μM, and IC_{90} values lower than 12.0 μM. The presence of a second aromatic ring in the molecule reduced the effectiveness of the compound NSC4493, increasing the virucidal concentrations, whereas the heterocyclic disulfide NSC677459 was totally unable to inactivate both viruses. The two dithianes, the *cis* and *trans* isomers of 1,2-dithiane-4,5-diol, 1,1-dioxide (NSC624151 and NSC624152, respectively) drastically inactivated both JUNV and TCRV at low concentrations.

The values of 50% effective concentration (EC_{50}) and 90% effective concentration (EC_{90}), as parameters of antiviral activity determined by a virus yield inhibition assay, were also included in Table 1 for comparative purposes. In general, the compound concentrations required to reduce virus yield from infected Vero cells highly exceeded the doses necessary to inactivate cell-free virions, with

Table 1
Inhibitory activities of disulfide-based compounds against JUNV and TCRV

Compound	JUNV			TCRV			
	IC_{50}[a]	IC_{90}[b]	EC_{50}[c]	IC_{50}[a]	IC_{90}[b]	EC_{50}[c]	CC_{50}[d]
Aromatic disulfides							
NSC20625	0.6	1.6	9.3	1.3	3.1	3.6	94.4
N2,100-6	1.8	5.7	19.8	1.6	3.1	12.5	143.1
14,304-9	5.0	12.0	55.1	1.6	6.3	37.5	> 400
NSC4493	4.2	100.0	36.5	5.9	21.3	20.6	363.2
NSC677459	> 50	> 50	100.0	> 50	> 50	84.8	> 400
Dithianes							
NSC624151	3.2	8.0	17.0	0.4	3.1	42.6	> 400
NSC624152	3.2	6.1	38.2	0.2	6.2	11.4	352.8

Values (expressed as μM) are the mean from duplicate independent tests.
[a] Inactivating concentration 50% = compound concentration required to inactivate virus by 50%.
[b] Inactivating concentration 90% = compound concentration required to inactivate virus by 90%.
[c] Effective concentration 50% = compound concentration required to reduce virus yield by 50%.
[d] Cytotoxic concentration 50% = compound concentration required to reduce cell viability by 50%.

the only exception of NSC677459, which showed a weak antiviral activity (EC_{50} values in the range 84.8–100 μM) without virucidal effect.

Cytotoxicity of the compounds was evaluated by the MTT assay in Vero cells and the values of CC_{50} were determined (Table 1). The ratios CC_{50}/IC_{50} were in the range 61–1764, except for NSC677459, marking the high level of selectivity of the disulfides.

Two active compounds representative of each structural type, the bis disulfide NSC20625 and the dithiane NSC624152, were chosen for further characterization of their virucidal action. Fig. 2A and B show the dose–response curves for virus inactivation. Both compounds showed a biphasic curve with a nearly lineal one-hit initial part at concentrations lower than 10 μM, characterized by a sharp reduction in virus titer, and then a very low variation on residual infectivity at higher concentrations. NSC20625 was confirmed as a more potent virucidal than the dithiane, since at high doses, the first compound reduced the infectious titer of JUNV and TCRV by about 5000–10 000-fold, whereas NSC624152 reduced the titer of JUNV by 1000-fold and TCRV by 200-fold, respectively.

Fig. 2C and D show the time course of virus killing by the two agents. Inactivation appeared to start immediately after addition of the compounds to the virion suspension and to proceed then with first-order kinetics, and again NSC20625 was the most effective inhibitor: inactivation reached 92–95% after 15–20 min, and 99.0–99.9% after 90 min of incubation at a compound concentration of 20 μM.

Temperature had also a strong influence on the virucidal activity, since inactivation did not occur at 4 °C (data not shown).

To gain some insight into the mechanism by which the two compounds inactivate arenaviruses, we tested the ability of drug-treated virus to perform several steps of the replication cycle. First, a radiolabeled virion binding assay was performed. Vero cells were exposed at 4 °C during 40 min to [^{35}S]-labeled virus samples previously treated with both compounds or MM at 37 °C during 1.5 h. Fig. 3A shows that the binding to Vero cells of JUNV or TCRV, after inactivation by compound treatment, was comparable to that of infectious virions.

Next, the ability of bound inactivated virus to undergo conformational changes leading to pH-dependent fusion in endosomes (Castilla et al., 1994) and virus internalization was evaluated. To this end, radiolabeled inactivated virions were bound to Vero cells at 4 °C as above, and then

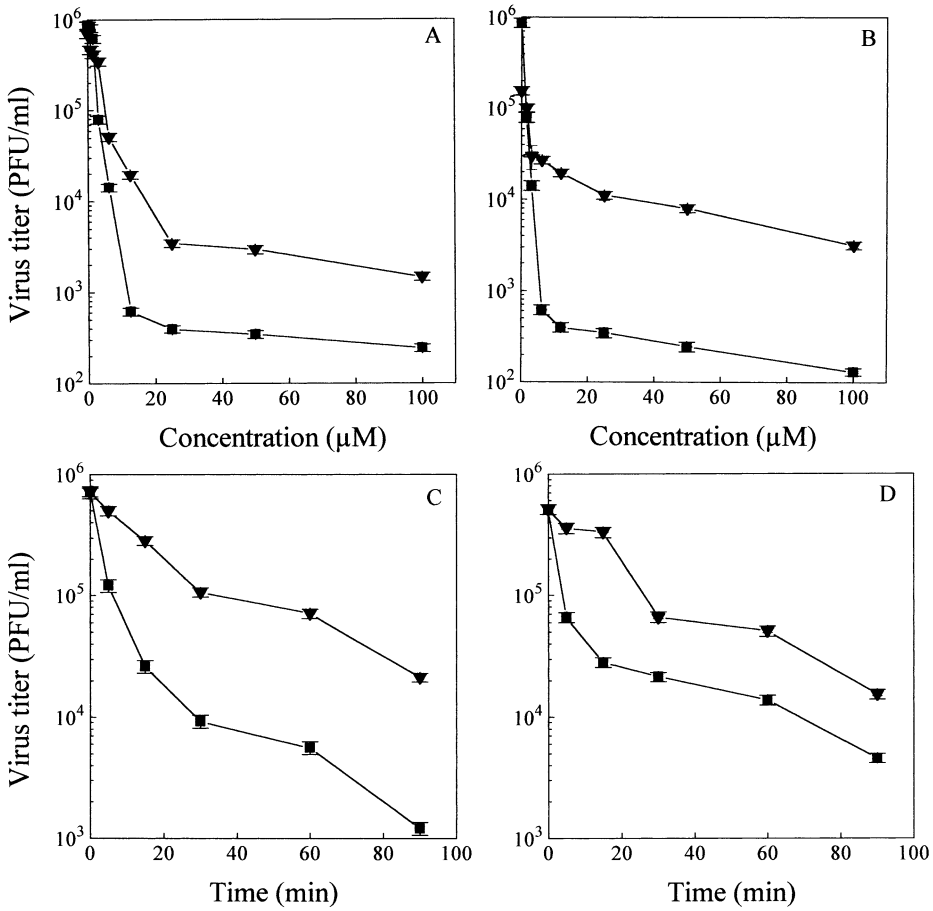

Fig. 2. Inactivation of arenaviruses with NSC20625 (■) and NSC624152 (▼). Dose–response curves: suspensions of JUNV (A) and TCRV (B) were treated with different concentrations of the compounds for 1.5 h at 37 °C, and then the samples were diluted and infectivity was determined by plaque formation in Vero cells. Kinetics of inactivation: suspensions of JUNV (C) and TCRV (D) were treated for different times at 37 °C with 20 μM of each compound, and then the samples were diluted and assayed for infectivity. Each point is the mean value of duplicate independent experiments ± S.D.

the temperature was raised to 37 °C, and incubation continued during 60 min to allow virus uptake. As seen in Fig. 3B, the amount of [^{35}S]-labeled internalized virus was similar for compound-treated and control virus preparations, indicating that virions inactivated by NSC20625 or NSC624152 treatment were able to mediate entry to Vero cells.

Once determined that inactivated virus was effectively internalized into Vero cells, the ability of such virus to synthesize viral proteins was analyzed. After 48 h of infection with inactivated and control infectious TCRV, no viral proteins were observed in cells infected with killed virus preparation whereas in cells infected with control virus the main viral proteins were detectable (Fig. 4). A similar pattern was obtained with inactivated JUNV (not shown). Thus, these compounds inactivate virion infectivity generating particles which entered the host cell but were unable to complete the viral biosynthetic pathway process. Although the inactivated virus preparation was ultracentrifuged after inactivation in order to separate virions and compound, it could not be discarded that residual drug added to the cells along with the inactivated virions prevented the

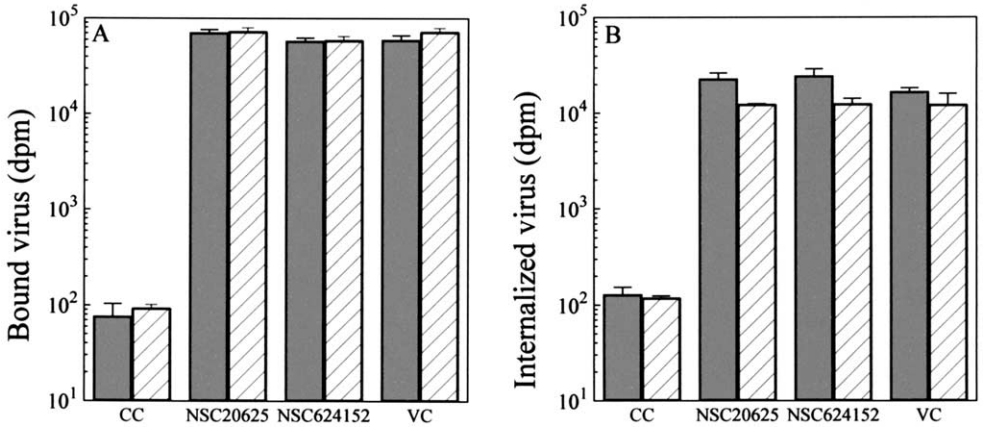

Fig. 3. Binding and internalization of radiolabeled purified virions of JUNV (grey solid bars) and TCRV (dashed bars). (A) Binding assay: Vero cells were incubated during 40 min at 4 °C with [^{35}S]-labeled virus samples previously treated with each compound at a concentration 20 μM or MM for 1.5 h at 37 °C, and then cell-bound radioactivity was determined. (B) Internalization assay: Vero cells were incubated with labeled infectious and inactivated virus samples during 40 min at 4 °C as above, then incubation followed during 60 min at 37 °C, and internalized virus was determined. CC, mock infected cell control; VC, infectious virion control treated with MM; NSC20625 and NSC624152, virion samples inactivated with each compound. Each value is the mean of duplicate independent experiments ± S.D.

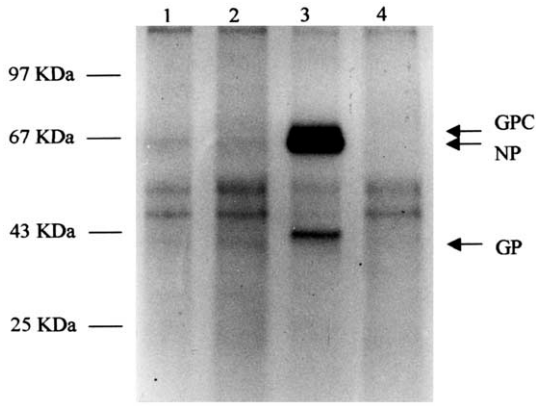

Fig. 4. Failure of inactivated TCRV to synthesize viral proteins in Vero cells. Suspensions of TCRV were incubated with MM or 20 μM of each compound for 1.5 h at 37 °C. Samples were then ultracentrifuged and pelleted virions were used to infect Vero cells. At 48 h post-infection cells were labeled with 100 μCi/ml of EXPRE^{35}S-^{35}S for 3.5 h, and then viral polypeptides were immunoprecipitated from cell lysates with anti-TCRV serum and analyzed by electrophoresis. Lanes: 1, TCRV treated with NSC20625; 2, TCRV treated with NSC624152; 3, TCRV treated with MM; 4, mock infection. The molecular mass markers are indicated in the left; arrows in the right indicate the positions of the main viral polypeptides.

synthesis of viral proteins after 48 h of infection. To test this possibility, cells were infected with untreated virus, after adsorption compound was added to infected cells, and then cell extracts were analyzed for the presence of viral proteins at 48 h post-infection. This control experiment demonstrated that the drug applied to the infected cells had no effect on the ability of infectious virus to synthesize viral proteins (data not shown).

4. Discussion

Results presented here have shown the effective inhibitory action against arenaviruses of several disulfide-based compounds. Two types of structural molecules, intermolecular aromatic disulfides and dithianes, were found to have antiviral and virucidal properties against both JUNV and TCRV: the compounds were able to inactivate virus by direct contact as well as to reduce virus yields from infected Vero cells. However, the comparison of the antiviral EC_{50} with the virucidal IC_{50} clearly indicates a major effectiveness of both

types of compounds to inactivate virions. The lack of stability of the compounds over the incubation period at 37 °C with the infected cells may be a probable explanation for this differential effect, but at present no data on stability of the compounds are available.

Both NSC20625 and NSC624152 were found to inactivate JUNV and TCRV in a concentration- and time-dependent manner. A drug concentration of 20 μM was able to kill virus after few minutes of contact, and in the case of NSC20625, the most active disulfide, more than 99.9% of infectivity was destroyed. The mode of inactivation by these compounds rendered non-infectious virus particles unable to complete the macromolecular viral biosynthetic pathway. The early stages of binding and internalization of inactivated virus took place with the same efficacy as for native virions, but the synthesis of virus proteins was totally abolished. These results suggest that infectivity was destroyed by NSC20625 and NSC624152, but apparently the conformational integrity of the virion envelope proteins GP1 and GP2, required to bind cellular receptors and to trigger the fusion between viral and endosome membranes during entry, was preserved (Castilla et al., 1994; Di Simone et al., 1994). It seems probable that the mechanism of virus inactivation by these compounds involves their diffusion across the lipid bilayer of the envelope into the viral particle to interact with an internal target.

The compounds assayed in this study against arenaviruses are included in a selected collection of electrophilic reagents with varied functional groups (aromatic C-nitroso compounds, disulfides, disulfoxides, maleimides, and others), which were found active as anti-human immunodeficiency virus type 1 (HIV-1) by mediating the chemical modification of the Zn–finger motif of the retroviral NP NCp7 (Rice et al., 1996; Witvrouw et al., 1997; Berthoux et al., 1999). This protein of lenti- and onco-retroviruses contains two highly conserved Zn-binding domains with three Cys residues and one His residue (CCHC) coordinating a Zn ion. It was demonstrated that the antiretroviral activity of compounds such as NSC20625, the dithianes NSC624151 and NSC624152, Aldrithiol-2, and other disulfides is associated to Zn ejection from NCp7 leading to cross-linkage of the protein and subsequent virion inactivation (Rein et al., 1996; Tummino et al., 1997; Rice et al., 1997; Rossio et al., 1998).

The presence of Zn-binding domains has also been described in Arenaviridae. The NP of LCMV and JUNV was found to bind Zn in vitro, and a classical Zn–finger motif was found near the carboxyl terminus of the NP sequence (Salvato and Shimomaye, 1989; Tortorici et al., 2001). This structural protein is essential for genome replication and nucleocapsid assembly. The small Z protein, encoded in the L RNA segment, contains a Cys3HisCys4 RING finger motif and also binds Zn in vitro (Salvato and Shimomaye, 1989). Z protein is packaged into virions (Salvato et al., 1992), but its precise function in arenavirus infection is poorly understood. Discrepant results were obtained about its role in genome replication and mRNA synthesis in TCRV and LCMV (Garcin et al., 1993; Lee et al. 2000; López et al., 2001), whereas other studies have suggested that Z might be the arenavirus counterpart of the matrix protein found in other RNA viruses as a bridge between the envelope and the nucleocapsid (Salvato, 1993). Recent findings have also shown that LCMV Z interacts with several cellular proteins (Campbell Dwyer et al., 2000). Further experiments should address the possibility that the coincidental effect of the disulfides to inactivate arenaviruses and retroviruses is associated with a similar interaction of the compounds with Zn-binding proteins, and, as a consequence of this compound-protein reaction, the treated arenaviruses proved to be unable to complete the replicative cycle.

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